UNIVERSITY OF ALBERTA

Characterisation of the Nature of Interaction of Two Antibodies Generated Against Prostate-Specific Antigen

BY

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Characterisation of the Nature of Interaction of Two Antibodies Generated Against Prostate-Specific Antigen

Hereby submitted by Diane C. Jette in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences - Pharmaceutical Biotechnology

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In memory of my mother

I. ABSTRACT

The epitopes recognized by two high-affinity anti-prostate specific antigen (PSA) monoclonal antibodies, B80 and B87, were characterised. These antibodies recognise two non-overlapping epitopes and can be used in a sandwich immunoassay to detect total PSA in serum. They were found to bind to both free PSA and PSA complexed with a_1-antichymotrypsin (ACT) and not to cross-react with porcine pancreatic kallikrein. In order to locate the epitopes, overlapping hexapeptides representing the entire PSA amino acid sequence were synthesized and screened in an immunoassay for their ability to bind with the two monoclonal antibodies. B80 showed specific binding to several peptides. B87 did not show binding to a specific peptide, suggesting that the B87 epitope may be non-linear. A bacteriophage peptide display library was used to select for peptide sequences that bind specifically to B80 and B87. Several consensus sequences were identified using this technique. One peptide sequence was found to be similar to a linear sequence on PSA (LGRHS), which was also identified as a possible B80 epitope by PSA hexapeptide scanning. Another peptide sequence, (WGFD), identified by affinity selection using a phage peptide display library, was found to bind specifically to B80 and is a potential B80 mimotope. A computer model of PSA was generated based on the sequence homology of PSA to other serine proteases of known structure. The model was used to identify regions unique to PSA which are located on the surface of the molecule not involved in the binding of ACT. The B80 epitope appears to be in one of these unique loop regions, away from the catalytic triad and the region masked by ACT.

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V LIST OF ABBREVIATIONS

Å	= Angstrom
Ab (A)	= absorbance
ABTS	= 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)
ACT	= α_1-antichymotrypsin

amino acids:

A (Ala)	= L-alanine
C (Cys)	= L-cysteine
D (Asp)	= L-aspartic acid
E (Glu)	= L-glutaMic acid
F (Phe)	= L-phenylalanine
G (Gly)	= L-glycine
H (His)	= L-histidine
I (Ile)	= L-isoleucine
K (Lys)	= L-lysine
L (Leu)	= L-leucine
M (Met)	= L-methionine
N (Asn)	= L-aspartic acid
P (Pro)	= L-proline
Q (Glu)	= L-glutamine
R (Arg)	= L-arginine
S (Ser)	= L-serine
T (Thr)	= L-threonine
V (Val)	= L-valine
W (Trp)	= L-tryptophan
Y (Tyr)	= L-tyrosine

Auto	= automated
BPH	= benign prostate hyperplasia
BDL	= biological detection limit
BSA	= bovine serum albumin
CaP	= cancer of the prostate
CDR	= complementarity-determining region
Ch	= chemiluminescence
CIA	= chemiluminescence immunoassay

Con A	= concanavalin A
CsCl	= cesium chloride
DMF	= N,N-dimethyl formamide
DNA	= deoxyribonucleic acid
DRE	= digital rectal examination
E. coli	= *Escherichia coli*
EDTA (ETA)	= ethylenediaminetetraacetic acid
ELISA	= enzyme-linked immunosorbent assay
FDA	= Food and Drug Administration
EIA	= enzyme-linked immunoassay
Fab	= antibody fragment
FIA	= fluorescence immunoassay
FI	= fluorescence
FMOC	= 9-fluorenylmethoxycarbonyl
f-PSA	= free prostate-specific antigen
hK1	= human pancreatic/ renal kallikrein
hK2	= human glandular kallikrein
hK3	= human prostate-specific antigen
HOBt	= 1- hydroxybenzotriazole
HRPO	= horseradish peroxidase
IgG	= immunoglobulin G
IT	= inter-alpha trypsin inhibitor
kDa	= kilodalton
L	= litre
LLD	= lower limit of detection
Mab	= monoclonal antibody
mg	= milligram
min	= minute
µg	= microgram
MG	= α_2-macroglobulin
µL	= microlitre
µM	= micromolar
µmol	= micromole
mL	= millilitre
mmol	= millimole

mM	= millimolar
MP	= microparticles
mRNA	= messenger ribonucleic acid
NPV	= negative predictive value
NMP	= 1-methyl 2-pyrolidinone
ng	= nanogram
nL	= nanolitre
nm	= nanometer
nM	= nanomolar
nmole	= nanomole
nucleic acid bases:	
A	= Adenine
C	= Cytosine
G	= Guanine
T	= Thymine
U	= Uracil
PAGE	= polyacrylamide gel electrophoresis
PBS	= phosphate buffered saline
PCI	= protein-C inhibitor
PEG	= polyethylene glycol
PENCE	= Protein Engineering Network Centre of Excellence
phage	= bacteriophage
pI	= iosoelectric point
pK2	= porcine pancreatic kallikrein
Poly	= polyclonal antibody
PPV	= positive predictive value
PSA	= prostate-specific antigen
PSA-ACT	= prostate-specific antigen - α_1-antichymotrypsin
PSA-AT	= prostate-specific antigen - α_1-antitrypsin
PSAD	= prostate-specific antigen density
PSA-DT	= prostate-specific antigen doubling time
PSA-IT	= prostate-specific antigen-inter-alpha trypsin inhibitor
PSA-MG	= prostate-specific antigen - α_2-macroglobulin
PSA-PCI	= prostate-specific antigen - protein-C inhibitor
R	= coefficient of regression

RIA	= radioimmunoassay
RNA	= ribonucleic acid
RPM	= revolutions per minute
SD	= standard deviation
SDS	= sodium dodecyl sulphate
SPOTS	= simple precise original test system
ssDNA	= single stranded DNA
tBU	= t-butyl ester
TBS	= Tris-buffered saline
TFA	= triethylamine
TIB silane	= triisobutylsilane
TRUS	= transrectal ultrasonography
TMB	= tetramethylbenzidene
t-PSA	= total prostate-specific antigen
TURP	= transurethral resection of the prostate
TW	= Tween 20
US	= ultra-sensitive

VI. INTRODUCTION

The measurement of prostate-specific antigen (PSA) in serum is used to detect and monitor the progress of prostate cancer (reviewed in Armbruster, 1993). Recently, a number of problems have been identified with the assays currently being used (reviewed in McCormack et al., 1995). These include differences in the recognition of the different isoforms of PSA, the inability to recognise PSA complexes, and cross-reactivity with homologous proteins. In order to address these issues, epitopes on PSA were characterised and identified using two anti-PSA monoclonal antibodies, designated B80 and B87. The identification and characterisation of epitopes on PSA may lead to the development of PSA assays that are more specific and also help to characterise the PSA assays currently in use.

In section one of this introduction, the biochemistry, physiology and clinical utility of PSA will be described. The problems associated with assays used for the measurement of PSA in serum will be described in section two. The nature of antigen-antibody interaction and cross-reactivity with protein and peptide epitopes, important in any discussion of immunoassays and protein antigens, will be discussed in section three. The tools that are currently available to locate and characterise protein epitopes such as topographical mapping, epitope scanning, phage display epitope libraries, and computer modelling, will be described in section four. Finally, two anti-PSA antibodies, B80 and B87, will be described in the last section of this introduction.

1.0 Prostate-specific Antigen

1.1 Prostate Cancer

Prostate carcinoma is a major cause of death in North America. It now exceeds deaths attributed to lung cancer (Ambruster, 1993). The incidence of prostate cancer in the U.S. over the last five years has doubled compared to the preceeding five years. The fastest rising segment of the population diagnosed with prostate cancer were patients 75 years or older (Boring et al., 1993; Silverberg et al., 1988, 1983). This increase in reported incidence can be attributed in part to the use of new diagnostic tools for the detection of prostate cancer, including PSA assays (after 1989) and the recent development of ultrasound. In Canada between 1970 and 1990 prostate cancer increased at an average annual rate of 3% (Levy, 1994). In 1990, prostate cancer surpassed lung

cancer as the most frequently diagnosed cancer in Canadian men. From 1990 to 1993 the incidence rose between 20% and 40%, at an annual rate of 10% to 15% per year (Levy, 1994). Mortality rates also increased during this period at an average annual rate of one percent per year. The five-year relative survival rate in three Canadian provinces (Saskatchewan, Alberta, and Ontario) was greater than 60% for all men diagnosed with prostate cancer in the early 1980's. In 1993, 3800 deaths out of 12,900 new cases (29%) were predicted. This decrease in mortality may be due to earlier detection of the disease, more effective treatment, or increased detection of clinically insignificant disease. Effective treatment of prostatic cancer is limited to androgen suppression therapy and aggressive treatment of localised disease with surgery and radiation therapy (Levy, 1994,).

The prostate is a small, encapsulated doughnut-shaped mass of glandular and muscular tissue, located directly below the bladder. It surrounds the urethra. It secretes an alkaline liquid into the urethra during ejaculation that aids in sperm mobility and creates conditions favourable to fertilisation. The prostate is not necessary for sexual function or a healthy life. After middle age the prostate can become enlarged due to benign prostatic hyperplasia (BPH) or cancerous tumours. Prostatitis, an inflammatory response to bacterial or mycobacterial infection, can also cause enlargement of the prostate (Schellhammer and Wright, 1993). This may result in prostatism, a partial or complete blockage of the urinary tract due to the squeezing of the urethra by the enlarged prostate. (Armbruster, 1993). The standard procedures used for the diagnosis of prostate disease include digital rectal examination (DRE), transrectal ultrasound (TRUS), TRUS guided needle biopsy, and immunoassays detecting serum markers such as prostate-specific antigen (PSA). An enlarged prostate can be treated using transurethral resection of the prostate (TURP) or radical prostatectomy (Armbruster, 1993).

1.2 History of Discovery

PSA was discovered simultaneously by several different investigators. The first report about a prostate-specific antigen was in Japan (Hara et al., 1971). The protein was named gamma-seminal protein. Later, Li and Beling isolated and purified a 31 kDalton protein called E1 antigen on the basis of its mobility by conventional electrophoresis (Li and Beling, 1973). In 1978 Sensabaugh isolated a seminal-specific, highly immunogenic protein with several sugar residues named p30 on the basis of this protein's estimated molecular weight (Sensabaugh et al., 1978). In 1979 Wang purified

and characterised a protein, prostate-specific antigen (PSA), found to be specific to prostate tissue (Wang et al. 1979). Papsidero developed the first serological test for PSA using polyclonal antibodies and proposed the use of PSA as a diagnostic marker for prostate cancer (Papsidero et al.,1980). Graves developed a latex particle agglutination assay for the detection of PSA and proposed the use of PSA as a forensic marker for the investigation of rape cases (Graves, 1985). The gene which codes for PSA was isolated and cloned by Henttu and Lundwall (Henttu et al.,1989; Lundwall and Lilja, 1987). Once the amino acid and DNA sequence were identified, it was determined that all of these investigators were studying the same protein, now most commonly called prostate-specific antigen (PSA).

1.3 Biochemistry of PSA

1.3.1 Homology with kallikreins

The PSA gene is a member of the human tissue kallikrein gene family. These genes encode for serine proteases, so-called because the mechanism of proteolytic cleavage involves a serine residue at the active site (Lilja, 1985; Lundwall and Lilja, 1987; Schaller et al., 1987; Watt et al., 1986). PSA is a protein product of a six-kilobase gene found on chromosome 19 in the region of q13.2-q13.4. The gene has four introns and five exons (Reigman et al., 1992) and has a greater than 84% nucleotide sequence homology with glandular kallikrien (hK2) and 73% homology with pancreatic kallikrien (hK1) indicating that there is a common ancestral gene (Berg et al., 1992; Carbini et al., 1993; Chung et al., 1986; Clements, 1994; Lundwall et al., 1987). Recently all three gene products have been renamed (Berg et al., 1992) as shown in Table 1.

Table 1: Nomenclature of kallikrein-family proteins[1]

Formal name	Common name	Description
hK1	PRK	human pancreatic/renal kallikrein, hKLK1 gene product
hK2	hGK-1	human glandular kallikrein, hKLK2 gene product
hK3	PSA	Prostate-specific antigen, hKLK3 gene product

[1] Table adapted from McCormack et al., 1995

The product of the hKLK1 gene is known as tissue kallikrein, glandular kallikrein, urinary kallikrein, pancreatic/renal kallikrein and hK1 (Carbini et al., 1993; Angermann et al., 1992). hK1 is a 238-amino acid glycoprotein expressed in the salivary glands, pancreas, and kidneys (Fukushima et al., 1985). It is not expressed in the prostate gland and is not considered a prostate cancer marker candidate. Since it has a 62% amino acid sequence identity with PSA (Figure 1), monoclonal antibodies to PSA may cross-react with this protein depending on the region on PSA which is recognised by the antibody. Assays using polyclonal antibodies, which recognise multiple epitopes on PSA, are more likely to cross-react with hK1 and may detect both PSA and hK1, thus providing an overestimation of the levels of PSA in serum. Biologically active recombinant hK1 has been produced and cross-reactivity of anti-PSA antibodies can now be evaluated (Rahn et al., 1992).

The gene product of the hKLK2 gene (Schedlich et al.,1987), hK2 is a 237 amino acid glycoprotein which has a 80% amino acid sequence identity with PSA (Lilja,1993) (Figure 1). The sequence for hK2 suggests that it is a trypsin-like protease (Schedlich et al., 1987), unlike PSA which has chymotrypsin-like activity (Lundwall and Lilja, 1987). It is likely, therefore, that hK2 has a different physiological role from that of PSA.

The hK2 protein has not been identified in human tissue or fluids but the messenger RNA (mRNA) has been identified in prostate tissue (Murtha et al., 1993; Young et al., 1992). These studies indicate that hK2, like PSA, is specific to the prostate. The expression of hK2 mRNA in human prostate is from 10% to 50% that of PSA mRNA (Chapdlaine et al., 1988; Henttu and Vihko, 1989; Young et al., 1992). Significant levels of hK2 are likely present in seminal plasma since PSA is present at a

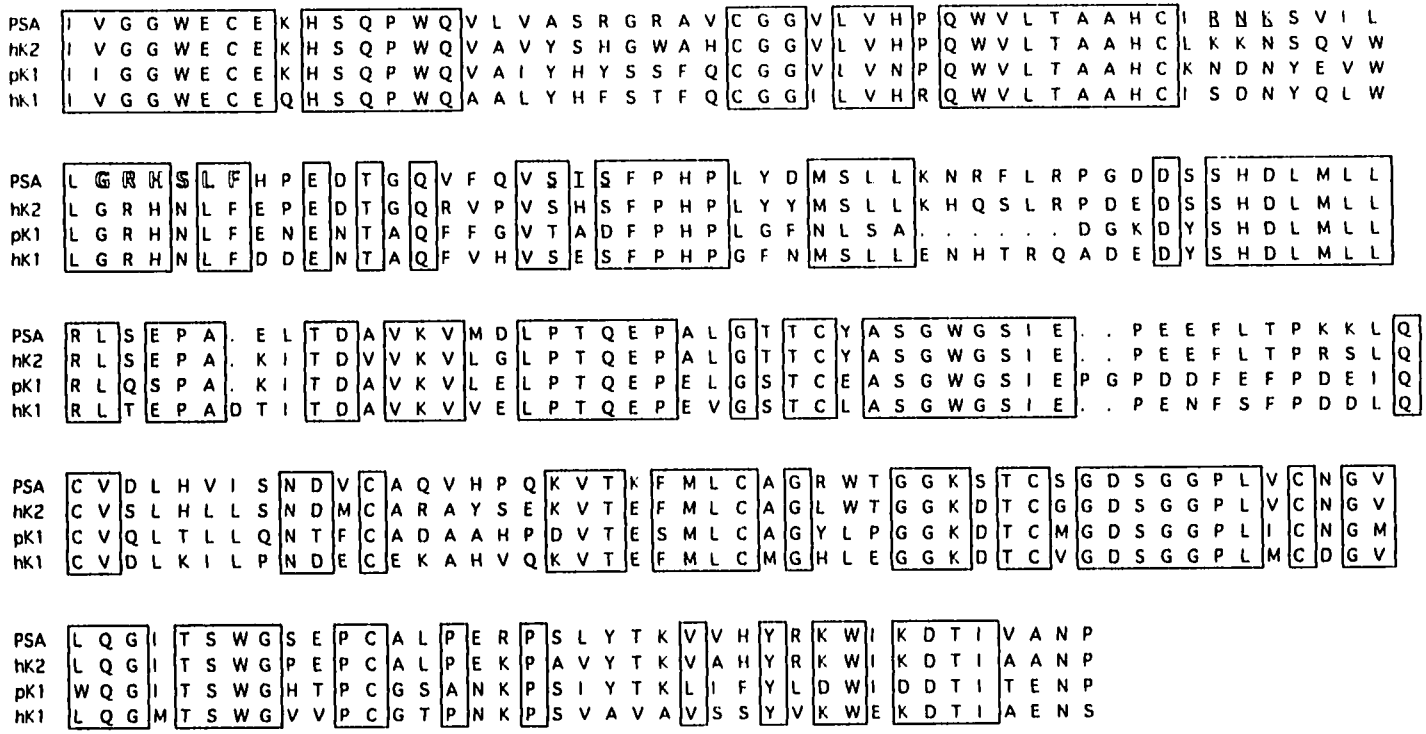


Figure 1: The amino acid sequence of PSA and kallikreins.

