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Ultrasensitive Immunoassays of Prostate-Specific Antigen Using Bispecific Antibodies.

By

Donald Robert Husereau ©

A thesis submitted to the Faculty of Graduate Studies and research in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

Department of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Fall, 2000

Canada

University of Alberta

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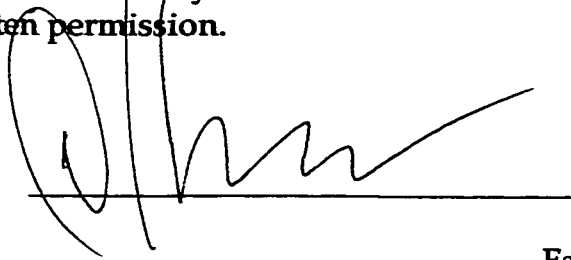
Title of Thesis: Ultrasensitive Immunoassay of Prostate-Specific
Antigen

Using Bispecific Antibodies.

Degree: Master of Science

Year this Degree Granted: 2000

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Abstract

Prostate-specific antigen (PSA) is secreted exclusively by prostate cells. Because of this, it was recognized that regular monitoring of PSA levels in serum could be beneficial in predicting the recurrence of prostate cancer post-radical prostatectomy.

To develop this assay, a number of steps were taken. First, a unique method for the bulk purification of PSA is described. This involved a positive-mode affinity purification using a Cibacron blue F3G-A dye affinity matrix.

Secondly, a unique method is described for purifying horseradish peroxidase (HRPO)-labeled bispecific antibodies using a benzhydroxamic-acid agarose column which binds to the active site of the HRPO enzyme.

Lastly, an evaluation of two immunoassays which measure PSA in the ultrasensitive range (< 0.1 ng/ml) is described. The benefits of added sensitivity are described herein. Both assays, one a monoclonal antibody (Mab)-based and the other, a bispecific monoclonal antibody (bsMab)-based, were capable of detecting PSA at concentrations < 0.006 ng/ml.

"Adieu, dit le renard. Voici mon secret. Il est très simple : on ne voit bien qu'avec le coeur. L'essentiel est invisible pour les yeux".

-- Antoine de Saint-Exupéry, *Le Petit Prince*

"Those who cannot remember the past are condemned to repeat it."

-- George Santayana
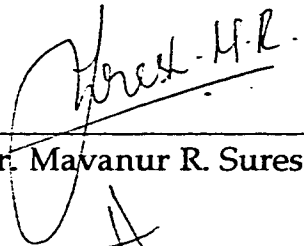
" I fancy you as coming to the acquisition of the myriad facts of medicine with little to tell you of the intellectual forces and historical sequences by which these facts have emerged"

-- Christian A. Herter

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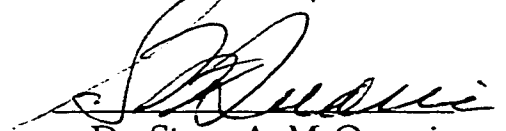
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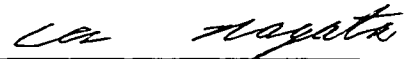
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List of Abbreviations

α2-MG	α-2-macroglobulin
A_{280}	Absorbance at 280 nm
AA	Amino Acid
Ab	Antibody
ACT	α-1-antichymotrypsin
AE	Acridinium Ester
AP	Alkaline Phosphatase
BDL	Biological Detection Limit
β-Gal	Beta-D-Galactosidase
BSA	Bovine Serum Albumin
BsMab	Bispecific Monoclonal Antibody
°C	Degrees Celsius
CaP	Prostate cancer (carcinoma)
CL	Chemiluminescence
COL	Colorimetry
CPRG	chlorophenol red-beta-D-galactopyranoside
Da	Dalton(s)
dBSA	Dialyzed BSA
DEAE	Diethylaminoethyl
DFP	Diisopropylfluorophosphate
EIA	Enzyme Immunoassay
ELFIA	Enzyme-linked Immunofluorometric Assay
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
Fc	Fraction of crystallizable. (Ig constant region)

FL	Fluorescence
FPLC	Fast performance Liquid Chromatography
hK2	Human Kallikrein 2
HPLC	High Performance Liquid Chromatography
HRPO	Horseradish Peroxidase
HSP	Human Seminal Plasma
IEX	Ion Exchange
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgG2b	Immunoglobulin G2b
IgG2c	Immunoglobulin G2c
IgG3	Immunoglobulin G3
IgG4	Immunoglobulin G4
IRMA	Immunoradiometric Assay
Kd	Dissociation constant
LLD	Lower Limit of Detection
Mab	Monoclonal Antibody
MW	Molecular Weight
O.D.	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PheMeSO$_2$F	Phenylmethylsulfonyl fluoride
PBS	Phosphate-buffered saline
PBS-BSA	BSA in PBS
PBS-T	0.05%-0.1% Tween 20 in PBS
pNpp	p-Nitrophenylphosphate

PSA	Prostate-specific Antigen
PSA-ACT	PSA-ACT complex
QAE	Quaternary ammonium Exchanger
RIA	Radioimmunoassay
RT	Room Temperature
SDS	Sodium dodecylsulphate
SE	Size Exclusion
TMB	3,3′,5,5′-tetramethylbenzidine
U	Units
UV	Ultraviolet
V/V	Volume by Volume
W/V	Weight by Volume

Chapter 1: Introduction

1.1 PSA

PSA Biochemistry. Prostate-specific antigen (PSA) is a glycoprotein (~33,000 Da.) transcribed by a single gene on the long arm of chromosome 19 and is secreted exclusively by prostate ductal epithelial and acinar cells (Riegman et al., 1992; Zhou et al., 1993). PSA has been characterized as a monomeric protein of 237 amino acids in length with approximately 8% carbohydrate content (4.84% hexose, 2.87% hexosamine, 0.25% sialic acid) (Zhang et al., 1995). Some research has indicated a single N-linked carbohydrate side chain while others have concluded there are four (Armbruster, 1993). It has been suggested that these different glycosylation patterns derive from at least 5 PSA isomers with pIs of 6.8-7.5. The pI of the major isoform is 6.9 (Wang et al., 1981; Zhang et al., 1995). A rigid meta-analysis of PSA extinction coefficients calculated by various purification techniques has concluded an assigned value of 1.84 ± 0.04 mL x mg^{-1} x cm^{-1} at 280nm (Stamey et al., 1995).

PSA has been categorized with the human kallikrein (hK) family of serine proteases (Watt et al., 1986). It has been shown to have a 57% amino acid sequence homology with that of human kallikrein 2 (hK2) (Watt et al., 1986). The enzymatic activity of PSA has been described as both trypsin-like (cleaving at basic residues) and chymotrypsin-like (cleaving at hydrophobic residues) (Watt et al., 1986; Sensabaugh and Blake, 1990). More recently, it was revealed that a contaminant, human kallikrein-2 (hK2), that was copurified with PSA contributed to the observed trypsin-like activity of the enzyme (Frenette et al., 1998). This trypsin-like serine protease has been postulated to play a role in PSA expression, hydrolyzing a prescursor (pPSA) to an active form (PSA) (Kumar et al., 1997). The kallikrein hK2 protein is putatively responsible for inaccurate

immunodiagnostic and immunohistochemical reports resulting from cross-characterization and cross-reactivity with PSA (Corey et al., 1997).

PSA is found in concentrations in semen between 0.5 – 5 mg/L (Average 0.7 mg/L) (Tessmer et al., 1995). PSA acts on the physiological substrate identified as high molecular mass seminal vesicle protein (HMM-SV-protein) or semenogelin. Two isomers of this protein, I and II, along with fibronectin have a major role in the formation of the seminal coagulum. Another protein, identified as seminal-vesicle specific antigen (SVSA) has also been demonstrated to be involved with this liquefaction process (Armbruster, 1993).

PSA is also found in human serum. It exists in both a free PSA and PSA-protease-inhibitor complexed form. A majority of PSA can be found complexed to α_1-antichymotrypsin (ACT) and to a much lesser degree α_2-macroglobulin (α_2-MG). A normal reference range for concentrations of PSA in serum in males is between 0 and 4 ng/mL (Armbruster, 1993). The serum half-life of PSA is estimated to be between 2.2 and 3.2 days (Armbruster, 1993). The interaction of PSA with corporeal fibronectin could be reasoned to play a major role in tumour metastasis in prostate cancer.

PSA purification. Initial interest and the discovery of PSA began as the search for a robust analyte in the forensic detection of semen ended in 1966. Two specific antigenic markers for semen, β-microseminoprotein and γ-seminoprotein, were discovered by Hara in 1971 (Hara et al., 1971). Further characterization of γ-seminoprotein was done by Koyanagi (Koyanagi et al., 1972). Later, Li, Schulman and Beling identified another semen-specific molecule as Protein E_1 (Li and Shulman, 1971; Li and Beling, 1973). Subsequently, Sensabaugh discovered a third seminal marker p30 (Sensabaugh, 1978). We now know that all of these molecules are one and the same: PSA.

The subsequent PSA purification by Sensabaugh (Sensabaugh, 1978) was undertaken to create a definitive marker for semen in forensic cases. This was

done by comparing the electrophoretic bands of seminal plasma and serum and identifying a positive marker, named p30, which could be purified and characterized through an immunological assay. The purification scheme entailed using two ion exchange columns and two identical gel filtration columns. The entire procedure was carried out at 4°C. This resulted in a protein which was estimated to be >98% pure by electrophoresis. A molecular weight estimate of 30,000 by size exclusion allowed them to identify their protein with that of Li's protein E_1. A percentage recovery was not published.

Interest in PSA was also spawned by the search for a tumour marker to indicate the presence of prostate cancer. Prostatic Acid Phosphatase (PAP, EC 3.1.3.2), a dimeric glycoprotein with a ~100,000 MW, had been discovered in 1936 but was not prostate specific. Interested in finding a specific tumour marker, Wang and Valenzuela purified PSA (called PA) in 1979 from prostatic tissue (Wang et al., 1979). Their purification involved an ammonium sulfate precipitation, ion exchange column, two gel filtration columns and a preparative PAGE. The purity by PAGE under reducing and non-reducing conditions was estimated to be 100%. A molecular weight of 33,000 by gel filtration on Sephadex G-75 and 34,000 by SDS-PAGE is published. No mention of either p30 or of protein E_1 occurs in their original publication. A final recovery of 2.7% was reported.

A simplified modification of the original Wang and Valenzuela method was published three years later (Wang et al., 1982). It used both prostatic tissue *and* seminal plasma as its source materials for PSA (still called PA). It again used an ammonium sulfate precipitation followed by ion exchange and gel filtration but required a second DEAE step instead of another gel filtration. A recovery of 17.0 % was reported from seminal plasma with 7.3% recovery from prostatic tissues. The MW was again estimated to be between 33,000 and 34,000 by Sephadex G-75 gel filtration. The first occurrence of isozymes (different pI

values) is alluded to in this paper. Final protein purity is again estimated to be 100% by SDS-PAGE. The entire procedure took 8-9 days.

In an effort to characterize the newly discovered tumour marker, Watt et. al. reported the amino acid sequence and enzymatic activity of PSA (still called PA) (Watt et al., 1986). It was purified from human seminal plasma using the original method described by Wang and Valenzuela. A final purification step using a large-pore Vydac (Hesperia, CA) C_4 column was performed. Although recovery and yield data was not reported, the purity should be assumed to be >99%. The PA protein was discovered to be a single polypeptide chain of 240 amino acids with proteolytic activity similar to that of chymotrypsin and trypsin. The inactivation of enzymatic activity using serine-protease inhibitors (PMSF and DFP) suggested it was a serine-protease. Comparisons of the AA sequence with that of serine proteases demonstrated a strong homology of PA with various enzymes in the kallikrein family (57%).

While research into PSA isolation, purification and characterization became more popular, continued efforts on characterizing γ-seminoprotein (γ-SM) continued in the mid-1980s based on the initial efforts of Hara and Koyanagi. Schaller was able to purify this "important marker in the detection of prostatic cancer and of semen stains in forensic medicine" by size exclusion and ion exchange methods (Schaller et al., 1987). Proteolytic activity and an amino acid sequence analysis were undertaken. Previously, γ-SM had been shown to be heterogeneous. Schaller isolated a main form, which was named fraction M. The MW was determined by SDS-PAGE and estimated to be 33,000. Amino acid analysis concluded the protein was made up of 237 aa and had sequence homology with human and porcine kallikreins (61 and 55% respectively). No kallikrein-like proteolytic activity was determined by substrate-directed experimentation but it was concluded to most likely be a serine protease because of sequence homology. While comparing this sequence analysis with that of Watt, Schaller erroneously concluded there were too many structural and

functional differences between the M fraction of γ-SM and PSA for them to be the same protein.

What began as an effective method to purify human prostatic acid phosphatase was the basis for the purification of PSA described in this thesis. Rusciano published a method for purifying PAP from seminal fluid because it could now be immunologically identified and measured (Rusciano et al., 1988a). Its clinical significance, however, was still unknown. The original purification was carried out by simply using a non-preparative HPLC column. Subsequently, a concomitant purification of PAP and PSA was published using a Cibacron blue F3G-A (Affi-Gel™ Blue gel, Bio Rad Labs, Richmond, CA, USA) dye affinity column and a semipreparative HPLC column (Rusciano et al., 1988b). Cibacron blue F3G-A was shown to have a high affinity for both PSA and albumin. The reported recovery was 45% for PSA with a ~96% purity. This recovery of PSA far exceeded the ~17% maximum which had been reported up till that time. PAP was also 60% recovered in an almost pure form. The molecular weight of PSA was reported to be 34 kDa using PAGE under reducing conditions but 30 kDa under non-reducing conditions. This discrepancy was explained by varying amounts of sialic acid residues on the protein.

To simplify PSA purification, Sensabaugh and Blake published a procedure based on the original method by Sensabaugh (Sensabaugh and Blake, 1990). The seminal plasma source material was dialyzed and applied to a CM-Sephadex ion exchange column. The pooled fractions were concentrated and subjected to two runs on a Sephacryl S-200 size exclusion column. Although the purity of the final product was reported to be >98%, the final recovery was not reported but could be estimated to be 25%. This report also suggests identity between p30, PSA, and γ-SM based on chromatographic data. In a subsequent paper, Graves et al. furthered this claim by using an identical purification

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method, and reported a yield of 12 mg from 100 mL seminal plasma with a recovery of 14% (Graves et al., 1990).

By 1990, it was recognized that a majority of PSA occurred as an ACT-complexed form in human serum rather than a free form. Efforts to measure PSA-ACT and PSA demonstrated greater clinical sensitivity in pCa prediction (Stenman et al., 1991). To this effect, reports describing purification of the PSA-ACT complex or post-purification complexation before measurement were published (Chen et al., 1995; Wu et al., 1995).

A new purification scheme, developed by Zhang and colleagues utilized ammonium sulfate precipitation, hydrophobic interaction chromatography, gel filtration and anion-exchange chromatography for purification of PSA from seminal plasma prior to *in vitro* complexation (Zhang et al., 1995). The scheme achieved a 30.1% recovery of PSA with a 99.5% purity. Approximately 65% of PSA purified from seminal plasma was enzymatically active and capable of forming these complexes. Other reports describe purification from serum of the PSA-ACT complex itself. A unique purification scheme involving SDS-PAGE and electroelution was published in 1995 (Tessmer et al., 1995).

1.2 Bispecific and Bifunctional Antibodies

Antibodies are proteins synthesized by B lymphocytes due to an immune response. They possess unique high-affinity binding properties and similar structural characteristics. Together, all antibodies form a family of plasma proteins known as immunoglobulins (Ig). The basic structure of an antibody is a tetrameric complex of two heavy (H) and two light (L) chains which form a Y-shaped structure. The two chains are present in an equimolar ratio and are linked by disulfide bonds. The amino terminal domain of the H and L chains combine to form two antigen binding regions, or paratopes. This binding region can have structural variability from B lymphocyte to B lymphocyte because of prior genetic rearrangement giving each antibody a unique binding specificity.

Various subclasses, or isotypes, of antibodies have been characterized by structural differences in the constant regions of their H chains. These structural differences confer differences in immune system functionality and allow some isotypes to form polymers.

The most abundantly secreted antibody in human plasma is the IgG isotype. When a foreign protein is introduced into a host, the immune system will respond by amplifying the production of those IgG antibodies that have affinity for it. The IgG antibodies not only bind and neutralize the foreign protein, but signal other immune system components to a complete immune response. The ability of the researcher to manipulate this response and to produce and utilize antibodies of a particular binding specificity has had a profound impact on the modern sciences.

Quite surprisingly, techniques have existed for the production of antibodies over 100 years (Silverstein, 1989). Traditionally, production of polyclonal antibodies was managed by the inoculation of an animal with an injected antigen. The animal was bled and the antiserum was used as a reagent. The presence of impurities in the antisera as well as the reproducibility from animal to animal limited the use of the antibodies produced by this technique. In 1975, Milstein and Kohler created a hybridoma – fusing the characteristics of specific binding from an individual antibody-secreting cell and the immortality of a myeloma cell (Kohler and Milstein, 1975). This technique eliminated the problems of specificity and reproducibility in one instance.

Unlike polyclonal antisera, monoclonal antibodies are typically produced by ascites fluid or mammalian tissue culture techniques. In some applications, it is perfectly feasible to use monoclonal and polyclonal antibodies in their impure form (Goding, 1980). In some other applications, the absolute quantity of antibody must be known and purification is necessary. This occurs when the antibody must undergo chemical modification or labeling, is used in plate-coating, or is used in *in vivo* applications (Campbell, 1996). Purification is also of

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paramount importance in bsMab production (Kricka, 1994), and will be discussed later in this chapter.

The strategy for purification of an antibody relies on its species and isotype (Table 1.1). For purposes of simplicity, only the purification of the commonly used G class of murine immunoglobulins (IgG) will be discussed here. The purification strategy should also take into consideration the intended use of the antibody. The simplest purification technique of IgG relies on the ability of ammonium sulfate to precipitate proteins. Saturated ammonium sulfate solution is added to the ascites or cell culture supernatant until a 45% (w/v) concentration in solution is achieved. This precipitates the antibody which is then centrifuged, resuspended and dialysed for further purification or immediate use. Typically this technique is coupled with size exclusion (SE) but can be used by itself to achieve >50% yields (Andrew and Titus, 1997).

Protein A and G purification techniques are also commonly employed. These proteins are commercially available in an agarose-coupled matrix. Protein A is a cell wall component produced by several strains of Staphylococcus aureus; and binds specifically to the Fc region of IgG for which it has four high-affinity binding sites (Andrew and Titus, 1997). Protein G is a bacterial cell wall protein isolated from group G streptococci. Protein A and G are extremely robust and can be exposed to a variety of denaturing conditions. Because some IgG1 subclass antibodies will not bind to protein A, consideration of the antibody isotype must be given before purification.

	Ammonium Sulfate	Protein A	Protein G	Ion Exchange
Rabbit IgG	++	++	++	++
Mouse IgG1	++	+	++	++
Mouse IgG2a	++	+	++	++
Mouse IgG2b	++	++	+	++
Mouse IgG3	++	-	+	++
Rat IgG1	++	-	-	++
Rat IgG2a	++	-	++	++
Rat IgG2b	++	-	-	++
Rat IgG2c	++	+	+	++
Human IgG1	++	++	++	++
Human IgG2	++	+	++	++
Human IgG3	++	-	++	++
Human IgG4	++	++	++	++

Table 1.1. The usefulness of different strategies to purify various allotypes and isotypes of IgG. The relative usefulness of each strategy is denoted by a symbol: Good = ++; Moderate = + Weak = - . Modeled after a table by A.M. Campbell in *Immunoassay*.

Protein G is considerably better at purifying this IgG1 subclass (Table 1.1). Because of harsh binding and eluting conditions, other milder purification methods may need to be considered before using this technique.

Ion exchange (IEX) chromatography relies on positively charged matrices to remove negatively charged protein (e.g. albumin) and retain antibody, which is eluted. Diethylaminoethyl (DEAE) and Quaternary Ammonium Exchanger (QAE) anion exchange resins are most commonly used. These are generally coupled to cellulose, cross-linked dextran, agarose, or acrylamide. FPLC matrices are also available (e.g. Mono Q or S). These resins are pseudospecific in nature (i.e. they will coelute some albumin) and other techniques (i.e. SE) may

be needed if highly pure (>99%) antibody is required. The mild binding and eluting conditions of this technique can be advantageous. As well, DE52 (DEAE-Cellulose, Whatman Ltd., London, UK) has an enormous capacity for binding protein (130mg/mL) and can be used effectively for concentrating dilute protein solutions (Andrew and Titus, 1997).

BsMab Purification. Unlike traditional monospecific antibodies, bsMabs have non-identical antigen binding sites to any two desired antigens. These bsMabs can be generated in various ways – by chemically coupling two distinct antibodies or Fab fragments, through genetic engineering (e.g. diabodies), or by somatic fusion to generate quadromas or triomas (Figure 1.1). A

Mab   **bsMab**
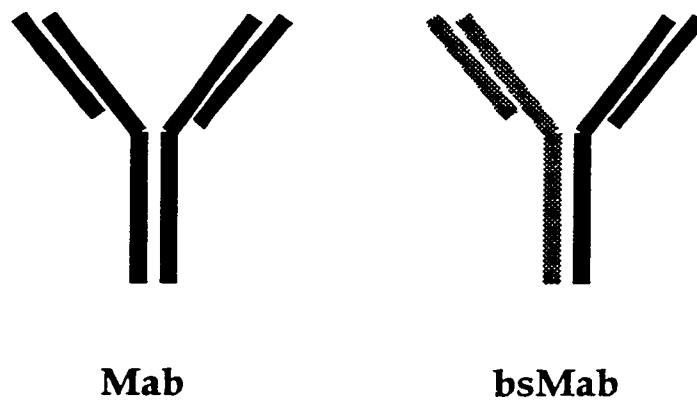
Figure 1.1: Mabs and bsMabs. Unlike polyclonal antibodies, monoclonal antibodies (Mabs) harbor a unique specificity for a sterically and spatially distinct epitope on a protein which can be reproduced. Bispecific monoclonal antibodies (bsMabs) generated by a fusion cell-culture method resemble Mabs structurally but contain specificities for two unique epitopes.

bsMab-secreting quadroma is generated by fusing the two monoclonal antibody (Mab)-producing hybridoma cell lines. This, however, presents some special problems. Post-translational random association of heavy and light chains in the quadroma generates the desired bispecific antibody along with other contaminating species including the parental monospecific antibodies (Suresh et al., 1986). This makes purification of the bispecific antibody the single greatest obstacle in the production and use of bsMabs from quadromas and triomas (Suresh et al., 1986; Kricka, 1994).

No one purification method exists for bsMabs. Typical purification schemes use a combination of protein A, ion exchange, and/or size exclusion. These non-specific methods are subject to trial and error and can be time consuming or problematic due to harsh eluting conditions. Dual (or double) affinity chomatography can be utilized by immobilizing the antigen if enough pure antigen is available (Cao and Suresh, 1998). Hydroxylapatite chromatography will elute proteins based on their isoelectric point and has been used to purify bsMabs with variable results (Karawajew et al., 1987; Karawajew et al., 1988). HPLC-Hydrophobic interaction (Manzke et al., 1997) and HPLC-SE chromatography (Takahashi and Fuller, 1988) have been used successfully to resolve the various bsMab isoforms. All of these techniques, however, are limited in terms of consistency, purity, and or yield.

Previous work in our laboratory has resulted in the development of several bsMabs with anti-tumor antigen specificity in one arm and anti-enzyme label specificity in the second arm. To purify these bsMabs we have developed various new techniques. Thiophilic adsorption chromatography was first described in 1985 by Porath (Porath et al., 1985). In the presence of a salt, thiophilic-gel (T-Gel™ , Pierce, Rockford IL) will preferentially bind to some sulfur-containing moieties in proteins. In the case of antibody purification, this has been postulated to be the interchain disulfide linkages in the Fc region (Kreutz et al., 1998). Given the hypothesis that bispecifics have fewer complete

disulfide linkages and a higher free sulfhydryl content due to a heterologous heavy chain association (i.e. non-matched sulfur-containing residues between chains), this technique coupled with a gradient elution has been used to successfully resolve bsMabs from their parental contaminants under mild eluting conditions (Kreutz et al., 1998).

Mimetic affinity chromatography is commonly used in the isolation and purification of various enzymes. Often a dye-ligand is used to immobilize an enzyme and separate it from other biological components. One such dye-ligand, MIMETIC blue A6XL, has traditionally been used to purify alkaline phosphatase from calf intestinal mucosa extracts (Lindner et al., 1989). It was shown that this same dye-ligand would be suitable for purifying bsMabs complexed with AP. To demonstrate this, two bsMabs, anti-PSA x anti-AP (P105) and anti-CA125 x anti-AP (P104) were successfully purified from mouse ascites using a MIMETIC blue A6XL dye-affinity column (Xu et al., 1998). This *single-mode* affinity purification technique retains all immunoglobulin heavy/light chain associations that are capable of binding to the enzyme marker while those that can bind to the tumor marker are discarded. These tumor marker-binding antibodies, especially the anti-PSA/CA125 monospecific, greatly reduce the specific activity of the crude ascites. The desired bsMab is the only antibody in the affinity column eluate capable of binding to the tumor marker and generating an enzymatic signal. The simplicity of this technique and the mild elution conditions make it highly desirable for purifying bsMab in immunoassay and immunohistochemical applications.

A Mimetic Red 3 – based method was also attempted as a "negative" mode purification method for HRPO-based immunoconjugates (Xu et al., 1998); that is, contaminants would remain on the column and the desired HRPO-labeled bsMab would be found in the unbound fraction. An SDS-PAGE revealed most, but not all, of the contaminants were removed and the resultant fractions could be used in an immunoassay but had a low specific activity. Hence, we set

out to develop a "positive" mode affinity chromatography of HRPO-based antibody immunoconjugates in this thesis.

Because *single-mode* affinity purification had been effectively demonstrated using the AP binding MIMETIC blue A6XL matrix , it was thought that a similar method could be carried out using a mimetic ligand for HPRO. Original work on this problem was accomplished using an HRPO-agarose column in a dual affinity system (Cao et al., 1998). As in the MIMETIC blue A6XL scheme, all tumor marker-binding antibodies in the crude would be washed from the column in the unbound fraction. To avoid harsh eluting conditions, it was shown that the HRPO-binding antibodies could be eluted by the simple addition of HRPO (Cao et al., 1998). The column, however, was neither economical nor robust and we sought another method. Hydroxamic acids had been previously demonstrated to bind to plant peroxidases (Reimann and Schonbaum, 1978). By using a hydroxamic acid-affinity column we speculated that we could purify HRPO-labeled bsMabs. The results of these experiments are the subject of Chapter 3.

1.3 Ultrasensitive Immunoassays

Immunoassays continue to be one of the most sensitive and specific analytical techniques utilized in a number of biomedical and non-medical settings. The sensitivity and specificity of an immunoassay are derived from the ability of an antibody to recognize and bind to a particular antigen, or analyte, and elicit a signal that qualitatively and quantitatively indicates the presence of the analyte to the observer (Fig 1.2). In a majority of immunoassays in use today, this signal is generated by an enzyme (Table 1.2).

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Fig 1.2.1: Directly labeled monoclonal sandwich assay

substrate   detectable product

- Colorimetric – e.g. pnPP, TMB, CPRG

- Fluorometric – e.g. Rhodamine, Coumarin, and Fluorescein derivatives. Phycobiliproteins. Lanthanides chelates.

- Chemiluminometric – e.g. acridinium and luminol analogues

- Bioluminometric – e.g. luciferin/luciferase

- Catalyzed Reporter Deposition

- Enzymatic Cascades

SOLID PHASE

Figure 1.2: The use of enzyme labels in immunoassay. In a bispecific antibody–based two-site noncompetitive (sandwich type) immunoassay, the enzyme label is attached by one paratope while the other paratope binds to the analyte (e.g. PSA). The enzyme can then be detected by reacting with a substrate which elicits a signal. Some detection systems use enzyme catalysis to amplify the final signal. A standard monoclonal sandwich assay differs only in that the enzyme is chemically coupled to the antibody (see Fig 1.2.1, inset).

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Assay Sensitivity. Sensitivity has been defined in many ways, but can be practically defined as the *amount of an analyte required to produce a change in the response which is significantly different from the response obtained in the absence of analyte (zero dose of analyte)* (Grotjan and Keel, 1996). This value has also been referred to as the lower limit of detection (LLD) and is usually calculated by taking 10 or 12 replicates of a zero value in a standard curve and extrapolating an analyte concentration value from the mean + 2 SD Although assays with low analytical sensitivity can be used in the laboratory, it was recognized that the LLD had to be reproducible in the clinical laboratory. The LLD was strictly a measurement of the analytical sensitivity of any given assay, or its intra-assay variability. The LLD is also referred to as the minimal detectable dose (MDD) or minimal detectable concentration (MDC).

To take into account variability from assay to assay at low levels of detection, and to minimize false positive detection, a measure of interassay variability and reproducibility, termed the biological detection limit (BDL), was devised for biological samples containing PSA (Graves, 1992; Vessella et al., 1992). It was calculated by establishing a confidence limit for multiple tests at a near-zero analyte concentration, and adding 2 SD of this interassay variation to the LLD. Improving sensitivity has several benefits including allowing the use of smaller samples for testing, as well as the analysis of substances that are only present at very low concentrations. Efforts to improve immunoassay sensitivities range from increasing signal generation and detection capabilities to reducing background noise.