PSA = Prostate-specific antigen, hK2 = human serum kallikrein (or human glandular kallikrein),
pK1 = porcine pancreatic kallikrein, and hK1 = human pancreatic kallikrein
Residues common to protein sequences are boxed.
Possible N-linked glycosylation sites for PSA are underlined.
The B80 epitope, identified by epitope scanning and affinity selection in this study, is shown in outlined letters.

concentration of five to 0.5 mg/mL (Sensabaugh, 1978). Due to the high degree of homology with PSA it is likely that some anti-PSA monoclonal antibodies could cross react with hK2 and thus over estimate the levels of PSA present in serum. The specificity of hK2 to prostate tissue makes this glycoprotein a potential new marker for prostate cancer. Antibodies specific to hK2 which do not cross-react with PSA are needed. Recombinant hK2 has been expressed (Saedi et al., 1995) and could be used for immunisation.

1.3.2 Physiological properties of PSA

PSA is produced only in male primates in the columnar epithelial cells of the prostate and periurethral glands (Henttu and Vihko, 1994; Sinha et al., 1994). It is found in seminal fluid at a concentration of 0.5 to 5 mg/mL and in serum at a concentration of 0 to 4 ng/mL (Sensabaugh, 1978). The production of PSA by the periurethral glands does not significantly contribute to serum PSA levels. Periurethral PSA is significant in urine especially after a radical prostatectomy (Iwakiri, et al., 1993; Takayama et al., 1994). The cellular localisation of PSA is actually higher in normal and benign tissue than in malignant prostate tissue (Chu, 1992; Papsidero et al.,1980; Wang et al., 1979). PSA mRNA is also expressed at higher levels in benign tissue (Qiu, et al., 1990). PSA is not synthesised by other tissues of the body except in low levels by malignant breast tumours (Yu et al., 1994) and human endometrium (Clements and Mukhtar, 1994). PSA is useful as a tumour marker because trauma or disease causes leakage of PSA out of the prostate into the bloodstream with a serum half life of 2.2-3.2 days (Stamey et al., 1987; Oesterling et al., 1988). Recently, the reverse transcriptase-based polymerase chain reaction method was used to detect PSA and the presence of malignant prostate cells in serum (Israeli et al., 1994; Katz et al., 1994; Vessella et al., 1994).

The physiological role of PSA is to liquefy seminal coagulum by rapidly cleaving semenogelin I and II, also called high-molecular-mass seminal vesicle protein (HMM-SV protein), which is the major structural protein of seminal coagulum (Lilja, 1985, 1993; Lilja and Lundwall, 1992). PSA has been reported to cleave insulin-like growth factor/binding protein, which may be involved in modulating prostate cancer (Cohen et al., 1992). Also, PSA may release a bioactive kinin-like substance that has a role in fertilisation (Fichtner et al., 1994).

1.3.3 Molecular forms of PSA

PSA was found to have a molecular weight of 30,000 Daltons by immunoelectrophoresis and 32,000 to 34,000 Daltons by gel filtration and gel electrophoresis (Wang et al., 1981). PSA was originally described from amino acid sequencing studies as a protein of 240 amino acid residues with a calculated molecular weight of 26.5 kDa (Watt et al., 1986) and later as a 237-residue protein with a molecular weight of 26.1 kDa (Schaller et al., 1987). cDNA sequence analysis (Lundwall et al., 1987) confirmed this later study. Recently the molecular weight of PSA was determined by mass spectrometry to be 28.5 kDa (Belanger et al., 1995). This discrepancy in molecular weight is due to the presence of an N-linked oligosaccharide on asparagine-45. PSA is made up of 8% carbohydrate with one N-linked side chain (Belanger et al., 1995). The carbohydrate chain consists of 4.84% hexose, 2.87% hexosamine and 0.25% sialic acid (Van Helbeek et al., 1985). The oligosaccharide chain accounts for the concanavalin binding properties of PSA (Van Dieijen-Visser et al., 1988; Lilja, 1985; Van Halbeek et al., 1985) PSA displays micro-heterogeneity and has at least five isoelectric points ranging from 6.8 to 8.0, the major isoform having a pI of 6.9. This heterogeneity is believed to be caused by the differing sialic acid content of the carbohydrate chains and not by variability in amino acid content of the molecule. The clinical and biological relevancy of the different PSA isoforms has been studied but found not to be useful in distinguishing between BPH and prostate cancer (Van Dieijen-Visser et al., 1988).

PSA has five disulfide bonds, believed to be homologous to those seen in chymotrypsin (Lundwall et al., 1987). cys-7 to cys-152, cys-26 to cys-42 (Histidine loop), cys-128 to cys-198, cys-188 to cys-213 (Methionine loop) and cys-188 to cys-213 (primary and secondary binding loops). Ser-186, homologous to chymotrypsin Ser-189 along with the active site residue, His-41, Asp-96 and Ser-192, determines the substrate specificity. These residues correspond to residues found in other serine proteases (Neurath and Walsh , 1976; Neurath, 1986).

Knowledge of the processing of PSA is based on the sequence of PSA (Gauthier et al., 1993; Lilja, 1985; Lundwall and Lilja, 1987; Schaller et al., 1987) and studies on other serine proteases (Angermann et al., 1992; Neurath and Walsh, 1976; Neurath, 1986; MacDonald et al., 1988). As a serine protease, PSA can occur in many different forms, including an active enzyme, an enzyme precursor, a zymogen, and an internally cleaved (or clipped) inactive enzyme. PSA purified from seminal plasma consists of

60% to 70% active enzyme (Christensson et al., 1990; Sensagaugh and Blake, 1990; Watt et al., 1986). The cleaving of PSA between residues 145 and 146 leads to inactivation of the enzyme (Christensson et al., 1990). This clipped form of PSA remains connected by the internal disulfide bonds and co-migrates with intact PSA on electrophoresis. Both the enzymatically active and clipped forms of PSA are referred to as free PSA (Table 2). It is presumed that free PSA in serum is the inactive clipped form which does not form a complex with protease inhibitors such as α1-antichymotrypsin, α2-macroglobulin or protein C inhibitor. It is also possible that uncomplexed PSA may be hK2. The cleavage site of the clipped form of PSA suggests trypsin-like activity perhaps due to autocatalysis of hK2 (reviewed in McCormack et al., 1995). Treatment with aprotinin removes this trypsin-like activity from seminal plasma PSA preparations (Chistensson et al., 1990).

PSA is synthesised in prostate epithelial cells as a precursor with an additional 24 amino acids on the N-terminal of the enzyme. The 17 amino acid hydrophobic leader peptide is first removed. Then a trypsin-like protease, not yet identified, removes the next 7 residues and converts the zymogen into the enzymatically active mature form of PSA. The new N-terminal Ile-1 forms an ionic bond with Asp-194, altering the conformational structure to expose the active site.

1.3.4 PSA complexes

PSA occurs as a number of different complexes in seminal plasma and in serum. The formation of these complexes will affect the level of PSA determined by an immunoassay. The PSA complexes that have been identified to date are listed in Table 2.

PSA forms a complex with protein C inhibitor (PCI), a single chain 57 kDa glycoprotein serpin, present in relatively high concentration in seminal plasma (Christensson and Lilja, 1994; Espana et al., 1993). Although PSA-PCI complexes occur, the PCI concentration is not high enough to regulate PSA activity on an equimolar basis.

Table 2: PSA complexes[1].

Complex	Description
t-PSA (Total-PSA)	All immunodetectable forms in serum, primarily f-PSA and PSA-ACT
f-PSA (Free-PSA)	Noncomplexed PSA; may be proteolytically active or inactive in seminal fluid and only inactive in serum
PSA-ACT	PSA covalently bound to α_1-antichymotrypsin inhibitor; synonymous with PSA complex
PSA-MG	PSA covalently linked and encapsulated by α_2-macroglobulin; not detected in immunoassay: synonymous with occult PSA
PSA-PCI	PSA covalently bound to protein C inhibitor; minor component in seminal fluid; not detected in serum
PSA-AT	PSA covalently bound to α1-antitrypsin; trace component in serum
PSA-IT	PSA covalently bound to inter-alpha trypsin inhibitor; trace component in serum

[1] Table adapted from McCormack et al., 1995

In serum, PSA can form stable complexes with two major serum protease inhibitors, α_2- macroglobulin (MG) and α_1-antichymotrypsin (ACT) (Stenman et al., 1991; Lilja et al., 1991; Christensson et al., 1993). When PSA is complexed to MG it is thought to be completely encapsulated with no epitopes accessible for immunodetection (Christensson et al., 1993). MG has a molecular weight of 725,000 Da and is composed of two subunits, joined by a disulfide bond, in a globular assembly resembling a cross (reviewed in Jensen-Scottrup, 1989). A bait region, containing substrate sequences for

proteases, is located in the centre of the dimer. When cleaved by a protease a major reorganisation takes place and the rod-like dimers become U-shaped and form massive walls that firmly hold the protease near the centre of the molecule. One or two cross links may form. MG can enclose two 50 Å spheres and, therefore, two protease molecules at once. The conformational change reveals previously concealed receptor recognition sites at the top of the walls of the MG molecule. The exposure of these sites allows for rapid clearance of the protease MG complex from circulation (Jensen-Scottrup, 1989).

PSA covalently binds to α_1-antichymotrypsin (ACT) in a 1:1 molar ratio (Stenman et al., 1991). The complex remains intact even after treatment with SDS and polyacrylamide gel electrophoresis (PAGE). As with chymotrypsin, an internal peptide cleavage is seen at Lys-358 on ACT. The rate of complexation of PSA to ACT in serum is slow compared to other proteases. The PSA-ACT complex is very stable but the turnover rate in blood is slower than for the PSA-MG complex. The half-life of the PSA-ACT complex in serum is 2 to 3 days. Unlike PSA, the clearance of PSA-ACT is not renal. Hepatic serpin (a class of protease inhibitors) enzyme complex receptors can eliminate ACT complexes and this is one possible mechanism for clearance of PSA-ACT (McCormack et al., 1995). It has been reported that the proportion of PSA-ACT in patients with prostate cancer is greater than in patients with BPH (Christensson et. al, 1993; Stenman et al., 1991; 1994). This may be due to the fact that PSA-producing prostatic epithelium cells also produce ACT (Bjartell, 1993; Bjork, 1993). ACT is normally excreted into the serum from the liver. Cancerous cells may be producing ACT while hyperplastic cells may not. The proportion of free PSA is greater in BPH than in cancer of the prostate despite a more than 1000-fold molar excess of ACT and MG in serum. The free form is likely enzymatically inactive and unreactive with protease inhibitors. When PSA is complexed to ACT, some epitopes near the active site are masked while others are available for binding to anti-PSA antibodies. (Christensson et. al, 1993; Stenman et al., 1991; 1994)

1.4 Clinical utility of PSA as a marker for prostate cancer

The ideal tumour marker will have the following characteristics;ilt will be specific for a particular tissue or tumour or both, it will be released from the tumour into blood or urine, the concentration in serum will be proportional to tumour burden or malignant potential, it will have the potential for early detection and monitoring of

the cancer, it will have a short serum half-life so that concentration changes quickly in response to therapy, it will have the ability to indicate the presence of a tumour before clinical detection is possible, and it will have 100% sensitivity and 100% specificity (Armbruster, 1993). PSA fulfills some, but not all, of the above criteria.

The clinical utility of PSA has been extensively reviewed (Armbruster, 1993; Oesterling, 1991; Ploch and Brawer, 1994; Ruckle et al., 1994; Schellhammer et al., 1993; Takayama, et al., 1994; Vessella and Lange, 1993) A complete literature review of the clinical data is beyond the scope of this study. The following is a brief summary of the clinical uses of PSA. PSA serum levels are used to monitor therapeutic efficacy, staging of cancer, tumor volume evaluation, detection of recurrent disease, differential diagnosis, conformation of metastasis of prostatic origin, and screening and early diagnosis of prostate cancer

PSA is released into serum from prostate tumours and the concentration is proportional to stage of disease. It has been used for early detection and monitoring of cancer and can detect the presence of a tumour in advance of clinical detection. PSA, however, is not cancer-specific. Some circulating PSA is found in normal males. Also, other conditions of the prostate, especially BPH, can elevate PSA values. As a result, specificity of this marker is low. PSA levels increase with age, probably due to the corresponding increase in the size of the prostate gland. For this reason, age-related cutoff values have been proposed.

PSA screening of the general male population has been proposed. However, there are many problems associated with such screening. PSA levels may not be a good indication of early disease in the general population and detection of clinically insignificant disease may cause over treatment. Also, not all prostate cancer will be detected. Suggested improvements to PSA screening are PSA density, age specific cutoffs, PSA velocity, the measurement of PSA complexes or isoforms and the use of ultrasensitive assays to monitor patients who have undergone a radical prostatectamy (reviewed in Armbruster, 1993; Oesterling, 1991; Ploch and Brawer, 1994; Ruckle et al., 1994; Schellhammer et al., 1993; Takayama, et al., 1994; Vessella and Lange, 1993).

A summary of the positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity of PSA assays using a variety of parameters is shown in Table 3.

Table 3: Clinical performance of PSA assays[1]

Reference	n	Assay type	PPV	NPV	Sensitivity	Specificity
Cooner et al., 1990	1807	PSA > 4	35	96	80	75
Catalona et al., 1991	235	PSA > 4	40	89	79	59
Muschenheim et al., 1991	565	PSA > 4	36	94	53	89
		PSA + DRE	66	92	28	98
Stenman et al., 1991	30	PSA > 8.4	nr	nr	70	70
		PSA > 12.5	nr	nr	63	80
		PSA > 16.5	nr	nr	61	90
		PSA-ACT > 4.5	nr	nr	79	70
		PSA-ACT > 7.6	nr	nr	70	80
		PSA-ACT > 10	nr	nr	66	90
		% PSA-ACT > 57	nr	nr	90	70
		% PSA-ACT > 59	nr	nr	88	80
		% PSA-ACT > 65	nr	nr	78	90
Baweret al., 1992	nr	PSA > 4	32	98	nr	nr
		PSA > 10	51	51	96	nr
Labrie et al., 1992	1002	PSA > 4	33	98	72	92
Powell et al., 1992	211	PSA > 4	47	nr	89	90

Table 3 (cont.)

Reference	n	Assay type	PPV	NPV	Sensitivity	Specificity
Christenson et al., 1993	nr	Free PSA/Total > 0.18	nr	nr	71	90
		PSA > 4	nr	nr	55	90
Leinonen, et al. 1993	1653	Total PSA >7.5	nr	nr	59	60
		Total PSA >10.1	nr	nr	48	70
		Total PSA >11.8	nr	nr	31	80
		Total PSA >13.7	nr	nr	28	90
		PSA-ACT> 0.7	nr	nr	93	60
		PSA-ACT> 0.76	nr	nr	79	70
		PSA-ACT> 0.85	nr	nr	62	80
		PSA-ACT> 0.92	nr	nr	38	90
Akdas et al., 1994	96	PSAD >0.1	92	48	nr	nr
		PSAD >0.6	60	98	nr	nr
Benson et al., 1994	68	PSAD > 0.15	67	90	nr	nr
Carter et al., 1994	nr	PSA > 4	nr	nr	78	60
		PSA velocity > 0.75	nr	nr	72	90
Mettlin et al., 1994	2011	PSA > 4	nr	nr	71	90
		PSAD > 0.1	nr	nr	74	85
		Age ref. PSA	nr	nr	67	90
		Velocity > 0.75	nr	nr	54	95
Seaman et al., 1994	107	PSAD > 0.35	70	90	nr	nr

Table 3 (cont.)