1.3.1 Increasing Signal

1.1.1.1 Assay design:

Increasing the immunoassay signal is very important when detection limits in the ultrasensitive range need to be achieved. Many factors need to be carefully considered. Proper consideration must first be given to the design of the assay: this includes the configuration of the assay; the antibodies being used in assay; the enzyme label selected; and optimization of reagent concentrations and incubation times must be performed. Choosing the correct substrate or enzyme/substrate combination will also lead to optimal sensitivities. For example, the HRPO colorimetric substrate ABTS is less sensitive than TMB. Even higher sensitivities using the HRPO enzyme label can be obtained with fluorescent and luminescent substrates. Other techniques, which can amplify the available signal from the immunoassay can be introduced and some of them are discussed here.

a) **Non-competitive versus competitive assays.** Immunoassays are generally categorized into two groups, the competitive immunoassay (limited reagent) and the noncompetitive immunoassay (reagent excess) (Christopoulos and Diamandis, 1996a). In a competitive design, labeled and non labeled- analyte are mixed with a limited amount of immobilized anti-analyte antibody. After incubation, the signal from the bound or free fraction is measured and the concentration of the analyte is extrapolated. In a noncompetitive design, an excess of labeled-antibody is used. After incubation, the signal from the bound fraction is measured and the concentration of the analyte is related to the signal. It has already been determined theoretically that noncompetitive assays offer higher sensitivities than competitive ones (Jackson and Ekins, 1986). Two-site noncompetitive (sandwich type) immunoassays also offer the benefit of two antibodies, which decreases the likelihood of cross-reactivity

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and increases the specificity of the immunoassay (Christopoulos and Diamandis, 1996a).

b) **Affinity of the antibody.** A second factor governing the ultimate sensitivity of the sandwich type immunoassay is the affinity constant of the antibody (Jackson and Ekins, 1986). The antibody pair should be selected for their high performance characteristics because overlapping epitopes will reduce sensitivity. The antibody with the higher affinity characteristics should be used for plate-coating (Porstmann and Kiessig, 1992). In general, an assay with moderate sensitivity (10^{-8} to 10^{-10} M of analyte) can be made from a matched antibody pair with affinities $>10^9 M^{-1}$. For ultrasensitive applications, antibody pairs with affinities $\geq 10^{10}$ should be employed (Diamandis et al., 1996).

c) **Enzymes.** Since the introduction of enzymes as alternatives to radioisotopes for signal generation in the early 1970s, enzyme immunoassay (EIA) methodology has flourished (Engvall and Perlman, 1971). In most assay designs, the enzyme acts by the chemical conversion of a substrate to a colorimetric, fluorometric, or chemiluminometric product (Gosling, 1996). The enzyme can also be used in coupled enzyme reaction schemes, or to generate more enzyme labels for signal generation (Fig 1.2). Because the detection limit of a sandwich type immunoassay is ultimately governed by the detection limit of the enzyme label, many efforts to improve the sensitivity focus on finding new enzyme labels, improving substrates or improving the ability to detect them (Kricka, 1994).

Of the varied enzyme labels, horseradish peroxidase (HRPO) and alkaline phosphatase (AP) continue to be the most popular. HRPO is used largely due to its better kinetics leading to faster assay development times, its suitability in conjugation procedures (e.g. its low molecular weight and

	Detection Limit, attomoles/assay		
Detection Method	COL	FL	CL
Enzyme			
Horseradish Peroxidase	25	1	0.1
Alkaline Phosphatase	50 0.011	0.5 0.1 1 molecule	0.1
β-D-Galactosidase	100	1 1 molecule	0.01
Glucose-6-phosphate dehydrogenase	-	-	1

Table 1.2: Enzymes used in immunoassay and their detection limits. Detection limits are categorized by enzyme and detection method. COL = Colorimetric. FL = Fluorimtetric. CL = Chemiluminescent. Detection limits are governing factors in but do not represent absolute sensitivities of immunoassays incorporating these enzymes. Other factors, such as antibody affinity and sample characteristics play a decisive role. *Adopted from Bronstein and Kricka.*(Bronstein and Kricka, 1989).

the "periodate" method of conjugation) and its detection limit (Gosling, 1996). A comparison of the detection limits of each enzyme label is shown in Table 1.2. Enzymes with high detectability and low non-specific binding should be considered when designing an ultrasensitive immunoassay.

d) **Assay optimization.** For all ultrasensitive applications, optimization of reagent concentrations and incubation time is of the utmost importance (Diamandis et al., 1996). Generally speaking, assays are optimized from the last step to the first step (Bayer and Wilchek, 1996). A standard optimization procedure involves comparing the signal (in duplicate or triplicate) to noise (no analyte) ratio at various antibody/enzyme label concentrations or incubation times. Because the antibody and analyte molecules are forming sandwich pairs (i.e. one-to-one binding), the optimal molar concentration of the labeled antibody should be based on the molar concentration of the analyte. Plate-coated antibodies are generally coated anywhere from 1-10 µg/mL (Engvall, 1980). It is important to bear in mind the nature of the substrate that is used. Colorimetric substrates often saturate after two magnitudes of concentration, so using them in an assay designed to have an LLD ≤ 0.01 ng/mL should require comparing the signals elicited from the assay at 1 ng/mL with background (0 ng/mL) (Khosravi et al., 1995). Fluorometric and chemiluminometric substrates will afford a larger concentration/response range and can be utilised accordingly (Diamandis, 1997).

Incubation times can vary from assay to assay. Antibodies may require incubation times anywhere from 1-3 h to reach saturation while a high affinity antibody may have no significant change in bound signal characteristics after 15 min (Kreutz and Suresh, 1997). Generally speaking, a 3 h incubation should ensure complete binding of the antibody to the analyte (Diamandis et al., 1996).

1.3.1.2 Substrate Detection:

a) Colorimetry. Substrates which convert to a chromogen in the presence of an enzyme label have distinct advantages in that they are (1) visible, (2) simple and relatively cheap, (3) stable after the colorimetric reaction is stopped (Porstmann and Kiessig, 1992). Not all colorimetric substrates are created equally, however. Because of differences in the enzymatic reaction rates and absorbance characteristics of each substrate, some have higher sensitivities than others. The proper choice of substrate is essential for assays requiring high sensitivities.

One of the most sensitive enzymatic detection systems for immunoassays is horseradish peroxidase (HRPO) combined with TMB (3,3',5,5'-tetramethylbenzidine) (Frey et al., 2000). Its detection limit is greater than that of either ABTS (2,2'azino-di(3-ethylbenzthiazoline)) or OPD (o-phenylenediamine) and its been shown to have a higher sensitivity than either alkaline phosphatase (AP) or β-D-Galactosidase (BGAL) under identical two-site EIA conditions (Porstmann and Kiessig, 1992). In the presence of H_2O_2 and HRPO, TMB is converted to an oxidized chromogenic dimer (Fig 1.3). which can be measured by an absorbance reading at 650 nm. Through the addition of a strong acid (pH < 1), the dimeric intermediate is converted to a yellow diamine form, which exhibits higher sensitivity and can be measured at 450nm

b) Fluorometry. Fluorometry is superior to colorimetry in terms of sensitivity and specificity (Christopoulos and Diamandis, 1996b). In general the sensitivity of fluorescence is 10-1000 times higher than colorimetry, although the practical sensitivity of the assay may only be increased by a factor of 2-10. (Porstmann and Kiessig, 1992). The potential sensitivity of fluorometric analysis has been typified by the record of single-molecule detection almost

exclusively being carried out with fluorometric compounds (Craig et al., 1995). The principle behind fluorescence is the measurement of the emission spectra of a fluorophore after excitation with a light source at a given wavelength. Typical fluorogenic substrates used in enzyme-linked fluorimmunoassay (ELFIA) procedures are usually
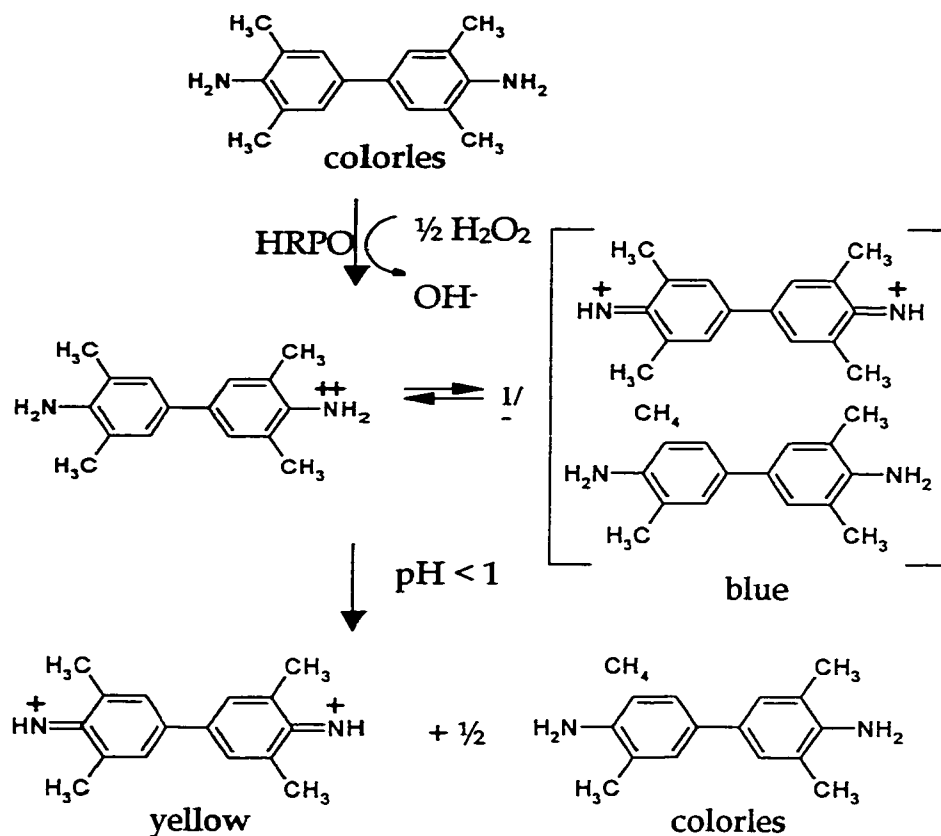
Figure 1.3: Horseradish peroxidase (HRPO) catalyzed oxidation of the 3,3'5,5'-tetramethylbenzidine (TMB) colorimetric substrate. HRPO and TMB in combination are one of the most sensitive detection methods available in colorimetric immunoassays.

fluorescein, rhodamine, or coumarin derivatives that are activated by enzymatic cleavage. p-Hydroxyphenyl derivatives have also been found to be excellent fluorogenic substrates for the detection of HRPO (Zaitsu and Ohkura, 1980). Fluorophores can also be used to label proteins directly but at the cost of lower sensitivities (Christopoulos and Diamandis, 1996b). Differences between fluorogenic substrates are outlined in Table 1.3. Higher sensitivity is usually determined by a larger difference between excitation and emission spectra, higher quantum yield, and high molecular extinction coefficients. Quantablu™ (3-(p-hydorxyphenyl)propionic acid, Pierce Chemical Company, Rockford, IL, USA), a fluorogenic substrate for HRPO detection, has been demonstrated to have a minimum detectable concentration of analyte 4.5-35 times lower than conventional TMB, OPD, or ABTS colorimetric-based assays (Pierce technical notes, 2000). One major limitation to fluorometry is that biological samples can have inherent fluorescence background (Christopoulos and Diamandis, 1996b). Time-resolved immunofluorometric assays (TRIFA), which use fluorophores that have long- lived excitation signals, overcome this limitation by allowing short-lived biological fluorescent signals to decay before measurement.

c) **Chemiluminescence.** Chemiluminescent labels were initially introduced as possible alternatives to radioisotopes in the 1970s (Kricka, 1996). Many chemiluminescent substrates are available for EIA. Often, the endpoints are more sensitive than the colorimetric or fluorometric alternatives (Kricka, 1996). The substrates used are often derivatives of cyclic diacyl hydrazides or acridinium esters. Because photon emission is often short-lived, the use of enhancers for "glow" type reactions is now commonplace. As with fluorometric enzyme substrates and their detection limits, the ultimate sensitivities of these new substrates are only one of the many factors of an optimized immunoassay.

Label type	Ex$_{max}$ (nm)	Em$_{max}$ (nm)	ε (LM^{-1}cm^{-1})	φ
Fluoresceins				
Fluorescein (FITC, DTAF)	492	516-525	72,000	0.3-0.85
Rhodamines				
TRITC G	535-545	570-580	107,000	
Coumarins				
4-MU	325	450	16,000	
Phycobiliproteins				
B-PE	545	575	2,410,000	0.59-0.58
C-PC	620	650	580,000	0.51
p-Hydroxyphenyls				
HPPA	320	425		

Table 1.3: Properties of various fluorogenic compounds. Substrates containing fluorescein, rhodamine, and coumarin derivates are generally activated by enzymatic cleavage. Phycobiliproteins can be chemically coupled to an antibody for direct detection. p-Hydroxyphenyl compounds are activated by HRPO only. These fluorogens exhibit lower detection limits than conventional colorimetri substrates. *Abbreviations used:* Ex$_{max}$ and Em$_{max}$, excitation and emission maxima; ε, molar extinction coefficient; φ, fluorescence yield; FITC, fluorescein isothiocyanate; DTAF, dichlorotriazinyl derivative of amino fluorescein; TRITC G, tetramethylrhodamine isothiocyanate, isomer G; 4-MU, 4-Methyl-Umbelliferone; B-PE, B-phycoerythrin; C-PC, C-phycocyanin; HPPA (3-(p-hydorxyphenyl)propionic acid.

The first chemiluminescent EIA used a horseradish peroxidase label acting on a cyclic diacylhydrazides (luminol) (Arakawa et al., 1979). The discovery of enhancers improved the sensitivity and utility of this assay by increasing light emission and reducing the assay reagent background. Acridinium esters have been used to calculate activity of peroxidase at a detection limit of 10^{-19} mol/L (Bronstein and Kricka, 1989). This same system has been applied in an immunoassay to detect human alphafetoprotein at a LLD of 1 pg/mL (Katsuragi et al., 2000).

The benefits of using chemiluminescent AP substrates versus colorimetric substrates has been demonstrated in several studies. In an immunoassay designed to measure concentrations of alphafetoprotein, a comparison of colorimetric and chemiluminescent substrates demonstrated a decrease in the detection limit from 2 to 0.03 ng/mL. The most sensitive substrates for AP are based on adamantyl 1,2-dioxetane aryl phosphates, such as disodium 3-(4-methoxyspirol[1,2-dioxetane-3,2'-tricyclo[3.3.1.13.7]decan-4-yl]phenylphosphate (AMPPD) and its 5-chloro substituted analogue(CSPD). The detection limit for the enzyme using this substrate is 1 zmol (10^{-21} mol) and light emission is long-lived.

1.3.1.3 Enzyme Amplification

a) **Polymers.** Homopolymers of enzyme-labels increase the relative amounts of enzyme available for signal generation for any given binding event. The signal generating substrate can then theoretically be catalyzed at a proportionally higher rate, which can lend to greater sensitivities. Commercially available poly[HRPO] and poly[AP] are joined by thioether linkages or by a dextran backbone. It is possible to increase the LLD 10 fold with this procedure (Kricka, 1994).

b) Coupled Enzyme Reactions. Coupled enzyme reactions usually harness the fast kinetics of NAD+ and FADP to recycle a chemical reaction which has a colorimetric or fluorometric endpoint (Kricka, 1994). Assay sensitivities using this technique can be very high, but limitations of the assay stem from expensive reagents and exacting assay conditions, which contribute to high interassay variability.

c) Catalyzed Reporter Deposition. This technique, which was originally described by Bobrow et al. (Bobrow et al., 1989) uses a biotin-tyramine substrate which reacts with HRPO to produce highly reactive biotin-tyramine free radicals. The free radicals then react with the enzyme label and surrounding protein. Because of the biotin tag, the addition of a biotin-binding streptavidin-HRPO label to the reaction well in essence amplifies the original HRPO label to many other enzyme (HRPO, or otherwise) labels. A recent solid phase enhancing technique allowed researchers to detect as little as 16 molecules of analyte in a Western blot experiment (Bhattacharya et al., 1999). The enzyme label can then be detected by a colorimetric, fluorometric or chemiluminometric substrate.

d) Immunocomplexes and ImmunoPCR. Immunocomplexation reactions use anti-enzyme labeled antibodies to amplify the existing amount of enzyme label. By attaching multiple streptavidin-[HRPO]$_3$ to the anti-enzyme label antibody, a proportional increase in HRPO is available for substrate reaction. Addition of a second step increases complexation and available HRPO for substrate catalysis. Comparison of a control assay with a complexation reaction resulted in an 81-fold (one layer of complex) or a 729-fold (two layers of complex) increase in sensitivity (Wilson and Easterbrook-Smith, 1993). ImmunoPCR reactions involve using a nucleic acid tag as a label that can then be amplified by PCR techniques. A sandwich immunoassay has been developed which can detect as little as 580 molecules (Sano et al., 1992).

1.3.2. Reducing Background Noise

Background noise is ultimately the result of conditions leading to an inconsistent or inappropriate amount of enzyme label being bound to the reaction well. Factors that need to be considered are the binding properties of the antibodies, as well as blocking, washing, and dilution of reagents.

Antibodies and solid phase. For highly sensitive assays, the antibodies must have high affinities (> 10^9 mol/L) (Diamandis et al., 1996). The plate-coating concentration of the antibody must be optimized as well as the plate-coating conditions. Purity of the antibody is very important and no other proteins should be present in the solution. Once an optimal antibody concentration is determined, plate-coating should be done under identical conditions for each subsequent assay.

Blocking and washing. In order to avoid non-specific binding of antibodies and enzymes, appropriate consideration of blocking and washing reagents should be taken. Some blocking reagents may exhibit non-specific binding in one assay design but not for another. The best reagent may need to be found by trial and error (Porstmann and Kiessig, 1992). Non-fat dry milk and BSA both contain biotin that could potentially interfere with assay sensitivity especially when it is incorporated into an assay using the streptavidin/biotin labeling system (Diamandis and Christopoulos, 1991). Low background blocking reagents are commercially available. Washing should be extensive (i.e. up to five repeated washes) and addition of Tween 20 to the wash buffer (0.05-0.1%) should be considered to reduce non-specific binding of protein which will increase background noise (Diamandis et al., 1996).

Dilution: Dilution of all reagents, with the exception of the plate-coating reagents, should be done in a protein solution. For most immunoassay procedures, 1.% BSA should be used. Addition of mouse serum to mouse antibody solutions can reduce non-specific binding but may introduce other confounding tfactors in an ultrasensitive assay. For ultrasensitive applications, the use of a 6% BSA solution or other physiological concentration of protein should be considered (Diamandis et al., 1996).

1.4   Ultrasensitivity and PSA

The use of a PSA test in the followup of patients undergoing radical prostatectomy was first proposed and evaluated by Oesterling and colleagues in 1988 (Oesterling et al., 1988). It was recognized that the PSA found in human serum was produced in the prostate gland (Papsidero et al., 1980). This fact implied that recurrence of pCa could be directly correlated with the presence of PSA. Oesterling et al. concluded that PSA was "a sensitive biochemical marker to detect recurrent prostatic cancer and it may be the most effective tool to follow patients after radical prostatectomy" (Oesterling et al., 1988). Further studies confirmed this assertion. Lange was able to predict recurrence in 59 out of 59 patients · who demonstrated levels of PSA > 0.4 ng/mL after a 3 to 6 month followup (Hudson et al., 1989; Lange et al., 1989) using a Hybritech Tandem-R immunoradiometric assay (Hybritech, La Jolla, CA, USA).

It was then suggested that an assay design with a lower BDL would have a greater positive predicitive value in predicting recurrence of pCa (Lange et al., 1989; Vessella et al., 1992). Interestingly, and contrary to predictions, evaluation of a more sensitive assay (Abbott IMx , Abbott Park, IL, USA: LLD=0.03 ng/mL, BDL=0.06ng/mL) did not seem to offer greater clinical sensitivity (Vessella et al., 1992). The assay employed a monoclonal antibody coated on microparticles to capture PSA, followed by addition of serum sample and an alkaline

phosphatase labeled-polyclonal antibody. The detection was done by conversion of the 4-methylumbelliferyl-phosphate substrate to (4-MUF-P) to the 4-MUF fluorogen. A retrospective study of 5 of 38 long-term cancer survivors after radical prostatectomy were shown to have levels of PSA exceeding 0.06 ng/mL but not greater than 0.1ng /mL. The authors concluded that PSA could be produced by benign prostatic tissue left behind from the operation, or from an extraprostatic source (Vessella et al., 1992; Takayama et al., 1993). Cross reactivity with PSA-like analytes like hK2 by polyclonal antibodies was not addressed, but could explain the reported detection of PSA. Furthermore, some patients who had levels below the BDL of 0.06 ng/mL progressed to a re-emergence of the disease. PSA levels did increase to > 0.1 ng/mL after re-emergence was detected with a bone scan. Because of its clinical significance, this 0.1 ng/mL level of PSA was arbitrarily defined as an upper limit for all subsequent ultrasensitive PSA assays (Graves, 1992; Vessella et al., 1992). This was a substantial decrease from the 4 ng/mL threshold level normally needed in assays to screen patients for pCa.

Other ultrasensitive assay designs were developed. A modification of the commercially available Yang Pros-Check radioimmunoassay was reported to have a BDL of 0.07 ng/mL[1] (Stamey et al., 1993). Based on these results, Stamey *et al.* concluded that an assay with a BDL of ≥ 0.1 ng/mL was able to detect cancer reccurence 310 days (mean) earlier than that of an assay with a BDL of ≥ 0.3 ng/mL (Stamey et al., 1993). Yu and Diamandis employed a monoclonal→PSA→polyclonal-AP sandwich design and time-resolved immunofluorometry using a terbium chelate to develop an assay with a BDL of 0.01 ng/mL (Yu and Diamandis, 1993). Using this data along with an analysis of

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[1] A confounding and short lived variable called the residual cancer detection limit (RCDL) was proposed in this analysis. It was defined as the mean + 3SDs of PSA levels in the sera of a statistically significant number of patients who are long term survivors after radical prostatectomy. It was supposed to reflect a biological LLD for the clinical laboratory and consequently was confused with the BDL on more than one occasion. References to RCDL are purposefully avoided in this thesis.

tumour-doubling times, Yu established prostate cancer relapse could be diagnosed 420 days earlier with this assay than with assays having a LLD of 0.1 ng/mL (Yu and Diamandis, 1993).

Subsequent to this, Khosravi and colleagues developed a polyclonal→PSA→monoclonal-HRP sandwich design using a colorimetric TMB substrate to develop an assay with a LLD of 0.003 ng/mL and a BDL of 0.009 ng/mL (Khosravi et al., 1995). A modified Hybritech Tandem-E (Hybritech, La Jolla, CA, USA) assay using matched monoclonal antibodies (monoclonal→PSA→monoclonal-AP) and LumiPhos 480 substrate (Lumigen, Southfield, MI: Formula: 4-methoxy-4-(3-phosphatephenyl) spiro [1,2-dioxetane-3,2'-adamantane])) reported a BDL of 0.008 ng/mL (Ellis et al., 1997). Using an AP-labeled bsMab-based sandwich assay and a substrate amplification system, Xu was able to develop an assay with a reported LLD of 0.25 pg/mL (Xu, Thesis, 1997) . The limitations of the practical use of this assay, however, stem from high interassay variability, exacting reagent concentrations and the prohibitive cost of the reagents. Then, Yu *et al.* modified their original immunofluorometric assay to include matched monoclonals (monoclonal→PSA→moncolonal-AP) in both fluorometric and chemiluminescent systems (Yu et al., 1997). The reported BDLs for these assays were 0.002 ng/mL and 0.004 ng/mL respectively. Using these and other assays, it was discovered that residual levels of PSA were measured in men who had prostate removal and were disease free (long-term). Levels were also consistently detected in women. Because of the possibility of cross-reactivity with other biological antigens, the existence and source of this PSA is still being debated.

Although ultrasensitive assays for PSA definitively addressed the issue of early detection of recurrent disease, the question of prognosis remained. Could this early detection improve patient outcomes or did it predict inevitable

failure? A report by van Iersel *et al.* concluded that "a PSA value > 0.04 ng/mL after radical prostatectomy... provides more leadtime...but there is no clear evidence that this gain is necessarily of benefit to the patient." (van Iersel et al., 1996). Schild *et al.* addressed the issue by irradiating patients who demonstrated PSA levels >0.3 ng/mL post-operatively. They concluded "isolated elevations of PSA following prostatectomy reflected residual disease" and were able to improve freedom from failure rates in 50% of patients treated (Schild et al., 1996). Other research groups have debated whether ultrasensitive PSA testing is of any benefit (Haab et al., 1995; Junker et al., 1999) but small patient samples and differences in treatment methodologies have continued the debate (Diamandis, 1997).

1.5 Hypothesis and Objectives

The central focus of this thesis is PSA, a tumor marker that has shown great utility in predicting both the occurrence and recurrence of prostate cancer. Prostate cancer continues to be the most commonly diagnosed cancer and the second highest cause of cancer death in North American men. As the population ages, the number of deaths in Canada are estimated to climb from 3,424 in 1991 to 7,800 by the year 2016 (Morrison et al., 1995). The use of the PSA test, then, will continue to be as important as surgical and radiological techniques in years to come. The intent of this thesis was to develop an ultrasensitive PSA test using bsMabs that could be useful in the management of prostate cancer after prostate removal. I specifically wanted to investigate the use of bispecific monoclonal antibodies in such a test, to determine if these unique immunoprobes would serve as functional tools for the clinical chemist. To accomplish this, I first had to obtain a source of PSA for quantitative comparison. In Chapter 2, I describe a modified method of a published protocol for the purification of PSA that is performed under mild conditions. The second part of this thesis was concerned with purifying the antibody reagents, including the labeled bispecific antibody

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reagents, which present a unique challenge in protein purification. Chapter 3 describes the exploration of a completely new technique for accomplishing this goal. Finally, an exploration of PSA tests using monoclonal and bispecific antibodies is described in Chapter 4.

Based on these objectives, I hypothesized:

1) PSA could be purified in bulk amounts using affinity methods including a Cibacron blue F3G-A agarose column and a bacitracin-agarose column as well as standard size exclusion and ion exchange techniques.

2) A previously published PSA purification method could be modified by altering the buffer conditions and adding a linear gradient elution to improve the initial yield of PSA when compared with a standard elution.

3) Bispecific antibodies, which present a unique challenge in protein purification could be purified by the use of a benzhydroxamic-acid agarose (BHA) column. The affinity of this matrix for the active site of HRPO would provide a unique method for the single-mode purification of HRPO-labeled bispecifics.

4) The BHA column could also be used to purify monoclonal and polyclonal antibodies which are HRPO-labeled by chemical cross linking.

5) A previously characterized standard sandwich assay for PSA using the P57 bispecific antibody, could be modified to measure PSA in an ultrasensitive range (between 0.001 and 0.1 ng/mL). This would be accomplished by altering parameters that contribute to sensitivity including substrate conditions, incubation times and coating/blocking conditions.

6) Bispecific antibodies could be utilized as key immunoprobes in PSA ultrasensitive assays. Because of their unique one-to-one labeling and batch-to-batch consistency, they could perform comparable to or better than a standard sandwich assay using chemically labeled monoclonal antibodies.

Chapter 2: Bulk Prostate-Specific Antigen Purification Techniques

2.1 INTRODUCTION

In order to develop an immunoassay for the quantitative estimation of PSA concentration in human serum, standards for comparison in an ELISA were required. Currently, PSA standards are available from very few sources at a prohibitive cost. Because of this, a reliable and reproducible method was sought out to purify PSA in bulk amounts.

PSA is most easily purified from seminal plasma, where it is found in concentrations between 0.5 and 5 mg/L (Tessmer et al., 1995). A number of methods have previously been described to purify PSA from this source (Sensabaugh, 1978; Wang et al., 1982; Schaller et al., 1987; Rusciano et al., 1988b; Graves et al., 1990; Sensabaugh and Blake, 1990; Tessmer et al., 1995; Zhang et al., 1995). All of them include ammonium sulfate precipitation, ion exchange, and/or size exclusion. Previously, a method for purification using an immunoaffinity column (agarose-bound PSA Mabs) had been used in our lab but subjected the PSA protein to harsh elution conditions (Jette et al., 1996). Because of this, the column was neither robust nor reliable. This chapter describes a modification of a method by Rusciano and colleagues which is robust, reliable, and economical for the bulk purification of PSA.

2.2 MATERIALS AND METHODS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 10 %) was performed according to previously published (LaemmLi, 1970) using a Bio-Rad Mini-protein II dual slab cell along with prestained broad range

molecular weight markers (New England Biolabs, Inc. , MA, USA or Biorad Labs, Richmond, CA, USA). Bands were revealed using Coomassie blue.

2.2.1 Mabs and cell Lines

B87.1 and B80.3 are a matched anti-PSA monoclonal antibody pair that have been described previously (Jette et al., 1996; Kreutz and Suresh, 1997; Xu et al., 1998) and together, B87.1 and B80.3 can form a heterosandwich with PSA either in its free form or when complexed with α-1-antichymotrypsin (ACT) (Kreutz and Suresh, 1997). Each monoclonal antibody binds to a unique epitope of PSA (Jette et al., 1996). B87.1 and B80.3 were generously donated by Biomira, Inc.

P57 is a well characterized, bsMab secreting, quadroma developed from the B80.3 (anti-PSA) and YP4 (anti-HRPO) parental cell lines in our lab previously using cell fusion techniques (Kreutz and Suresh, 1997). This P57 quadroma was purified using a thiophilic gel (T-Gel™, Pierce, Rockford, IL, USA) gradient elution technique that is discussed in this thesis.

2.2.2 Preparation of human seminal plasma.

Pooled ejaculates from healthy donors (courtesy of Dr. Tony Szott, Fertlility Clinic, Royal Alexandria Hospital, Edmonton, AB, Canada) were allowed to liquefy and centrifuged for 10 min at 600 x g at RT to remove the cellular fraction, then dialyzed for 24 hrs against a buffer. For Cibacron Blue F3G-A affinity chromatography, 200 volumes of 50 mmol/L citrate-phosphate buffer, pH 6.4 with 0.75 M NaCl (citrate-PO_4/NaCl), with one change of buffer was used. For bacitracin agarose, 200 volumes of 0.1 M ammonium acetate buffer with 10 mM Ca^{2+}, with one change of buffer was used. The dialysate was then centrifuged for 30 min at 15,000 x g to remove any particulate matter. This was then stored at –20°C until needed.

2.2.3 Assays

The activity of HRPO was determined by measuring the oxidized colorimetric product of 3, 3', 5, 5' – tetramethylbenzidine (TMB) at 650 nm or, after stopping the reaction with 1M H_3PO_4, at 450 nm using a commercially available substrate (K&P, Gaithersburg, MD, USA).

BsMab sandwich ELISA to detect the presence of PSA in purified fractions (Qualitative). To monitor for the presence of PSA in the chromatographic fractions, 100μL of PBS containing B87.1 (1 μg / mL) was added to each well of a 96-well microtiter plate and incubated overnight (16 h) at 4°C. The plate was subsequently washed 3 times with 0.05% PBS-Tween 20 (PBS-T) and 200μL of PBS containing BSA (1%) was added to each well to block non-specific binding sites for 1 h at 37°C. It was then washed as before and either used immediately or stored at 4°C for a maximum of 2 weeks. To each well, 10 μL of the fractions were added to 90 μL of PBS. The plate was put on the shaker for 1 h at RT. The plate was again washed 3 times with PBS-T and 50 μL of T-gel purified P57 (diluted 1:10 in PBS-BSA 1%) was added to each well for 30 minutes on a shaker at RT. The plate was then washed with PBS-T and 100 μL of PBS containing 1% BSA and 5 μg / mL HRPO was added to label the P57 antibody. After a 30 minute incubation on a shaker at RT, the plate was washed 3 times with PBS-T and developed with the commercially available TMB substrate (Figure 2.1).

substrate   *signal

substrate   *signal

HRPO

HRPO

SOLID PHASE   SOLID PHASE

Direct Binding   **Bridge**
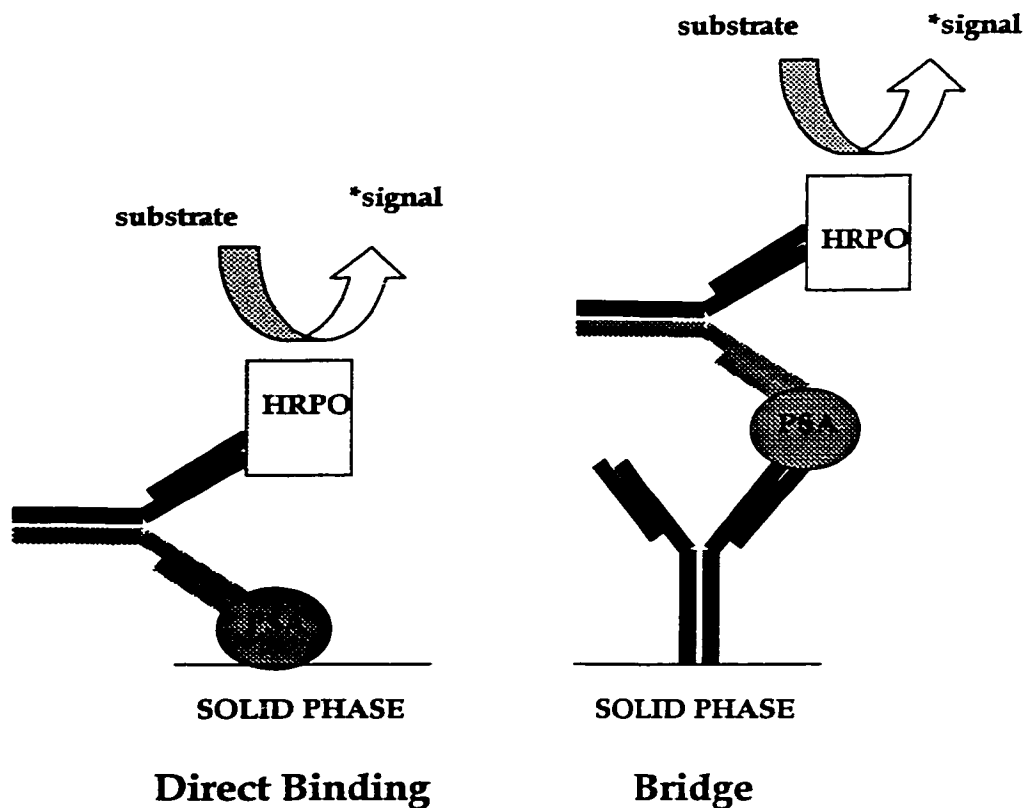
Figure 2.1: Configurations of two immunoassays for the detection of PSA. A less-sensitive, direct binding assay (on left) was utilized when too much PSA in the pooled fractions led to observed signal in all fractions. In the bridge assay, the solid phase 96 well microtiter plate is coated previously with an anti-PSA Mab (B87.1) and blocked before addition of the fractions of interest. In the direct-binding assay, fractions are applied directly to the solid-phase and blocked before detection with the HRPO-labeled P57 bsMab.

Direct binding ELISA: If the bridge assay was too sensitive (i.e. relative amounts of PSA could not be distinguished), a less sensitive direct assay was performed by putting 10 µL of each fraction directly onto a plate overnight at 4°C and repeating the procedure while omitting the B87.1 coating step. Briefly, the P57 bsMab and HRPO were incubated and formed an immunocomplex with PSA in the purified fractions, which could be detected (Figure 2.1).

Quantitative ELISA of PSA. To quantitate PSA, the sandwich assay was performed as described above using pooled fraction samples diluted to an optimal concentration range (0-100 ng/mL) in duplicate or triplicate for testing. PSA standards (Hybritech, Inc., San Diego, CA, USA) were added in triplicate on the same plate and concentrations of PSA were estimated from a comparison of the O.D. readings to a standard curve.

2.2.4 Protein concentration

Protein concentrating was accomplished using a sucrose method. Fractions were collected and placed in dialysis tubing (MW cutoff 8,000) which had been soaked in water and tied or clipped at both ends. The tubing was then placed in a beaker and surrounded with sucrose in order to draw solvent from the tubing osmotically. This was done overnight at 4°C.

2.2.5 Chromatography

Bacitracin A Agarose (UFC Bacitracin Agarose) was obtained from UpFront Chromatography (UpFront Chromatography, Copenhagen, Denmark). Cibacron blue F3G-A (Affi-Gel® Blue gel) was obtained from Bio-Rad Labs (Bio-Rad Labs, Richmond, CA, USA). Sephadex G-50 (fine), Sephadex G-75 (superfine), Sephadex G-100 (fine), and DEAE-Sephacel were all obtained from Amersham Pharmacia Biotech (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, CANADA).

2.2.5.1 UFC Bacitracin Agarose Serine Protease Affinity

Chromatography with the bacitracin A agarose column was first accomplished by loading 10 mL of the centrifuged dialysate onto 10 mL of UFC bacitracin agarose previously packed into a 1.25 x 15 cm column and equilibrated previously with a 0.1 M ammonium acetate buffer with 10 mM Ca^{2+}. The column flow rate was set to 0.5 mL/min at $4^\circ C$ and was washed until no significant absorbance at A_{280} was seen (approx 4 h). An elution buffer which consisted of ammonium acetate 0.1 M with 10 mM Ca^{2+} plus 1 M NaCl and 20% isopropanol was then added to elute the protease. The elution fractions were then analyzed using SDS-PAGE and ELISA. The structure of bacitracin A is seen in Figure 2.2.

2.2.5.2 Cibacron blue F3G-A dye affinity chromatography
a) Batch wise elution

Purification of PSA from seminal plasma on Cibacron Blue F3G-A was initially purified without an elution gradient. Twenty mL of the centrifuged dialysate was loaded onto 90 mL of Cibacron blue F3G-A (see Figure 2.3) packed in a column (2.5 cm x 25 cm) previously equilibrated with citrate-PO_4/NaCl, pH 6.4. The column flow rate was 0.65 mL/min (39 mL/h). The column was then washed with a minimum of 300 mL of the equilibrium buffer at which point no significant absorbance at 280nm could be detected. The column was then eluted with citrate-PO_4/NaCl with 0.5 M KSCN added and 4.0 mL fractions were collected. Fractions were then analyzed for purity using SDS-PAGE. This and all subsequent purifications were performed at RT.
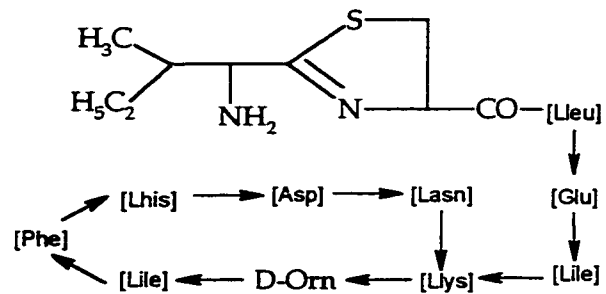
Figure 2.2: Structure of bacitracin A. Bacitracin A agarose
(UFC Bacitracin Agarose, UpFront Chromatography,
Copenhagen, Denmark) has been used as an affinity matrix
for the purification of various proteases, including serine
proteases.

R₁ = H or SO₂ONa
R₂ = H or SO₂ONa
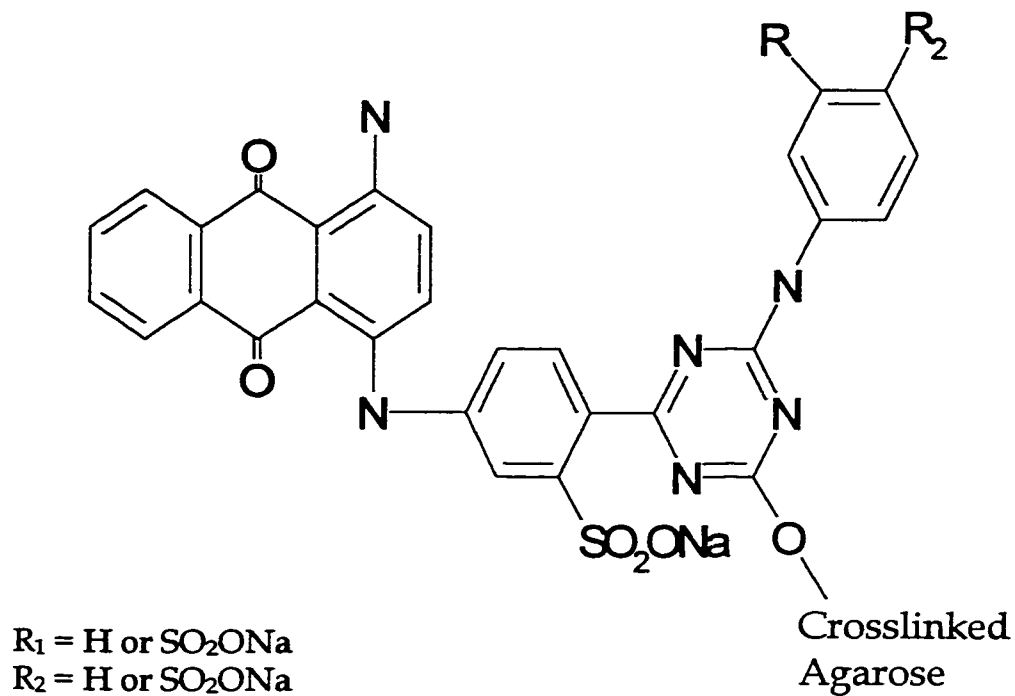
Figure 2.3: Structure of Cibacron blue F3GA. Cibacron blue F3G-A (Affi-Gel® Blue gel, Biorad Labs, Richmond CA, USA) is a covalently coupled Reactive blue 2 sulfonated dye ligand which purifies protein by ion exchange, hydrophobic, and affinity binding mechanisms. PSA has been shown to bind tightly to this matrix and can be eluted with KSCN.

b) Gradient elution

Purification of PSA from seminal plasma on Cibacron blue F3G-A gel was subsequently performed using an elution gradient. Twenty mL of the centrifuged dialysate was loaded onto 90 mL of Cibacron blue F3G-A was packed in a column (2.5 cm x 25 cm) previously equilibrated with citrate-PO_4/NaCl, pH 6.4. The column flow rate was 0.65 mL/min (39 mL/h). The column was then washed with at least 300 mL of the equilibrium buffer at which point no significant absorbance at 280nm could be detected. The column was then subjected to a linear gradient elution of 0 to 1 M KSCN using citrate-PO_4/NaCl. This purification was highly reliable and repeatable and eventually automated using the FPLC system (Pharmacia LKB Biotechnology AB) and FPLCmanager. Both unbound and eluted fractions were then analyzed using SDS-PAGE and/or ELISA. The programmed protocol is in Table 2.1.

2.2.5.3   Size exclusion methods.

Purification of PSA using a Sephadex G-50 (fine) column. Fractions after Cibacron blue F3G-A gel purification (using a gradient elution) determined by SDS-PAGE to have the highest concentrations of PSA were pooled and concentrated overnight at 4°C using sucrose. Sephadex G-50 (fine) (20 g) was swelled overnight in citrate-phosphate buffer, pH 6.4 and packed in a column (2.5 x 110 cm) yielding a 200 mL bed volume. The column was then equilibrated with 600 mL (3 volumes) of the same citrate-phosphate buffer. From the 3 mL of the pooled concentrated PSA, 1 mL was carefully applied to the top of the column. The column flow rate was 0.3 mL/min (18 mL/h). Fractions were collected at 7 minute intervals and the absorbance of protein at 280 nm was recorded. Peak fractions were then analyzed for purity using SDS-PAGE and/or ELISA.

Time	Function	Setting	Comment
0.00	CONC%B	0.0	Start with 100 % equilibrium buffer.
0.00	ML/MIN	0.65	Set column flow rate to 0.65 mL /min
0.00	CM/ML	0.03	Set chart recorder at 0.03 cm / mL
0.00	VALVE.POS	4.1	Direct equilibrium buffer to pumps
0.00	VALVE.POS	2.3	Redirect pumped buffer to column
0.00	VALVE.POS	3.3	Allow flow from column.
0.00	VALVE.POS	5.1	Direct column output to fraction collector/wash.
0.00	VALVE.POS	1.1	Main valve set to "load"
200.0	VALVE.POS	1.2	Main valve set to "inject sample"
220.0	VALVE.POS	1.1	Finish injection. Reset to "load"
528.0	PORT.SET	6.1	Start fraction collector.
528.0	CONC%B	0.0	Start gradient elution.
750.0	CONC%B	100.0	Finish gradient elution.
770.0	CONC%B	100.0	Allow 20 additional mL of elution buffer.
771.0	PORT.SET	6.0	Stop fraction collecting.
771.0	ML/MIN	0.02	Stop fast flow (zero value not allowed).
971.0	VALVE.POS	1.3	Redirect flow from column.
Fraction collector (FRAC-100) set at 10 minute samples.			

Table 2.1: Programmed protocol on FPLCManager for gradient elution purification of PSA on Affi-Gel-Blue gel. This LC control protocol was programmed for batch-to-batch reproducibility and reliability when producing bulk samples of PSA. Optimal volumes/times were pre-determined. The protocol was saved as *'Affi-Gel'* in the *'Suresh1'* databank in FPLCManager.

Purification of PSA using Sephadex G-75 (superfine) and Sephadex G-100 (fine) columns. The Sephadex G-50 protocol was used for the remaining 2 mL of pooled, concentrated, semi-purified PSA using both Sephadex G-75 (superfine) and Sephadex G-100 (fine) columns for a comparative evaluation. Each of these matrices were swelled to achieve a final 200 mL bed volume. A modification in flow rate (0.15 mL/min) was necessary for the Sephadex G-75 (superfine) matrix. Fraction collection time was doubled to 14 minutes to compensate for this flow rate difference. A comparison of the properties of these size exclusion matrices can be seen in Table 2.2.

2.2.5.4   Ion exchange methods

Purification of PSA using a DEAE-Sephacel ion exchange column. Fractions containing PSA were dialysed against 20 volumes Tris-HCl 0.01 M buffer, pH 7.8 for 48 hr with 5 changes of buffer. DEAE-Sephacel was allowed to swell in the same buffer overnight and 75 mL of the matrix was packed into a column (2.5 x 15 cm). The column was then cleaned with 75 mL of Tris-HCl 0.01 M, pH 7.8 with 1 M NaCl as per the manufacturer's instructions. The column flow rate was set to 0.4 mL/ min and 3 column volumes of Tris-HCl 0.01 M buffer, pH 7.8 were passed through the column to equilibrate it. Ten mL of a pooled sample of Cibacron blue F3G-A gel/Sephadex-purified PSA was loaded onto the column. Another three column volumes were then passed through the

Gel	Working pH Range	Particle size range wet bead (μm)	Bed Volume (mL/g) dry Sephadex	Fractionation range (Da) for Globular Proteins	Exclusion Limit (Da)	Approx. max. flow rate – (mL/ min)
Sephadex G-50 (fine)	2-10	40-160	9-11	$1.5 \times 10^3 - 3 \times 10^4$	3×10^4	D*
Sephadex G-75 (superfine)	2-10	23-92	12-15	$1 \times 10^3 - 5 \times 10^4$	5×10^4	1.5
Sephadex G-100 (fine)	2-10	103-311	15-20	$1 \times 10^3 - 1 \times 10^5$	1×10^5	4.2

Table 2.2: Properties of three size exclusion matrices used in the purification of PSA. Size exclusion was used to purify higher molecular weight contaminants including albumin after purification on the Cibacron blue F3G-A dye column. Each matrix was made up to a 200 mL bed volume and 1 mL of concentrated pooled eluent was applied in parallel experiments to determine which was optimal for separation. D* = Darcy's Law: The flow rate is proportional to the pressure drop over the bed, inveresely proportional to the bed height, and virtually independent of the column diameter.

column during a wash step before a linear gradient elution from 0 to 0.15 M NaCl in 0.01M pH 7.8 Tris-HCl was started. The gradient elution was accomplished using two P-500 high precision pumps and a mixer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Fractions were collected every ten minutes and the absorbance of protein at 280 nm was recorded. The fractions were then analysed by SDS-PAGE and ELISA for the presence of PSA.

2.2.6 Enzyme Activity

Assay to determine PSA chymotrypsin-like activity. S-2686 Chymotrypsin chromogenic substrate (Helena Laboratories, Mississauga, OT, Canada) was used to determine the enzymatic activity of PSA after each purification step. The structure is shown in Figure 2.4. A working solution (600 µg/mL H_2O) of this chromogenic peptide was made and 100 µL was introduced to the wells of a microtiter plate. To this, approximately 1 µg of PSA (as previously determined by quantitative ELISA) was added to each well at RT. An optical density reading at 405 nm was taken after an overnight incubation to determine enzymatic activity.

2.3   RESULTS AND DISCUSSION

Overall purification scheme. The ability to purify PSA to a high degree of purity is important when the protein is required for labelling, protein characterization, or other applications where the quantity of pure protein must be known. A method was sought which could purify bulk amounts of PSA while reducing overall cost and time and which was accessible to researchers internationally. Previously LnCaP cells were used in our lab as a source material for PSA purification (Kreutz and Suresh, 1997). For the purification scheme outlined here, human seminal fluid was chosen as a starting material because it is a richer source of PSA (Rusciano et al., 1988b).
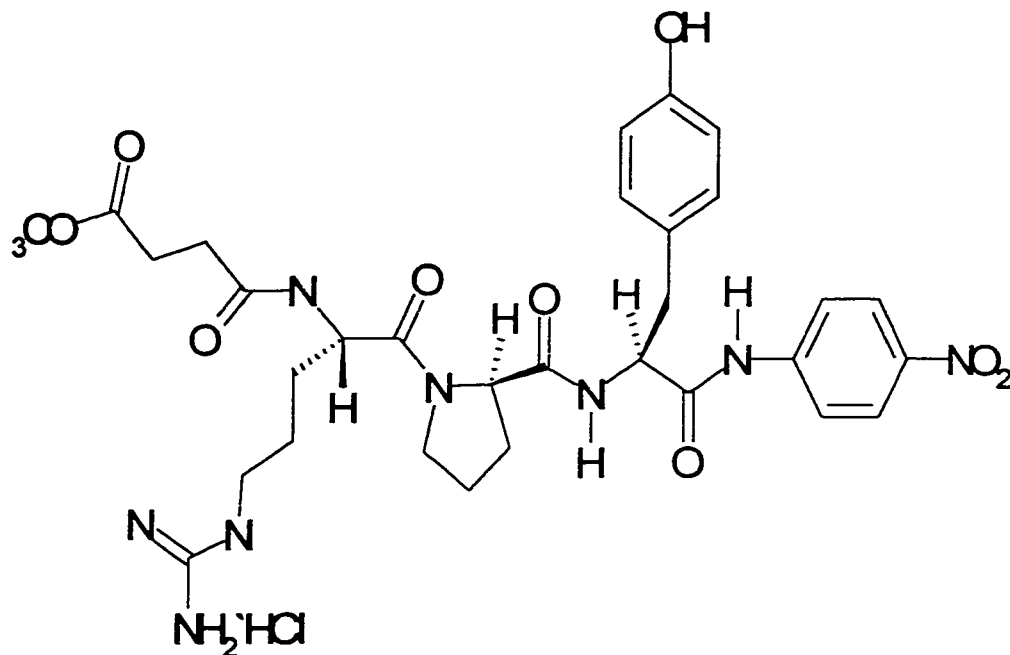
Formula: MeO-Suc-Arg-Pro-Tyr-pNA•HCl

Figure 2.4: Structure of S-2686 Chymotrypsin Chromogenic Substrate. PSA exhibits chymotrypsin-like enzymatic activity which will cleave the carboxyl side of basic residues and free the p-nitroanilide chromogenic moiety from the substrate. Enzymatic avtivity can then be measured using a colorimetric plate reader set at 405nm.

Due to the excellent recovery and yield characteristics of the Cibacron Blue F3G-A column, it was decided that this would make a practical first step. The overall time of this scheme developed in this thesis is relatively short (~ 2 weeks). A time line is shown in Figure 2.5. Purification schemes previously have taken anywhere from 1 to 3 weeks, whereas this scheme will optimally take two. A comparison of percentage recovery with other methods does not reveal any distinct advantages or disadvantages of this purification method (Table 2.3). Instead, this scheme emphasizes economy and reliability when the bulk purification of PSA is required.

2.3.1 UFC Bacitracin Agarose Serine Protease Affinity

It was hypothesized that bacitracin-agarose, a known serine-protease binder, could purify PSA from human seminal plasma. However, attempts to develop an affinity method for serine protease purification were not succesful. PSA did not appear to bind to the matrix under the conditions used and even small amounts of PSA were not detected in the eluted fractions by sensitive ELISA methods. Due to their preliminary nature, these data are not shown. However, given the previously published reports on affinity methods for serine proteases, this could potentially be an area for future research.

2.3.2 Cibacron blue F3G-A dye affinity chromatography

a) Batch wise elution

Purification of PSA from seminal plasma on Cibacron blue F3G-A gel was performed without an elution gradient. The original purification scheme by Rusciano et al. demonstrated the concomitant purification of PAP from PSA using two separate purification modes (Rusciano et al., 1988b). PAP was purified via a negative mode affinity method while PSA was purified by a positive mode affinity method. Cibacron blue F3G-A functions as an ionic,

WEEK	DAY				
	1	*2*	*3*	*4*	*5*
I	*Seminal Plasma Dialysis*	*Begin Cibacron Blue Column*	▨▨▨		*Begin Gel Filtration for weekend.*
II	▨▨▨		*Begin Sephacel Purificatio n*	▨▨▨	

▨▨▨ = Symbolizes *collecting fractions, analyzing* relevant fractions under SDS-PAGE, *determining purity* in each fraction, pooling those with high relative concentration of PSA and *dialysing* or *concentrating* those fractions if necessary.

Figure 2.5. Timeline for the purification of PSA.
Purification of PSA to 98% purity can be achieved using three matrices over a two-week period. After each step, the relevant fractions are assessed for purity by SDS-PAGE, then pooled and dialyzed/concentrated.

	Total Volume (mL)	Total Protein (mg)	Total PSA (mg)	Fold Purity	Recovery (%)
Seminal Plasma	70	447.1	35.91	1	100
Cibacron blue F3G-A	80	104.04	19.86	2.38	55.30
Sephadex G-100	53.5	6.76	5.82	10.72	16.20
DEAE-Sephacel	105*	5.05	4.95	12.20	13.78
* Only 1/5th of this volume was actually applied to DEAE column. Calculations have been extrapolated.					

Table 2.3: Purification table for the purification of PSA. PSA was measured by a quantitative bsMab sandwich assay described previously (Kreutz and Suresh, 1997). PSA was purified to essentially 98% purity by using three columns sequentially.

hydrophobic, aromatic, or sterically active binding matrix in various applications with binding or releasing proteins with a high degree of specificity depending on buffer conditions. PAP was shown to pass through the column initially using the citrate-PO$_4$, pH 6.4 equilibrium buffer and NaCl was added to dissociate all but the most tightly bound proteins from the matrix. The final elution step, using the strongly chaotropic KSCN buffer, removed PSA from the Cibacron blue F3G-A column. Because PSA was the only protein of interest in my purification scheme, a modification of the original scheme included the addition of the milder NaCl eluent into the original equilibrium buffer, allowing all but PSA and other, tightly bound proteins to pass through the column before a final elution with KSCN. A large amount of protein when measured by absorbance at 280nm can be seen in several peaks in the unbound fractions and a sharp peak is seen after elution (Figure 2.6). When each fraction was subjected to an ELISA, a majority of PSA was seen in both the largest unbound peak and the eluted peak (Figure 2.6). Observation of PSA in this peak is consistent with the original published purification (Rusciano et al., 1988b). A gel electrophoresis under reducing conditions (data not shown) revealed the presence of two major protein bands in the bound fraction at ~33,000 Da and ~70,000 Da with three additional bands at the >80,000 Da range. The 33,000 Da protein corresponds with the same 33,000 Da protein in the electrophoretic profile of human seminal plasma, and together with the ELISA data can be inferred to be PSA. To support this even further, the electrophoretic profile of semipurified PSA has identity with that of the published profile by Rusciano et al. (Rusciano et al., 1988b). Some additional material can be seen as a "fuzzy" band in the 22,000-27,000 Da and low molecular weight (<10,000 Da) ranges. Based on previous reports, the 22,000-27,000 Da protein can be inferred to be the "nicked" forms of PSA (Zhang et al., 1995). Based on the original description of human
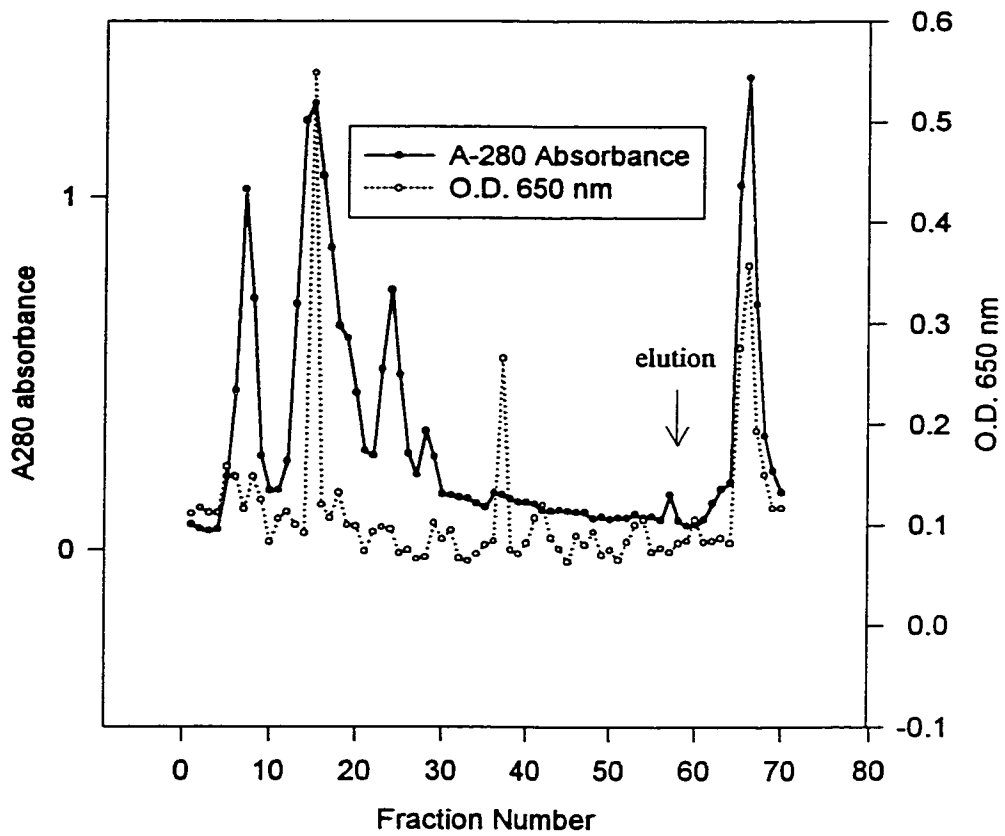
Figure 2.6: Purification of PSA on a Cibacron blue F3G-A gel column without gradient elution. The graph shows the A_{280} absorbance and ELISA data for Cibacron blue F3G-A purification without gradient elution. PSA was eluted using KSCN 0.5M added to the original equilibrium buffer.

seminal plasma under SDS-PAGE conditions by Sensabaugh, and the ability of Cibacron Blue F3GA to tightly bind both PSA and albumin, the ~70,000 Da protein could reasonably be concluded to be albumin (67,000 Da) (Sensabaugh, 1978; Rusciano et al., 1988b). The major presence of this larger molecular weight albumin contaminant was the basis of attempting a gradient elution and using a subsequent size exclusion column during the next step of purification.

b) Gradient elution

Purification of PSA from seminal plasma on Cibacron blue F3G-A gel was subsequently attempted using an elution gradient. Possible differences in the affinity of the Cibacron blue F3GA matrix for PSA and other contaminating proteins such as albumin were the basis of the rationale for attempting elution of PSA using a linear gradient elution. Gradient elution can exploit subtle differences in binding affinity by first eluting less tightly bound proteins at lower ionic strengths from the matrix. When comparing the absorbance profiles of the gradient vs. non-gradient techniques no significant differences can be seen (Figures 2.6 and 2.7). Although the unbound fraction profile looks different in comparison, this profile was consistently non-uniform between batches. When these fractions were subjected to SDS-PAGE analysis, PSA can be seen to be eluted about halfway through the elution gradient (~0.5M KSCN) and approximately at the center of the peak (Figure 2.8). These pooled fractions still harboured enzymatic activity. Of the three > 80,000 Da bands that existed in the non-elution gradient scheme, only one (80,000 Da) remained co-eluted with PSA using the gradient elution method. Unfortunately, efforts to eliminate the presence of the major 70,000 Da contaminant were not successful, as it was still co-eluted with PSA. This contaminant was shown by SDS-PAGE to still be
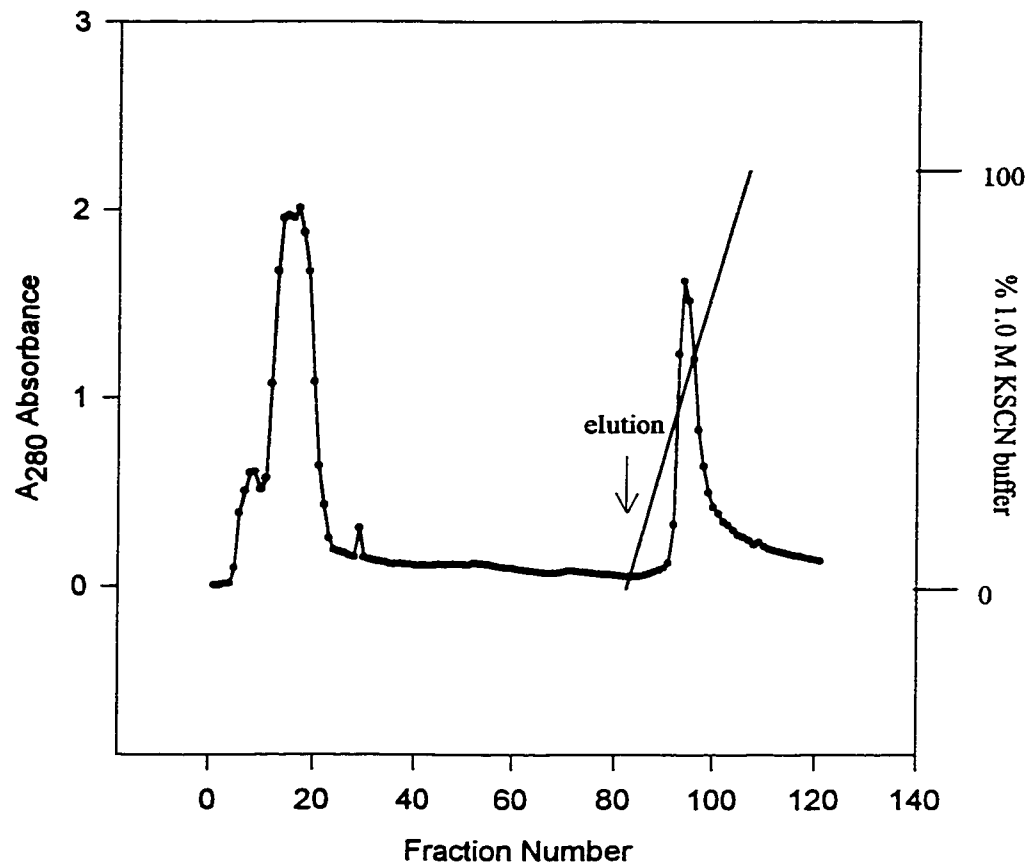
Figure 2.7 : Purification of PSA on a Cibacron blue
F3G-A gel column with gradient elution. The graph
shows the A_{280} absorbance data for each fraction.
Gradient elution was carried out from 0 to 1.0 M KSCN
to achieve a higher purity after Affigel-Blue Gel
purification (see Fig 2.8).