Reference	n	Assay type	PPV	NPV	Sensitivity	Specificity
Semjonour et al., 1994	229	PSA >4	27	nr	89	35
		PSA >10	36	nr	59	71
		PSAD > 0.15	36	nr	93	56
		PSAD > 0.25	52	nr	75	82
		PSAD > 0.4	79	nr	59	96
Shinohara et al., 1994	nr	PSA >4	27	88	95	13
		PSA >6	30	91	92	28
		PSA >12	39	83	55	71
		PSA >20	39	78	24	88
		PSAD >0.1	29	93	95	24
		PSAD >0.3	50	82	45	85
		PSAD >0.5	65	80	29	95
Stenman et al., 1994	7204	PSA > 2.0	nr	nr	82	80
		PSA > 4	nr	nr	57	97
		PSA >10	nr	nr	30	100
Wolff et al., 1994	85	PSA >4	47	nr	51	89
		PSAD >0.4	94	nr	64	98
		PSAD >0.2	74	nr	82	86

[1]PSA units = ng/mL, PSAD units = (ng/mL)/ cc, Velocity units =(ng/mL)/y, PSA/ACT units = proportion or %
PPV = Positive predictive value, NPV = Negative predictive value

Clinical sensitivity is defined as the number of men designated to be positive by the test performed on men who have prostate cancer. Specificity is defined as the number found to be negative by the test among all men without cancer. The positive predictive value is the total number of men with a positive test and prostate cancer (true positive) divided by the number of men with a positive test result. The negative predictive value is the number of true negatives divided by the total number of men with negative test results (Crawford and DeAntoni, 1993).

Stamey and Kabalin (1989) proposed the use of PSA density (PSAD) or PSA level/volume of the prostate, as a more accurate indication of prostate cancer, especially in the region of 4 to 10 ng/mL PSA. In this range of PSA values there is an overlap with prostate cancer and BPH. Serum concentration of PSA was found to be proportional to the volume of the prostate in healthy individuals.

PSA velocity is a measure of the change of PSA serum levels over time. Carter et al. (1992) observed a gradual increase in PSA values with BPH and an exponential increase with cancer of the prostate. However, this group concluded that there was no increase in sensitivity between prostate cancer and BPH when patients had serum levels greater then 4 ng/mL. Catalona et al (1994) using a velocity cutoff value of 0.75 ng/mL/year saw no improvement in sensitivity when the initial PSA value was > 4 ng/mL. However, they did conclude that a positive slope of greater than 0.75 ng/mL/year is a useful indicator of disease if viewed over several years. PSA levels are proportional to disease state but are not solely used for staging (reviewed in Ruckle et al., 1994). PSA levels can differentiate metastatic from other stages. However, with very advanced cancer there is a loss of PSA concentration possibly due to destruction of prostate tissue.

PSA can be used to monitor disease recurrence after a radical prostatectomy using ultrasensitive assays (reviewed in Takayama, 1994). Removal of the prostate will lower PSA levels to undetectable levels since all of the source tissue is removed. The decrease of PSA in serum depends on the half-life of PSA in serum and on the original PSA value. Any PSA above the lower limit of detection indicates some remaining tissue or metastasis.

Stenman et al. (1991) developed assays to measure both free and complexed PSA using three antibodies with different PSA epitopes, one of which is masked when PSA is complexed to ACT. They showed that the concentration of PSA complexed to ACT was higher in men with prostate cancer than in men with BPH only. Lilja et al. (1991) demonstrated that the PSA-ACT complex is the immunodominant form of PSA in serum

representing approximately 80% of the detectable PSA. Christensson et al., (1993) measured total PSA, free PSA, and complexed PSA in patients with BPH and prostate cancer. In both groups the PSA-ACT complex is the immunodominant form in serum (60% to 70%). They reported that the ratio of PSA to total PSA is significantly smaller with prostate cancer than with BPH, and that PSA-ACT complex is greater in prostate cancer. This may be due to the fact that prostate cancer cells produce more ACT than do epithelial cells in benign hyperplastic tissue (Bjartell et al., 1993; Bjork et al., 1993). With the development of new assays for complexed PSA, the clinical utility of PSA-ACT complex is being evaluated (Lilja et al., 1994; Oesterling et al., 1994).

RT-PCR has been used to detect metastatic prostate cancer cells in peripheral blood, lymph nodes, and bone marrow (Vessella et al., 1992; Moreno et al., 1992). mRNA gene transcripts are used to produce cDNA, which is amplified by PCR 10 to 100 million fold. The products are detected by gel electrophoresis. One prostate cancer cell among 1 to 10 million lymphoid cells can be detected, allowing for the detection of micrometastasis. The detection of so few cells does not necessarily predict the development of metastatic tumours. A minimum number of cells are needed to seed a tumour. Some groups have proposed the use of other gene products such as CD44 in addition to PSA to detect aggressive growth potential (Takayama et al., 1994).

It is difficult to summarise the clinical data for PSA due to differences in each study. Many parameters were investigated, such as PSA, PSAD, and PSA-DT. A number of investigators used different assays to measure PSA and a variety of methods to measure other parameters such as prostate volume. A wide range of patient populations was used with different PSA levels. No standard statistical methods were used in all cases and data was reported differently. However, based on the data from references listed in Table 3, some general conclusions can be made and are listed below:

1. The positive predictive value (PPV) for PSA of > 4 ng/mL ranges from 33% to 47% and for PSA greater than 10 ng/mL from 36% to 51%.

2. The specificity of this assay is limited by the overlap of BPH and prostate cancer at PSA levels of 4 ng/mL. At a specificity of 88% to 90%, the sensitivity of PSA > 4 ng/mL ranges from 46% to 89%,

3. Using PSAD significantly improves sensitivity or specificity. However, variation is introduced by measuring the volume of the prostate by TRUS. Age related cutoffs, which factor in enlargement of the prostate gland with age, can be used instead.

4. Measuring PSA velocity can be useful if the patient is monitored over several

years, especially with patients that have undergone a radical prostatectomy. It can also be useful in differentiating between metastatic and localised disease. This parameter may not be useful for screening the general population due to normal day-to-day variation of PSA levels.

5. Currently, the staging of prostate cancer is inadequate. PSA may assist in differentiating between localised and advanced metastatic disease. A rise in PSA after a radical prostatectomy can signal recurrence of disease in advance of any clinical evidence. The development of ultrasensitive assays and the use of RT-PCR may improve the utility of PSA as a early marker of metastatic disease.

6. The measurement of PSA complexes can improve accuracy in the PSA level range of 4 to 20 ng/mL. The ratio of free-to-complexed PSA is dependent on PSA concentration at higher PSA levels. There are problems with assay standardisation associated with the formation of PSA complexes in serum.

7. The usefulness of screening the general male population for prostate cancer is limited by the cost effectiveness of the screen and the possibility of over diagnosis and over treatment of subclinical disease.

8. In spite of its many problems, PSA is still the best available marker for the detection and monitoring of prostate cancer.

2.0 PSA assays

The first assays to measure PSA were based on isoelectric focusing, immunodiffusion, immunoelectrophoresis and rocket immunoelectrophoresis (Papsidero et al., 1980, 1981; Kuriyama et al., 1982). An enzyme linked immunosorbent assay using polyclonal antibodies was first developed by Wang et al. (1981, 1982). The lower limit of detection of this assay was 0.1 ng/mL. Other groups developed two and three site RIAs and ELISAs using polyclonal and monoclonal antibodies. A list of PSA assays and their performance characteristics is shown in Table 4.

There is no standard approach to determine performance measures for immunoassays, but a number of standards for the evaluation of PSA assays have been proposed (Graves et al.,1990; Vessella and Lange, 1993). The lower limit of detection (LLD) is defined as the lowest value that can be distinguished from 0 with 95% confidence under ideal conditions. It is a function of assay design, signal strength, and intra-assay precision. LLD is usually expressed as the average ± 2 standard deviations (SD) of 20 or more repetitive intra-assay measurements of a buffer solution. The

assays are repeated using serum with no analyte. The two sets of values should be identical. This parameter is not always useful clinically because of variability of the assay from day-to-day (inter-assay precision). The biological detection limit (BDL) is defined as the PSA concentration above which there is a 95% confidence that on repeat testing of the same sample on a subsequent run, the PSA value will remain above the LLD. This parameter is precision dependent.

To measure the BDL for an assay with an LLD of 0.05 ng/mL, samples with PSA values from 0.06 to 0.10 ng/mL are selected. The inter-assay precision with >10 independent assays is determined and the average and standard deviation calculated. The biological limit is LLD + 2 SD. A more clinically relevant parameter is the clinical threshold or residual cancer detection limit (RCDL). This parameter is difficult to determine for PSA and is set by the values obtained from "cured" patient serum.

Other assay performance criteria commonly used include intra-assay precision, inter-assay precision, dilution of linearity, and spike and recovery (reviewed in Vessella and Lange, 1993). Intra-assay precision is a measure of the variability of a PSA value within an assay. A least 20 replicates of a sample are assayed in one run and the average and standard deviation calculated. A SD of < 5% is usually acceptable. Inter-assay precision is a measure of between assay variability. The same samples are run by a number of different operators in at least 10 different assays. An SD of < 5% for values in the high and medium range and a SD of < 10% for values in the low range is normally acceptable. Regression analysis along the entire standard curve should yield a slope of 1.00. This is referred to as the linearity of dilution. Non-linear slopes can be caused by matrix effects.

Interfering or cross-reacting substances may alter PSA values. The PSA values should be within 95% of the value obtained from control serum in the presence of spiked interfering substances. Substances usually tested include common blood proteins like bilirubin and haemoglobin, chemotherapeutic drugs, and homologous proteins such as kallikrein.

A spike and recovery test is performed by adding a known amount of PSA to control serum. The assay should recover 100% of the PSA value. The formation of complexes in sera not detected by the assay or degradation of PSA by serum enzymes will result in low recovery of PSA.

The high dose hook effect occurs with many PSA assays including Tandem R and IRMA Count (Alfthan and Stenman, 1988; Nomura et al., 1983; Rodbard et al., 1987; Vaidya et al., 1988; Wolf et al., 1989). In these assays, unusually high samples give

falsely low values. Excess antigen saturates the solution and solid phase antibody binding sites, preventing sandwich formation. This is also called a prozone (or prezone) effect. The high dose hook may also be due to heterogeneity of affinity of the binding site of polyclonal antibodies, autodigestion resulting in fragments which will not sandwich, and inadequate washing. Solutions to this problem include the use of a two step assay and dilution of serum at two or more concentrations (Bodor et al., 1989; Ryall et al., 1982). In the Tandem R assay a high dose will give a result which is above the linear range of the assay, requiring further dilution. The IMx assay from Abbott addresses this problem with a two-step immunoassay, which incorporates a second wash step before the addition of second antibody (Vessella et al., 1992).

Heterophile antibodies, such as human anti-mouse antibodies (HAMA), may influence PSA values (Thompson et al., 1986). Excess normal mouse IgG, added to the reaction buffer, can reduce this effect. The HAMA response is especially important with patients undergoing radioscintigraphic scans with labelled murine antibodies (Morton et al., 1988; Primus et al., 1988). Assays which use a double Mab design can form a sandwich with immobilised and tracer antibodies and produce a false high signal. Alternatively, antibodies can sterically hinder PSA binding to antibodies and lower the signal.

Recently many authors have discussed the need to standardise PSA assays (Graves, 1990; Stamey et al., 1994; Takayama et al., 1994; Vessella et al., 1993; Wu, 1995). Table 5 shows linear regression data of serum samples analysed for PSA in different assays. There are some major discrepancies between assays, especially between Pros-check and Tandem R. The serum value obtained in a Tandem R assay is 1.8 times that obtained in a Pros-check assay. However, this calibration factor is not accurate throughout the linear range of the assays, which makes it difficult to compare values between assays. The normal range determined by the Proscheck assay is 0 to 2.5 ng/mL, for Tandem R it is 0 to 4.0 ng/mL. Even among the FDA-approved assays there are differences in values obtained with clinical samples. These discrepancies may be due to the use of polyclonal vs. monoclonal antibodies, antibodies directed against different epitopes, differences in recognition of free and complexed PSA, and equilibrium vs. kinetic assay design.

At present, there is no international standard for PSA (Graves et al., 1993). In order to develop one, a number of factors need to be considered. The method of purification is important and the method used by Sensabaugh (Sensabaugh and Blake, 1990) has been proposed as a standard method to purify an international PSA standard

(Graves et al., 1993). Sensabaugh (Sensabaugh and Blake, 1990) reported an extinction coefficient of 1.42. A calculated value of 1.69 based on the amino acid sequence and the amount of glycosylation has been proposed (Lundwall et al., 1987; Schaller et al., 1987).

Since PSA in serum is complexed to ACT or MG, concentration expressed as ng/mL is not a valid measure of immunogenic PSA concentration. A standard using units/mL may be more relevant (Graves et al., 1993; Vessella and Lange, 1993). It would be difficult to make a PSA-ACT standard since all of the PSA used must be enzymatically active to complex completely. The use of purified PSA-ACT as a standard has been proposed (Stamey et al., 1994; Wu, 1995). This strategy will not standardise the existing commercial assays if the antibodies used in the assays recognise free PSA only.

In order to determine the specificity of anti-PSA antibodies, the epitopes recognised by these antibodies need to be characterised. The following sections will discuss the nature of antibody-antigen recognition, the use of peptides as mimotopes, and the methods used to locate and characterise epitopes.

Table 4: PSA assays

Assay Name	Manufacturer	FDA appro.	Assay Type	LLD (BDL) at lowest STD ng/mL	Inter-assay %CV	Linear Range ng/mL	% Recovery range
[1]Pros-check	Yang Laboritories	no	RIA, poly.	0.25	5 - 7	0 - 100	96 - 116
[1]Tandem R PSA	Hybritech	yes	RIA, Mab	0.1-0.2	1.3 - 5	0 - 100	97 - 102
[1]Tandem E PSA	Hybritech	yes	EIA, Mab	0.3	<5	0 - 100	99 - 108
[1]IMx™	Abbott	yes	EIA, Mab, Auto, MP	0.24	<5	0 - 100	nr
[1]Stratus™ PSA	Dade/Hybritech	yes	FIA, poly, Auto	0.1	7	0 - 200	nr
[1]AIA-1200™	Tosoh	yes	RIA, Mab, Auto	0.2	5	0 - 200	nr
[1]ACS180™ PSA	CIBA-Corning	yes	CIA, Mab/poly	0.2	nr	2 - 60	nr
[1]IRMA Count PSA	Diagnostic Products	no	RIA, Mab	0.18	nr	nr	nr
[1]Zundel, 1990	non-commercial	no	EIA, Mab	0.05	4	0.1 - 60	96 - 109
Vihko et al., 1990	non-commercial	no	EIA, Mab	0.005 (0.008)	20	0.15 - 0.5	101 - 125
Equi-Molar PSA	CanAg® Diag.	no	FIA, Mab, PSA-ACT	0.009	13	0.01 - 1.0	90 - 98
Klee et al., 1994	non-commercial	no	CIA, Mab	0.03	5 - 8	0.03 - 500	66 - 113
Liedthe et al., 1993	modified Tandom E	no	EIA, Mab	0.16	10 - 14	0.16 - 450	53 - 115
Cattini et al., 1993	Serono Diagnostics	no	EIA, Mab, Auto	0.1	7 - 12	0 - 100	92 - 104
Yu, et al.1993	non-commercial	no	EIA, Mab/poly, TRF	0.002 (0.01)	14	0.01 - 0.1	nr

[1]data from Armbruster, 1993

TRF = Time resolved fluorscence, CIA = Chemiluminescence immunoassay,Ab = Absorbance, FI = Fluorescence, Ch = Chemiluninescence, US = ultra-sensitive, RIA = Radioimmunoassay, EIA = enzyme-linked immunoassay, FIA = Flourescence immunoassay, Mab = monoclonal antibody, Poly. = polyclonal antibody, Auto = automated, MP = microparticles, FDA = Food and Drug Administration, USA, approval. PSA-ACT = Prostate-specific antigen complexed with α1-antichymotrypsin.

Table 5: Calibration of PSA assays using linear regression (y = ax - b).

Assay y	Assay x	Slope (a)	Intercept (b)	r
[1]Tandem R	Pros-Check	1.85	-0.41	0.988
[1]Tandem E	Tandem R	0.977	-0.231	0.997
[1]IMx™	Tandem R	0.9	-0.231	nr
[1]IRMA Count	Tandem R	1.4	0.42	0.995
[1]ACS180™	IMx™	1.81	-0.04	0.997
[1]AIA-1200™	Tandem R	1.3	nr	nr
[1]Equi-Molar PSA	Tandem R	0.952	0.096	0.99
Klee et al., 1994	IMx™	0.90	nr	0.94
Klee et al., 1994, Urinary PSA	IMx™	1.46	nr	0.982
Liedthe et al., 1993	Tandem E	0.99	-0.003	1.00
Cattini et al., 1993	Tandem E	1.13	-1.98	0.98
Yu et al., 1993	Tandem E	1.031	0.10	0.96
Yu et al., 1993	IMx™	1.013	0.205	0.99

* nr = not reported, r = coefficient of regression

[1]data from Armbruster, 1993

3.0 Antigen-antibody recognition

3.1 Structure and function of antibodies

Twenty years ago the first high resolution crystal structures of antibody fragments were determined (reviewed in Bentley, 1994). Since that time a great deal has been learned about the structure and function of antibody-antigen recognition (reviewed in Padlan, 1992). The antibody molecule consists of constant and variable domains which exhibit a characteristic basic folding unit of approximately 100 amino acids. Three highly variable segments, found in the N-terminal domain of each chain, are responsible for the diversity of antigen recognition by antibodies. These segments are widely separated in the primary structure but are brought into close proximity at the surface of the antibody by the folding of the polypeptide chains. The immunoglobulin fold, which produces this effect, is a double β-sheet sandwich pinned together by a disulfide bridge, made up of cystine residues located in conserved positions in the sequence. The strands of the β-sheet are connected by loops formed by hairpin turns. The strands need not be connected to neighbouring strands but can be linked by a loop to a strand on the opposite sheet or to a distant strand on the same sheet. These loops define the topography of the domain. The conservation of the surface topography allows different heavy and light chains to combine freely and interchangeably and contributes to the diversity of the binding site of the immunoglobulin molecule. The immunoglobulin fold is the same in all known immunoglobulin domains and is also found in other molecules of the immune system belonging to what is now known as the immunoglobulin superfamily.

The IgG molecule consists of two 214 amino acid light chains (L) and two 446-amino acid heavy chains (H) joined by disulfide bridges. Cleavage by enzymes can produce antibody fragments such as Fab, F(ab')2, and Fc. Three loops on the light (VL) chain (L1, L2, L3) and three loops on the heavy (VH) chain (H1, H2, H3) of the variable domain form the antigen binding site. The amino acid sequence of these loops is hypervariable and is responsible for the specificity and binding affinity of the antibody. The loops are also called the complementarity-determining region and are named CDR 1, CDR2, CDR3, CDR4, CDR5, and CDR6, in order of their appearance in the amino acid sequence. The double β-sheet frameworks of VL and VH domains together form a scaffolding of constant structure on which the antigen-antibody site or paratope is erected. Residues on the β-sheet which are not part of the loops are called the field residues. These residues play an important role in stabilising the conformation of the

loops and consequently of the antibody antigen binding site.

The usual conformation of a protein is an α-helix or β-strand. Loops appear on the surface of proteins and reverse the direction of the polypeptide chain. Three or four residues can form hairpin loops with conformation that is outside of the norm. Residues such as Gly, Asn, or Pro are often associated with loops and allow the unusual conformation to occur. In immunoglobulin molecules the loops are longer than normal and hairpin loops are not common. The conformation in this case is not intrinsic to the amino acid sequence of the loop but involves tertiary interactions with H-bonding and packing (Chothia and Lesk,1987). The framework or field residues in the conserved sequences can determine the conformation of the loop. Several canonical structures of the loops found in immunoglobulins have been identified (Chothia and Lesk,1987; Chothia et al.,1989). The structure is determined by a few key residues and the length of the loop itself. Five of the hypervariable regions have only a few main chain conformations or canonical structures. None have yet been identified for the H3 or CDR6 loop. Sequence variations in the loops only modify the surface of the binding site by altering the side chains on the same canonical main chain structure. However, sequence changes at a few specific sets of positions switch the main chain to a different canonical conformation. Hypervariable regions on different molecules that have the same residues at these sites can have very similar conformation. This is exploited in the technique of chimerization of murine antibodies to humanise certain domains by grafting murine CRD regions onto human framework regions (reviewed in Lesk and Tramontano, 1992).

3.2 Paratope diversity

In order to characterise epitopes on PSA, the diversity, affinity, and specificity of antibody-antigen recognition must be considered. In the following two sections the structure and function of antibody binding sites (paratope) and epitopes are discussed.

Somatic mutations or point mutations accumulate in the variable region of the antibody and are associated with altered affinity (reviewed in Colman, 1994). Examples show that a very conservative substitution may abolish binding (Colman, 1994; Knossow et al., 1994: Varghese et al., 1988; Tulip et al., 1992). Other examples show that a non-conservative change may not affect binding affinity (Colman, 1994). The potential number of antibodies using the genetically coded amino acids is 10^{18}. Assuming that there are only 4 or 5 different amino acids defined in structural terms

for folding purposes, the number of different antibodies possible is 10^8. Assuming that the surface size of the epitope is about 15 amino acids, the number of different epitopes possible is 10^{19} for 20 amino acids and 10^{10} for five classes of amino acids (reviewed in Colman, 1994). Genetic diversity of antibodies is only part of the story. Side chain rearrangements and main chain changes within the loop structure also contribute to diversity of antibodies (Bhah et al, 1990; Herron et al., 1991; Rini et al., 1993; Stanfield et al., 1990; Stanfield and Wilson, 1993). Global changes in the VL-VH interface of CDRs have been observed during engagement with an antigen. These rearrangements and changes are specific to the antigen. Different antigens may induce different structural responses in the same antibody. The net result is an increase in diversity. Affinity increases with time through somatic mutation and may eventually reach saturation. Due to the structural restrictions of the paratope imposed by canonical combinations it becomes increasingly difficult to mutate towards further improved binding. Genetic shift towards other germ line gene combinations is observed in the later stages of the immune response (reviewed in Colman, 1994).

The study of escape mutants has shown that changes in the antigen that altered the shape of the epitope prevent binding if the other residues in the epitope are rigid (Tulip et al., 1990). Loss of a key hydrogen bond can also prevent binding or lower affinity (Bhat et al.,1990). Antigen-antibody systems are more sensitive to amino acid changes than other systems. A key factor appears to be the inflexibility of the surrounding structure as well as the physico-chemical environment. Looser packing density of atoms is seen with antigen-antibody interfaces compared to protein-protein interfaces or to the interior of folded protein molecules (Tulip et al.,1992)

3.3 Epitopes

Epitopes or antigenic determinants are recognised by specific regions of immunoglobulin molecules called paratopes. The affinity constants for the epitope-paratope interaction can range from 10^3 to 10^{11} L/mole (reviewed in Van Regenmortel, 1992). Epitopes can be divided into two main groups, continuous and discontinuous epitopes (Atassi and Smith, 1978). Continuous epitopes are identified by short linear peptide fragments that are able to bind to antibodies raised against the intact protein. These antibodies cross-react, albeit weakly, with free linear peptides. The free peptide is unlikely to mimic exactly the conformational structure of the epitope on the

protein where it is constrained. A short peptide is unconstrained and may represent a small portion of a larger more complex epitope. Continuous epitopes have been found to contain as few as 5 to 8 amino acids with 2 to 3 contact residues (Geysen et al., 1984, 1988; Schoofs et al., 1988: Trifilieff et al., 1991). The minimum number of contact residues in an epitope has not yet been determined. Some antibodies can recognise a single C-terminal residue, while others have been found to bind specifically to dipeptides. Larger peptides may have only a few contact residues. The remaining residues contribute to binding by providing a scaffold to hold the contact residues in a favourable conformation.

A discontinuous epitope is made up of residues not continuous in sequence, brought together by the folding of the polypeptide chain. This type of epitope is believed to occur more frequently than continuous epitopes (reviewed in Van Regenmortel, 1992). The ability of an antibody to bind to a discontinuous epitope depends on the conformation of the protein. Antibodies which recognise discontinuous epitopes will not bind to denatured proteins or peptide fragments derived from the amino acid sequence of the native antigen. The majority of monoclonal antibodies studied recognise discontinuous epitopes (Quesniaux et al., 1990). Only about 10% of monoclonal antibodies or 10% of the antibodies in polyclonal antiserum will recognise linear epitopes and cross-react with peptide fragments. Peptides can cross-react with antibodies directed to discontinuous epitopes by mimicking the conformation of the epitope. The amino acid sequence of the peptide may not resemble the sequence found on the epitope on the protein but the shape of the epitope is approximated so that binding can occur. This type of peptide antigen is referred to as a mimotope (Geyson et al.,1986).

Laver et al. (1990) proposed that native proteins do not contain linear epitopes. Antibodies may bind to unfolded regions of the antigen called unfoldons that are recognised by antibodies raised against denatured proteins. The antigen used for immunisation may have been denatured or the antibodies selected with an assay that uses antigen-adsorbed on a solid support, a process known to alter the conformation of proteins (reviewed in Butler, 1992). There is evidence, however, that true linear epitopes exist. Some anti-peptide antibodies can neutralise the function of protein antigens. This would not occur if the antibody only recognised denatured proteins. Also, peptides have been used successfully as vaccines in animal studies to produce antibodies which cross-react with native protein (Anderer and Schlumberger, 1965; Emini et al., 1985; McCray and Werner, 1987: Parry et al., 1988; Roehrig et al., 1989; Smyth et al., 1990).

3.4 Structural vs. functional epitopes

Epitopes can be defined in either structural or functional terms (reviewed in Smith-Gill, 1994). The structural epitope, determined by X-ray crystallography is the topographical area of the protein surface that is in contact with the antibody. There are many common features of a structurally defined epitope. Typically 15 to 20 residues are in contact on both the antigen and antibody. There is a high degree of complementarity of the two surfaces in topography, polarity, and charge. The buried surface areas are about 750 Å for both the paratope and epitope. Usually all six CDRs are in contact with the antigen. The degree of contact and regions of predominant interactions vary for each complex. Discontinuous epitopes including segments from at least two different portions of the protein antigen are involved in binding. Typically, 10 to 15 hydrogen bonds and 100 van der Waals contacts involving both side-chain and main chain interactions, are seen. The relative importance of hydrogen bond, van der Waals interactions and salt links vary for each antigen-antibody complex (Davies et al., 1990; Gestoff et al., 1988; Laver et al., 1990).

Functional epitopes are defined by functional assays, including binding to antibodies or to antigens with point mutations, cross-reactivity to homologous proteins, and amino acid replacement analysis (Smith-Gill, 1994). These and other functional assays identify unique sets of 4 to 12 exposed amino acids that are involved in binding. Functional epitopes usually include fewer residues than structural epitopes. The presence of more overlapping epitopes seen with functional studies suggests that multiple antibodies may bind an antigen simultaneously (Davies et al., 1990). These epitopes are much more closely positioned than would have been predicted using the structural epitope model. The clustering of epitopes, determined by antibody inhibition assays, can be used to determine regional localizations of epitopes and indicate antigenic areas on proteins. Antigenic regions may be larger than both individual structural or functional epitopes and may contain multiple epitopes that can overlap to varying degrees (Newman et al., 1992).

The failure of most monoclonal antibodies to bind to peptides designed to contain critical residues suggests that the surrounding amino acid residues are essential for binding. These residues contribute to the overall physical and chemical complementarity, even though their energetic contribution cannot be immunochemically detected. (Smith-Gill, 1994)

3.5 Antibody-peptide interactions

Antibody-peptide interactions have been extensively studied by Wilson et al (reviewed in Wilson et al., 1994). In these studies the crystal structure of the antibody-peptide complexes of Fab fragments of antibodies raised against peptides was determined. The size of the epitope on a peptide ranged from 7 to 12 residues with 7 to 8 residues being average. The size of the buried interface surface is 480 $Å^2$ for peptide antigens and 550 $Å^2$ for antibodies. This was smaller than the buried interface for protein antigen-antibody complexes which range from 700 $Å^2$ to 900 $Å^2$. Peptides, then, access only about 50% to 70% of the possible Fab surface that can interface with a protein epitope. Stanfield and Wilson (1993) found that the shapes of antibody binding sites for anti-peptide antibodies resemble the clefts of grooves seen for haptenic ligands. Anti-protein antibodies tend to have flatter, more undulatory, surfaces that correspond more to the shape of a globular protein. Little correlation is seen with the area of antibody buried by the ligand and the relative affinity. A very small ligand which buries comparatively little surface (~280 $Å^2$) can have nanomolar binding affinities (reviewed in Davies et al., 1990; Colman, 1994; Stanfield and Wilson, 1993; Wilson and Stanfield, 1993).

Conformational changes, or induced fit, hve been observed in the case of antibody-peptide interactions. Due to the small number of residues involved in a peptide antigen, high affinity binding can only be achieved if the peptide is substantially buried in the antibody binding site. For anti-peptide antibodies, antigen mediated changes are seen which include small segmental shifts, side chain shifts of 1 to 2 Å, CDR loop changes, large rearrangements of the H3 loop, and global rearrangements in the VL-VH interface (Bhat et al., 1990; Herron et al., 1991; Rini et al., 1993; Stanfield et al., 1990; Stanfield et al., 1993).

An example of induced fit of a peptide antigen is shown in studies of anti-menadione (Vitamin K3) antibodies (reviewed in Edmundson, 1994). The antibody binding pocket was found to be too small for the antigen. The walls of the pocket were displaced by the ligand, and after days of soaking, the ligand was funnelled down into the solvent channel into a deep pocket. Once the deep pocket is occupied, the original binding site is no longer capable of binding. Free peptides in solution differ in structure from bound peptides. Peptides often show a preference for turn structures when bound to

antibodies (Edmonson, 1994). For peptide antigens, induced fit may play a more important role than for protein antigens.

3.6 Cross-reactivity

Shared reactivity can occur when two multi-determinant antigens share one or more identical epitopes (reviewed in Bentley et al., 1994). An example of this phenomenon is seen with antibodies to CA19.9, a cancer-associated antigen found on the surface of mucin molecules. The epitope occurs many times on the same molecule and on a variety of different mucin molecules. Highly homologous proteins such as PSA and kallikrein share identical amino acid sequences in some regions. An antibody directed against this common epitope will recognise both of these antigens and will have reduced immunospecificity (reviewed in Bentley, et al. 1994; Berzofsky and Schechter, 1981; Harper et al.,1987; Van Regenmortel, 1992).

True cross-reactivity is the recognition of an epitope that is different in amino acid sequence but structurally related. These epitopes share a similar conformational shape that 'fits' the antibody paratope. This type of cross-reactivity is called heterospecificity or heteroclitic binding. It is possible to find an epitope that is bound more strongly than the epitope on the antigen used for immunisation.

Small peptides which are recognised by antibodies directed to an epitope on a protein will bind with lower affinity than the native protein. One reason for this may be that there are fewer contact points with the CDRs on the antibody and fewer CDRs are involved in binding. If only a few CDRs are in contact with the protein epitope it is more likely to cross-react with an epitope not similar to the original. High non-specific binding to unrelated proteins peptides may be enhanced in immunoassays with a high density of antigen immobilised on a solid support or with molecules with a high density of epitopes.

Cross-reactivity is affected by the contact residues. Two antibodies raised against the same epitope may have different contact residues. An amino acid substitution at one position on the epitope may affect binding for one antibody and not another, causing one antibody to cross-react and not the other. Residues in the antigen-antibody complex may be exploited in different ways. A side chain may form a hydrogen-bond in one complex and be involved in van der Waals contacts in another. The exploitation of residues forming the antigen-binding site can vary with cross-reacting antigens even when the conformation is unchanged. The solution to complementarity for antigen-antibody

complexes is not unique and can vary with different antibodies to the same epitope. Water molecules can be trapped within the antibody-antigen interface, occupying voids where complementarity between antigen and antibody is less than perfect. Buried solvent can be displaced to allow alternative modes of achieving binding. Readaption of the peptide conformation at the interface of the cross-reaction complex can occur. High affinity does not necessarily coincide with high specificity (reviewed in Bentley et al., 1994; Berzofsky and Schechter, 1981; Harper et al.,1987).

4.0 Methods for localising epitopes

A number of methods have been developed to locate epitopes on protein antigens including X-ray crystallography of antigen-Fab complexes; use of peptide fragments as cross-reactive antigenic probes; identification of critical residues in peptide fragments by systematic replacement studies; use of fusion proteins and peptides; study of mutants, analogs and homologous proteins; and topographic mapping by competitive binding assay (reviewed in Atassi, 1984; Benjamin et al., 1984; Berzofsky, 1985; Jemmerson and Paterson, 1986; Van Regenmortel, 1984, 1989, 1992). Many of these techniques were used in this study to characterise the epitopes on PSA.

4.1 Synthetic peptides and peptide fragments

A fragment of a protein antigen, or a synthetic peptide derived from a native protein's sequence, which cross-reacts with the antibody can identify a continuous epitope. The binding affinity to the peptide is usually very small except for peptides at the chain termini (Absolom and Van Regenmortel, 1977; Milton and Van Regenmortel, 1979; Taimer et al., 1985; Walter, 1986; Westhof et al., 1984). The termini of the protein are more mobile and surface-oriented. A small peptide is better able to mimic the conformation of this epitope than an epitope located in the middle of the protein antigen.

The affinity constant of an antibody-peptide complex is usually found to be several orders of magnitude lower than for the protein antigen. This is due in part to the fact that a linear peptide does not accurately mimic the complete structure of the epitope and may represent only a part of a larger discontinuous epitope on the protein. Berzofsky et al. (1981) proposed three mechanisms that would account for this apparent low affinity. The first possibility is that functional groups on the peptide are

arrayed in a way that results in less than maximal complementarity with the antibody combining site. Secondly, only a sub-population of peptide molecules is in a sufficiently native-like conformation at any given time to bind effectively. The binding in this case will be dependent on the conformational equilibrium constant of the peptide. Lastly, an intermediate model was proposed where the initial binding of a non-native conformation with less than maximal complementarity was followed by subsequent changes in the peptide which resulted in a native-like conformation. The microenvironment at the surface of the carrier or solid support may induce the peptide to adopt a more suitable conformation for antibody recognition. Ionisable groups at the termini of the peptide are not present in the polypeptide chain and may inhibit binding. Amidation and acetylation of the termini can eliminate this problem but removing the charge can also reduce reactivity, especially with antibodies that recognise an epitope at the C-termini (Gras-Masse et al., 1986; Hodges et al., 1988).