3

2   Elution gradient

A_{280} Absorbance

1   Elution peak

0

85   90   95   100   105   110

Fraction Number

83 —
62 —

47.7 —

32.5 —

25 —

16.5 —
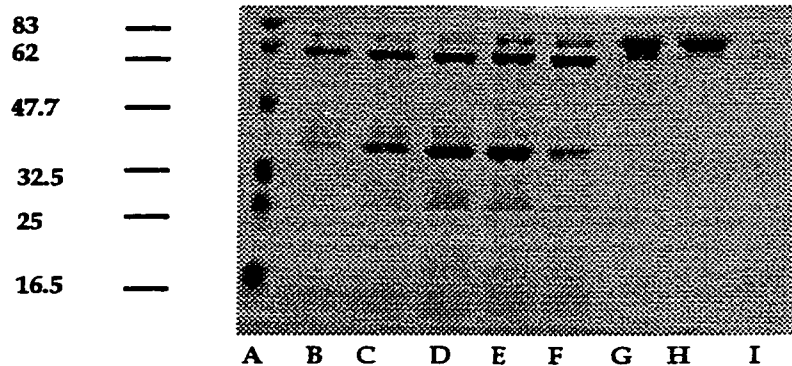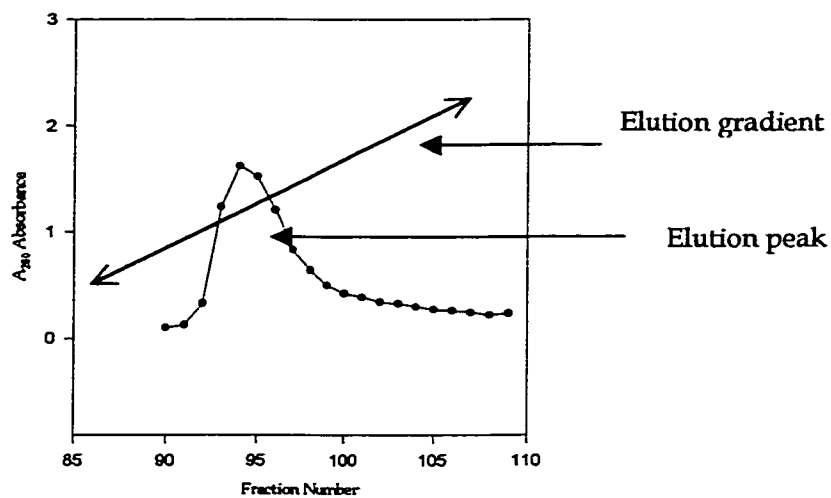
A   B   C   D   E   F   G   H   I

Figure 2.8. Sodium dodecyl sulfate-polyacrylamide (10%) electrophoresis of Affi-Gel-Blue Column. Lane A : molecular mass standards (N.E.Biolabs Inc.); Lane B,C,D, E,F,G,H,I: Fractions 91,93,95,97, 99,101, 103,105 from the elution peak. Coomassie Blue-stained PSA is seen in fractions 93-99 coeluted with albumin. The 80,000 Da contaminant is seen in the final elution fractions when the elution gradient reached its maximum concentration of KSCN.

present in fractions well after PSA was eluted. Interestingly, the higher molecular weight contaminant (80,000 Da), which could be lactoferrin (a known component of seminal plasma (Sensabaugh, 1978; Rusciano et al., 1988b)) was shown to appear in increasingly greater concentration in the final elution fractions after the elution of the major 70,000 Da contaminant was complete and the column was fully equilibrated at 1.0 M KSCN (Figure 2.7). This protein was not seen in the Cibacron blue F3G-A purification without gradient elution likely because of the lower molarity of KSCN used during elution. Ultimately, the relative amounts of PSA in the gradient eluted fractions were observed to be higher using SDS-PAGE than those fractions from batch elution and this technique was used with all subsequent PSA purifications.

2.3.3 Size exclusion methods.

Further purification of PSA was accomplished using a size exclusion techniques. Size exclusion (SE) or gel filtration (GF) techniques separate proteins solely based on their molecular weights. Because of significant differences in molecular weight between the desired PSA protein and the higher molecular weight contaminants, size exclusion was reasoned to be a good choice for the further purification of PSA. An ideal matrix should have an exclusion limit approaching, but not below that of the molecule of interest. Secondary purification of PSA using size exclusion was initially undertaken using a Sephadex G-100 column. This was selected based on the ideal fractionation range described in its specifications. Two large, not fully resolved peaks were evident in the protein A_{280} absorbance profile (see Figure 2.9). When analyzing the peaks using gel electrophoresis under reducing conditions, the separation of the major 70,000 Da contaminant and the higher molecular weight contaminants was achieved to near completeness from PSA and some other, lower molecular weight proteins (Figure 2.10).
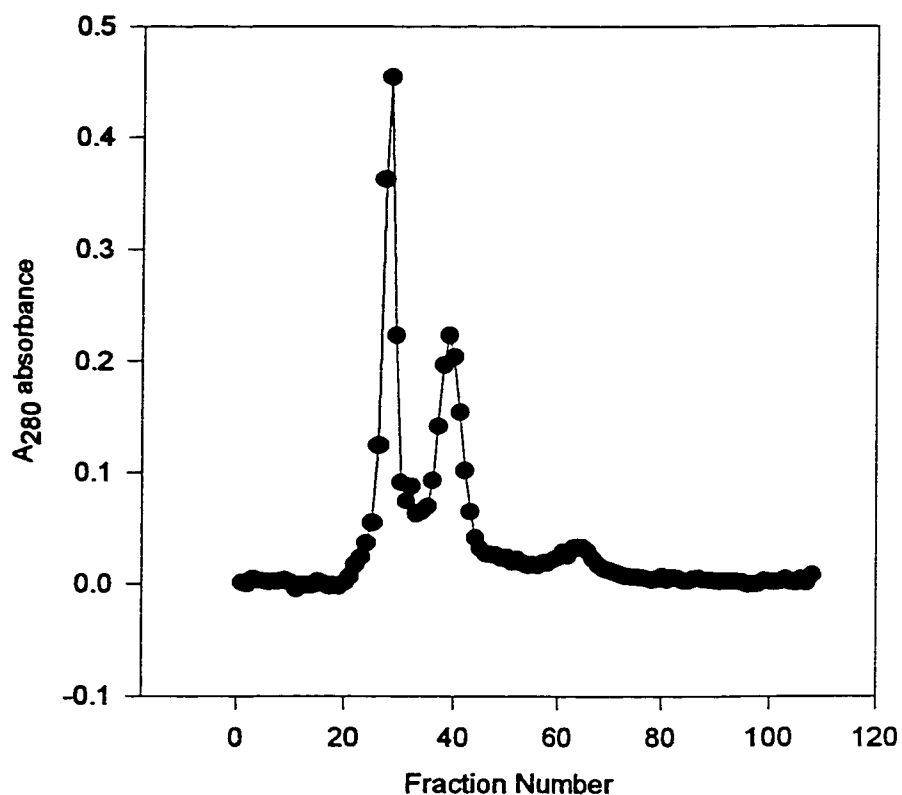
Figure 2.9: A_{280} **absorbance profile of PSA purified from a Sephadex G-100 (fine) column.** Sephadex G-100 (fine) was swelled to 200 mL and equilibrated. One mL of concentrated, pooled fractions from the Affi-Gel blue purification was loaded on top of the column. Almost complete separation was achieved with the larger molecular weight contaminants eluting before the smaller molecular weight proteins, which predominantly included PSA (see Figure 2.9). Fraction size 2.1 mL. Column flow rate 0.3 mL/min.
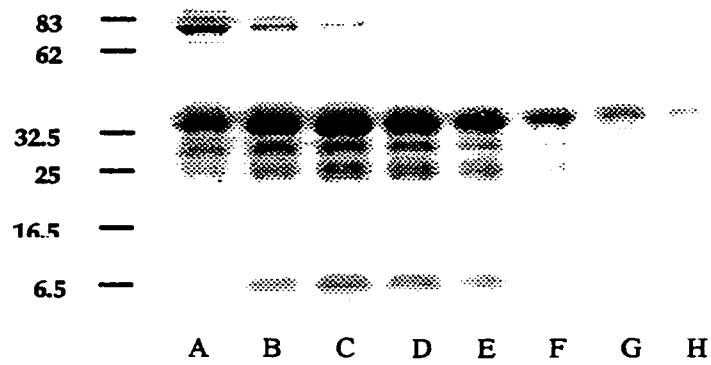
Fig 2.10: SDS-PAGE of fractions after purification on a Sephadex G-100 (fine) size exclusion column. Lane A : molecular mass standards (N.E.Biolabs Inc.);Lane B,C,D, E,F,G,H: Fractions 37,38,39,40,41,42,43 from the elution peak. PSA is seen in all fractions with albumin and other higher molecular weight proteins being separated after the third fraction.

Alternate matrices were tried when it was discovered that their exclusion limits were closer to the MW of PSA. Although the MW of PSA is slightly higher than the exclusion limit for the Sephadex G-50 crosslinked dextran matrix, it was hypothesized that some separation of PSA from its higher molecular weight contaminants would still occur. As in the Sephadex G-100 profile, two unresolved peaks are observed in the A_{280} absorbance profile (Figure 2.11). When analyzed by gel electrophoresis under reducing conditions, the first peak was shown to contain a large amount of albumin and larger molecular weight contaminants and have no PSA (Figure 2.12). The second peak was observed to contain PSA with a small amount of coeluted albumin. Because of the presence of small amounts of albumin, the Sephadex G-50 matrix could not be deemed better than the Sephadex G-100 matrix. The pooled PSA fractions were still shown to be enzymatically active against the chymotrypsin substrate, S-2586.

Because Sephadex G-75 has the most favorable separation characteristics, with proteins > 50,000 Da excluded from the crosslinked dextran matrix, this matrix was utilised as a method of comparison. The A_{280} absorbance profile is markedly different, with a short, wide first peak resolved from a sharp second peak (Figure 2.13). SDS-PAGE analysis reveals a highly pure PSA eluted in the second peak along with some lower molecular weight proteins, while albumin and other higher molecular weight contaminants are found in the first peak (Figure 2.14). Because a superfine matrix was used for purification and the flow rate had to be reduced by half, the entire purification using this matrix took twice as long. Column packing was also problematic as the smaller particles took longer to settle. Because this matrix is not particularly suited to large column, fast flow applications, consideration of the use of a less fine (e.g. Sephadex G-75 (fine)) matrix should be undertaken in the future. BioRad Labs also makes a crosslinked dextran matrix (BioRad P-60

50-150 mesh) with a similar exclusion limit (60,000 MW) which would most likely be suitable for carrying out this size exclusion step in the future.
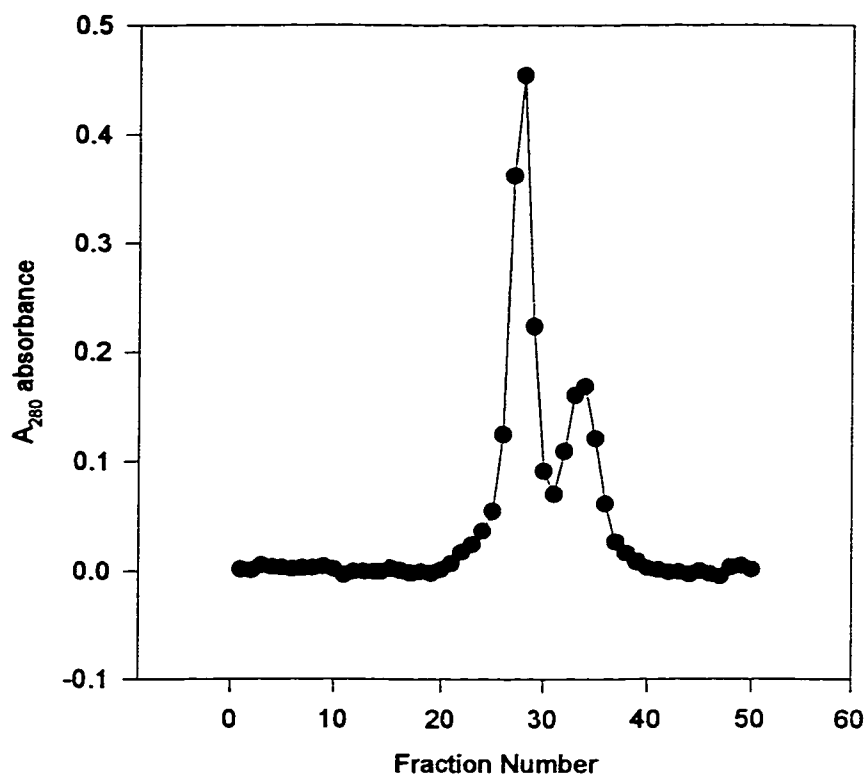
Figure 2.11: A_{280} absorbance profile of PSA purified from a Sephadex G-50 (fine) column. Separation was good but not as complete with Sephadex G-100 (fine) upon comparison. This is likely attributable to the exclusion limit (30,000 Da - see Table 2.2) being too close to the molecular weight of PSA (~33,000 Da). The SDS-PAGE analysis of this separation can be seen in Figure 2.12. Fraction size 2.1 mL. Column flow rate 0.3 mL/min.

175	—
81	—
47.7	—
32.5	—
25	—
16.5	—
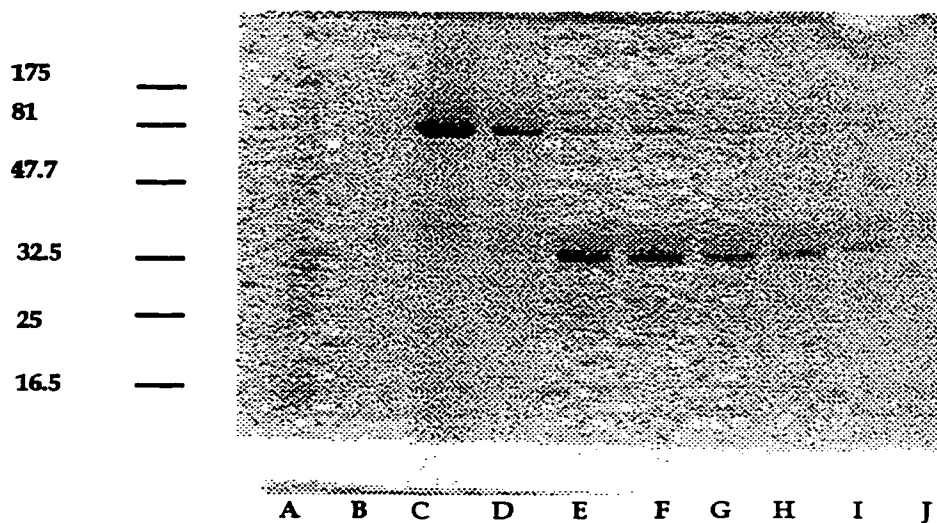
A   B   C   D   E   F   G   H   I   J

Fig 2.12. SDS-PAGE of PSA containing fractions from the Sephadex G-50 (fine) Column. Lane A:molecular mass standards (N.E.Biolabs Inc.);Lane B,C,D,E,F,G,H,I,J: Fractions 14,25,28,32,33,34,35,36,and 44. PSA can be seen in fractions 32-36 which corresponds with the elution peak. A majority, but not all, of albumin and other higher molecular weight contaminants were successfully separated from PSA using this purification scheme.
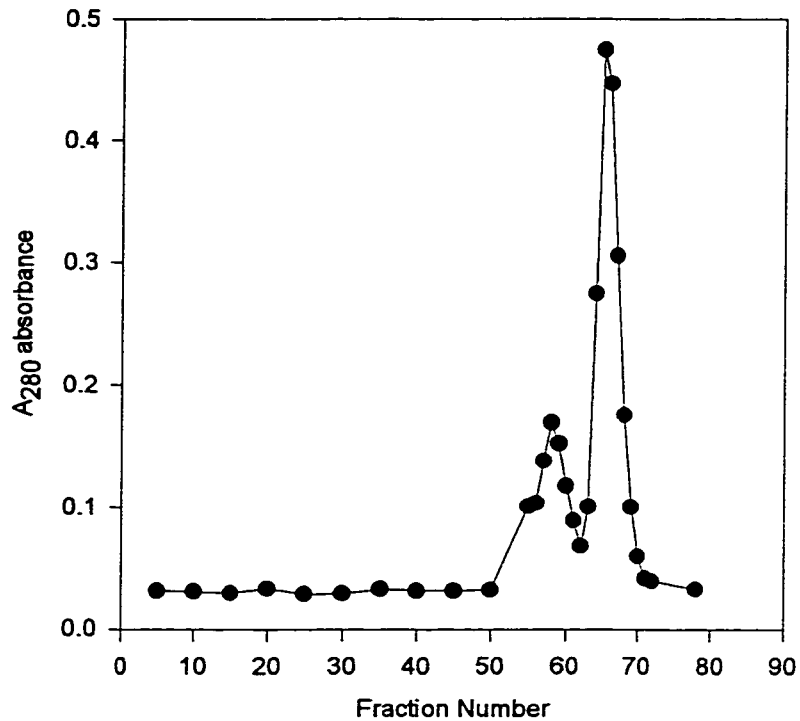
Figure 2.13: A_{280} absorbance profile of PSA purified from a Sephadex G-75 (superfine) column.
Sephadex G-75 (superfine) had the most favorable exclusion limit of the three size exclusion matrices used. Larger molecular weight contaminants were eluted in the first short, broad peak followed by PSA in a sharp tall peak (see Figure 2.14). Because of flow rate restrictions, this separation was performed at 0.15 mL/min. Fraction volume is 2.1 mL.
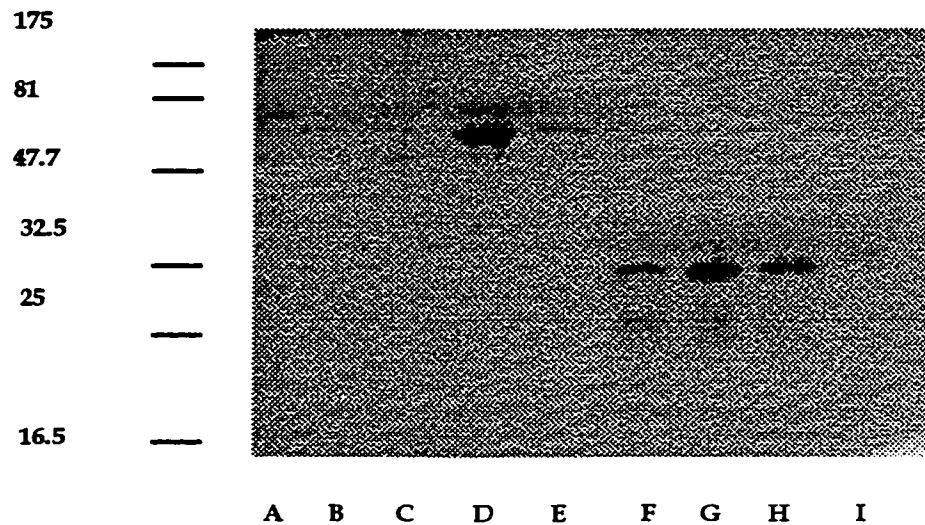
Fig 2.14: SDS-PAGE of fractions from the Sephadex G-75 (ultrafine) column purification. Lane A:molecular mass standards (N.E.Biolabs Inc.);Lane B,C,D,E,F,G,H,I: Fractions 40,50,55,62,64,65,67, and 70. The 70,000 Da major contaminant and other higher molecular weight contaminants can be seen in fractions 55 and 62. PSA can be seen in fractions 64-70 with no contaminants present

2.3.4 Ion exchange methods

Purification of PSA using a DEAE-Sephacel ion exchange column. DEAE-Sephacel was used in a manner similar to Wang et al. as a final "clean-up" of the remaining proteins in the PSA purified fractions (Wang et al., 1982). DEAE Ion exchange has been demonstrated to have different affinity characteristics for both PSA and albumin (Wang et al., 1982; Sensabaugh and Blake, 1990). Because of this, these proteins have been shown to be effectively resolved using a linear elution gradient. A small test sample was applied to the DEAE-Sephacel column to assess it. Because of the small amount of sample load, no protein was detectable by UV at 280 nm when analyzed. ELISA data reveal, however, the strong presence of PSA late in the elution (Figure 2.15). When these purified fractions were analyzed under SDS-PAGE, a single band at ~33,000 Da is evident. (data not shown).

Some easily remedied problems are evident with this scheme. PSA samples were placed in dialysis tubing and concentrated by using sucrose to draw solvent osmotically. This is simple and highly economical but generally resulted in a concentrated pooled sample which was too large (60 mL seminal plasma yielded ~10 mL of concentrate) to be placed on the 200 mL bed volume size exclusion column. This means that multiple runs were necessary, lengthening the overall time of production. The sucrose concentration step also resulted in a drastic decrease in overall PSA recovery (see Table 2.3) To correct both of these problems, lyophilization of the pooled Cibacron blue F3G-A samples could be used as an alternative step prior to size exclusion. It should be noted that all purifications were done at RT as a matter of convenience, which could also theoretically increase PSA degradation. Repeating this scheme at 4°C could have a positive impact on the overall PSA yield. Some other aspects of this scheme remain to be explored. If albumin is
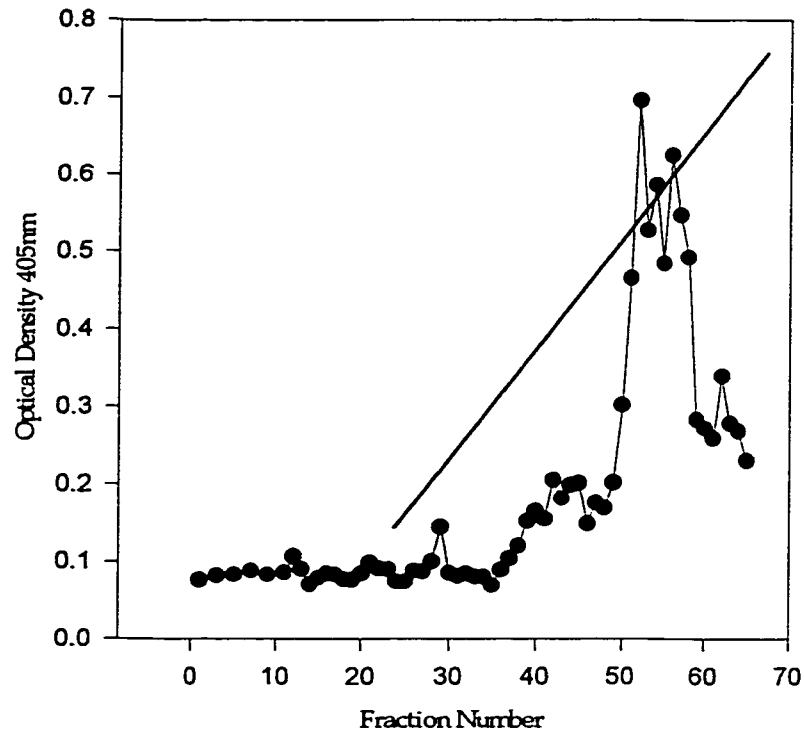
Figure 2.15: Optical density values of PSA in fractions purified by DEAE-Sephacel gradient elution. The above data represent the gradient elution fractions. Gradient elution was accomplished by incrementing the NaCl concentration from 0 to 0.15M in a 0.01 M Tris-HCl pH 7.8 buffer. Fractions were evaluated by direct coating. The column flow rate was 0.4 mL/min. Fractions were 10 mL each.

positively identified as the major 70,000 Da contaminant, it could be theoretically removed using other popular methods, including lectin (e.g. Con A) affinity and ion exchange methodologies.

2.4   CONCLUSION

In summary, the use of the modified method of Rusciano *et al.* was effective for reliably and reproducibly purifying bulk amounts of PSA in a relatively short amount of time without prohibitive costs. The matrices used were easily obtainable and the purification methods were straightforward. In addition, the purification conditions are mild. Using this method, enough PSA can be purified in about 2 weeks to provide standards for ~ 330 plates of a standard assay and 16,500 plates for an assay measuring PSA in the ultrasensitive range[1]. Purification of PSA was an important first step for developing the immunoassays that will be described in the remainder of this thesis.

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[1] The standard assay assumes 50 µL of 0 to 50 ng/mL standards applied in triplicate. The ultrasensitive assay assumes 0 to 1 ng/mL standards applied in triplicate.

Chapter 3: A General Affinity Method To Purify Peroxidase-Tagged Antibodies[2]

1.1 Introduction

Antibodies with enzyme tags are used extensively in biochemical and immunochemical applications. The ability of an enzyme-tagged antibody to localize and quickly convert a substrate to a quantifiable product is the basis of immunoassay (Oellerich, 1984; Ekins and Chu, 1997), immunohistochemical (Mao et al., 1999) and prodrug-based immunotherapeutic applications(Chouchane et al., 1990). Although a great number of enzymes have been used in immunoassays, horseradish peroxidase (HRPO) and alkaline phosphatase (AP) continue to be the most widely used among several described in the literature. The enzyme-tag is generally attached to a monoclonal or polyclonal antibody by a covalent linker (Hermanson, 1996). This chemical cross-linking technique can be problematic because of variable and partial inactivation of either the enzyme or the antibody, competition from unreacted species resulting in low specific activity, decreased shelf-life, and variations in the size and properties of the conjugate from one batch to the next (Milstein and Cuello, 1983; Kricka, 1994).

To overcome this problem, bispecific antibodies (bsMabs) have been developed(Milstein and Cuello, 1983; Cao and Suresh, 1998). Unlike traditional monospecific antibodies, bsMabs have non-identical antigen binding sites to any two desired antigens. Previous work in our laboratory has resulted in the development of several bsMabs with anti-tumor antigen specificity in one arm and anti-enzyme label specificity in the second arm(Kreutz and Suresh, 1997; Cao et al., 1998; Xu et al., 1998). These bsMabs can be generated in various ways

– by chemically coupling two distinct antibodies or Fab fragments, though genetic engineering (e.g. diabodies), or by somatic fusion to generate quadromas or triomas. The enzyme-tag in this scenario is attached non-covalently, by one antibody binding arm. A bsMab-secreting quadroma is generated by fusing the two monoclonal antibody (Mab)-producing hybridoma cell lines. This, however, presents some special problems. Post-translational random association of heavy and light chains in the quadroma generates the desired bispecific antibody along with other contaminating species including the parental monospecific antibodies(Suresh et al., 1986). This makes purification the bispecific antibody the single greatest obstacle in the production and use of bsMabs from quadromas and triomas.

Purification of HRPO-conjugated antibodies has been previously accomplished with a Concanavalin A (Con A)-Sepharose column (Arends, 1979). The group specific nature of Con A binding to various other glycoproteins made it less than ideal for our purposes. We have previously purified HRPO-labelled bsMabs using Mimetic Red 3 (Xu et al., 1998) matrix and the uncomplexed bsMAb on an HRPO-agarose column (Cao et al., 1998) but sought a more reliable and economical method. The Mimetic Red method involved negative adsorption of HRPO and HRPO-Mab derivatives and was not often useful since several contaminants were also found in the unbound fraction. Hence, we set out to develop a positive mode affinity co-chromatography of HRPO-tagged antibodies. Hydroxamic acids have been previously demonstrated to bind to plant peroxidases (Reimann and Schonbaum, 1978). By using a hydroxamic acid-affinity column we speculated that we could purify HRPO-labelled bsMabs. In this paper we report the purification of HRPO-labelled bsMabs for their application in immunoassay by using a hydroxamic acid-affinity column. For clarity, in this manuscript the

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suffixes conjugated, labelled (complex), and tagged denote covalent, non-covalent and either (generic) form of the antibody, respectively.

3.2 Materials and Methods

3.2.1 Reagents

Benzhydroxamic acid-agarose was obtained from UpFront Chomatography (Copenhagen, Denmark). Prostate-specific antigen (PSA) was purified in our lab based on a modification of a published method (Rusciano et al., 1988). Horseradish Peroxidase (HRPO, Type I, EC 1.11.1.7), goat-anti-mouse IgG antibodies, streptavidin-HRPO, and goat-anti-mouse IgG HRPO-conjugated antibodies were obtained from Sigma (St.Louis, MO, USA). Dialysis tubing (8000 MW cutoff) was obtained from BioDesign Inc. (Carmel, NY, USA). The 96-well microtiter plates were obtained from Nunc (Naperville, IL, USA). All buffers were made to 50 mM unless otherwise stated.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 10%) was performed according to previously published methods (Laemmli, 1970) using a Bio-Rad Mini-protein II dual slab cell along with prestained broad range molecular weight markers (New England Biolabs, Inc., MA, USA). Proteins were stained using Coomassie Blue.

3.2.2 Mabs and cell lines.

P57 is a well characterized, bsMab secreting, quadroma developed in our lab previously (Kreutz and Suresh, 1997) which binds to HRPO and PSA. This P57 quadroma was grown in RPMI-1640 with 5% FBS, 500 ml of the supernatant was harvested and the immunoglobulins were precipitated with a 45% final ammonium sulfate concentration. The pellet was centrifuged at 20,000 x g for 30 minutes at room temperature, dissolved in 30 ml of phosphate buffer pH 7.0 and

dialyzed against 3 changes of 1L phosphate buffer for 24 hours prior to purification on the benzhydroxamic acid column.

B87.1 and B80.3 are matched anti-PSA monoclonal antibody sandwich pair that have been described previously (Jette et al., 1996; Kreutz et al., 1998; Xu et al., 1998). B80.3 is a parent cell line of the P57 quadroma. Together, B87.1 and P57 can form a heterosandwich with PSA either in its free form or when complexed with α-1-antichymotrypsin (ACT) (Kreutz and Suresh, 1997).

3.2.3 Assays

The activity of HRPO was determined by measuring the oxidized colorimetric product of 3, 3', 5, 5' – tetramethylbenzidine (TMB) at 650 nm or, after stopping the reaction with 1M H_3PO_4, at 450 nm using a commercially available substrate (K&P, Gaithersburg, MD, USA).

3.2.3.1 ELISA

ELISA to detect the presence of HRPO-labelled P57 bispecific antibodies.

To monitor the presence of bispecific antibody, 100μL of PBS containing B87 (1 μg/ml) was added to each well of a 96-well microtiter plate and incubated overnight (16 h) at 4°C. The plate was subsequently washed 3 times with 0.05% PBS-Tween 20 (PBS-T) and 200μL of PBS containing BSA (1%) was added to each well for 1 h at 37°C to block non-specific binding sites. It was then washed as before and stored at 4°C for 1 week. To each well, 100 μL of a PSA standard (100 ng/ml) was added. The plate was put on the shaker for 1 h at RT. The plate was again washed 3 times with PBS-T and 10 μL of each fraction putatively containing the bsMab labelled with HRPO was added to 90 μL PBS in each well for a minimum of 30 minutes. Finally the plate was washed with PBS-T and developed with the TMB substrate. The principle of this bridge assay (Suresh et al., 1986) is shown in Fig 3.1.

■ PSA   ○ HRPO

1. Anti-PSA antibody is coated on a microtiter plate and blocked. Prostate Specific Antigen is added.

2. Eluted fractions are applied to the plate. Only the bispecific monoclonal antibody is capable of binding to PSA on the plate. The plate is washed.

wash

3. Peroxidase activity is measured with TMB substrate. Activity correlates with presence of bispecific antibody.

Figure 3.1- ELISA for the detection of PSA using the purified HRPO-labelled bispecific antibodies from the benzhydroxamic acid-agarose (BHA) column. This ELISA configuration was used to both detect the presence of HRPO-labelled bsMabs from the BHA column and subsequently for the quantitative detection of PSA.

ELISA to detect the presence of unlabelled P57 bispecific antibodies.

To detect the presence of unlabelled P57 antibodies, 100 μL of PBS containing goat-anti-mouse (at the manufacturer's recommended dilution) was added to each well of a 96-well microtiter plate and incubated overnight (16h) at 4°C. The plate was subsequently washed 3 times with PBS-T (0.05%) and 200 μL of PBS containing BSA (1%) was added to each well to block non-specific binding sites for 1 h at 37°C. To each well, 10 μL of each fraction putatively containing the unlabelled bsMab was added to 90 μL PBS for 30 minutes. The plate was washed 3 times with PBS-T and developed with TMB substrate for 15 minutes to monitor the presence of any background activity. An optical density reading was obtained. The plate was again washed 3 times with PBS-T and 100 μL of a 1 mg/ml HRPO in PBS solution was added to each well and allowed to incubate for 15 minutes. Finally the plate was washed 3 times with PBS-T and developed with the TMB substrate.

3.2.4   Chromatography

Affinity purification of HRPO with the benzhydroxamic acid-agarose column.

Crude HRPO (Type I) was dissolved in sodium phosphate buffer pH 7.0 to a final concentration of 1 mg/ml (5 ml) and loaded onto a 1.25 x 30 cm column containing 25 ml of a benzhydroxamic acid-agarose matrix. The column was previously equilibrated with phosphate buffer pH 7.0. The column flow rate was 18 ml/h. After loading, the column was washed with approximately 4 column volumes of phosphate buffer and eluted with borate buffer, pH 9.0. Unbound and eluted fractions (1.5ml) were collected.

Affinity purification of HRPO-labelled bsMabs with the benzhydroxamic-acid agarose column. Half of the antibody supernatant from the P57 quadroma which had been previously precipitated with ammonium sulfate was resuspended with 25 ml phosphate buffer and dialyzed. This crude antibody was combined with 10 mg of HRPO dissolved in 5 ml phosphate buffer pH 7.0, allowed to incubate for 15 minutes, and loaded onto a 1.25 x 30 cm column containing 10 ml of a benzhydroxamic acid-agarose matrix. The column flow rate was 18 ml/h. After loading, the column was washed with approximately 4 column volumes of phosphate buffer and the bound proteins were eluted with borate buffer, pH 9.0. The unbound and eluted fractions (1.5 ml) were collected.

Affinity purification of HRPO-conjugated goat-anti-mouse IgG polyclonal antibodies and streptavidin-HRPO complex. A 100 μL volume of HRPO-conjugated polyclonal goat-anti-mouse IgG antibody was dissolved in 5 ml of a 1% BSA in sodium phosphate buffer pH 7.0 and the affinity chomatography procedure was carried out as before. A 10 μL portion of each fraction was added to a murine monoclonal B87.1-coated and blocked plate. The fractions were allowed to incubate for 1 h and then washed and developed using TMB as a substrate. A 20 μL volume of streptavidin-HRPO was applied to the column under identical experimental conditions. Fractions were collected and assessed as described in Section 3.3.

3.3   Results

Affinity purification of HRPO. Initially, HRPO was purified alone in order to test the column and establish the affinity purification method and its parameters as described previously (Reimann and Schonbaum, 1978). The absorbance of each collected fraction was recorded then each fraction was diluted 1:20 in a 96-well microtiter plate and the plate was diluted 1:20 again in order to assay the

relative peroxidase activity in each fraction. Protein absorbance and activity were seen in both the unbound and eluted fraction, which came off as a sharp peak (Fig. 3.2). The SDS-PAGE revealed retention of the active 44,000 MW species in the eluted fraction with loss of a major ~30,000 MW species in the unbound (data not shown). A much greater amount of peroxidase activity was seen in the eluted fractions when compared with the unbound fractions.

Affinity Co-purification of HRPO-labelled P57 bsMabs (anti-PSA x anti HRPO).

A schematic of the affinity co-purification of HRPO-labelled antibodies is shown in Fig. 3.3. It is pertinent to mention that the crude antibody sample was incubated with an excess of HRPO(10 mg) prior to initiating affinity co-chromatography. The chomatographic profile of the labelled P57 antibody shows a large amount of protein which did not bind to the column (Fig. 3.4). Very little peroxidase acitivity was seen in this unbound fraction when it was subjected to the ELISA to detect labelled-antibodies. The A_{280} absorbance peak in the spectrometric data is seen corresponding with a single sharp peak of peroxidase activity in the eluted fractions. The ELISA data show the co-purification of the bsMAb and HRPO enzyme associated with it exclusively in the eluted peak. This pattern of affinity purification with a high yield was reproduced in three separate runs. The pooled eluted fractions were consequently dialyzed against PBS and stored at $-20^{\circ}C$.

Chomatography of unlabelled P57. To rule out non-specific binding of the antibody to the affinity matrix, P57 was loaded onto the column in a parallel experiment without prior incubation with HRPO. Fractions were collected and 10 μL portions were added to 90μL PBS in a 96-well microtiter plate and further diluted 1:10 on a goat-anti-mouse antibody coated plate as described previously. Unlike the data shown in Figure 4, very little protein was observed by

spectrometry in the eluted fraction. After incubation with the TMB substrate no peroxidase activity was observed in any fraction (Fig. 3.5). This step was
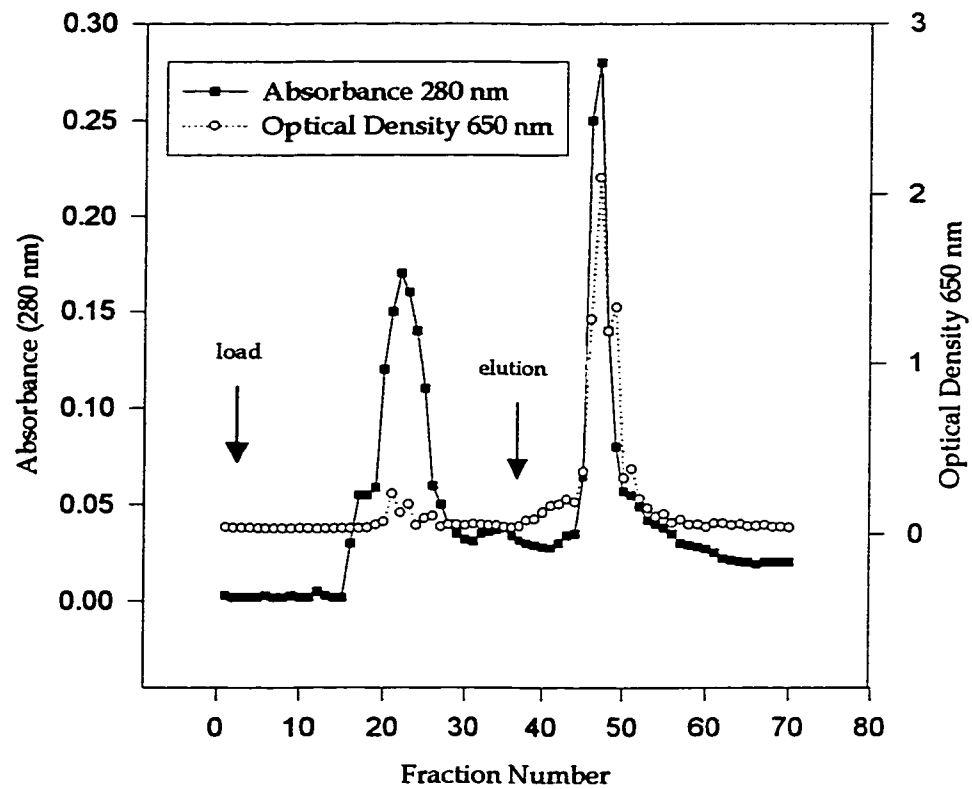
Figure 3.2. Purification of crude HRPO on a benzhydroxamic acid – agarose column. Each fraction represents 1.5 mL volume. Each fractions A_{280} value was measured and then diluted 1:400 to assess its peroxidase activity.
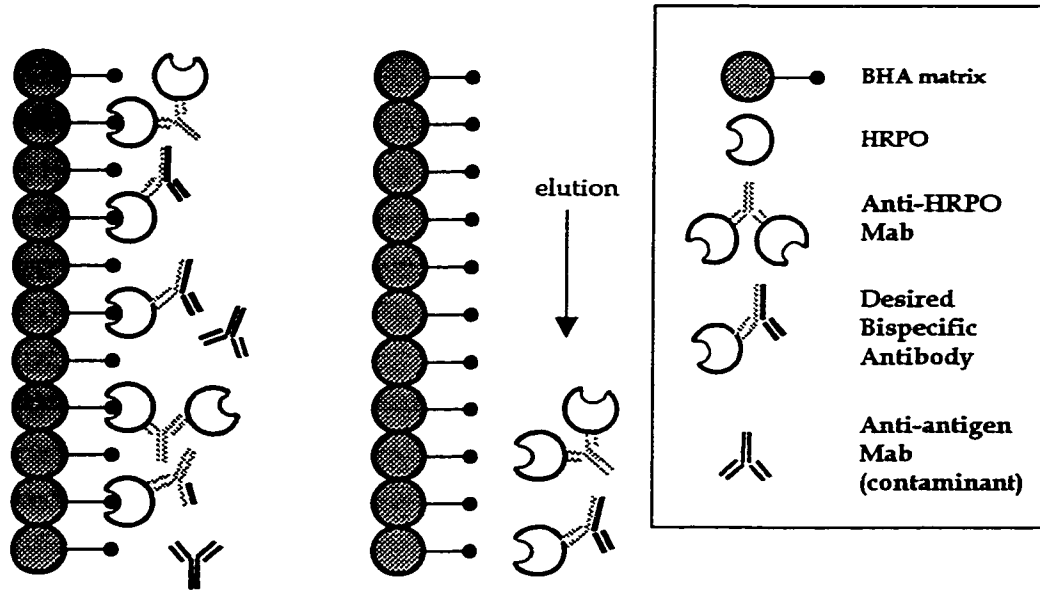
Figure 3.3 - Purification of bispecific monoclonal antibody on the benzhydroxamic acid -agarose (BHA) column matrix - All antibodies with a homologous heavy/light chain association for binding HRPO are captured on the HRPO-binding BHA matrix. All other antibodies, some of which could potentially lower specific activity of the purified fraction, are washed from the column.
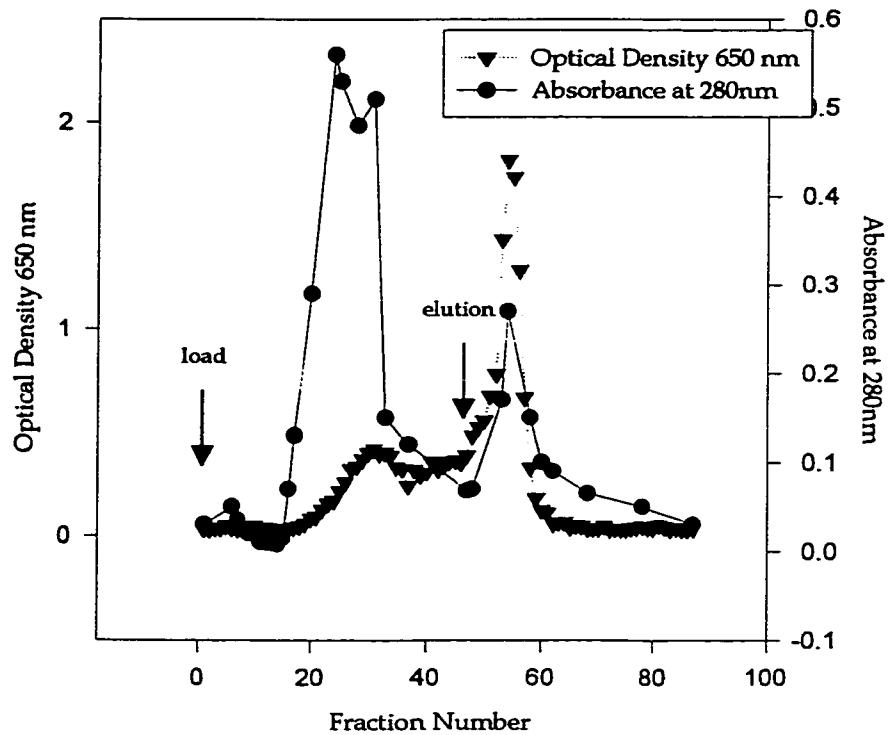
Figure 3.4. Purification of HRPO-labelled bsMabs from cell culture supernatant on a benzhydroxamic acid-agrose column. The A_{280} absorbance of each fraction was measured. Optical density (O.D.) is represented by the peroxidase activity after ELISA (as shown in Figure 3.1).
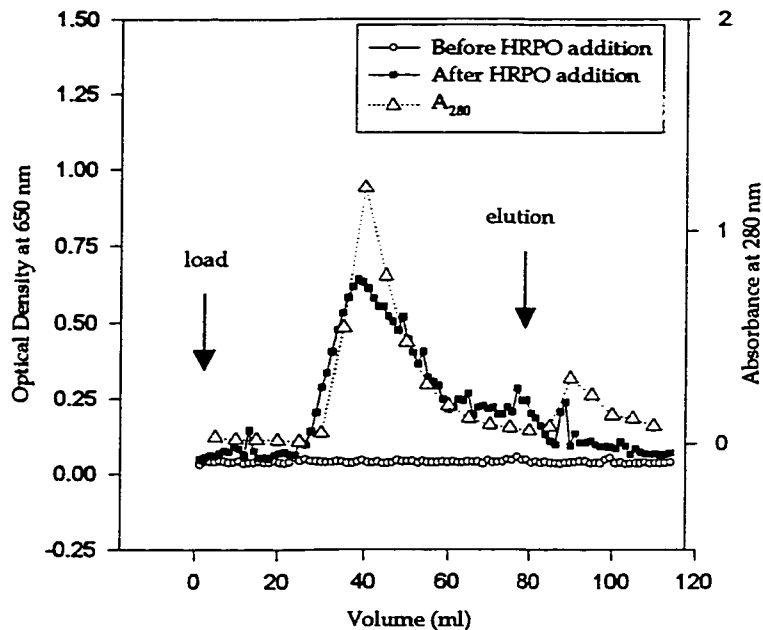
Figure 3.5. Chromatography of unlabelled bispecific antibody. Unlabelled P57 subjected to same experimental conditions as in Figure 4. A_{280} absorbance of each fraction is taken. ELISA is performed as before to check for endogenous peroxidase activity. An additional measurement was taken after HRPO addition to indicate which fractions contain the bsMab.

undertaken to rule out any idiosyncratic or pseudoperoxidase background activity in the eluted fractions by contaminating bovine hemoglobin or endogenous peroxidase in the FBS-enriched culture supernatant. After washing, incubation of the microtiter plate with HRPO, and a subsequent wash step, peroxidase activity was observed in the unbound fractions upon addition of the TMB substrate (Fig. 3.5). Virtually no peroxidase activity was seen in the borate eluted fractions.

Affinity purification of HRPO-conjugated goat-Anti-Mouse IgG polyclonal antibodies and streptavidin-HRPO complex. The covalently coupled HRPO-polyclonal antibody immunoconjugates had a similar elution profile to the previous bispecific Mab purification with non-covalently labelled HRPO. Very little absorbance at 280 nm (data not shown) was seen in the unbound fractions, however, a large amount of peroxidase activity could be seen in the eluted fractions (Fig. 3.6). The streptavidin-HRPO activity profile (data not shown) was measured by assessing the relative peroxidase activity in each fraction. Peroxidase activity was seen predominantly in the eluted fractions.
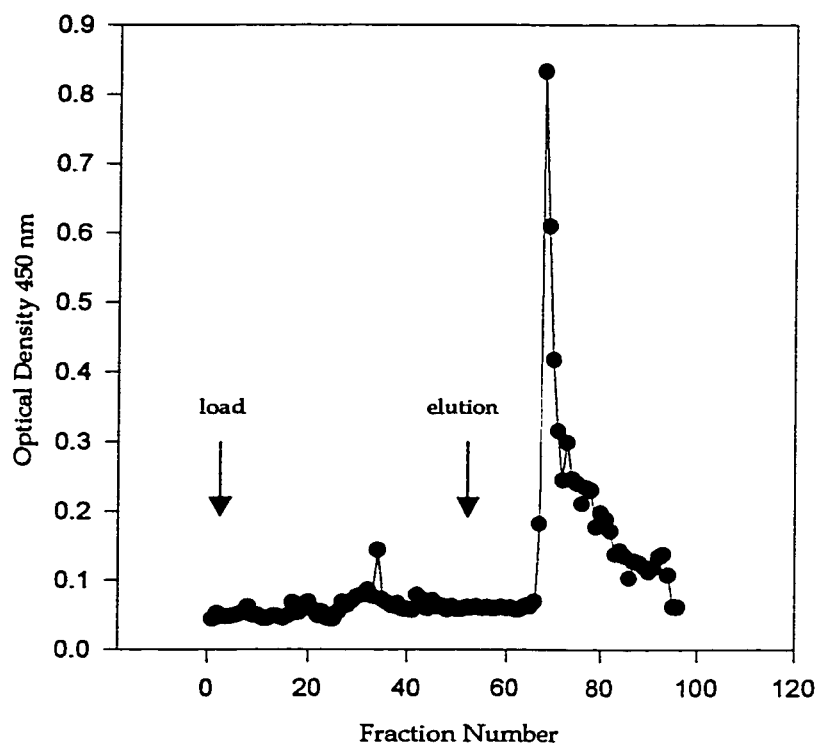
Figure 3.6. Purification of commercial polyclonal goat-anti-mouse IgG HRPO-conjugated antibodies on a benzhydroxamic acid agrose column. A_{280} absorbance was insignificant and not shown. Optical density (O.D.) is represented by the peroxidase activity after ELISA.

SDS-PAGE analysis of HRPO, P57, and commercial Antibodies. An SDS-polyacrylamide gel electrophoretic analysis was performed to observe the nature of the various fractions. The gel was stained with Coomassie Blue to observe proteins from both the unbound and eluted fractions. Prior to purification, the crude P57 supernatant was seen to have both a heavy (~50,000 MW) and two faint light chain bands (~27,000 MW) along with albumin (data omitted). The two light chain bands in both the crude and purified P57 lanes are characteristic of their bispecific nature (Suresh et al., 1986). Crude HRPO (Type I) is seen to have many bands including the active 44,000 MW band. This same band could be observed along with the heavy and light antibody chains in the labelled P57 antibody eluted peak .

3.4   Discussion

In order for an application involving enzyme-labelled or enzyme-conjugated antibodies to be successful, the antibodies must be able to bind to a target and produce an enzymatic signal (Engvall and Perlman, 1971; Hermanson, 1996). When monoclonal or polyclonal antibodies are covalently bound to HRPO, some inactivation of antibody, peroxidase or both can occur, thus, reducing the specific activity of both species if a purification method to resolve the active species is not adopted. High backgrounds can also be a concern, perhaps due to random conjugations and aggregate formation. Antibodies with inactivated binding sites will lower the batch specific activity but will not interfere with the application as they cannot bind and will generally be discarded in any subsequent wash step (Fig. 3.1). Antibodies conjugated to inactivated HRPO, however, will interfere with assay function as they will bind without eliciting a signal and could potentially reduce the sensitivity of the assay when used. These dysfunctional immunoprobes will likely be copurified with their active counterparts after covalent coupling by regular size exclusion and ion-exchange purification techniques.

We investigated the potential of using a benzhydroxamic acid agarose as a matrix for purifying antibodies with covalent or non-covalently bound HRPO to eliminate the presence of enzymatically inactive immunoprobes. The binding of hydroxmates and bezhydroxamic acid (BHA) to peroxidase has been extensively studied, and characterized on a molecular level (Aviram, 1981; Veitch and Williams, 1995; Henriksen et al., 1998). This BHA-agarose matrix has been shown to be specific for the heme-active site of HRPO (de Ropp et al., 1999). Some isozyme specificity has also been demonstrated (Veitch and Williams, 1995), which could be due to differences in active site exposure after protein folding. This might explain some of the peroxidase activity that was observed in the unbound fractions (Fig. 3.2). Because of this active site specificity, purification using the BHA agarose matrix will inevitably yield antibodies which are bound to enzymatically active HRPO and have a higher specific activity (Fig 3.3). Commercial and in-house polyclonal-HRPO immunoconjugates and streptavidin-HRPO-based complexes also be quantitatively purified by this affinity procedure. This column could be benefical for quality control/assurance procedures in-house or by diagnostic reagent manufacturers.

We believe combining bispecfic antibodies with this purification technique has distinct advantages. Although generating these second generation probes requires a greater initial effort, bsMabs eliminate the problems of chemical inactivation and aggregation associated with enzyme conjugation by chemical coupling. In addition, bispecific antibodies labelled with HRPO potentially have a consistent one-to-one labelling of enzyme to antibody, which lessens batch-to-batch variation and increases overall reliability (Kricka, 1994). In addition, no one purification method exists for bsMabs. Typical purification schemes have used a combination of protein A, ion exchange, and/or size exclusion. These non-specific methods are subject to trial and error and can be time consuming or problematic due to harsh eluting conditions. Dual affinity

chomatography can be devloped by immobilization of the antigen if enough pure antigen is available (Cao et al., 1998). Thiophilic gradient elution and hydrophobic interaction chromatography have been used successfully to resolve the various Mab isoforms or species and have been previously described (Manzke et al., 1997; Kreutz et al., 1998).

There are some further advantages to this new purification method. Using only a small amount (250 mL) of antibody supernatant, we were able to purify enough HRPO-labelled bispecific antibody for 30 ELISA assay plates or 3000 wells. The entire procedure from the initial thawing of quadroma cells to purification took only about 10 days. Although we employed a fairly large column volume, a much smaller volume could be used in theory since the binding capacity of benzhydroxamic acid-agarose claimed by the manufacturer is 5-10 mg of HRPO per 1 ml of the matrix. Dilution of the labelled-antibodies in the pool due to the larger column was of little concern since further dilution would be required in any subsequent assay. Nevertheless, the eluted peroxidase-labelled fraction demonstrated a sharp absorbance peak. We suggest that pooled eluted fractions be dialyzed against PBS to remove the borate buffer which seemed to have a detrimental effect on peroxidase activity (Fig 3.7). We also suggest that a carrier protein be added (1% BSA) as we have had the most success storing bsMabs in this way.

It should be noted that when purifying bsMabs, free HRPO and monospecific antibodies directed toward HRPO will also be copurified. These copurified contaminants should be of little concern as neither will bind to the target antigen and will be eliminated in any subsequent wash steps (Fig. 3.1 and 3.3). They could theoretically contribute to low levels of non-specific binding and higher background levels in some immunoapplications. We have, however, performed ultrasensitive assays for PSA with detection limits below < 0.01 ng/ml using our purified fractions without any observed elevations in

background (unpublished data). Three unidentified co-eluted protein contaminants (>70,000 MW) were also observed in the SDS-PAGE analysis.
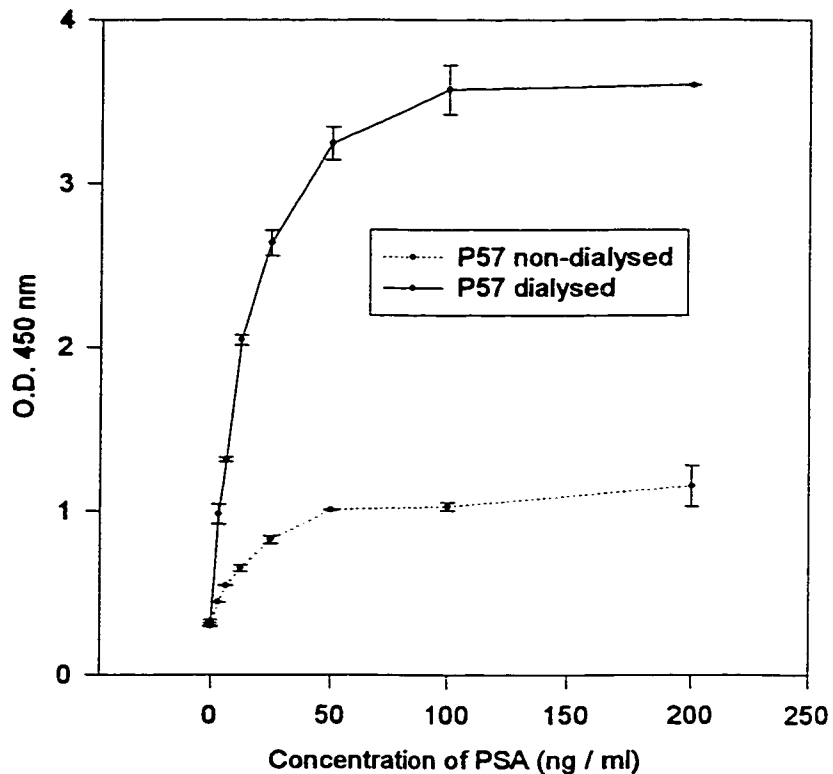
Figure 3.7: A comparison of P57 antibody performance with and without dialysis after purification on BHA column. A bridge assay was carried out as previously described using purified P57 which had either been dialysed against PBS or stored in borate buffer. Although antibody dilutions of 1:50 were both done in PBS, the antibody stored in the higher pH, borate buffer performed poorly when compared with the dialysed, purified antibody.

Although not demonstrating a detrimental effect in the subsequent ELISA, the coelution of these contaminants could be due to the group specific nature of the BHA protein matrix. Although BHA is able to purify HRPO by binding to the heme-active site, it may also specifically bind to other proteins which have metal cations. These contaminants could potentially be eliminated by using a more pure HRPO, e.g. Type III or IV, with an RZ value of greater than 3.0 or by varying the components of the cell culture media .

Other advantages are evident in this purification scheme. The elution conditions are mild and suitable for biological applications. The same procedure can be exploited for the affinity purification of peroxidase attached to any protein: Fab fragments; polyclonal and monoclonal antibodies; and genetically engineered antibodies or diabodies. Previous studies in our laboratory had demonstrated the successful purification of alkaline phosphatase immunoconjugates (Xu et al., 1998) using a phosphate analog-based mimetic Blue A6XL column. Benzhydroxamic acid agarose affinity is a perfect companion to this and allows us to quickly and easily purify bsMabs labelled with the versatile HRPO reporter enzyme for a broad range of biochemical and immunochemical applications.

3.5   Conclusion

The use of benzhydroxamic agarose is an ideal method to purify HRPO-conjugated or labelled antibodies. When coupled with bispecific monoclonal antibody applications, the result is an immunoprobe with high specific activity and batch to batch consistency of enzyme labeling.

Chapter 4: Comparison of bsMab- and Mab-based ultrasensitive assays for the measurement of PSA.

4.1 Introduction

Previously, a high-affinity solid-phase monoclonal, PSA, solution-phase bispecific-HRPO sandwich assay had been developed in our lab. It demonstrated rapid kinetics and high sensitivity (15 minute incubation, 0.028 ng/mL LLD) using a TMB-based colorimetric assay (Kreutz and Suresh, 1997). Although this sensitivity was already in the < 0.1 ng/mL ultrasensitive range, it was hypothesised that a greater sensitivity could be achieved by modifying some of the conditions of the assay, including the incubation times and substrate conditions.

4.2 Materials and Methods

4.2.1 Reagents

PSA was purified based on a modified method of Rusciano et al. as described in Chapter 2. PSA standards were made in a 6% BSA in PBS, pH 7.2, solution and the concentrations were independently verified by Sheila Stelmaschuk, Pathology Department, Cross Cancer Institute. BSA, tyramine, biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester (NHS-LC-Biotin), and streptavidin-HRPO were obtained from Sigma (Sigma, St. Louis, MO, USA). Dialysis tubing (8000 MW cutoff) was obtained from BioDesign Inc. (Carmel, NY, USA). The 96-well microtiter plates were obtained from Nunc (Naperville, IL, USA). Eppendorf tubes were obtained from Sarstedt, Inc. (Newton, NC, USA). TMB colorimetric substrate was obtained from Kirkegaard and Perry (K&P, Gaithersburg, MD, USA). Quantablu™ fluorometric substrate was obtained from Pierce (Pierce, Rockford, IL, USA). All buffers were made to 50 mM unless otherwise stated.

4.2.2   Mabs and Cell Lines

P57 is a well-characterized, bsMab secreting, quadroma developed in our lab previously (Kreutz and Suresh, 1997) which binds to HRPO and PSA. This P57 quadroma was purified using a benzhydroxamic acid (UFC Benzhydroxamic Agarose S, UpFront Chromatography, Copenhagen, Denmark) column using a procedure that was discussed in Chapter 3. B87.1 and B80.3 are a matched anti-PSA Mab pair that have been described previously (Jette et al., 1996; Kreutz and Suresh, 1997; Xu et al., 1998) and together, B87.1 and B80.3 can form a heterosandwich with PSA either in its free form or when complexed with α-1-antichymotrypsin (ACT) (Kreutz and Suresh, 1997). B87.1 was generously donated by Biomira, Inc.