Synthesising a longer peptide will not necessarily result in higher affinity (Jemmerson, 1987; Wilson et al., 1984). The longer chain may induce folding which is different from the native protein. A smaller and more flexible peptide, on the other hand, may be able to fold into the correct configuration. Conformational mimicry can be increased by stabilising the structure of the peptide by cyclization. Information on the three-dimensional structure of the epitope is required for this (reviewed in Vuilleumier and Mutter, 1992). Peptides can be designed to mimic other structures found in proteins such as α helixes, β turns, and β sheets (reviewed in Kemp, 1990).

Peptides can be tested on the solid phase itself. Pepscan, developed by Geyson et al. (1986) has been used to identify a number of protein epitopes. There are limitations to this technique. Only 6 to 10 amino acid peptides can be synthesised, covalent attachment may impair antigenic activity, and some sequences give rise to non-specific binding. Bivalent binding, caused by a high density of epitopes on the pin, favours cross-reactivity.

Once a peptide fragment has been identified, the epitope can be further characterised using size analysis and replacement set analysis. In size analysis a series of peptides of decreasing length is made and tested until binding is no longer seen. Using this method, the minimum residues needed for binding can be determined. In replacement analysis each residue in the peptide is replaced in turn with the 19 other naturally occuring amino acids. In this way, the contact residues, the amino acids necessary for binding, can be identified. Residues which are contributing to the scaffold are identified by replacement of residues with side chains that are either smaller (Gly or Ala), larger

(Typ or Tyr), or have a different charge from the original amino acid.

Antipeptide antibodies which cross-react with the native protein can be used to localise epitopes (reviewed in Van Regenmortel, 1992). The antibodies are developed by immunising with peptide fragments and then tested for cross-reactivity with the native protein. An order-disorder paradox is seen with this kind of study (Dyson et al., 1988). Antibodies raised against highly disordered peptides recognise ordered epitopes on proteins with good affinity. The reverse in not usually true. The cause may be that a preferred conformation becomes stabilised on the carrier protein by the B-cell receptor during immune stimulation (Dyson et al., 1988).

4.2 Protein analogs and homologous proteins

Mutants, analogy, and homologous proteins can help identify and characterize epitopes (reviewed in Coleman, 1994; Van Regenmortel, 1992). Mutants with known amino acid substitutions can be used to determine the influence of single amino acids to antigenic recognition. The substitution of a single amino acid in a protein usually only causes local change;, no long-range structural alterations occur (Coleman, 1994; Van Regenmortel, 1992). Antibodies directed to adjacent epitopes are unaffected by the change. Site-directed chemical modifications or site-directed mutagenesis can be used to create a library of mutants. Infectious agents with neutralising antibodies have been used to select for "escape mutants." Identification of the substitution that leads to the loss of neutralisation will reveal the location of the epitope.

4.3 Topographical mapping

Topographic mapping of a protein using competitive binding assays with pairs of monoclonal antibodies can determine the relative positions of epitopes on the surface of the protein (Van Regenmortel, 1992). Steric hindrance may occur if the epitopes are too close to one another. A Fab fragment covers ~ 700 Å on the surface of the antigen, while the epitope may be smaller. With a large number of antibodies a continium of epitopes is sometimes seen (Mathews and Roehrig, 1984; Underwood, 1982). Competing antibodies can also enhance binding due to allosteric effects (Cecilia et al., 1988; Heinz et al., 1984).

4.4 Prediction of continuous epitopes

Some attempt has been made to establish empirical rules to predict the position of epitopes from the primary or secondary structure of the protein antigen (reviewed in Pellequer, et al., 1994). Hydrophilic residues tend to be surface-orientated rather than buried inside the molecule. Hydrophilicity and hydrophobicity profiles on proteins have been used to identify portions of the protein that are located at the surface of the molecule. This approach is not absolute since both hydrophilic and hydrophobic residues are observed in epitopes (Pellequer, et al., 1994).

Motions in polypeptide chains may be due to the rapid flipping of side chains or by the flexibility of the main chain. Atomic temperature factors, B values of Debye-Waller factors, have been used to predict the position of epitopes (Westhof and Alshuh, 1984). Correlation between the location of continuous epitopes and the mobile regions of proteins was seen if only main chain atoms were used. Mobility in proteins is mostly found on the surface loops and in turns of the polypeptide chain. There are examples of surface loops that are not mobile and not antigenic (Westhof and Alshuh, 1984).

In a recent study (Pellequer et al., 1991), hydrophobicity and hydrophilicity scales were found to be only 51% to 57 % correct, accessibility scales 46% to 50% correct, and scales which predict turns 53% to 61% correct. These results are a further indication that an epitope is not an intrinsic feature of a protein molecule but is defined only in terms of antigenicity and immunogenicity (Van Regenmortel, 1992).

4.5 Antigen mimicry with peptides (Mimotopes)

There are two basic approaches to developing peptides or peptide mimetics which mimic epitopes. The structural, or rational design, approach examines the structure and conformation of the protein epitope and attempts to create a peptide or mimetic which mimics the shape and charge characteristics of the epitope. This approach requires some knowledge of the three-dimensional shape of the epitope. In order to mimic secondary structural elements and discontinuous epitopes with a small flexible molecule the conformational space of the peptide must be restricted in some way.

Using the structural approach, peptides that mimic loops have been created by synthesising cyclic peptides using disulfide bonds. This approach is not always successful. Dorow (Dorow et al., 1985) synthesised myoglobin peptide analogs with D-cysteins at the N and C termini. Both open chain and cyclized peptides were used as

immunogens. Antibodies raised against the cyclic form cross-reacted poorly with native and linear peptides. A detailed study of the effect of loop size on antigenicity was made by Plaue (Plaue et al., 1990). Peptides derived from the same hemagglutinin loop were synthesised using linkers of different lengths. Antibodies raised against these peptides neutralised influenza virus in mice (Muller et al., 1990).

Other secondary structures can also be mimicked. Amphilic peptides which contain separate regions of hydrophobic and hydrophilic residues have been synthesised (reviewed in Kaiser, 1987; DeGrado, 1988; Mutter and Vuilleumier, 1989). These peptides allow for the formation of a protein-like hydrophobic core and hydrophilic surface. β-Turns have been mimicked using four residues which have a β-turn-forming potential inserted between two short sequences of β-sheet-forming amino acids (Beyreuther et al., 1987). Peptides which mimic epitopes on α-helixes have been created using the relevant residues on a helix face alternating with alanine (Beyreuther et al., 1987; Von Grunigen and Schneider, 1989).

Discontinuous epitopes have been mimicked with a linear peptide with two tandemly joined stretches of sequence from α and β subunits of human choriogonadotropin (Bidart et al., 1990). Site-specific antibodies were elicited which recognised the protein in solution when the peptide was used for immunisation. Kaumaya (Kaumaya et al., 1990) created peptides which contained a four-helix bundle. This study shows the importance of conformational structure on antibody recognition. Mutter (Mutter et al.,1989) covalently attached peptides to a well-defined common synthetic carrier. The peptide was attached to block anchoring points on the synthetic scaffold. These complexes are called template-assembled synthetic proteins (TASP). Standard method of peptide synthesis is used and a variety of secondary structures can be mimicked, including βαβ, 4 α, 4 α, 4 β, etc. (Ernest et al., 1990; Mutter and Vuilleumier, 1989; Rivier et al., 1990). Controlled construction of synthetic peptide immunogens is possible using this technique.

Peptide mimetics have side chains found on amino acids supported on a hydrocarbon backbone. The possible molecular shapes that can be created are limited only by the synthetic chemistry involved. The hydrocarbon backbone makes mimetics more rigid than peptides and mimetics are not as easily degraded by protolytic enzymes. Once a peptide which mimics an epitope is identified, a mimetic can be synthesised which has the same shape and side chain conformation as the peptide (reviewed in Moore, 1994).

The functional approach requires that the peptide mimics the function of the

target antigen. Knowledge of the structure of the epitope is not required. Peptides are synthesised and tested with continued modifications until the desired effect is seen. Alternatively, a library of peptides representing a large diversity of possible conformation is screened and peptides that show binding to the target antibody are selected. Random peptide libraries are discussed in the next section.

4.6 Random Peptide Libraries and Diversity

The recent development of recombinant random peptide libraries has facilitated the identification of peptide sequences that specifically bind to antibodies (Cwirla et al., 1990; Devlin et al., 1990; Scott et al., 1990). Libraries can be made synthetically or by using recombinant techniques. The power of a peptide library is determined by the diversity that can be generated. Scanning of structural space is possible using random peptide sequences. For a basic set of 20 naturally occurring amino acids the following number of peptides is required in order for the library to represent all possible combinations of sequences (Gallop et al., 1994).

3-mer peptide library	= 20^3 combinations	= 8000 peptides
4-mer peptide library	= 20^4 combinations	=160,000 peptides
5-mer peptide library	= 20^5 combinations	= 3.2 million peptides
6-mer peptide library	= 20^6 combinations	= 64 million peptides

The molecular diversity possible with peptide libraries is shown in Table 6. Recombinant libraries have the greatest degree of diversity with a maximum library size of 10^{12} structures (Gordon et al., 1994). A phage pIII peptide library, for example, can contain 10^7 to 10^8 phage clones.

Table 6: Molecular diversity and peptide libraries[1]

Type of library	Number in library	Uses of library
Recombinant peptide libraries identification	10^{12} to 10^{6}	Lead
Combinatorial synthetic peptide libraries	10^{11} to 10^{5}	Lead identification peptide analoging
Spatially Addressable libraries	10^{5} to 10^{1}	Lead identification peptide analoging
Multiple peptide libraries	10^{3} to 10^{1}	Analoging and fine tuning
Serial medicinal chemistry	10^{1} to 1	Fine tuning

[1]Adapted from Gordon et al., 1994

4.7 Recombinant peptide libraries

Using recombinant libraries, peptides can be constrained within the framework of a larger peptide or protein. Flexibility is reduced and tighter binding to the antibody is possible. Many types of fusion proteins have been used for peptide libraries (reviewed in Dower and Cwirla, 1993; Scott, 1992, 1994). fd filamentous bacteriophage has been engineered to display a random peptide sequence in the minor pIII protein. The epitope is inserted at or near the N-terminus of the mature protein. The amino terminal is more flexible, more exposed, more likely to tolerate epitope insertion and more accessible for recognition by the antibody. An epitope located at or near the amino terminus is less likely to be structurally influenced by the coat protein itself (Scott, 1992, 1994). A list of antibody or protein specific peptide sequences identified in studies using pIII phage display libraries is given in Table 7. These libraries are well characterised and easy to work with. The library can be readily amplified by infecting E. coli cells, and large libraries of greater than 10^{8} to 10^{9} can be constructed and screened. The fact that the peptide is displayed on four or five pIII proteins on each phage particle allows for multivalent interactions and increased avidity. The size of

peptides displayed by this type of library ranges from six to 36 residues (Kay et al., 1993). Cyclic peptide libraries, incorporating cysteine residues at each end of the random peptide sequence, have also been used (Hoess et al., 1993; McLafferty et al., 1993).

The major pVIII coat protein has also been used to display peptides on phage. pVIII is located on the body of the phage and while four or five copies of pIII are present there are several thousand copies of pVIII on each phage particle. This allows for high avidity binding to ligands with weak binding affinities. The size of the peptide that can be displayed on this protein is limited to five or six residues (Greenwood et al., 1991). Larger peptides can be displayed if they occur in combination with wild type pVIII proteins.

Other fusion proteins have been used to construct random peptide libraries. The Lac I repressor protein has been used (Cull et al., 1992; Schatz, 1993). In this system the epitope is displayed near the C terminus. The lam B gene product, a E. coli surface recognition receptor for phage λ and maltose, has been also used (Brown, 1992). Here the peptide is located on a surface-exposed loop. Peptides have been displayed on polysomes derived from wheat-germ extracts (Kawasaki, 1991). The peptides are displayed by cyclohexamide-treated ribosomes which have been stopped during translation by a library of mRNA. Human growth hormone (hGH) has been tethered to the amino terminus of pIII by a random peptide region that links the two domains of the hormone (Matthews and Wells, 1993). The results obtained using these alternate recombinant libraries are shown in Table 8.

In all of the above recombinant libraries the peptides were encoded by degenerate oligonucleotides which are chemically synthesised. The oligonucleotides are either inserted into the phage or plasmid genome or converted to RNA for expression of the library. The peptides are identified by isolating the carrier by affinity purification using the target molecule. The clone containing the antibody-binding peptide is amplified using phage or cells or PCR and the peptide sequence is deduced from the nucleotide sequence.

4.8 Synthetic peptide libraries

Synthetic peptide libraries consist of short peptides of six to 10 residues in length. These short peptides are highly flexible and are able to mimic a large number of structures. Chemical libraries can incorporate non-natural amino acids and non-amino

acid backbone structures, allowing for more diversity in both the backbone and side chains.

Multiple peptide synthesis is used to produce synthetic peptide libraries. The milder Fmoc/tBu chemistry and hydroxybenzotriazole-based coupling agents are preferred. The peptides can be synthesised on a variety of solid matrices in nMol to mMol quantities. Geysen et al. (1984) developed a system to synthese peptide libraries by parallel synthesis on polyacrylic acid-grafted polyethylene pins arrayed in a 96-well microtitre plate format. Up to 50 nmole of peptide can be synthesised on each pin. A direct-binding ELISA is used to detect peptides that bind the target molecule. The tethered peptides can be reused in many assays. Geyson claims to synthesise and screen thousands of peptides per week in his laboratory using this system. Linkers have been developed to allow for cleaved peptides in aqueous solution for competitive binding assays or functional bioassays. The most significant application of this type of library is antibody epitope analysis.

Epitopes are mapped by synthesising a complete set of overlapping peptides derived from the primary structure of the antigen. An ELISA is used to determine binding. Replacement set analysis is used to identify the critical residues for binding. Each amino acid in the peptide sequence is replaced in turn with all 20 amino acids or alanine to determine the effect on antibody recognition of the substitution within the epitope. Residues which poorly tolerate substitutions are implicated as being directly involved in the antigen-antibody interaction (Getzoff et al., 1988; Geysen et al., 1986).

The "Tea bag" method is used to synthesise peptide on resin sealed in porous polypropylene bags (Houghten, 1991). One amino acid is coupled to the resin in each bag. Up to 150 different peptides, each as long as 15 residues, can be synthesised in 500 umole amounts in one batch. This system gives greater synthetic flexibility as the reaction conditions for the coupling can be altered for each amino acid.

Non-traditional solid supports have been developed, such as cellulose, paper disks, or cotton fragments for the synthesis of peptide libraries (Blankemeyer-Menge and Frank, 1988; Eichler, et al., 1989; Frank and Doring, 1988). From one to three μmole of peptide per cm^2 of matrix can be produced. Peptides synthesised on functionalized cellulose sheets are particularly useful for screening in ELISAs. Like pins, the immobilised peptides can be re-assayed a number of times (SPOTs). Automated multiple peptide synthesis has also been used to develop peptide libraries (Gausepohl et al., 1992; Schnorrenberg et al., 1989; Zuckermann et al.,1992). Pipetting robots are

used with 48 to 96 reaction vessels to produce 10 to 50 μmol of peptide. The automated methods use continuous flow principles for peptide synthesis.

Combinatorial synthetic peptide libraries have been produced (Houghten et al., 1991, 1993; Pinilla et al., 1992; Dooley et al., 1993). Within each chemical coupling step, multiple compounds are generated simultaneously such that each synthesis cycle results in an exponential increase in library size. Mixtures of activated monomers are coupled to one or more solid supports at each cycle of the synthesis using split synthesis design. The support is divided, coupled, recombined and redivided for each coupling step. The result is a library of great diversity.

Combinatorial libraries can be produced on pins (Geysen, 1984, 1986). Two positions of the amino acid sequence are defined and a library of 20^2 peptides is synthesised and screened. A binding peptide is identified and new libraries are made in which the third position is defined. The new library is then screened for binding to the target molecule. The process is repeated until all positions have been defined and a binding peptide identified. The peptide identified in this way often does not contain sequences found in the original antigen and is called a mimotope. This method is particularly suited for the development of mimotopes to non-peptide epitopes and to epitopes that have not been well defined.