Biotinylation of the B80.3 Mab. Biotinylation of the B80.3 Mab was carried out according to a published protocol (Hermanson, 1996). The B80.3 Mab (1.42 mg/mL) was first prepared by dialysing it for 24 hr against 1000 volumes of PBS, pH 7.2 with one change of buffer. Then, 3.4 mg of the water soluble sulfo-biotin derivative (NHS-LC-biotin) was dissolved in 1 mL H$_2$O and quickly added to the 1 mL dialysed antibody solution. The resultant mixture was allowed to react for 1 hr then dialysed against 1000 mL PBS for 72 hrs to eliminate the presence of the reactive NHS-LC-biotin. The dialysed biotinylated antibody was then stored at -20°C in 1% BSA for further use.

4.2.3   ELISA

4.2.3.1   ELISA Optimization

Standard Assay Format. All assays were based on a noncompetitive sandwich type assay format (Fig 4.1). The B87.1 Mab was first coated on the plate, blocked, and PSA was added at a given concentration. Then P57, or HRPO-labeled B80.3

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was used to detect the presence of PSA by the addition of a substrate. In the case of the catalyzed reporter deposition (CARD) assay, BT substrate and then streptavidin-labeled enzyme were added prior to development with a substrate to amplify the signal (Fig 4.2). A list of all assays performed is in Table 4.1.

Substrates

a) **Colorimetry.** The activity of HRPO was determined by measuring absorbance of the oxidized colorimetric product of 3, 3', 5, 5' – tetramethylbenzidine (TMB) at 650 nm or, after stopping the reaction with 1M H_3PO_4, at 450 nm using a commercially available substrate according to the manufacturer's directions (K&P, Gaithersburg, MD, USA).

b) **Fluorometry.** Fluorometric analysis was carried out using a FL-600 fluorometric plate reader set at a 5 mm probe width, and solid black, 96 well, flat bottom, opaque microtiter plates. The activity of HRPO was determined by measuring the fluorescence of a commercially available substrate, Quantablu™ (Pierce, Rockford, IL, USA), with the excitation spectrum set at 360 (40 bandwidth) and the emission spectrum set at 460 (40 bandwidth).

Dialysis of blocking and carrier solution. 6% BSA was dialysed against 20 volumes of PBS, pH 7.2 with 5 changes of buffer. The 1% BSA/PBS blocking solution was made from this 6% solution by diluting it in PBS 1:5. A direct comparison of dialysed 1% BSA (dBSA) versus non-dialysed BSA was carried out using two P57 sandwich assays under identical conditions. BSA and dBSA

	Detection Antibody	
Assay Format	P57	B80.3
Colorimetric		
1hr	+	+
3hr	+	+
Fluorometric		
1hr	+	+
3hr	+	+
CARD amplified		
1hr	+	-
3hr	+	-

Table 4.1. Summary of all assays performed. Both P57 and B80.3 were utilized to detect PSA at ultrasensitive ($< 0.1ng/mL$) levels. The CARD amplification assay was only performed along with the P57 assay.
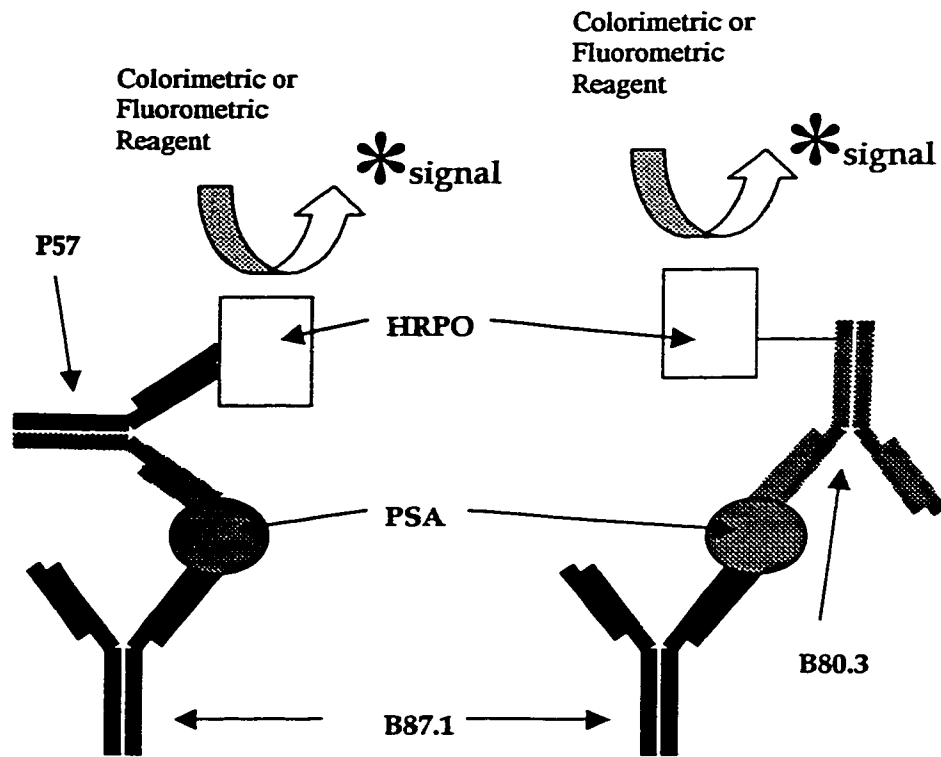
Figure 4.1. Configuration of the bsMab-based and Mab-based sandwich assays. The B87.1 Mab is coated on the plate to capture PSA. After the plate is blocked and washed, any PSA in the sample will complex with either the Mab-HRPO or bsMab0HRPO immunoprobe which is then detected with either a colorimetric or fluorometric substrate. The CARD assay amplification is shown in Figure 4.2.
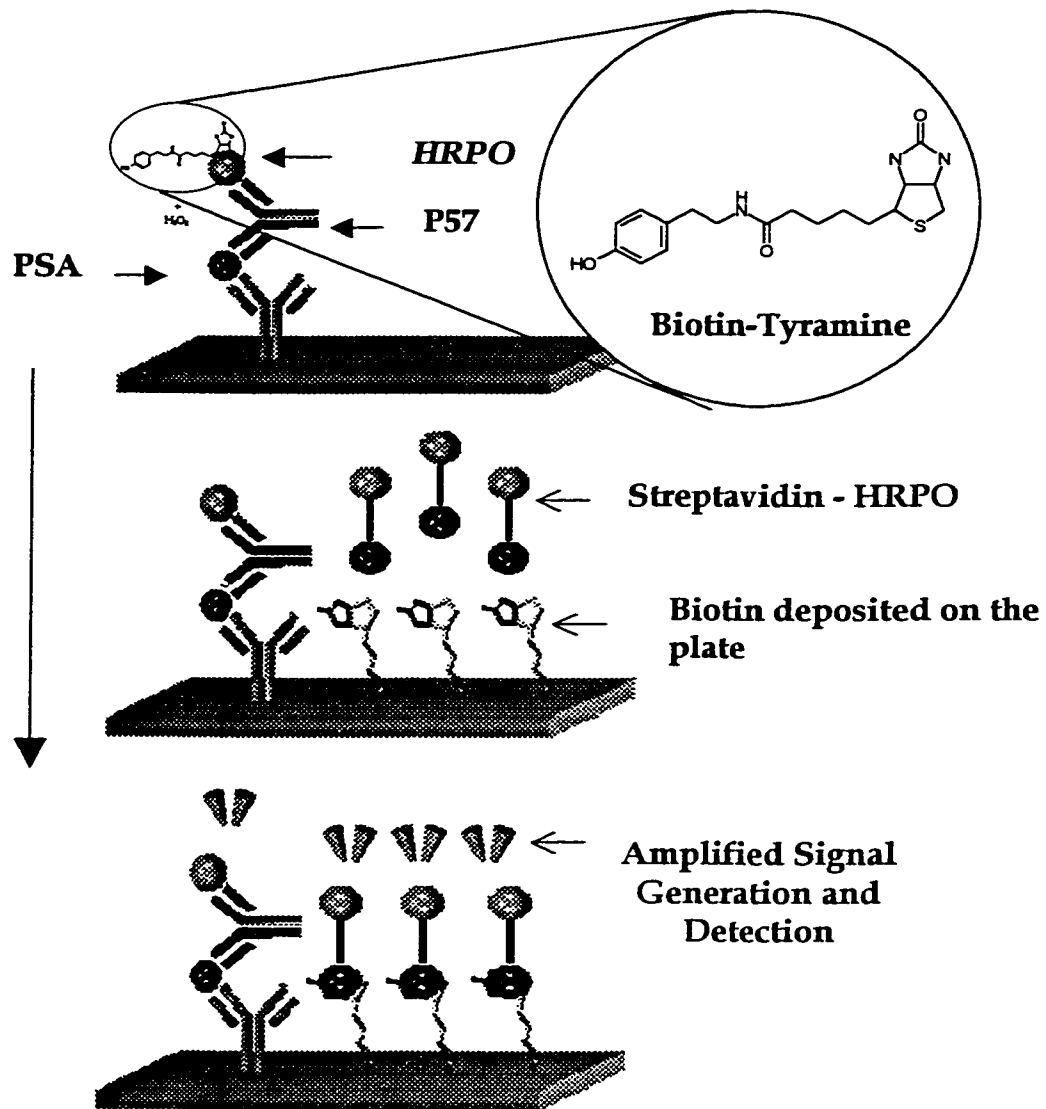
Figure 4.2. Catalyzed Reporter Depostion (CARD) – based immunoassay. In this enzyme amplification scheme, presence of HRPO catalyzes the deposition of a biotin-tyramine on the plate. The biotin is then available for binding in a subsequent streptavidin-enzyme label addition step.

were used both as blocking proteins and carrier proteins in all assay steps. In each well of a 96 well microtiter plate, 100 μL of B87.1 Mab (10 μg/mL) was incubated overnight at 4°C (16hr). The plate was washed twice with PBS-T. To one set of wells, 250 μL of 1% BSA was added, to the other set of wells, 250 μL of 1 % dBSA was added and allowed to incubate for 1 hr at 37°C. The plate was washed 3 times with PBS-T. To the dialysed and non-dialysed BSA-blocked wells, 50 μL of PSA standard (2 ng /mL) was added in triplicate and a zero standard (6% BSA or dBSA) was added in replicates of ten. Then 50 μl of P57 (1:50 in 6% BSA or dBSA) was added to each well and the plate was shaken for 30 min at RT. After incubation, the plate was washed 3 times with PBS-T and developed with TMB. The plate was stopped by the addition of 1 M H₃PO₄ after 5 minutes and the absorbance was read at 450 nm.

Reagent concentrations. Optimization of reagent concentrations was generally done starting from the last step of the sandwich assay and proceeding to the first. Each well was previously incubated with 100 μL of a 10 μg/mL solution of the B87.1 monoclonal antibody overnight at 4°C, then washed twice with PBS-T, blocked with 1% BSA for 1 hr at 37°C, washed 3 times with PBS-T, incubated with 10 ng/mL or 0 ng/mL of PSA in 6% BSA for 30 minutes, and then finally washed 3 times with PBS-T.

a) **Labeled-Monoclonal antibody optimization** Streptavidin-HRPO and the biotinylated B80.3 Mab were assessed for their optimal concentrations by using varying dilutions (streptavidin-HRPO: 1:200, 1:400, 1:800 and 1:1600) in a checkerboard assay against varying dilutions of the biotinylated Mab B80.3 (0.25, 0.5, 1, 10, 20 μg/mL). After a 15 min incubation period the streptavidin-HRPO labels were developed with a TMB substrate. Measurements for each streptavidin-label/biotinylated B80.3 were done in triplicate and compared with one background measurement (0 ng/mL PSA).

Combined concentrations of the streptavidin-label/biotinylated Mab

demonstrating the highest signal to noise ratio (average of three

measurements divided by the background) were used in all subsequent Mab

assays. The plate configuration of this assay is shown in Figure 4.3.

	Concentration of biotinylated Mab B80.3 (μg/mL)		
Conc. Of streptavidi n-HRPO	1	10	20
1:200	neg	neg	neg
1:400	neg	neg	neg
1:800	neg	neg	neg
1:1600	neg	neg	neg

Figure 4.3. Optimal concentration of streptavidin-HRPO label versus biotinylated antibody. To test the optimal concentration of the streptavidin-HRPO label and biotinylated Mabs, a checkerboard assay design was employed. After a 15 minute incubation period, optimal concentrations were assessed by averaging the three signal-generating wells and dividing by the background at each concentration.

b) **Optimal concentration of the labeled P57 antibody.** After each purification

of P57 antibody from the benzhydroxamic agarose column, an optimal

concentration was calculated by comparing the signal generated in a

standard sandwich assay using varying concentrations of P57. To each well of a 96 well microtiter plate, 100 µL of B87.1 Mab (10 µg/mL) was added and allowed to incubate overnight at 4°C. The plate was then washed twice with PBS-T and 250 µL of 1% BSA was added to each well for 1 hr at 37°C. To each well, 50 µL of the pooled purified P57 fraction was added at various concentrations (neat, 1:10, 1:50, 1:250, 1:1250, 1:6250 and 0 in 6% BSA/PBS). PSA (5ng/mL in 6% BSA) was added to half of the wells (50µL) and a negative control (6% BSA) was added to the other half. Both the test and negative control wells were performed in duplicate. After 30 minutes incubation, the plate was washed 3 times with PBS-T and developed with the commercially available TMB substrate.

c) **Optimal concentration of B87.1 capture-antibody for plate coating.** The optimal concentration of B87.1 was done last using a concentration range defined by Engvall (Engvall and Perlman, 1971). B87.1 was coated on a 96 well microtiter plate at 1, 5, and 10 µg/mL in PBS overnight at 4°C. The plate was blocked with 1% BSA and incubated for 1 hr at 37°C. The plate was then washed 3 times with PBS-T and PSA standards (50 µL of 0, 5, 10, 25, 50 and 100ng/mL in 6% BSA/PBS) were added to the wells in triplicate for each of the individual B87.1 concentrations. P57 was then added (50µL/well) at a previously determined optimal concentration (1:50 in 6% BSA/PBS) and the plate was allowed to incubate at RT for 30 minutes. The plate was then washed 3 times with PBS-T and developed with a commercially available TMB substrate. In this instance, the substrate reaction was not stopped but measured at 650 nm after 15 minutes.

PSA/P57 incubation time. To demonstrate differences in signal to noise for the detection of PSA with P57 at various incubation times, a 96 well microtiter plate

was incubated overnight at 4°C with B87.1 (5 µg/mL, 100µL /well). The plate was washed with PBS-T and 250 µL of 1% BSA/PBS was added to each well for 1 h at 37°C. The plate was then washed 3 times with PBS-T and 50 µL of P57 (at 1:10, 1:50, and 1:100 dilution) was added in triplicate to three sets of wells. Then 50 µL of PSA (1 ng/mL in 6% BSA) was added to half of each set and a negative control (6% BSA) to the other half. This was done at periodic intervals which represented 3, 2, 1, and 0.5 h incubation times. After incubation the plate was washed 3 times with PBS-T and developed with TMB. The TMB substrate reaction was stopped after 15 min and the absorbance at 450 nm was measured.

PSA standard curve to assess sensitivity. Standard curves for the ultrasensitive analysis of PSA were generally carried out using a calibrated 5 ng/mL standard which was successively doubly diluted in Eppendorf tubes in 6% BSA (d) prior to use. Each concentration of PSA was carried out in triplicate and the negative (0 ng/mL) controls were done using a minimum of 10 replicates so that the sensitivity of the assay could be assessed.

4.2.3.2   Catalyzed Reporter Deposition (CARD) Amplified Assay

Synthesis of the biotin-tyramine (BT) substrate. The BT reagent was synthesized from the addition of 0.1 mmol equivalents of NHS-LC-Biotin and tyramine added to 200µL DMSO and stirred overnight at RT according to a method by Bobrow and colleagues (Bobrow et al., 1989). The resultant mixture was aliquoted and stored at -20°C until it was needed further.

BT substrate optimal concentration determination. To determine the optimal concentration for of the synthesized BT reagent, 250 µL of 1% BSA was added to each well of a 96 well mcirotiter plate and incubated for 1 hr at 37°C. The plate

was then washed 3 times with PBS-T and 50 μL doubling dilutions of BT

substrate solution (doubling dilutions started with 30μL/mL of original DMSO

reaction mixture in Tris-HCl pH 8.0 with H_2O_2, 0.01% (v/v)) were added in

triplicate to the wells along with a 25 μg/mL HRPO in 1%BSA/PBS solution

which was added to the positive control wells. The plate was incubated at RT

for 15 minutes. The plate was then again washed 3 times with PBS-T and 100 μL

of a 1:5000 solution of streptavidin-HRPO in 6% BSA/PBS-T was added. The

plate was again shaken at RT for 15 minutes. The plate was then washed 5 times

with PBS-T and developed by the addition of the commercially available

colorimetric TMB substrate. A measurement was taken after 2 minutes. The

average of the three replicates was calculated and divided by the negative

control to give a signal to noise ratio. The concentration of BT substrate which

gave the highest signal to noise ratio was used for all subsequent assays.

**Determination of the optimal concentration of the streptavidin-HRPO label in
the CARD amplified assay.** Once the optimal amount of BT substrate was

determined, an optimal amount of streptavidin labeled HRPO was determined

by comparing the assay signal to noise for 5 ng/mL of PSA versus 0 ng/mL PSA

at various dilutions of the reporter enzyme. A 96 well microtiter plate was

coated with the B87.1 monoclonal capture antibody (5 μg/mL) overnight at 4°C.

The plate was then washed 3 times with PBS-T. To each well 250 μL of 1% dBSA

was added for 1 hr at 37°C. The plate was again washed 3 times with PBS-T. To

the positive control wells, 5 ng/mL of PSA standard in 6% dBSA was added and

6% dBSA without PSA was added to the negative control wells. The plate was

allowed to incubate on a shaker for 30 minutes at RT. The plate was then

washed 3 times with PBS-T. The two concentrations which previously showed

the highest signal to noise ratio were chosen. To half of the positive and negative

control wells, one of the previously determined optimal concentration (0.23

μL/mL) of BT substrate was added (predetermined optimal concentration of

DMSO reaction mixture in Tris-HCl pH 8.0 with 0.01% (v/v) H_2O_2). To the other half of the wells, the other previously determined optimal concentration (0.46µL/mL) was added. The catalyzed reporter deposition reaction was allowed to proceed for 15 min at RT (no shaker). The plate was then again washed 3 times with PBS-T and 100 µL of streptavidin-HRPO in 6% BSA PBS-T at 7 concentrations (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400) were added. The plate was shaken at RT for 15 minutes. The plate was then washed 5 times with PBS-T and developed by the addition of a colorimetric TMB substrate. The reaction was stopped by the addition of 1M H_3PO_4 (100µL) after 6 minutes. The average of the two replicates at each streptavidin-HRPO concentration was calculated and divided by the negative control to give a signal to noise ratio. The concentration of streptavidin-HRPO substrate which gave the highest signal to noise ratio was used for all subsequent assays for any particular batch of streptavidin-HRPO. Streptavidin-AP activity was assessed in the same manner.

Dialysis of the BSA Blocking Solution in the CARD assay. A comparison of the CARD amplified assay using dialysed and non-dialysed 1% BSA in PBS was carried out in a similar manner to the previous standard ELISA assay for comparison. 6% BSA was dialysed against 20 volumes of PBS, pH 7.2 with 5 changes of buffer. 1% BSA for blocking was made from this 6% solution by diluting in PBS 1:5. A direct comparison of dialysed 1% dBSA versus non-dialysed BSA was carried out in the blocking step using two P57 CARD-sandwich assays under identical conditions. The optimal concentrations of both P57 and B87.1 had been previously determined. In each well of a 96 well microtiter plate, 100 µL of B87.1 Mab (10 µg/mL) was incubated overnight at 4°C (16hr). The plate was washed twice with PBS-T. To one set of wells, 250 µL of 1% BSA was added, to the other set of wells, 250 µL of 1% dBSA was added and allowed to incubate for 1 hr at 37°C. The plate was washed 3 times with PBS-T. PSA (2ng/mL) was then added in triplicate to the dBSA and BSA

blocked wells. Dialysed BSA 6% was then added to 10 wells as a negative control for each blocking condition. Then 50 μl of P57 (1:50 in 6% dBSA) was added to each well and the plate was shaken for 30 min at RT. After incubation, the plate was washed 3 times and the BT substrate (pre-determined optimal concentration in Tris-HCl buffer, pH 8.0 with 0.01% H_2O_2 (v/v)) was added and allowed to incubate at RT for 15 minutes. The plate was then washed 3 times with PBS-T and streptavidin-HRPO (1:500) in 6% dBSA/PBS was added for 15 minutes at RT. The plate was then washed 5 times with PBS-T and developed with TMB. The plate was stopped by the addition of 1M H_3PO_4 after 5 minutes and the absorbance was read at 450nm.

BT substrate incubation time. Although the original recommendation for incubation time of the BT substrate was 15 minutes (Bobrow et al., 1989), a comparison of longer incubation times with the goal of achieving heightened sensitivity was carried out. A 96 well microtiter plate was coated with 250 μL 1% BSA for 1 hr at 37°C. The plate was washed 3 times with PBS-T. To three sets of wells 50 μL of HRPO 25 μg/mL in 1% BSA was added in successive doubling dilutions. To one set of wells, 50 μL of BT substrate at a predetermined optimal concentration (DMSO reaction mixture diluted in Tris-HCl, pH 8.0 with 0.01% (v/v) H_2O_2) was added and allowed to incubate for 60 minutes at RT (no shaker). Subsequently BT substrate was added for 30 minutes and 15 minutes for the remaining two sets of wells. After incubation the plate was washed 3 times with PBS-T. To each well, 100 μL of streptavidin-HRPO (1:5000 in 6% BSA/PBS-T) was added and shaken at RT for 15 minutes. The plate was then washed 5 times with PBS-T and developed with the commercially available TMB substrate. An absorbance measurement at 650 nm was taken after 2 minutes.

Incubation time for the CARD assay. Two CARD assays were run in parallel to determine the effect of longer incubation times of PSA and P57 on the assays overall sensitivity. A 96 well microtiter plate was coated with B87.1 monoclonal antibody overnight (~16 h) at 4°C. The plate washed twice with PBS-T and blocked with 1% dBSA for 1 hr at 37°C. The plate was then washed three times with PBS-T and PSA was added to the plate in triplicate at two concentration ranges. For the 3 hr incubation time, 50 μL of PSA (1.0, 0.5, and 0.05 ng/mL in 6% dBSA) was added in triplicate with the negative control added in replicates of 10. Two hours later, 50 μL of PSA (1.25, 0.625, and 0.3125 ng/mL in 6% dBSA) was added in triplicate with the negative control added in replicates of 10. The bsMab P57 (1:50, 50 μL/well) was added just prior to the addition of PSA in both instances. After incubation, the plate was washed 3 times with PBS-T and BT substrate (pre-determined optimal concentration in Tris-HCl with 0.01% v/v H_2O_2) was added for 15 minutes. The plate was again washed three times with PBS-T and 100 μL of streptavidin-HRPO (1:800 in 6% BSA/PBS-T) was added for 15 minutes. The plate was developed with the commercially available TMB substrate for 5 minutes and stopped by the addition of 1 M H_3PO_4. An absorbance measurement at 450 nm was then taken.

Catalyzed reporter deposition standard curve for PSA. Once the optimal reagent concentrations and for both BT and the streptavidin-enzyme label were elucidated, the assay was carried out to generate a standard curve using a concentration range of PSA from 0 to 5 ng/mL. Each concentration of PSA was carried out in triplicate and the negative (0 ng/mL) was done using 10 replicates to properly assess the assay sensitivity (LLD).

4.3 RESULTS AND DISCUSSION

4.3.1 Assay Development

The monitoring of PSA after radical prostatectomy represents one of the most important uses of the tumor marker assay to date. Theoretically, following the total removal of the prostate, serum PSA levels should be undetectable or close to zero. Very early recurrence of prostate cancer metastasis could be potentially detected several months ahead of time by an ultrasensitive assay compared to conventional current clinical PSA assays. Since the idea was first proposed in 1988 (Oesterling et al., 1988), a number of assays have demonstrated this (Graves, 1992; Graves et al., 1992; Stamey et al., 1993; Vessella, 1993; Yu and Diamandis, 1993; Khosravi et al., 1995; Yu et al., 1997). In particular, assays which have demonstrated LLDs in the ultrasensitive range (between 0.01ng/mL and 0.1 ng/mL) have been demonstrated to be effective in predicting the recurrence of disease in patients who have undergone radical prostatectomy (Schild et al., 1996; Diamandis, 1997). To achieve detection limits in this ultrasensitive range, a number of principles underlying immunoassay technology were explored using Mabs and bsMabs that were previously developed for the detection of PSA in our laboratory. These include evaluating the assay configuration, antibodies used, incubation times of reagents, reagent concentrations, and choice of substrate for development.

The detection of PSA using the P57 bsMab and the B80.3 Mab previously were all performed using a sandwich assay (two-site non-competitive) format and a colorimetric substrate (Jette et al., 1996; Kreutz and Suresh, 1997; Xu et al., 1998). The sandwich assay format was not changed for the design of the ultrasensitive assay. Sandwich assays employing matched monoclonal antibodies have previously been demonstrated both practically and in theory to offer the highest sensitivity when detecting an analyte. The affinity of the antibodies for the antigen as well as the ability of the antibodies to form a

sandwich pair (i.e. binding to different sterically and spatially distinct epitopes on the antigen) governs the sensitivity. The B87.1 and B80.3 Mabs were previously shown to bind to two separate epitopes on the PSA molecule with high affinities (Jette et al., 1996). They were ideally suited to be the basis of developing an assay achieving sensitivities in the ultrasensitive range. Both P57 and P105 quadromas were products of the B80.3 parental cell line and thus harboured the same B80.3 paratope region. Bispecific antibodies have a uniform 1:1 labeling of enzyme to antibody and batch-to-batch reproducibility and are generally able to demonstrate lower limits of detection equivalent to their Mab counterparts over equal or larger concentration ranges (Kricka, 1994). Because reconfiguring an immunoassay for heightened sensitivity often compromises the measurable concentration range of an analyte (Khosravi et al., 1995), it was reasoned that the development of an ultrasensitive assay using these highly consistent immunoprobes would have some distinct advantages.

Plate coating. The first step in all of the sandwich assays that were explored was the coating of a 96 well microtiter plate with the B87.1 antibody. Phosphate-buffered saline, pH 7.2-7.4, was used for all of the experiments and the plates were incubated consistently for a minimum of 16 h at 4°C as suggested by Engvall and Perlmann (Engvall and Perlman, 1971). Plates were never allowed to incubate longer than 24 h. An exploration of different buffer conditions was attempted. Both bicarbonate buffer (pH 8.0) and Tris-HCl (pH 7.8, as suggested by Diamandis and colleagues (Diamandis et al., 1996)) were utilized when coating conditions were suspect due to assay inconsistencies but no significant advantages were observed in these experiments with either buffer.

Blocking. Blocking is an essential step in any immunoassay as it saturates the remaining unoccupied binding sites in the polystyrene wells after the coating of the plate with Mab. This essential second step in all of the immunoassays

evaluated prevents absorption of analyte or reporter enzyme to the plate that could lead to high backgrounds and reduced sensitivity. Various proteins have been utilized for blocking in the past including BSA (1-5%), gelatin (0.02-0.1%), or casein (1%) alone or in combination with Tween-20 (Pathak et al., 1997). In the preliminary explorations of this PSA assay, dried milk powder (1 - 5%) was used to block the plates but was substituted with BSA (1%) when problems with dissolution (clumping in wells etc.) were discovered. The transparency of the BSA solution allowed for a visual assessment of the dissolution before adding it to the plate. All blocking was done for a duration of at least 1 but no more than 2 h at 37°C. BSA was thought to be an ideal choice for plate coating because it has been demonstrated that BSA post-coating and addition of BSA to serum diluent decreases background readings and increases specific activity (Schonheyder and Andersen, 1984; Pruslin et al., 1991). Because of the potential for contaminants such as bovine immunoglobulin, however, reservations about its use have been expressed (Smith et al., 1993).

To further diminish background when using BSA as blocking solution, dialyzing of the BSA solution before blocking was attempted. It has been reported that small molecules including biotin may contaminate BSA and could theoretically increase non-specific binding in immunoassays (Diamandis et al., 1996). This is a unique concern in assays that involve streptavidin-enzyme labels because they can directly bind bound or free biotin and lead to inconsistencies between assays. To test this hypothesis, two assays were compared: the P57 based assay, which does not involve the use of streptavidin labels; and the P57 CARD assay which relies on the biotin streptavidin interaction for producing an amplified signal. Under identical conditions, dialyzed BSA solution did make a difference in the CARD assay background but no difference was apparent in the regular P57 assay. (Figure 4.4)
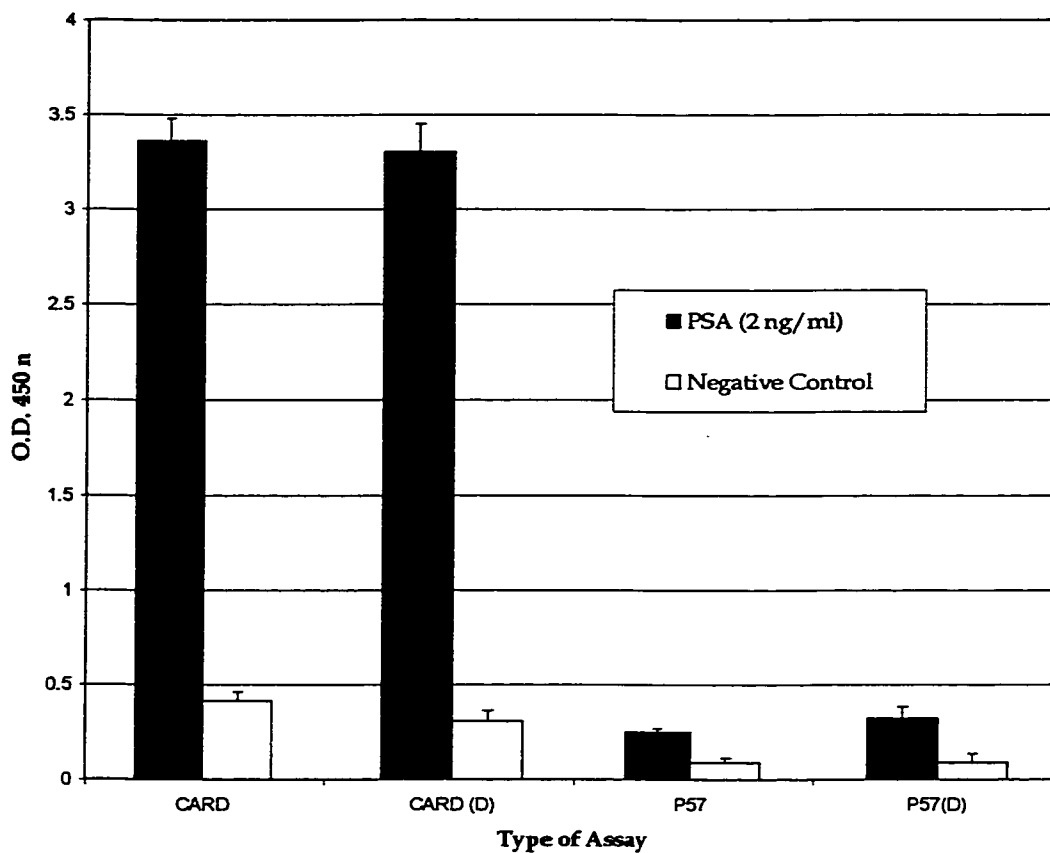
Figure 4.4. Comparison of Standard P57 and CARD assays using dialyzed vs. non-dialyzed BSA. Both P57 and CARD assays were compared on the same plate under identical conditions using either dialyzed or non-dialyzed BSA 1% as a blocking buffer. Dialysis of BS will eliminate BSA-bound biotin that could theoretically increase backgrounds of assays that involve streptavidin-enzyme labels. Each assay compared the signal generated by HRPO using TMB. After 5 minutes, the assays were stopped with the addition of 1 M, H_3PO_4 and measured at 450 nm.

P57-based sandwich assay. The P57-based sandwich assay uses the labeled, purified anti-HRPO x anti-PSA bsMab generated by the P57 hybrid-hybridoma which has previously been described (Kreutz and Suresh, 1997). A novel purification of P57 that is already bound to HRPO was accomplished using the benhydroxamic agarose column that is discussed in detail in Chapter 3. Because the HRPO label with full enzyme activity (Kreutz, 1997 Ph.D.Thesis) is already bound to one paratope of the bsMab, optimization of the detection reagent is straightforward. For each batch of purified bsMab, an assay is performed at a given concentration of PSA using a number of dilutions of the pooled, purified fractions. Figure 4.5 shows the result of one such assay that compares the pooled fractions from two runs. The optimal dilution is taken to be the point where the signal is saturated, that is an O.D. 450 nm absorbance reading of ~ 3.0.

As the ideal conditions of the assay were performed from the last step to the first, as suggested by Diamandis (Diamandis et al., 1996), an optimal concentration of the plate-coated B87.1 antibody was also performed. Using the concentration range of 1-10 µg/mL, and performing three standard curves at each plate-coat concentration, a comparison of the different coating conditions revealed no significant difference between the coating conditions (Figure 4.6). Although in theory this suggested 1 µg/mL was as effective as 10 µg/mL for plate coating, most subsequent assays were performed at a conservative 5-10 µg/mL concentration to ensure antigen binding.

Once the reagent conditions of the assay were determined, a final evaluation of incubation time was performed. Ultrasensitive assays developed for PSA generally have utilized incubation times anywhere from 11 min to 3
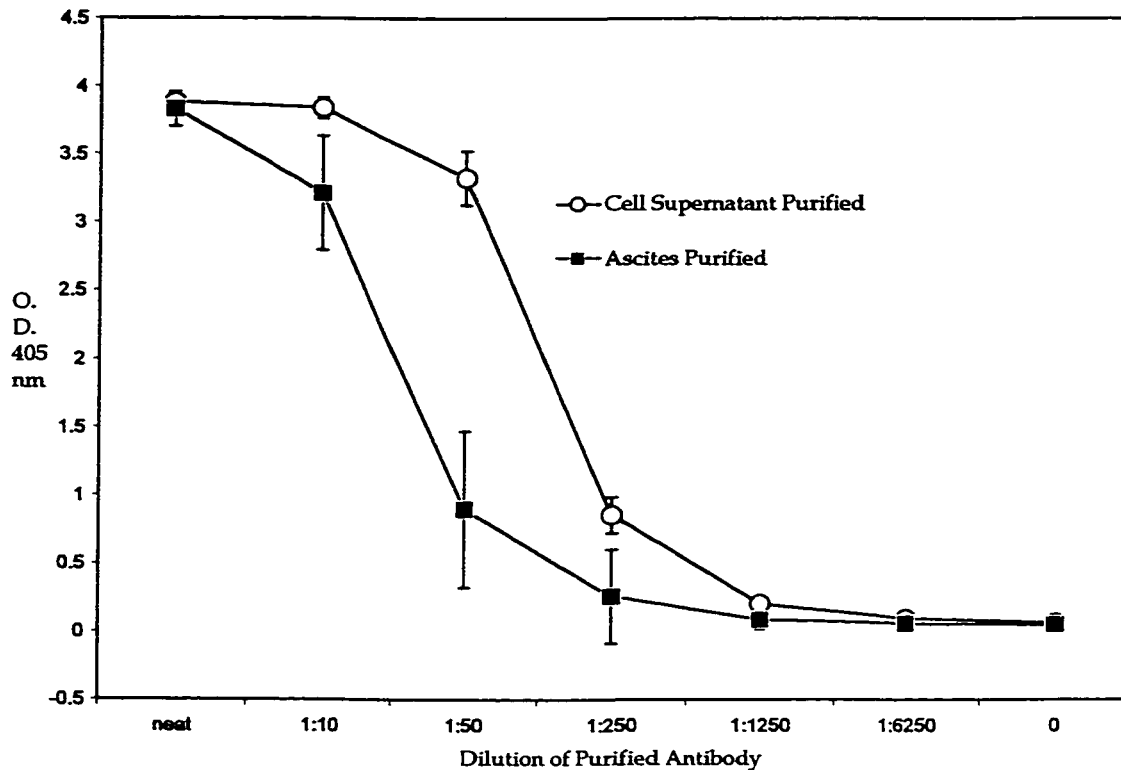
Figure 4.5. Optimization of labeled P57 antibody concentration. The optimal concentration of P57 antibody for each purified batch was estimated by performing a standard sandwich assay using varying amounts of purified antibody. Plates were coated with capture antibody and blocked in the usual way. PSA (10 ng/mL) was added to half of the wells and a negative control to the other half. The prepared antibody was then added to each well in triplicate at various dilutions. The assay was developed with TMB and stopped, and the absorbance at 450 nm was measured. An optical density above 3 was considered adequate for dilution of the antibody. The optimization of the alkaline-phosphatase-labeled P105 antibody was performed in a similar fashion.
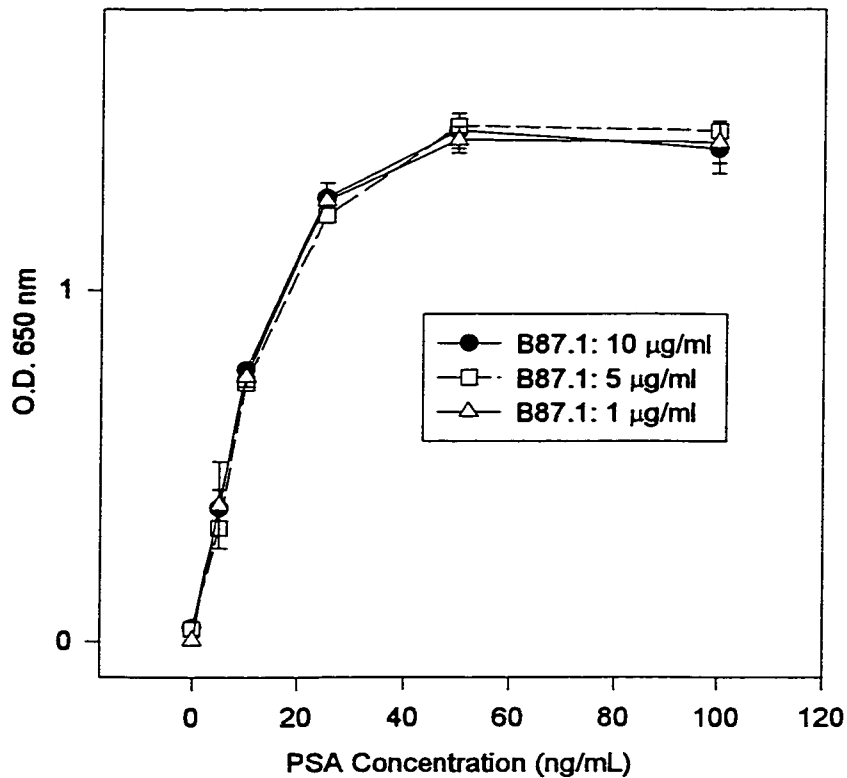
Figure 4.6. Optimization of B87.1 plate-coated monoclonal assay.
The optimal concentration of B87.1 for plate coating was determined
by three standard curve sandwich assays in parallel using three
different plate-coating conditions. Each of the assays used the P57
bispecific antibody for PSA detection and were developed with TMB.
The reaction was not stopped, but the absorbance measurement of the
650nm chromogen was taken after 15 minutes. The coating
concentrations were selected based on optimal concentrations
reported by Engvall (Engvall, 1971)

days, to ensure complete formation of the antibody-antigen immunocomplex (Prestigiacomo and Stamey, 1994). This assurance of complete binding contributes to the linearity and sensitivity of the assay. Increasing incubation time is one of the most simplistic ways to increase assay sensitivity (Khosravi et al., 1995; Diamandis et al., 1996). Figure 4.7 illustrates this point. No distinction can be made between 1 ng/mL and 0 ng/mL of PSA in the standard sandwich assay using colorimetric detection after just 30 minutes of complexation. After 3 hours however, the signal observed at 1 ng/mL is significantly larger than the background signal. When the 3 h incubation was applied to the sandwich assay using standards, a 10 fold increase in overall assay sensitivity was observed (Figure 4.8).

CARD amplified P57 assay. To further increase the signal observed in the standard P57 sandwich assay, a modification of the assay using catalyzed reporter deposition (CARD) was performed. Catalyzed reporter deposition was first described by Bobrow et al. (1989) and involves using the HRPO reporter enzyme to catalytically deposit biotin-tyramine on to the protein-coated well (Figure 4.2). The P57 bsMab was ideally suited for this assay because a consistent one-to-one labeling of the reporter enzyme to antibody should theoretically allow for more consistency in amplification.

The basis of this assay is the utilization of biotin-tyramine as a substrate. Tyramine is a hydroxyphenyl compound that was first observed to dimerize in the presence of H_2O_2 and HRPO during an investigation of potential HRPO fluorogenic substrates (Zaitsu and Ohkura, 1980). Theoretically, as the concentration of tyramine in the presence of $HRPO/H_2O_2$ is reduced, the highly reactive tyramine falls below a dimerization threshold and reacts with hydroxyphenyl compounds including tryptophan and tyrosine residues that are present or near the HRPO enzyme. By using a biotin-tyramine conjugate instead of tyramine alone, biotin-tyramine (BT) is deposited on the plate and can be
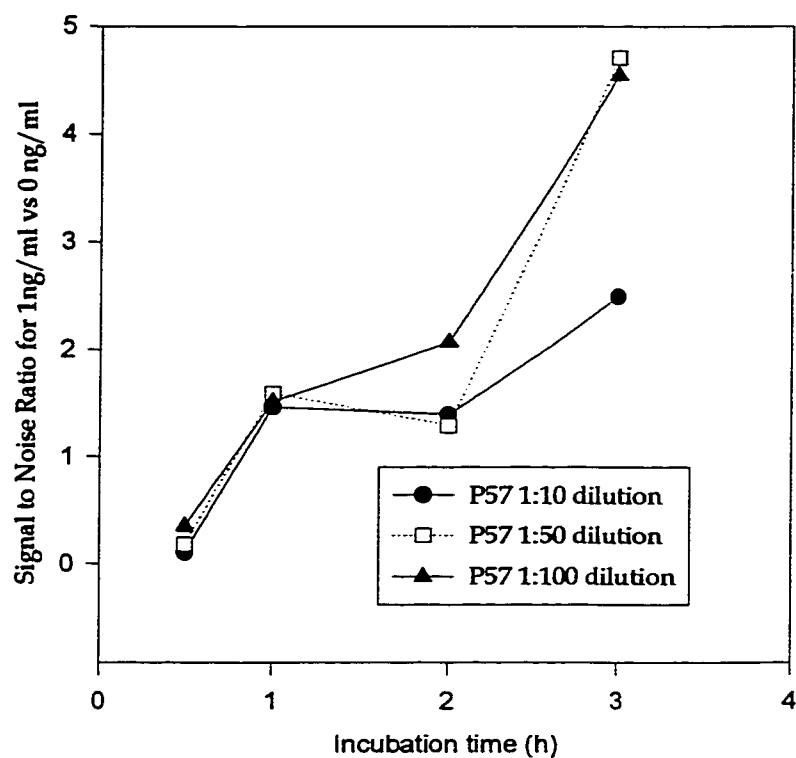
Figure 4.7. PSA incubation time and sensitivity. To demonstrate the effect of incubation time of PSA and the P57 bispecific detection antibody, a standard assay of 1 ng/mL PSA was performed using three different concentrations of P57 detection antibody and three different incubation times. The detection of PSA was accomplished using a TMB substrate, which was stopped with 1 M H_3PO_4 after 15 minutes. A signal to noise ratio was calculated by dividing the averaged measured optical density for 1 ng/mL PSA by the averaged measured optical density at 0 ng/mL PSA.
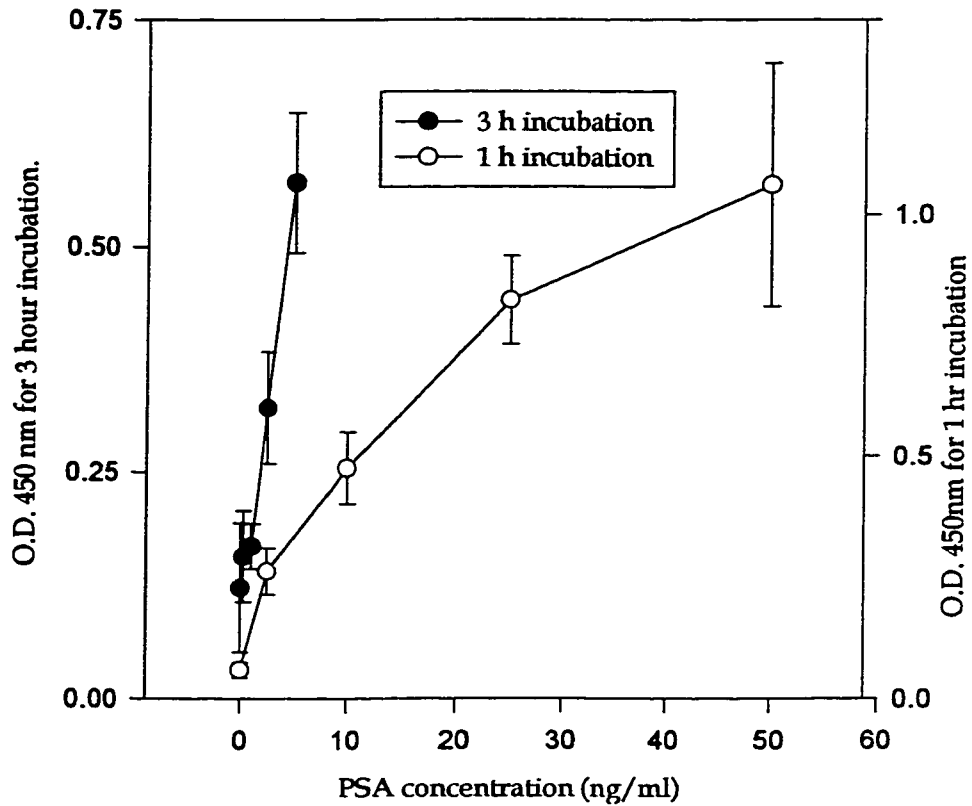
Figure 4.8. PSA incubation time and sensitivity. A higher signal to noise ratio after 3 hours results in a greater dynamic range at low PSA concentrations and a greater sensitivity. This standard assay was performed on the same plate using P57 at previously optimized concentrations.
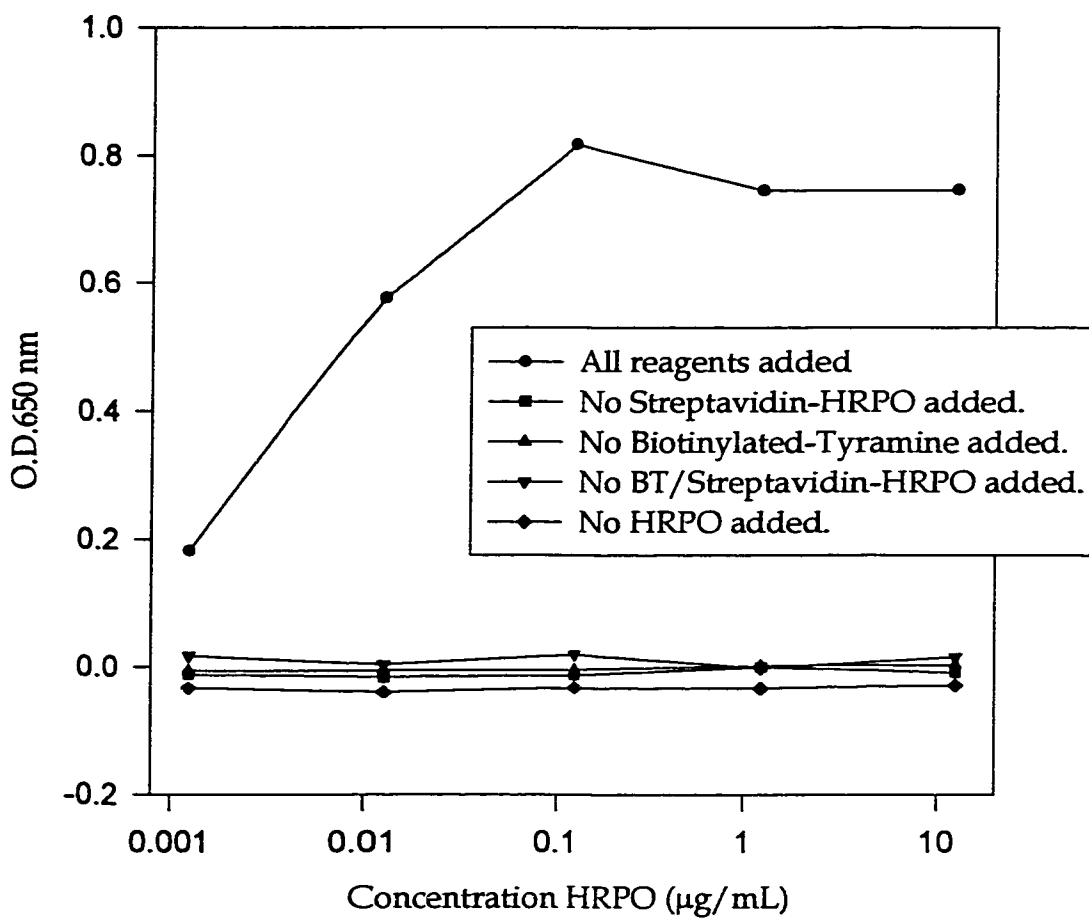
Figure 4.9. BT substrate negative controls. To demonstrate the importance of all components in the CARD assay system, a 96 well microtiter plate was first coated with a protein solution (1% BSA) and incubated with the BT substrate along with 10-fold dilutions of HRPO.

detected by the simple addition of a streptavidin-enzyme label. Figure 4.9 illustrates the effect of the variouscomponents on the ability to detect HRPO. If any one of the reagents is omitted, no signal is observed.

After synthesizing the BT substrate, the ideal concentration was determined by using a series of dilutions of BT in the presence or absence of HRPO. Doubling dilutions of the BT reagent carried out in triplicate revealed a peak in the signal to noise ratio at a very low concentration ($0.23 - 0.46$ $\mu l/mL$) of the reagent (Figure 4.10). Because of inconsistencies between batches of synthesized BT, and since pure BT was not isolated from the residual reactants, this optimization assay needed to be performed each time BT was synthesized. It should be noted that this optimal concentration of BT changed after only three months of storage at $-20^\circ C$, drastically affecting its performance, and had to be reassessed accordingly. After 1 year of storage at minus $20^\circ C$, the optimal concentration increased from $0.23 - 0.46$ $\mu l/mL$ to $1.25 - 2.5$ $\mu l/mL$ suggesting degradation with time.

To further evaluate the assay, a number of different dilutions of streptavidin-HRPO were utilized for a P57-CARD assay to compare the observed signal at 5 ng/mL PSA with that of 0 ng/mL. It was already observed that the background could be reduced slightly if dialyzed BSA was used instead of regular BSA for blocking (see Figure 4.4). It was hoped that a lower concentration of streptavidin-HRPO could reduce background noise levels even further without compromising the signal. Figure 4.11 demonstrates the effectiveness of lowering streptavidin-HRPO concentration while performing the P57-CARD assay in triplicate at the two different PSA concentrations. Decreasing streptavidin concentration only served to decrease the background slightly, but at the expense of diminished signal. From these data, little significant differences in streptavidin-HRPO concentration could be ascertained, but a 1:800 dilution gave the highest signal-to-noise ratio and was thought to be a reasonable choice.
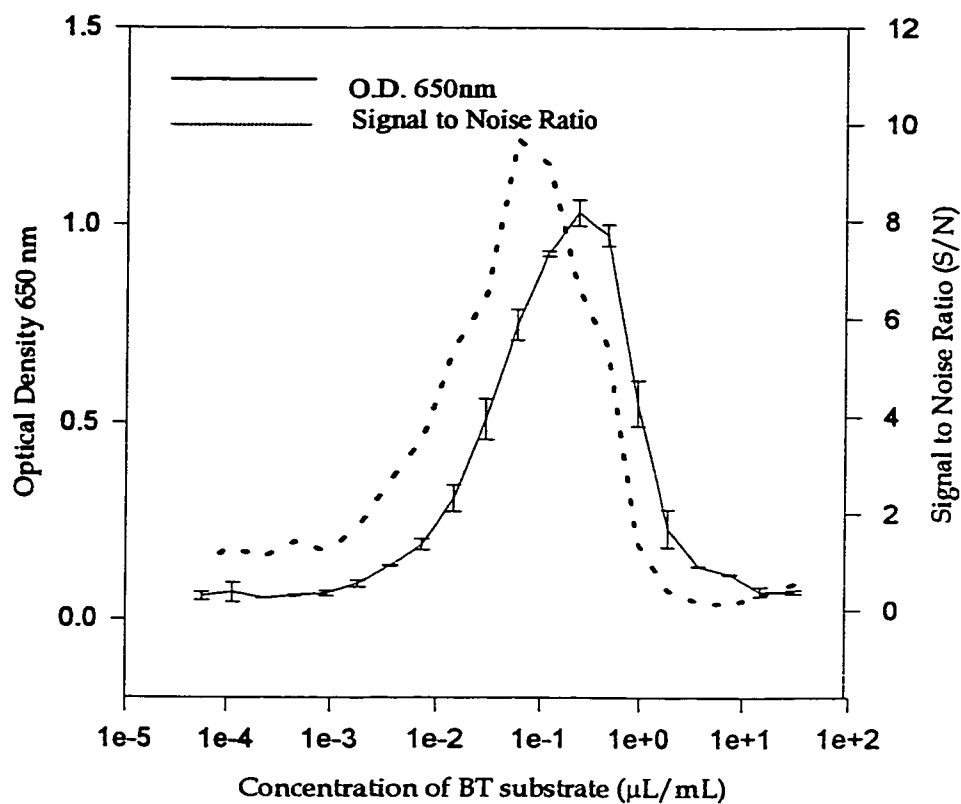
Figure 4.10. BT substrate concentration optimization. Synthesized BT substrate was added to a BSA-blocked 96-well microtiter plate containing either HRPO or a negative control. The BT substrate was doubly diluted on the plate starting with a 30 μL/mL concentration in Tris-HCl pH 8.0 buffer with 0.01% H_2O_2 added. An optimal concentration is theoretically achieved when the BT substrate falls below a dimerization threshold and begins to react with free hydroxyphenyl residues (Tyrosine, Phenylalanine) on the plate. The biotin-labeled protein on the plate can then be detected by streptavidin-labeled enzyme. In this case streptavidin-HRPO was used.
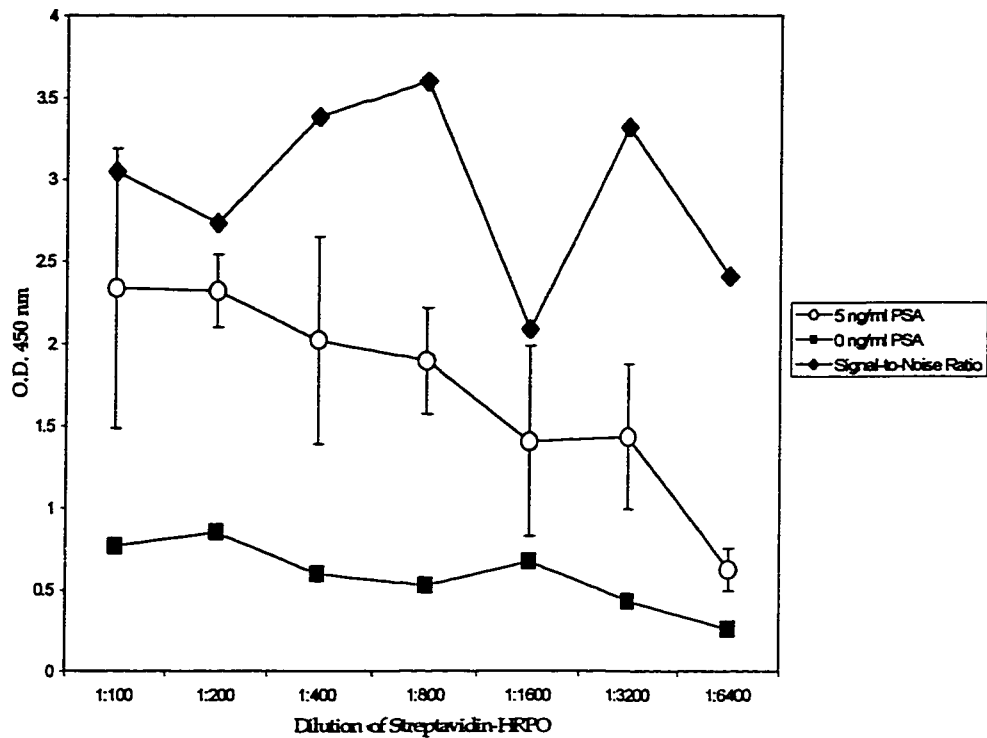
Figure 4.11 Streptavidin-HRPO Concentration for CARD. After the optimal concentration of BT substrate was determined, a test to determine the optimal concentration for streptavidin-labeled enzyme reporter was carried out. In this instance, the CARD assa was allowed to proceed in wells that contained either 5ng/mL PSA or 0 ng/mL PSA. After deposition of biotin on the plate, various concentrations of streptavidin-HRPO were added and allowed to incubate at RT for 15 minutes.

These data also show that the backgrounds in the P57-CARD assay are significantly higher when compared with those of the conventional assay. Typically, backgrounds in the CARD amplified assay are between 0.5 and 1.0 when using TMB stopped with H_3PO_4. Backgrounds for the conventional (no amplification) P57 assays are 10-fold less; often, 10 replicates of zero will average between 0.04 and 0.07 O.D. There may be several explanations for this including non-specific binding or catalysis of deposition by endogenous peroxidase. Diamandis (Diamandis, 1991) has suggested that a signal amplification of 10- to 30-fold could be achieved using the BT substrate system but the detection limit was only improved 3 to 6 fold because of an increase in background (Diamandis, 1991). This phenomenon was similarly observed when the P57 and P57-CARD assays were compared.

High backgrounds are further exaggerated when the BT substrate is allowed to incubate for longer times. Because the standard incubation time of BT was determined to be 15 min, a comparison of different $BT/HRPO/H_2O_2$ incubation times was carried out. Figure 4.12 shows the result of this comparison. It was hoped that longer incubation times would amplify the presence of HRPO at lower concentrations. Instead, higher background is seen when the BT substrate is allowed to react for 60 minutes compared with the similar, lower backgrounds observed at 15 and 30 minutes. No significant difference is observed between the 15 min and 30 min incubation times in the 3 log linear dynamic range. Because of this, 15 min was selected as the optimal time for incubation. The assay was then performed at 1 h and 3 h incubation times, just as in the P57 assay, for a matter of comparison. The data demonstrated heightened sensitivity and a reduced LLD in the 3 h assay when compared with the 1 h assay. (Figure 4.13)
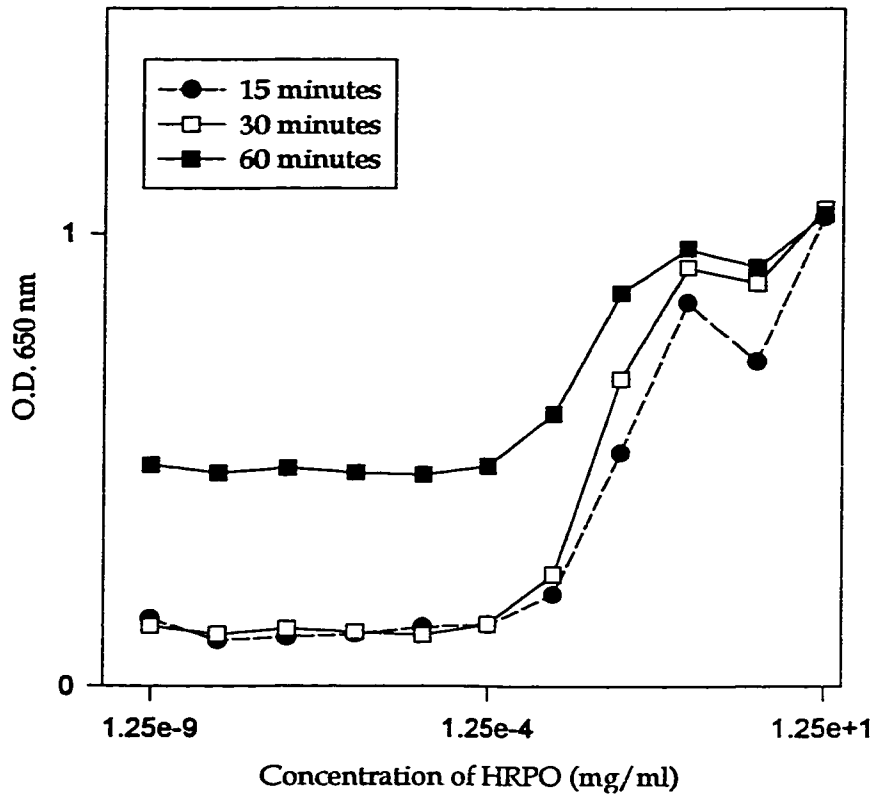
Figure 4.12. BT substrate incubation time. To determine if longer incubation times increase the linear range of detection, a 96 well microtiter plate was first coated with a protein solution (1% BSA) and incubated with the BT substrate along with doubling dilutions of HRPO.
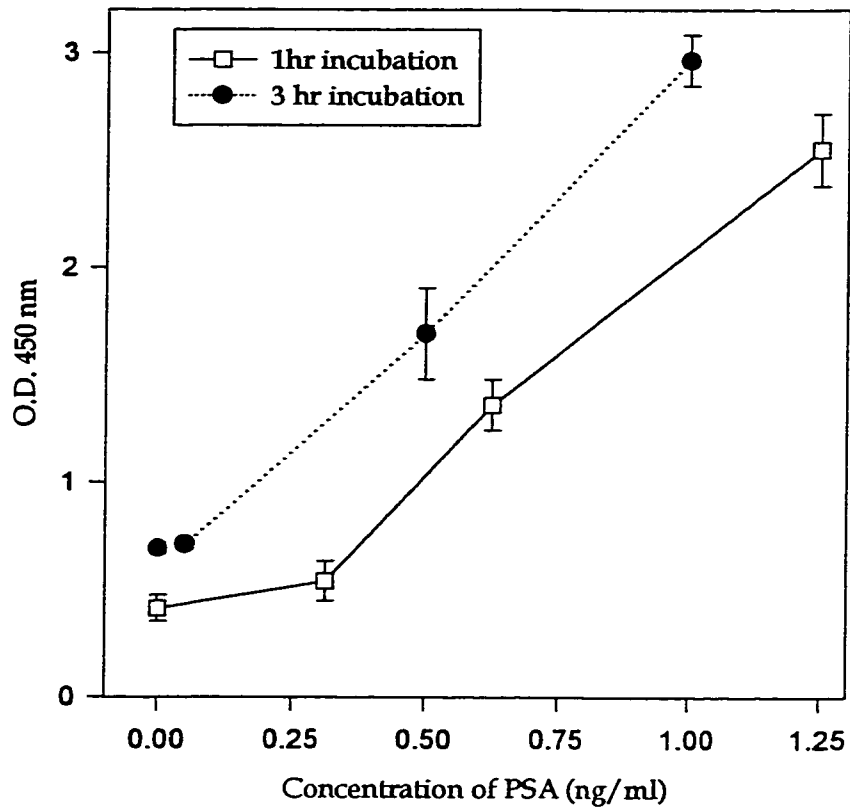
Figure 4.13: PSA incubation time and sensitivity. To demonstrate the effect of incubation time of PSA and the P57 bispecific detection antibody, two CARD assays were run on the same plate using 3hr and 1hr incubations of PSA and the P57 bispecific antibody. Both were then subjected to identical BT substrate and streptavidin-HRPO conditions and developed with TMB. An increase in background for the negative control was also seen. Each PSA concentration was performed in triplicate with the negative control (0ng/mL) being performed in replicates of 10.