Light-directed spatically addressable parallel chemical synthesis has been developed to create peptide libraries (Dower and Fodor, 1991; Fodor et al., 1991; Jacobs and Fodor, 1994). This technique combines photo lithography and solid phase peptide synthesis. A photolabile protecting group is used. A specific region on the matrix is unmasked and deprotected using light. The amino acid is coupled only at the unmasked region. A new region can then be unmasked and deprotected. Another amino acid is coupled, and the process repeated. This method produces an array of areas with peptides with known sequences. Peptides are screened for binding using a fluorescent labelled antibody and laser confocal fluorescence microscopy.

Peptide libraries have been coded with oligonucleotides (Needels et al., 1993; Lerner et al., 1993, patent; Brenner and Lerner, 1992; Nielsen et al., 1993; Kerr et al., 1993; Nikolaiev et al., 1993). PCR is used to amplify the oligonucleotide sequence and the amino acid sequence is deduced. The major advantage of this approach is that the sensitivity of the PCR reaction allows for the screening of a small amount of peptide. Non-natural amino acids or other organic compounds can be used as tags. Arochlores (chlorinated aromatic compounds) and hydrocarbons have also been used as tags. This

system employs a binary code and capillary gas chromatography to deduce the amino acid sequence of the peptide (Ohlmeyer et al, 1993). Results obtained using synthetic peptide libraries are shown in Table 9.

4.9 Screening of libraries

Four general approaches are used to screen peptide libraries (Gordon et al., 1994). Affinity selection is used to isolate the peptide ligand,and the peptides are then analysed singly or *en bloc*. Affinity techniques are also used to label carrier-bearing peptide ligands and the peptide is isolated using the label. The functional activity to the target can also be used to modify peptide ligands. The isolation of the peptide is based on the modification. Peptide ligands from a pool of mixed peptides can be screened and progressively less diverse mixtures are used to deduce the composition of the peptide ligand. This process is called iterative screening.

Iterative screening can be used for large synthetic libraries. A number of mixed pools are synthesised and tested for binding. One position in the peptide sequence is defined and the remaining positions are randomised. Once a peptide mix is identified a pool containing a second defined position is screened and a dimer is identified. This process is repeated until all of the amino acid residues in the peptide are defined and an antibody-binding peptide identified. Positional scanning can be used to identify binding peptides (Pinilla et al., 1992). Libraries of defined dipeptides at different positions are tested. One hundred and twenty different peptide pools, required for 20 amino acids of a hexapeptide, can be screened in a single assay. The results from this assay are used to deduce the sequence of the peptide with optimum binding. Similarly, iterative screening using limited pools can be screened. Pools with the highest binding are selected and new pools are made with less diversity (Geysen et al., 1986; Tribbick et al., 1989; Houghten et al., 1991, 1993; Pinilla et al., 1993). The process is repeated using pools with increasingly less diversity until a peptide sequence with optimum binding is identified.

Table 7: Recombinant pIII bacteriophage random peptide display libraries[1]

Reference	Peptide size and type	Target	Ligand	Concensus sequence
Scott, 1990	6-mer	Two Mabs	Peptide	DFL
Scott, 1992	6-mer	Con A	methyl α-D-mannopyranoside	YPY
	6-mer	Mab	Linear epitope	RHSV(V/I)
Stephen, 1992	6-mer	Streptavidin	Biotin	GDW/FXFI
	6-mer	Streptavidin	Biotin	PWXWL
Guegler, 1993	6-mer	Poly	Biotin	YYLH
Smith, 1993	6-mer	S-protein	Peptide	F/YNFEI/VL
Cwirla, 1990 & Barrett, 1992	6-mer	Mab	Peptide	YGGFL
Oldenberg, 1992	8-mer	Con A	methyl α-D-mannopyranoside	YPY
Hoess, 1993	8-mer	Mab	Lactodifucotetraose	PWLY
	CX_6C	Mab	Lactodifucotetraose	none
Blond-Elguindi,1993	8-mer and 12-mer	BiP	Linear Epitope	*W/X*X*X*X*
Hammer, 1992	9-mer	HLA-DRB1allele 0101	Peptide	YX_2MXAX_2L
	9-mer	HLA-DRB1allele 0401	Peptide	WX_2MxTLX_2
	9-mer	HLA-DRB1allele 1101	Peptide	WX_2MXRX_3
Christian, 1992	6-mer and 12-mer	Mab	Linear Eptiope	ESTRP/AM
Devlin, 1991	15-mer	Streptavidin	Biotin	HPQ
Keller, 1993	15-mer	Mab	Linear Epitope	Y/FGPGR
Kay, 1993	$X_{18}PGX_{18}$	Strepatvidin	Biotin	HPQG/V
	$X_{18}PGX_{18}$	Poly	Mouse IgG	RTISKP
O'Neil, 1992	CX6C	glycoprotein IIb/IIIa	Linear Epitope	R/KGD
	6-mer	glycoprotein IIb/IIIa	Linear Epitope	none
McLafferty, 1993	XCX_4CX	Mab	Peptide	YGG/AF
	XCX_4CX	Streptavidin	Biotin	HPQF

[1]Adapted from Scott, 1994

Table 8: Other recombinant random peptide display libraries[1]

Reference	Library Type	Peptide size and type	Target	Ligand	Concensus sequence
Felici, 1991	pVIII phage library	9-mer	Mab	Linear Epitope	SND/E
Felici, 1993	pVIII phage library	9-mer	Mab	Assembled epitope	GRXPNP
Luzzago, 1993	pVIII phage library	CXgC	Mab	Assembled Epitope	GSXF
Cull, 1992	lac repressor	9-mer	Mab	Peptide	RQFKV
Schatz, 1993		Multiple libraries	BirA protein	Linear Epitope	IFEAQKIEWR
Brown, 1992	D-Receptor	9-180 residues	Iron Oxide	Unknown	RRTVKHHVN
Matthews, 1993	Substrate phage	5-mer	Substilisin/H64A	Linear epitope	Contain H
	Substrate phage	5-mer	Factor Xa	Linear Epitope	Contain R

[1]Adapted from Scott, 1994

Table 9: Synthetic peptide libraries:[1]

Reference	Library Type	Peptide size and type	Target	Ligand	Concensus sequence
Geyson, 1986	Peptides on pins	5-mer/ immobilized	Mab	Assembled epitope	WQMGHS
Tribick, 1989	Peptides on pins	5-mer/ immobilized	B-JP B-JP	Unknown Unknown	QfHpβA VβAwMhβAH
Houghten, 1991	Free peptides	6-mer to 8-mer	Mab Bacteria	Peptide Unknown	DVPDY RRWWC
Houghten, 1992	Free peptides	6-mer to 8-mer	Mab Receptor Bacteria	Peptide Peptide Unknown	PYPNLP YGGFM FRWLL
Pinilla, 1993	Free peptides	6-mer to 8-mer	Mab	Peptide	YGGFM
Pinilla, 1992	Free peptides	6-mer/positional scanning	Mab MAB	Peptide Peptide	DVPDYA PYPNLL
Blake, 1992	Free peptides	4-mer 6-mer	Poly Mab	Peptide Linear epitope	YGGFL PQVGHD
Songyang, 1993	Free peptides	Phospho-Y-tri-peptides	SH2 Domains	Linear epitopes	Multiple
Lam, 1991 Lam, 1993	Peptides on beads Peptides on beads	5&8-mer/immobilized 5&8-mer/immobilized	Mab Streptavidin	Peptide Biotin	YGGFL HPQ
Needels, 1993	Encoded peptides	5-mer	Mab	Peptide	RQFVVT

[1] adapted from Scott, 1994

5.0 Anti-PSA monoclonal antibodies; B80.7 and 87.1

In this thesis, the issue of immunospecificity of anti-PSA antibodies at the molecular level was addressed by characterising the epitopes recognised by two of anti-PSA monoclonal antibodies designated B80.3 (B80) and B87.1 (B87) developed at Biomira Inc. These two murine monoclonal antibodies were raised against PSA. They have been evaluated in a sandwich format RIA for their clinical utility. In this assay immobilised B87 was used to capture PSA from solution and iodinated B80 was used as the tracer antibody to measure bound PSA. The PSA serum values were found to have a high correlation with the PSA values of serum from prostate cancer patients obtained using the Hybritech Tandem R PSA assay.

The B80 hybridoma cell line was fused with an anti-horseradish peroxidase (anti-HRPO) cell line to produce a hybrid-hybridoma or quadroma secreting a bispecific monoclonal antibody with one binding site directed against PSA and the other against HRPO. This useful reagent eliminates the need for biotinylated or secondary antibody based detection methods and potentially has the highest specific activity of HRPO generated signal. Further, this bispecific monoclonal antibody probe eliminates the high background often created by the secondary polyclonal antibody-enzyme conjugates used in conventional immunoassays.

Recently Wu et al. (1995) investigated the specificity of antibodies from the Tandem-E PSA kit, a Dako polyclonal anti-PSA antibody and B80.7 monoclonal antibody. PSA isoforms, and PSA-ACT were separated by PAGE and detected by Western Blot. Wu et al. found that all the antibodies recognised all of the isoforms of PSA as well as free PSA and PSA-ACT. None of the antibodies could detect PSA-MG in HPLC column fractions, probably because the epitopes on PSA are masked by the macroglobulin. The differences in binding between the antibodies tested were in the antibody titres required to see binding. While all of the antibodies recognised the PSA-ACT complex and all of the PSA isoforms, B80 was found to have the highest affinity for the various forms of PSA.

6.0 Aims and objectives

As alluded to in the previous section, problems exist with the consistency of results in the currently used PSA assays for diagnosing and monitoring prostate cancer. These discrepancies occur because each assay uses a different set of antibodies which putatively recognise different epitopes on PSA. Other factors may include differences in

antibody affinity, cross-reactivity (specificity), assay format, and the nature of the PSA calibrators used in the assay. Additional difficulties in PSA assays were introduced with the new understanding that the antigen exists in both free and complexed form with ACT and α_2MG. Characterising the interaction between the anti-PSA monoclonal antibodies and free or complexed PSA may help resolve the inconsistencies in the currently used PSA assays.

In this work two anti-PSA monoclonal antibodies were characterised using new techniques which have been developed to help identify and characterise protein epitopes.

The objectives of this work are:

i) To identify and characterise the nature of the interaction of the two anti-PSA monoclonal antibodies using the techniques of epitope mapping, epitope scanning, affinity selection using a phage display peptide library, and computer modelling of PSA.

ii) To develop immunoassays to detect free and complexed PSA and investigate of the cross-reactivity to related kallikreins.

iii) To synthesise overlapping hexapeptides representing the entire length of the PSA molecule to help identify of potential linear epitopes or fragments of discontinuous epitopes which are recognised by the two anti-PSA monoclonal antibodies.

iv) To exploit a decapeptide phage display library to identify a sequence representing potential epitopes or mimotopes which interact with the two anti-PSA monoclonal antibodies.

v) To use a computer generated model of PSA in order to spatially visualise the location of the putative epitopes identified in this study.

vi) to use the information gained in this study to understand the performance of the assay using the two anti-PSA monoclonal antibodies.

VII. MATERIALS AND METHODS

1.0 Materials.

Anti-PSA mouse IgG monoclonal antibodies B80 and B87 were obtained from Biomira Inc., of Edmonton, Alberta, and anti-PSA x anti-peroxidase (HRPO) bispecific mouse IgG monoclonal antibody (bispecific B80) was obtained courtesy of M. R. Suresh, Faculty of Pharmacy and Pharmaceutical Sciences, university of Alberta. Standard media used for cell culture, RPMI-1640 supplemented with 2 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin and 5% fetal bovine serum and tetracycline were obtained from Gibco BRL, Gaithersburg, MD. Bacto-triptone and Bacto-yeast were obtained from DIFCO, NY. Polyclonal rabbit anti-mouse IgG, rabbit anti-rat IgG, rabbit anti-goat IgG and goat anti-rabbit IgG peroxidase conjugate, cyanogen bromide activated Sepharose 4B, porcine pancreatic kallikrein, kallikrein from human plasma, and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic Acid) (ABTS) were purchased from Sigma, St. Louis, MO. Maxisorp™ 96-Well Immuno Plates were obtained from Nunc, Roskilde, Denmark. Spin concentrators were obtained from Amicon, USA. PSA standard was purchased from Scripps Laboratories, San Diego, CA. α1-antichymotrypsin from human plasma (ACT) and rabbit anti-ACT IgG were obtained from Calbiochem, Lajolla, CA. Molecular weight markers for polyacrylamide gel electrophoresis (SDS-PAGE), precast 4% to 15% gradient polyacrylamide gels, SDS buffer cartridges, and the Phast™ electrophoresis system were obtained from Pharmacia, Uppsala, Sweden. The Mimotope kit, used to synthesise the immobilised hexapeptides, and the SPOTs kit used to synthesis peptide mimotopes were obtained from Cambridge Research Biochemicals, Cheshire, UK. A Vmax micro plate reader and MAX Line Plate Washer from Molecular Devices, Menlo Park, CA, was used. Eschericha coli strain K 91 (Hfi-C thi) was used in conjunction with the bacteriophage (phage) decapeptide display library (24). Dideoxynucleotide sequencing was performed using the Sequenase 2.0 system obtained from United States Biochemicals, St Louis, MO. One Step TMB (tetramethylbenzidine) substrate, streptavidin-HRPO and NHS-LC-Biotin were obtained from Pierce Chemical Co, Rockford IL. The Peptide Companion software package was obtained from CSPS (CoshiSoft/PeptiSearch), Tucson, AZ.

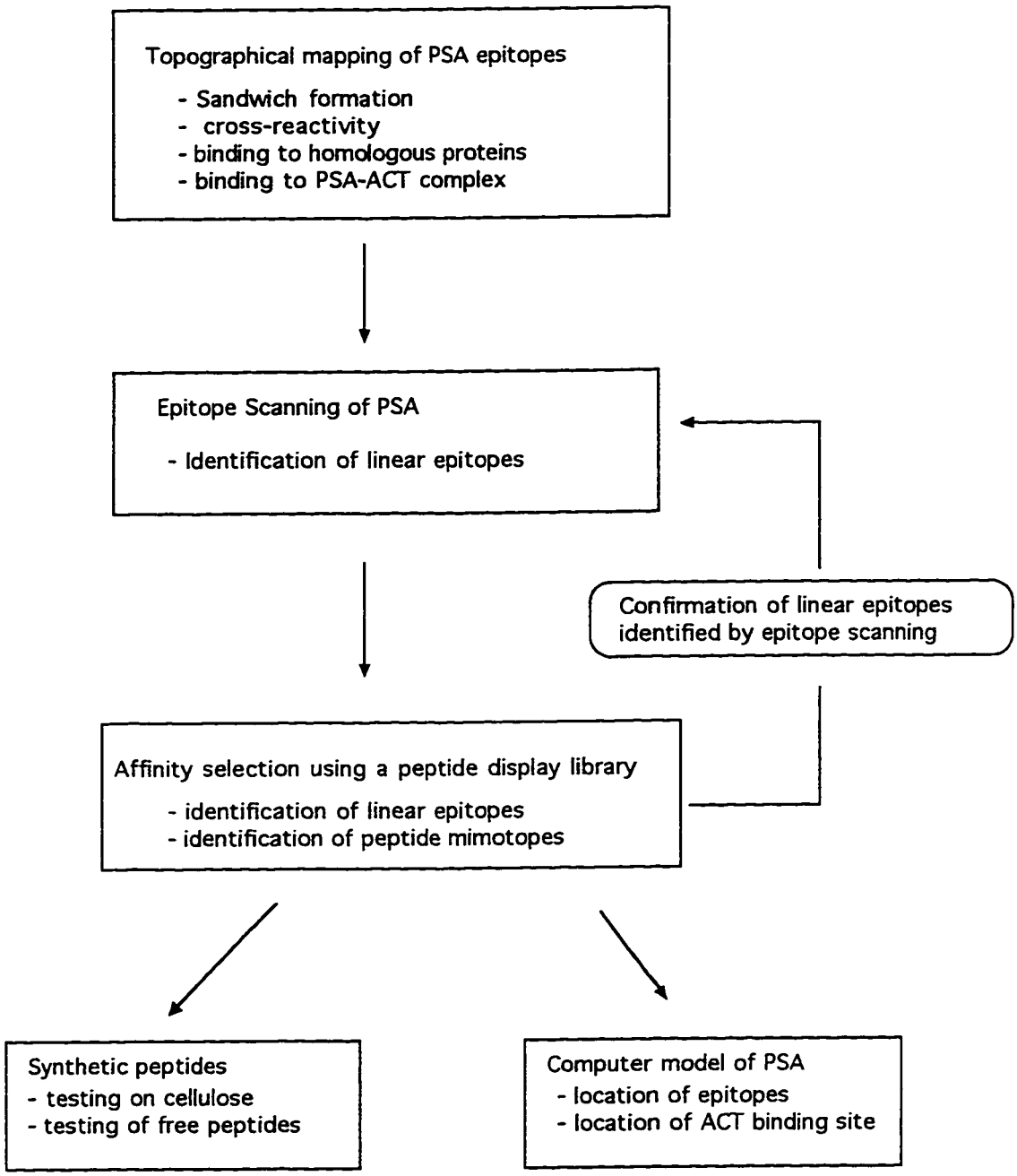


Figure 2: General strategy for mapping epitopes on PSA

2.0 General strategy

Figure 2 outlines the general strategy used to map the epitopes of PSA recognised by two anti-PSA monoclonal antibodies. The general characteristics of the epitopes of PSA were determined using immunoassays. The assays, described in detail below, were designed to determine three things: the ability of antibodies to form a sandwich with PSA, the degree of cross-reactivity with proteins which show a high degree of sequence homology with PSA, and the ability of anti-PSA antibodies to recognise PSA when complexed to ACT. Epitope scanning was used to locate possible linear epitopes for B80 and B87. A random peptide display library was used to identify peptide sequences that are specifically recognised by B80 and B87. Epitope predictions were visualized using a computer-generated model of the PSA molecule.