B80.3 Mab-based assay. Because of the extra preparation and equipment involved in producing them, bsMabs are not readily available to every researcher. For this reason, the B80.3 Mab, which was the original sandwich partner of B87.1, was also evaluated for its usefulness in an ultrasensitive immunoassay. There are some inherent advantages to using monoclonal antibodies. They are faster to produce, easier to purify, and, due to their bivalent nature, will usually have slightly higher avidities for the antigen than their bsMab progeny. The Mab also gives the researcher a choice

of reporter enzymes for labelling. There are, however, some disadvantages to using Mabs. Direct enzyme labelling techniques or indirect techniques such as biotinylation can modify the active binding site and decrease the affinity for the antigen (Kricka, 1994). In addition, the labelling process tends to be inconsistent, and variations from batch to batch are inevitable (Diamandis and Christopoulos, 1991). Because of its simplicity and reliability, biotinylation of the detecting antibody has become the most popular method of attaching an enzyme to an antibody. The use of biotin and the use of streptavidin could also result in steric hindrance, increased non-specific binding and inconsistencies in antibody-antigen binding. The scheme evaluated here is solid-phase→B87.1→PSA→B80.3-biotin→streptavidin-HRPO (B80-HRPO) and is shown in Figure 4.1.

In order to evaluate the utility of the assay, an evaluation of the reagent concentrations that would be useful for the detection of PSA at ultrasensitive levels was performed. As in the case of P57, evaluation was carried out from the last step to the first. In the case of B80-HRPO, there were two reagents involved in detection and a checkerboard assay was performed. The concentration range of B80.3 (1,10, and 20 µg/mL) was chosen based on an assumption that the plate coated Mab and detecting Mab should be roughly equimolar (10 µg/mL). The concentration range of streptavidin-HRPO (1:200 – 1:1600) was based on the

manufacturer's suggested concentration range (1:500). The results of the checkerboard assay in Figure 4.14 show a definite trend toward a higher signal to noise ratio at all B80.3 concentrations as the streptavidin-HRPO is increasingly diluted. This is explained by lower backgrounds in the raw data at lower concentrations of streptavidin-HRPO. Likely, the streptavidin-HRPO exhibits some degree of non-specific binding to the protein in the microtiter wells which is not rectified by washing with PBS-T. Interestingly, the higher concentrations of B80.3 exhibited a levelled or diminished signal at the lowest concentration of streptavidin-HRPO. The best signal-to-noise ratio was exhibited by the combination of 1μg/mL of the biotinylated B80.3 and a 1:1600 dilution (625 pg/mL) of the streptavidin-HRPO reagent and this was used for all subsequent assays involving B80-HRPO.

After the working concentrations of biotinylated B80.3 and streptavidin-HRPO were determined, it was assumed that the assay would behave in a similar fashion to the previous P57 assay and no significant differences would be seen between 1 and 10 μg/mL of the plate coated B87.1 antibody. The assay was then performed at 1 h and 3 h incubation times using colorimetric and fluorometric substrates, just as in the P57 assay, for a matter of comparison. The data once again demonstrate heightened sensitivity and a reduced LLD in the 3 h assay when compared with the 1 h assay. (Figure 4.15)
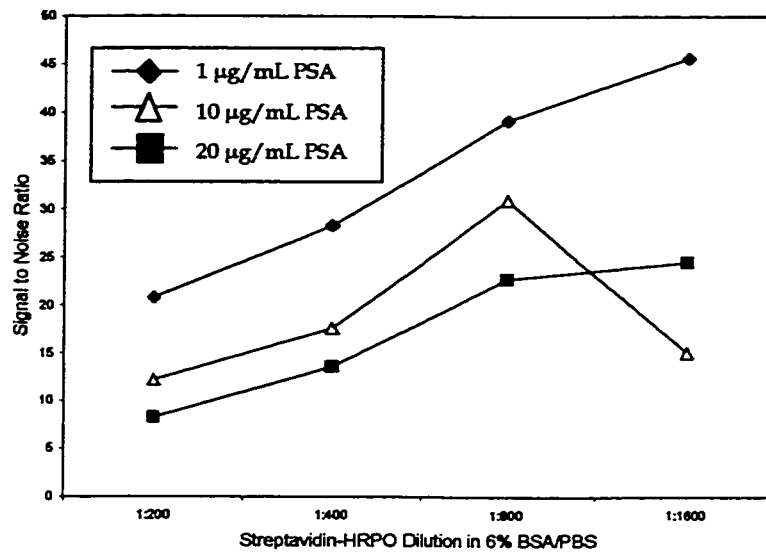
Figure 4.14. Optimization of labeled B80.3 and streptavidin-HRPO reagent concentrations. The optimal concentration of the biotin labeled B80.3 monoclonal and streptavidin-AP reporter enzyme was performed by a checkerboard assay. The signal generated from wells coated with B87.1 capture antibody, blocked with 1% BSA, and incubated with 10 ng/mL of PSA in triplicate were compared with negative controls under identical conditions. Optimal concentrations were then used for subsequent standard curve assays
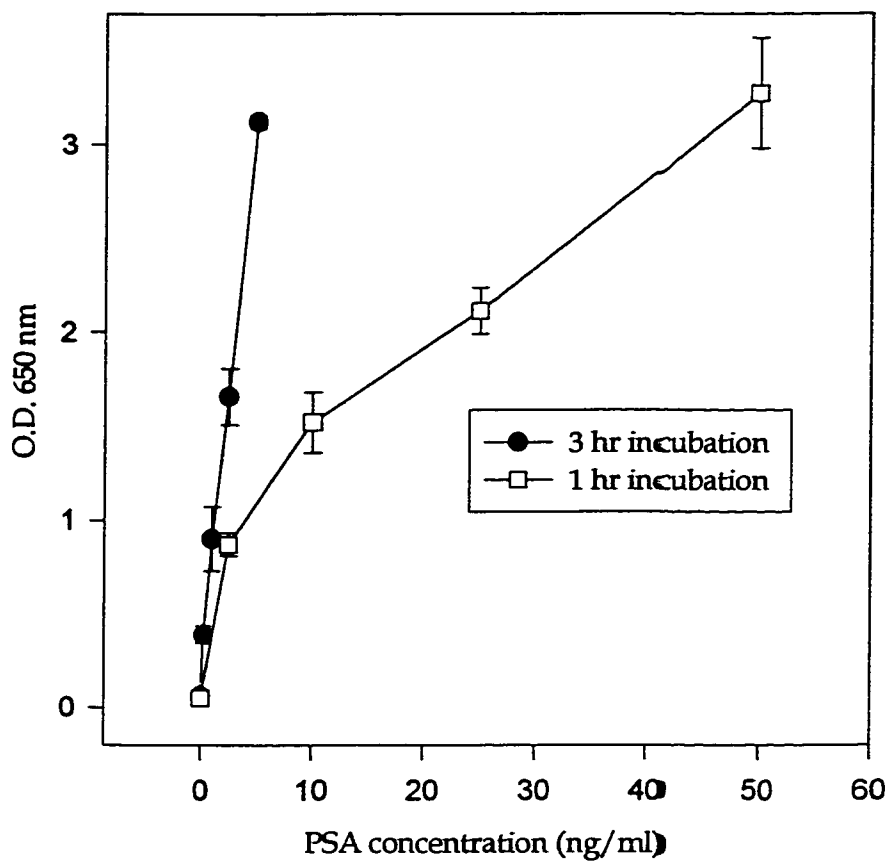
Figure 4.15. PSA incubation time and sensitivity for the B80.3-HRPO Mab-based assay. A higher signal to noise ratio after 3 hours results in a greater dynamic range at low PSA concentrations and a greater sensitivity. This standard assay was performed on the same plate using biotinylated B80.3 and streptavidin-HRPO at previously optimized concentrations.

Ultrasensitive PSA assay comparison						
Assay	Antibody Type	Substrate Type	Linear Range (ng/mL)	Incubation Time	Vol/ Assay (µl)	LLD (ng/mL)
P57	bsMab	Colorimetry	0 -25	1 h	50	0.056
B80.3	Mab	Colorimetry	0-10	1 h	50	0.047
B80.3	Mab	Colorimetry	0-5	3 h	50	0.024
P57	bsMab	Fluorometry	0-2.5	1 h	50	0.031
P57	bsMab	Fluorometry	0-0.1	3 h	50	<0.006
B80.3	Mab	Fluorometry	0-1.25	1 h	50	0.011
B80.3	Mab	Fluorometry	0-0.1	3 h	50	<0.006
P57-CARD	bsMab	Colorimetry	0-1.5	1 h	50	0.411
P57-CARD	bsMab	Colorimetry	0-1.5	3 h	50	0.070

Table 4.2. A comparison of assay performance for various bsMab-and Mab-based assays. Of all the assays that were evaluated, some general conclusions about their performance can be seen in the above table. Increases in sensitivity were typically seen with increases in incubation time and/or substitution of a fluorometric substrate for a colorimetric one. Decreases in the effective assay range correspond with heightened sensitivity. The colorimetric substrate was TMB, while the fluorometric substratewas Quantblu™.

4.3.2 Assay Performance.

Once the conditions for measuring PSA in the ultrasensitive range were determined for each assay, a standard curve was performed with each as a matter of comparison. If the assay demonstrated good linearity, the sensitivity (LLD) was calculated by taking the average value of a minimum of 10 replicates of the zero standard and adding 2 standard deviations of that value in the linear range. A summary of these assays is seen in Table 14.2.

When we compare the relative performance of these assays, two major factors were observed that contributed to heightened sensitivity: choice of substrate and incubation time.

Colorimetric and Fluorometric substrates. When comparing the two 1 h assays of PSA using the TMB colorimetric and Quantblu™ fluorometric substrates, the fluorometric substrate can immediately be seen to increase the sensitivity 2-4-fold (Table 4.2, Figure 4.16). This is consistent with the conclusion by Portsmann, who reported that in general the sensitivity of fluorescence is 10-1000 times higher than colorimetry, although the practical sensitivity of the assay may only be increased by a factor of 2-10 (Porstmann and Kiessig, 1992). This increase in sensitivity corresponds with a decrease in the range of the assay, which can be seen in the P57 1h colorimetric and fluorometric assays.

Incubation time. The second significant factor contributing to sensitivity in the evaluation of these assays was incubation time. As mentioned previously, incubation time allows for more complete immunocomplexation of antibody and analyte, allowing for greater signal at a lower concentration range. A comparison of 1 h and 3 h colorimetric B80.3 demonstrates this effectively. Sensitivity is increased 2-fold (0.047→0.024 ng/mL) by simply allowing

TMB colorimetric

Quantblu™ fluorometric
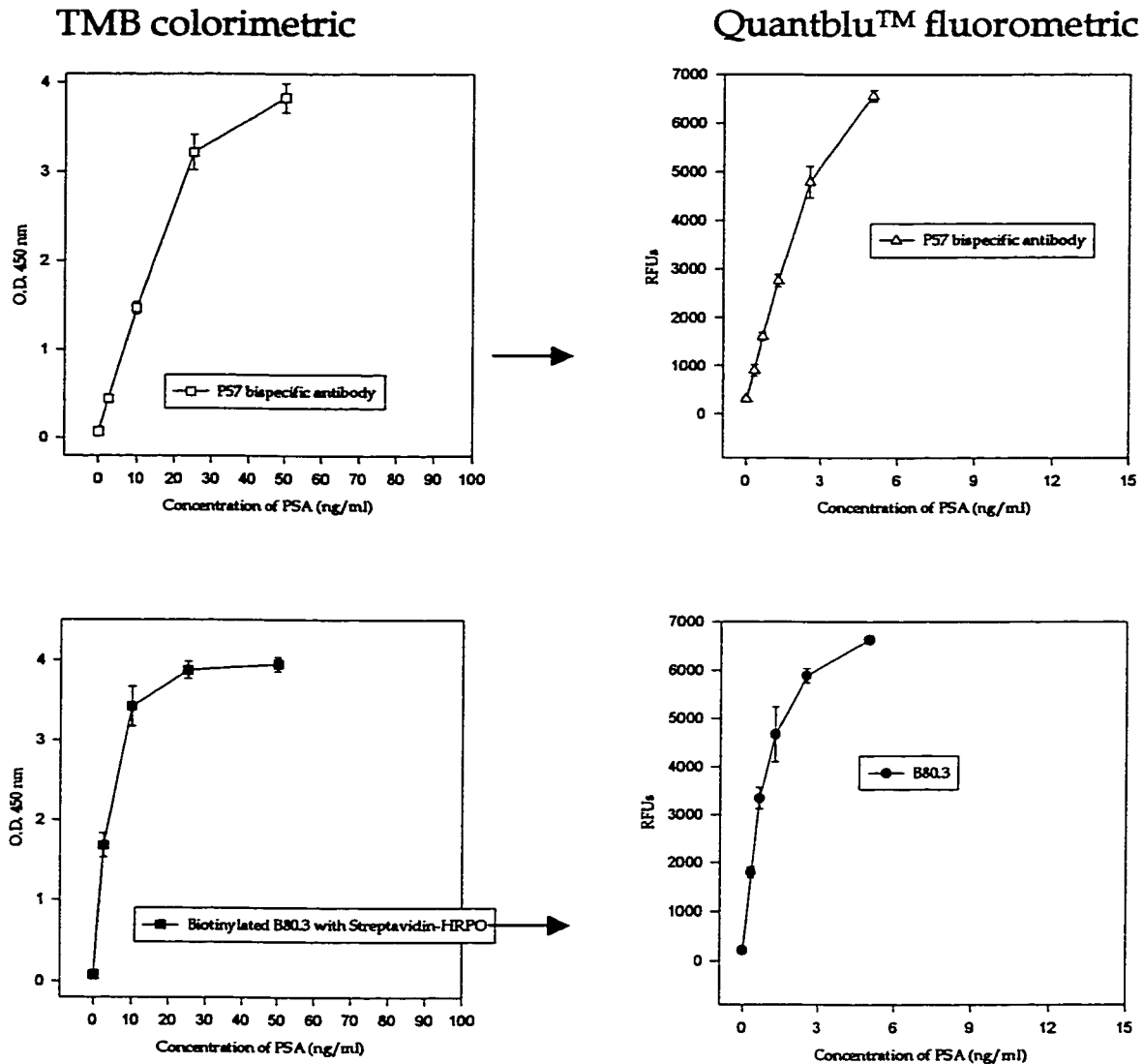
Figure 4.16. Evaluation of fluorometric substrates. In both the B80.3 Mab-based and P57 bsMab-based assays, the substitution of a fluorometric substrate for a colorimetric substrate demonstrated an increased sensitivity. This subsequently decreased the effective range of measurement for the assay. The P57 bsMab assay, although slightly less sensitive, demonstrated greater linearity in both the colorimetric and fluorometric assays. This is likely due to consistent one-to-one labeling of the enzyme reporter to the antibody.

incubation to proceed an extra 2 hours. The same phenomenon can be seen with the CARD 1 h and 3 h assays; although the sensitivity of the original assay was not increased by amplification, increased sensitivity was seen after a longer incubation time. It was not demonstrated in the evaluation of these assays if incubation times longer than 3 h would increase sensitivity even further. This is yet to be explored.

Effective range of measurement. A consistent observation with all of the immunoassays evaluated was a decrease in the effective range of PSA measurement when sensitivity was increased. An example of this can be seen with the P57 1h colorimetric and fluorometric assays. Although a 2-fold increase in sensitivity was achieved by substituting the Quantblu™ fluorometric substrate for the TMB colorimetric one, the effective range of measurement for this assay was decreased 10-fold (0 – 25 ng/mL to 0 – 2.5 ng/mL). In practical terms, this should not be a concern. The design of this assay is to monitor increases in PSA that theoretically start at or near zero. The effective range was also seen to be consistently larger in the P57 bsMab-based assays when compared with the B80.3 Mab assays (Table 4.2) when assays were performed under identical conditions. This corresponds with a slight decrease in sensitivity in the bsMab-based assay when compared with the Mab-based assay.

BsMab vs. Mab-based assay. It was hypothesized that bispecific antibodies would be able to perform comparable to or better than their monoclonal antibody counterparts under ultrasensitive conditions. A comparison of the lower limits of detection shows the sensitivities are similar in magnitude but Mab-based assays consistently demonstrated slightly increased sensitivities. The reasons for this may be twofold. Firstly, two analyte binding sites on the Mab compared to only one on the bsMab double the avidity of the antibody for the antigen. This is presumably why differences in sensitivities between both assay formats are more exaggerated in the 1 h vs 3 h assay formats. Secondly,

chemical labeling of the Mab allows for the attachment of multiple enzyme reporters to a single antibody. This translates to higher signal for every binding event in the microtiter well.

CARD Assay. Although the CARD amplification system was meant to amplify signal by proportionally increasing the amount of enzyme reporter on the plate, no practical increase in sensitivity was observed. In actuality, the sensitivity was lessened when P57 and P57-CARD assays were subjected to identical conditions. In, part, this is due to substantially higher backgrounds in the CARD amplification system. Attempts to rectify this problem, including dialysis of blocking and diluent buffers, only allowed for a moderate decrease in background noise but not, however, enough to validate its use. These lessened sensitivities were also due in part to more erratic background levels that increased the standard deviation from background replicates. These inconsistencies are not surprising, as each microtiter well must undergo the catalyzed deposition reaction in wells that may have subtle differences in protein blocking, washing, etc. The slightest variation from well-to-well would subsequently be amplified. In addition, development of the substrate is accelerated, leaving a smaller window of opportunity to stop the substrate reaction when it is saturated (i.e. blue TMB chromogen at 650 nm absorbance ~ 1.0). This is also unsuitable when assay-to-assay variation becomes a concern, as in the routine measurements of biological samples. Overall, the CARD assay system may be of little use in quantitation for the detection of low analyte concentrations. This limitation has been recognized by Diamandis (Diamandis, 1991). However, its use as a qualitative method has already been adopted as a common practical usage of this technology, especially in immunohistochemical applications.

When a combination of increased incubation time and a fluorometric substrate was attempted, a dramatically sensitive assay (LLD < 0.006 ng/mL) over a small range of measurement was the result (Figure 4.17). The range is

also dependent on substrate development time and was observed to decrease when the development time was increased. The effective range was similar to that of the proposed practical range for the ultrasensitive measurement of PSA (Yu et al., 1997). With a 3 h incubation time and fluorometric substrate, the assay could easily distinguish between background and the lowest standard concentration (6.25 pg/mL). In order to determine its practicality in the routine measurement of patient samples, however, further optimization of the assay and an assessment of its biological detection limit would have to be carried out. Unlike amplification schemes, however, the simplicity of this assay should allow for consistent assay performance. A shorter incubation time (i.e. < 3 h) may need to be considered if the effective range is impractical (i.e. unable to monitor patients > 0.1 ng/mL).

4.4   CONCLUSION

Monclonal and bispecific antibodies are very useful tools for the ultrasensitive measurement of PSA. Both exhibited similar sensitivities, although the bsMab-based assay demonstrated a greater linear dynamic range. A sandwich assay consisting of matched Mabs or a bsMab/Mab pair using HRPO and a fluorometric substrate was able to provide adequate lower limits of detection with an assay that was simple to perform. Increased incubation time of the antibody/analyte resulted in dramatic increases in sensitivity. Exploration of this assay to assess its usefulness in a clinical setting will determine if very early recurrence of prostate cancer can be detected for earlier treatment options and better outcomes.
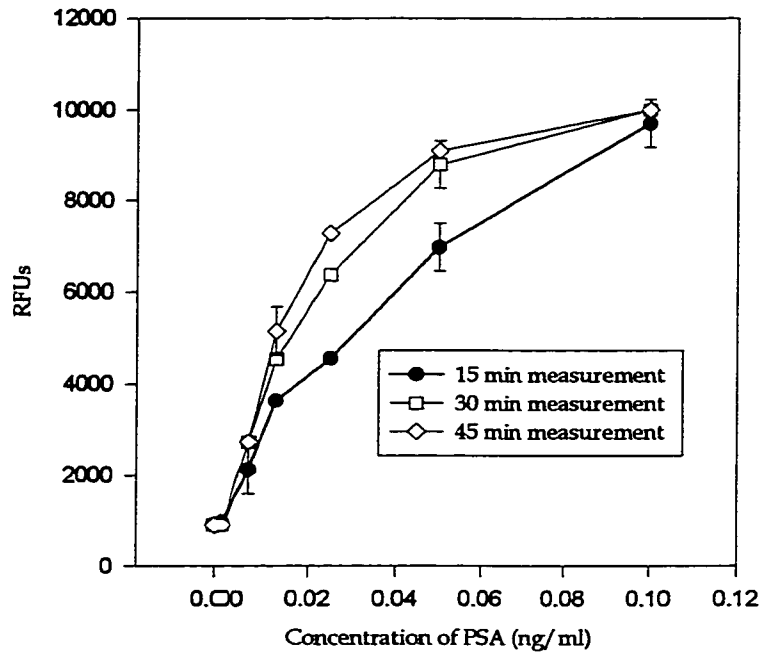
Figure 4.17. The P57 bsMab-based fluorometric assay using a 3 h incubation time. Using the combined techniques of increased incubation time and fluorometry, the practical sensitivity of this assay was increased dramatically. As multiple readings were taken, the saturation of the substrate decreased the linear range of measurement even further. Further evaluation of this assay is needed to determine its usefulness in a practical setting.

Chapter 5: Summary and Future Directions

In Chapters 1 and 2, past methods of PSA purification are described along with a new method, which is a modification of a published method of Rusciano *et al.* (1988b). The serendipitous discovery of the strong binding potential of PSA to the Cibacron blue F3G-A dye matrix provided a good first step toward purifying bulk amounts of PSA. Bulk purification is important in that it provides ample material for both immunoassay standards and purified antigen for the creation of new monoclonal antibodies. In addition to PSA purification, the original published method was also concerned with the negative-mode purification of prostatic-acid phosphatase, a putative tumor marker that has since been shown to lack clinical sensitivity. It was hypothesized that buffer conditions could be modified to increase the amount of contaminant protein in the unbound, without decreasing the PSA yield. This proved correct. In just one column run, PSA purity increased 2 fold. The hypothesis that the addition of a linear gradient elution would enhance this effect further also proved correct and the purity of PSA was increased from 8% of the total amount of protein to 19% in a single step.

Large molecular weight contaminants in the pooled fractions were subsequently separated by size exclusion techniques, using three cross-linked dextran matrices with different separation characteristics. Of the three, Sephadex G-75 (superfine) provided the best separation, but has certain limitations in purification due to difficulties in column packing and diminished flow rates. It can be speculated that Sephadex G-75 (fine) or an equivalent, due to better flow characteristics could be used as alternative matrices. As mentioned in Chapter 2, the major large MW contaminant is suspected to be albumin, known to bind strongly to the Cibacron blue F3G-A dye, and a known component of seminal plasma (Sensabaugh, 1978; Rusciano et al., 1988b). This

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fact, discovered in hindsight, may also open up various pathways for the discovery of better PSA purification as many methods exist for the efficient separation of albumin from protein mixtures, including glycan-binding lectin columns (e.g. Concanavalin A). In addition, DEAE ion exchange, which was used as a final step in this purification, may have proven useful as a second step, given its ability to separate albumin from PSA efficiently (Wang et al., 1982).

PSA purified by the method described in Chapter 2 is enzymatically active, which is important for both enzymological research and the development of complexed PSA standards (Christensson et al., 1990; Chen et al., 1995). It is unknown whether hK2, a structurally similar protein and possible contaminant in PSA purification methods was co-purified using this method. This, too, remains to be tested. Ultimately, the purification method outlined here is reliable, reproducible and relatively inexpensive, using robust matrices and mild eluting conditions.

In Chapter 3, a novel method for the purification of HRPO-labeled antibodies is described. Unlike other published protocols, this method exploits the ability of benzhydroxamic acid to bind to the active heme site of the HRPO reporter enzyme (de Ropp et al., 1999). Because this allows for the direct purification of active HRPO it was hypothesized that by attaching antibody to the enzyme, the indirect purification of active HRPO-antibody conjugates would occur. The results of these succesfull experiments confirmed this initial hypothesis.

This technique was especially useful for purifying HRPO-labeled bispecific antibodies. Unlike their monoclonal antibody counterparts, bispecific antibodies developed by cell fusion techniques have presented a unique problem in purification due to random heavy and light chain association which introduces contaminating analyte-binding antibodies into the cellular supernatant. By passing this supernatant through a benzhydroxamic acid-

agarose column in the presence of HRPO, most contaminants were washed from the column and the eluted fractions contained HRPO-labeled bispecifics with a high specific yield. Antibodies capable of binding to HRPO would also be co-eluted but this did not present any problems in PSA assays with proper washing.

This new technique developed in this thesis has universal applications. Not only does it solve the unique problem of bispecific antibody purification, it is theoretically able to purify any HRPO-labeled protein. Because of its active-site specificity, specific activity of the purified batch will always be high. Because benzhydroxamic acid-agarose was shown to have no affinity for antibodies, researchers could use this technique to purify HRPO-labeled-polyclonals and monoclonals. It also allows for standardization of different batches by purifying only active conjugates. It may also find application in purifying labeled mRNA probes, DNA probes and HRPO-fusion proteins.

Finally, Chapter 4 describes the evaluation of modifications of a sandwich immunoassay for PSA testing originally developed by Kreutz and Suresh (1997). The modifications all focus on increasing the sensitivity of the original assay for testing PSA. This increased sensitivity is important, as it has been demonstrated to be more predictive of prostate cancer recurrence (van Iersel et al., 1996; Diamandis, 1997; Yu et al., 1997; Vassilikos et al., 2000) and has demonstrated an initial positive impact on clinical outcomes (Schild et al., 1996).

Steps to increase the sensitivity involved using different substrates, increasing reagent incubation times, evaluating reagent concentrations, using an enzyme amplification system and exploring different blocking/washing conditions. The hypothesis that a fluorometric substrate would improve detection limits was confirmed and was seen to improve detection limits 2 to 4 fold. Increasing the incubation time of the PSA and detection reagents from 1 to 3 h also contributed to heightened sensitivity. It remains to be tested whether extending incubation times beyond 3 h will even further increase the sensitivity.

Certain modifications were seen to have either a negligible effect on assay sensitivity. Dialyzing BSA may hypothetically decrease background levels by eliminating biotin or other contaminants that can contribute to non-specific bindling in the microtiter wells. No significant effect was seen using this intervention. Tween-20 and 6% BSA were also used because they were reported to decrease background (Diamandis et al., 1996). Testing various concentrations of the B87.1 plate-coated antibody based on concentrations recommended by Engwall (Engvall and Perlman, 1971) had a negligible effect.

Some modifications had a negative effect on increasing assay sensitivity. It was hypothesized that an enzyme amplification technique may increase sensitivity. One technique, a catalyzed-reporter deposition assay based on a method by Bobrow (Bobrow et al., 1989) actually reduced the sensitivity of this assay by consistently increasing background levels. It was also hypothesized that a chemiluminescent substrate would greatly improve sensitivity. Because of this, a luminol-based chemiluminescent substrate was also briefly explored but high backgrounds and consistently observed cross-talk when the signal was measured in a 96-well microplate chemiluminometer hindered the use of this technique. These data were not included in this thesis because of their preliminary nature. Whether the problem was assay-based or instrumental in nature remains to be investigated.

Hypothesizing that bispecific antibodies, with their batch-to-batch reproducibility and unique one-to-one binding characteristics for both analyte and reporter enzyme, would prove to be as effective as monoclonal antibodies for the ultrasensitive detection of PSA, a series of assays were undertaken. Because these unique immunoprobes have shown sensitivities and kinetics in immunoassays comparable to monoclonal antibody-based immunoassays in the past, it was thought that they should perform similarly under ultrasensitive conditions. During shorter 1 h incubation times, these antibodies proved to be less sensitive, perhaps owing to better avidity of the HRPO-labeled monoclonal

antibody for the analyte. After three hours, however, and employing fluorometric substrates, the bsMab- and Mab-based assays demonstrated standard curves that were comparable to each other. The bsMab and Mab colorimetric 3 h standard curves also demonstrated similar slope but problems with a diminished overall signal (compared to other assays) contributed to decreased sensitivity in this bispecific-based assay.

In the end, a PSA test was developed which measures in the desired ultrasensitive range. It combined the use of a fluorometric substrate with a longer (3 h) incubation time. It required roughly 4 h to perform (3 h incubation plus 45 min substrate development) and can effectively measure PSA with a lower limit of detection less than 6 pg/mL and an upper limit of 0.1 ng/mL. This assay could be used to monitor prostate cancer patients after prostate removal, to improve patient outcomes, and have a positive impact on health in general.

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