3.0 Purification of PSA

A human prostate cancer cell line (LNcap) was used as a source of PSA. The cells were grown (standard media, 275 mm^2 tissue culture flask) and the supernatant was collected and pooled every three to five days. An ELISA determined that he concentration of PSA in the unpurified supernatant was from 0.9 μg/mL to 1.5 ug/mL. PSA was purified from one litre of cell supernatant using an affinity column with immobilised anti-PSA antibodies B80 and B87. The column was made by first washing Sepharose 4B (one gram in 3.5 mL, cyanogen bromide activated) with two volumes of buffer (0.5 M phosphate, pH 6.8). A mixture of B80 and B87 (10 mg in three mL phosphate buffered saline, pH 7.2 (PBS) was dialysed overnight against three changes of buffer. The antibodies were added to the Sepharose gel and gently agitated at room temperature. The amount of antibody remaining in solution was determined by monitoring the reaction mixture absorbance at 280 nm (A280). The reaction was stopped when approximately 90% of the antibody was bound. The column was then washed twice with PBS (10 mL). Ethanolamine (10 mL of 100 nM in distilled water) was added to block any unbound reaction sites on the Sepharose (two hours at room temperature).

Prior to use, the Mab affinity column was washed twice (10 mL of 10 mM phosphate buffer, pH 6.4). Cell supernatant containing 1.5 ug/mL PSA (one litre) was cycled for 24 hours through the column using a peristaltic pump (1.7 mL/min.) so that the total volume of supernatant passed through the column three times. After washing the

column with buffer, the PSA was eluted with glycine in water (100 mM, pH 2.5). Column fractions (0.5 mL) were collected into tubes containing buffer (50 μL of 1M phosphate, pH 8.0) in order to neutralise the eluates. The protein concentration of each fraction was determined by measuring the absorbance at 280 nm and the appropriate fractions were pooled. After pooling, an ELISA was performed as described below to determine the concentration of purified PSA. The column, which can be reused, was washed (0.1% sodium azide in 10 mM phosphate) and stored at 4 °C until needed.

4.0 Preparation and analysis of PSA-ACT Complex

The complex was prepared using the method described by Lilja et al. (1991). Purified PSA (7μg) was added to purified ACT (50 μg) in reaction buffer (50 μL of 50 nM TRIS-HCl, pH 7.8, with 0.1 M NaCl) and incubated for six hours at 37 °C. The degree of complex formation was analysed by SDS-PAGE electrophoresis. A Phast™ electrophoresis system (Pharmacia) was used to analyse free and complexed PSA. A precast acrylamide gel (4% to 15 % gradient) was run under standard conditions (10 mAmps, 250 volts in SDS barbital buffer) as per the manufacturer's instructions. Samples containing four μL of purified PSA, complexed PSA and unbound ACT along with low range molecular weight markers, were analysed.

5.0 Preparation and analysis of biotinylated antibodies

One mg of the anti-PSA antibody was biotinylated using 20 ug of NHS-LC-Biotin (1:5 molar ratio) in 50 mM of carbonate buffer at pH 8.5 for two hours at 4°C. The biotinylated antibody was dialysed three times against PBS, using a spin concentrator (molecular weight cutoff of 30,000 Daltons, 4000 RPM, 12 to 15 min), to remove unreacted biotin.

The biotinylated antibodies were tested for biotinylation by directly coating the antibodies on a microtitre plate (4°C, 16 hours) starting at five ug per well and diluting in twofold dilution for 11 steps. After blocking, the wells were incubated with anti-mouse IgG-HRPO or streptavidin-HRPO and washed. ABTS substrate was then added. The immunological activity of the antibodies was tested in a sandwich PSA ELISA as described below. In this case Streptavidin-HRPO was used instead of anti-mouse IgG-HRPO as the secondary reagent (Figure 3).

6.0 Immunoassays

6.1. PSA Sandwich ELISA using bispecific anti-PSA antibody

A sandwich ELISA (Figure 4) using bispecific B80 and B87 was used to measure total PSA. Microtitre wells were coated with Mab B87 (one μg in PBS,16 h, 4 °C). The plate was then blocked (1% bovine serum albumin in PBS (BSA/PBS), one h, 37 °C). Samples or standards (50 μL) were added to each well along with bs B80 (10 ng) and HRPO 300 ng in 50 μL of PBS). The plate was incubated with shaking (30 minutes at room temperature). After washing (three x 200 μL PBS containing 0.05% Tween 20 (PBS/TW)), ABTS substrate (100 μL of 0.5 mg/mL ABTS, 0.5 μL of 30% H_2O_2, in 0.1M citrate with 0.1M phosphate, pH 4.0) was added to each well and the amount of PSA present in the sample was determined by measuring the absorbance at 405 nm using a micro plate reader. The assay was calibrated for PSA concentration using commercially available purified PSA.

6.2. PSA-ACT ELISA.

This assay (Figure 5) was designed to detect PSA only when it is complexed to ACT. Microtitre wells were coated with either B80 or B87 (one ug in PBS, overnight, 4 °C). and blocked as above. PSA-ACT complex was titrated from 100 ng/well (100 μL BSA/PBS) in twofold serial dilutions. The plate was incubated (shaking, 1.5 h, room temperature) and then washed. Anti-ACT Rabbit IgG polyclonal antiserum was diluted (100 μL, one to 1000 in BSA/PBS) and added to each well. The plate was again washed. Anti rabbit IgG-HRPO was diluted as above and added to each well. The plate was incubated for one hour and washed. ABTS substrate was added and the amount of immobilised PSA-ACT complex present in the sample was determined by measuring the absorbance at 405 nm.

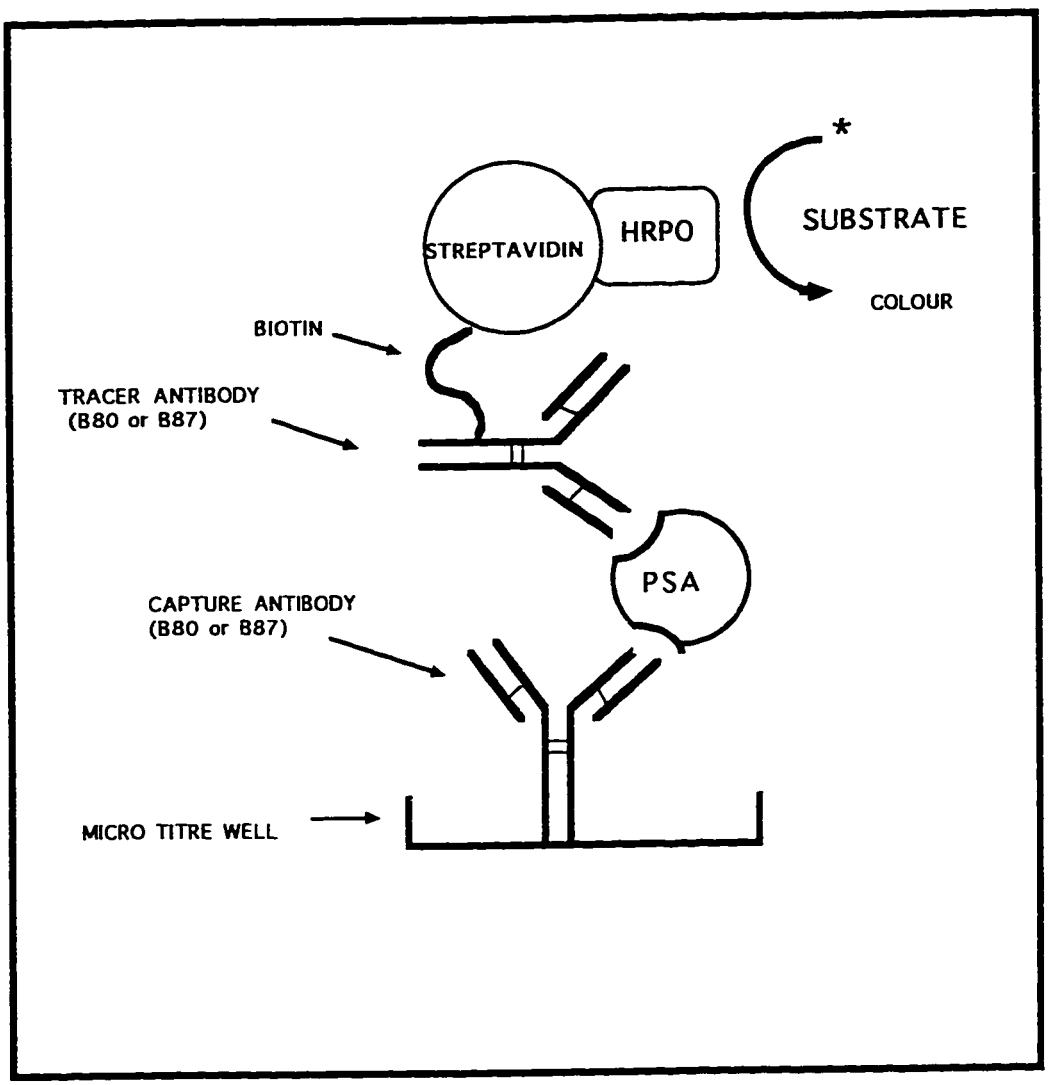


Figure 3: Sandwich ELISA of PSA using biotinylated monoclonal antibodies. An anti-PSA monoclonal antibody, the capture antibody, is immobilized on a microtitre plate. PSA is incubated on the plate and a biotinylated antibody, the tracer antibody, is used to detect bound PSA. Streptavidin complexed to HRP is used to detect the tracer antibody.

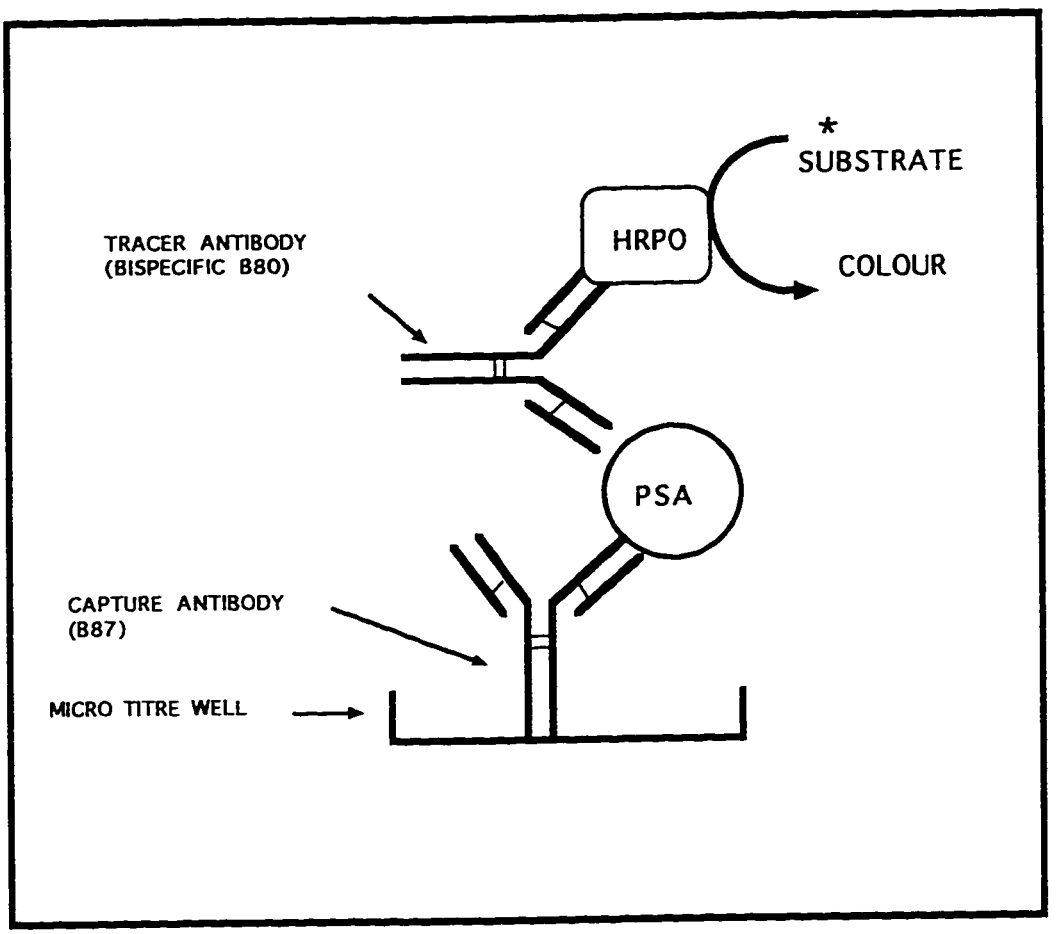


Figure 4: Sandwich ELISA with bispecific monoclonal antibody. An anti-PSA antibody, the capture antibody, is immobilized on a microtitre plate. PSA is incubated on the plate and an anti-PSA anti-HRPO bispecific antibody, the tracer antibody, is used to detect bound PSA. No secondary antibody is required in this assay format.

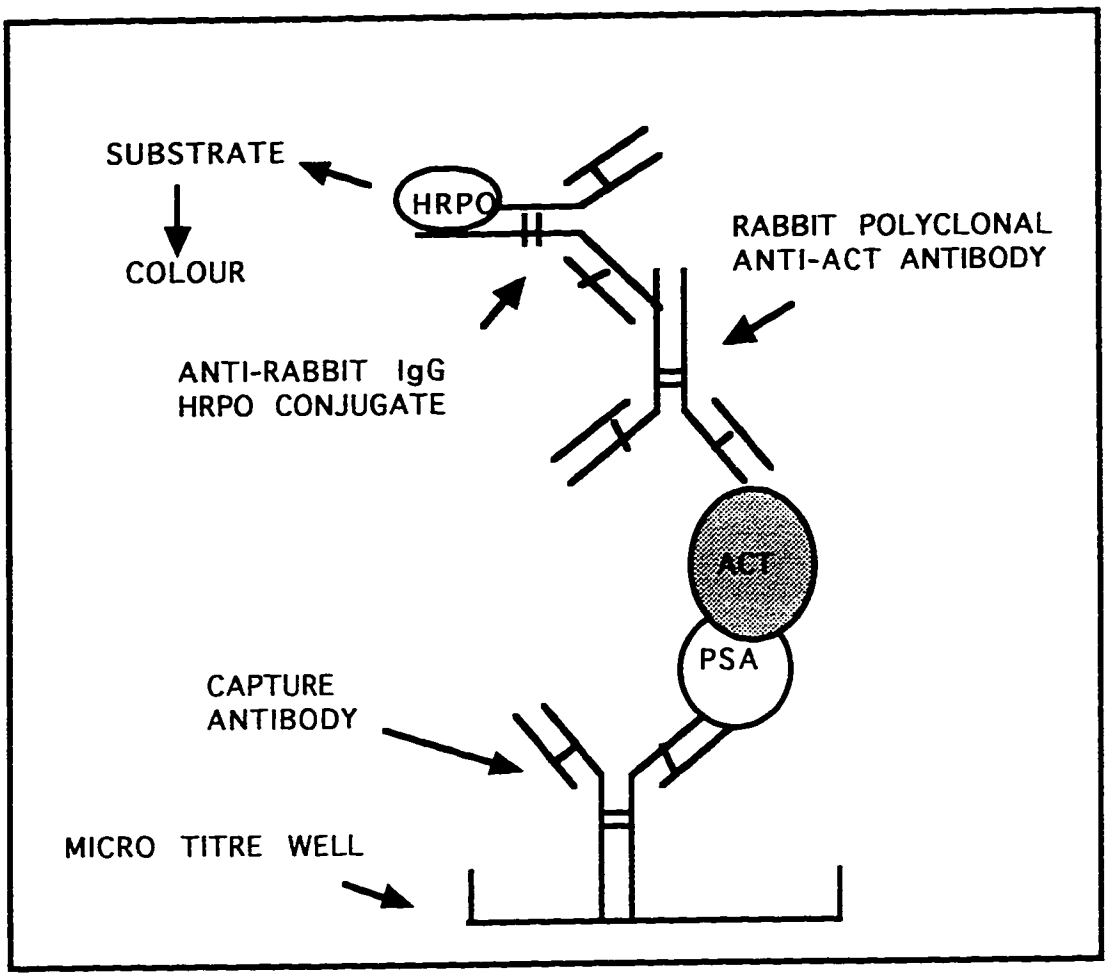


Figure 5: ELISA of PSA-ACT complex. Anti-PSA monoclonal antibody was used as a capture antibody and was immobilized on a microtitre plate. PSA/ACT complex was incubated with the antibody and anti-ACT antibody was used to detect the presence of bound complex. Anti-rabbit IgG complexed with HRPO was used to detect the presence of the rabbit anti-ACT antibody.

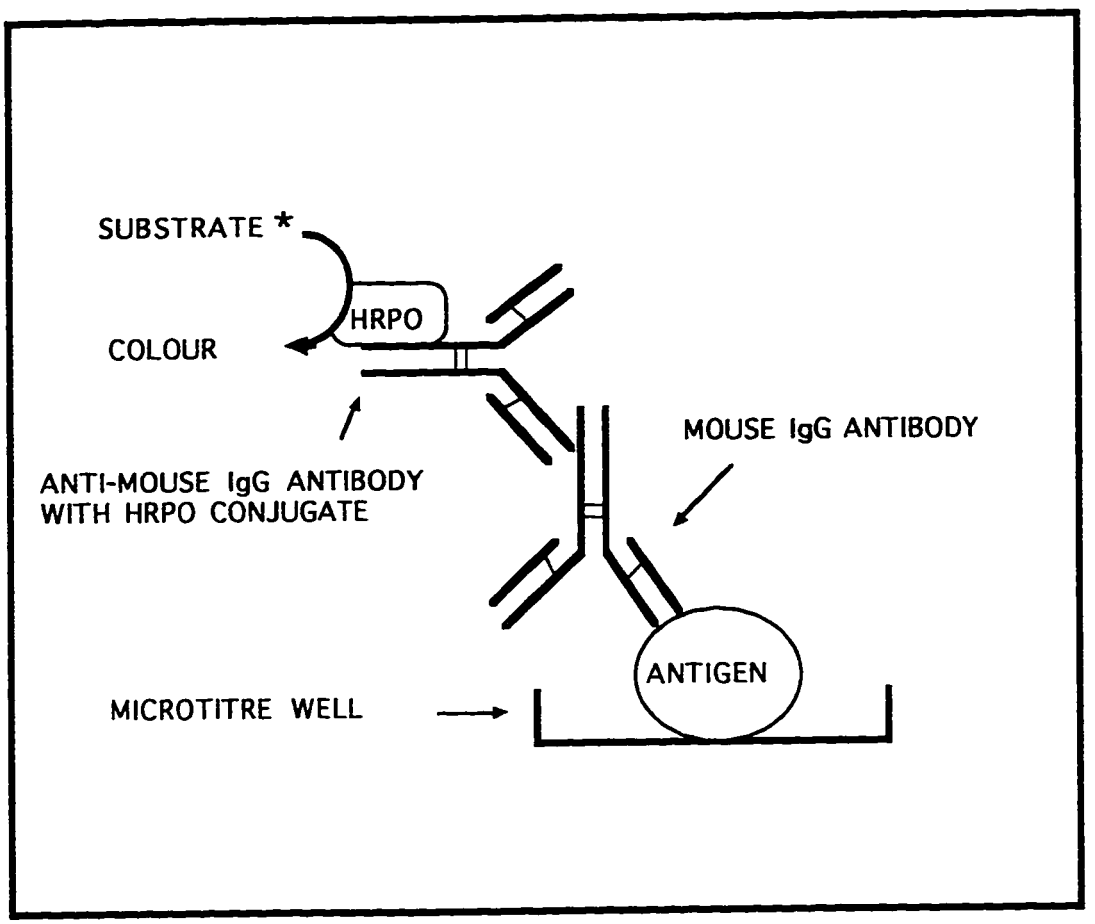


Figure 6: Direct binding ELISA. In this assay antigen is immobilized on a microtitre plate. Anti-antigen monoclonal antibody is incubated on the plate and, after washing, anti-mouse IgG conjugated with HRPO is used to detect bound antibody.

6.3. Cross-reactivity Analysis.

This assay (Figure 6) was designed to measure the degree of cross-reactivity of anti-PSA antibodies to proteins which exhibit a high degree of sequence homology with PSA. Microtitre wells were coated with either purified PSA, human plasma kallikrein, or porcine pancreatic kallikrein (200 ng, PBS, overnight, 4 °C). The plates were blocked as described above, and B80 or B87 was titrated from 1 μg/well in BSA/PBS in twofold dilutions. The plate was incubated for three hours, then washed. Anti-mouse IgG-HRPO was diluted as above and added to each well. The plate was incubated for one hour and washed. ABTS substrate was added and the degree of cross-reactivity of antibody binding to kallikreins or PSA was determined by measuring the absorbance at 405 nm.

7.0 Epitope scanning

7.1 The epitope scanning and mimotope design kit

The 240 overlapping hexapeptides representing the entire PSA sequence were synthesised on pins using the Mimotope Kit. The kit contained computer software for generating synthesis schedules, blocks of derivatised pins for peptide synthesis and derivatised amino acids for use in the synthesis, reaction trays, and 1-hydroxybenzotriazole.

The software package made it pessible to enter and store protein sequences. Selecting the General Net (GNET) option generated a schedule for the synthesis of every overlapping peptide of a specified length. In this study overlapping hexapeptides derived from the PSA sequence were selected. Two control peptides, PLAQ and GLAQ, were synthesised. A monoclonal antibody which specifically recognises the positive control peptide, PLAQ was included in the kit. The negative control peptide, GLAQ, differed from the positive control peptide by only one residue but is not recognised by the control antibody. The weight of each amino acid required for each cycle of the synthesis and the wells into which each amino acid was to be pipetted into was listed by the synthesis schedule.

The pins consist of polyethylene that have been grafted with acrylic acid by radiation. Mono-t-butyl oxycarbonyl-1,6-diaminohexane was coupled to the grafted polyacrylic acid matrix. After the temporary t-butyloxycarbonyl amino protecting group was removed, β-alanine was added as a spacer group. All peptides had an alanine residue at the carboxy terminus (Figure 7). The pins were held in a polypropylene

block that is arranged in the same format as a 96-well microtitre plate. Individual pins could be removed. Common procedures such as blocking were performed in polypropylene trays.

The amino acids used in the synthesis had 9-fluorenylmethyloxycarbonyl (Fmoc) on their α-amino group and were in the activated form as a pentafluorophenyl ester except serine and threonine which were supplied as oxo-benzotriazine esters. Side chain groups were protected: t-butyl ester for serine, threonine, and tyrosine; t-butyl ester for aspartic acid and glutamic acid; t-butyloxycarbonyl for lysine and histidine; 4-methoxy-2,3,6-trimethylbenezenesulphonyl for arginine; and triyl for cystine.

7.2 Synthesis of peptides on pins

Standard solid-phase peptide synthesis using Fmoc-amino acid active esters was used to build each hexapeptide on the functionalized pins. Peptides were synthesised, from the carboxy terminus by repetitive cycles of Fmoc deprotection (20% (v/v) piperidine in DMF, 30 min), washing (DMF 5 min, methanol 2 min, methanol 5 min, air dry 10 min, and DMF 2 min), and coupling the next amino acid (30 mM with 60 mM HOBt in purified DMF, overnight at 30 °C in a sealed plastic bag) in its protected and activated form to the newly exposed amino group. DMF used for coupling was purified to remove amines by treating with activated 4 Å molecular sieves (20 g/litre, 48 hours). After the final Fmoc group was removed, the terminal amino acid was capped by acetylation (DMF:acetic anhydride:triethylamine 5:2:1 by volume, 90 min at 30°C in a sealed plastic bag). The side-chain protecting groups were removed by trifluroroacetic acid containing scavengers (Trifluroacetic acid:Anisole:Ethanedithiol 95:2.5:2.5 by volume, 4 hours at room temperature in a sealed plastic bag). The pins were then thoroughly washed before use to remove contaminants.

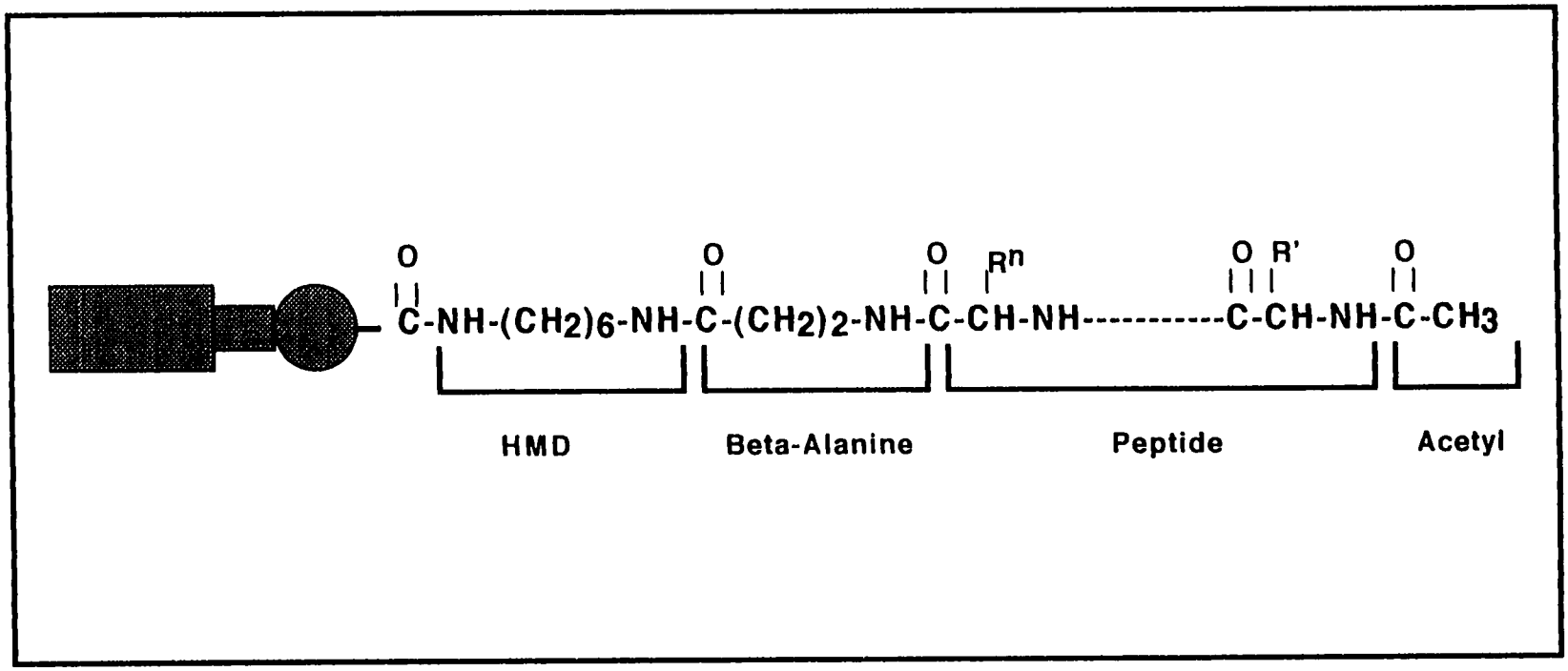


Figure 7: Peptide synthesis on pins. The peptides were synthsised on beta-alanine which is coupled to a linker attached to the polyacrylic acid matrix. The pins are arranged in a microtitre plate format and can be regenerated and reassayed a number of times.

7.3 Immunological screening of peptides on pins

A standard ELISA was used to screen the PSA-derived peptides for binding to B87 and bispecific B80. The pins were blocked in a 96-well micotirer plate. For screening with B87, the antibody (100 ng/well, 200 μL of BSA/PBS) was added to each well of a microtitre plate and the pins incubated in this solution for three hours and washed. Anti-mouse IgG-HRPO was diluted and added to each well of a microtitre plate. The pins were again incubated and washed. ABTS substrate was added and the relative amount of antibody binding to immobilised peptide was determined by measuring the absorbance at 405 nm. For screening with B80, bs Mab B80x HRPO (10 ng) and HRPO (300 ng in 200 μL of PBS) were added to each well. The pins were incubated for one hour and subsequently washed. ABTS substrate was added and the absorbance at 405 nm was measured. Control pins were analysed with each block of peptide pins. An assay with a second antibody only (anti-mouse IgG conjugated to HRPO) was run to determine the degree of non-specific binding.

The peptides on pins can be assayed repeatedly (the manufacturer claims up to 50 times) with some loss of binding efficiency. The bound antibody must be completely removed before each use by sonication and disruption (0.1 M sodium dihydrogen orthophosphate, pH 7.2, 1% SDS. 0.1% 2-mercapthanol, 60 °C, 10 min). The pins were washed in boiling methanol and air dried. The pins were screened at least three times for each experiment described in the result section.

8.0 Random phage peptide display library.

8.1 The phage display library system

The recombinant peptide library used in this study (Christian et al. , 1992) contained 4 x 10^8 independent clones into which a random decapeptide insert was cloned into the pIII protein gene of bacteriophage fd. The phage contain a tetracycline resistance gene which allows infected cells to grow in media with tetracycline.

In order to construct the phage library each variable amino acid in the epitope region was randomly encoded by a degenerate codon. The codon was in the form of NNK (all, all, G and T). All 20 amino acids and one stop codon were encoded by the 32 different codons. A degenerate oligonucleotide was synthesised by first encoding the

invariant sequence that surrounds the variable region. Equimolar mixtures of nucleotides NNK were then used where random amino acids were to be encoded. These degenerate oligonucleotides were inserted into appropriate sites in the coat protein gene of the plasmid vector. The appropriate ends for ligation with the vector could be generated by restriction endonuclease digestion. The ligated product was introduced into bacterial cells by electroporation. The phage were grown in the cells, collected and purified.

In order to isolate phage which express the desired epitope, a technique known as biopanning was used (Figure 8). An immobilised monoclonal antibody was incubated with the phage library. The antibody-bound phage were eluted with lowered pH or with excess ligand and amplified by growth in bacterial cells. New cycles of biopanning were used to enrich the phage with high affinity binding epitopes. The amino acid sequence of the epitope was deduced from sequencing the single-stranded phage DNA. The selection of the peptide epitopes was made on the basis of the presence of a unique consensus sequence among independent phage clones.

8.2 Amplification and purification of phage library

An aliquot of the library containing 2 x 10^9 phage was added to K91 E coli (Hfr-C thi) cells in the growth phase in 2 x YT media (1.6% Bacto-triptone, 1% Bacto-yeast, 0.5% sodium chloride, pH 7) for 10 minutes at room temperature, without shaking. The number of cells present in the media was estimated by measuring a 1/10 dilution of cells at an absorbance of 600 nm. The cells were grown until the A600 is 0.5. Approximately 5 x 10^8 cells/ mL will produce an A600 of 0.5. Tetracycline was then added (0.2 μg/mL) to the media and the infected cells were incubated (37 °C, on shaker) for 30 minutes to allow them to express tetracycline resistance. After one hour, the cells were added to 1 litre of medium containing 20 μg/mL tetracycline. The cells were then incubated overnight (16 hours, 37 °C, with shaking).

8.3 Harvesting of phage

A one litre culture of phage-infected cells was centrifuged in four 250 mL tubes (12,000 g) for 10 minutes at 4 °C. The supernatant-containing phage was recentrifuged in new tubes. The phage was precipitated (overnight at 4 °C) with 0.2

volumes of 20% polyethylene glycol (PEG, average molecular weight 8000) with 2.5 M NaCl. The precipitate was centrifuged for 40 minutes (15,000 g, 4 °C). The pellet was resuspended in eight mL of TBS (150 mM NaCl, 50 mM Trisma base, pH 7.4) and titred.

8.4 Phage titreing

In order to determine the number of phage particles recovered the phage were diluted in TBS/glycine (0.1%) to cover a range of 3 x 10^5 to 3 x 10^6 virons/mL. 10 μL of diluted phage were added to10 μL of K91 cells (OD_{600} of 0.4 to 0.6). The cells and phage were incubated without shaking at room temperature for 10 minutes. Tetracycline was then added (0.2 ug/mL) to the media and the infected cells were incubated for 20 to 40 minutes (37 °C, on shaker). Two hundred μL of media were added and the cells plated on LB-tet agar plates (1% Bacto- tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl, 20 μg/mL tetracycline, 15 g/l Bacto-agar). The plates were incubated at 37 °C overnight. The tetracycline-resistant colonies present on the plates were counted and the total number of phage per mL calculated. The titre of the stock phage is expressed as TU/mL.

8.5 Purification of phage by CsCl gradient

A 31% w/w CsCl solution was made by adding 4.83 g of CsCl to a tared 50 mL tube. Phage in TBS was added to bring the weight to 10.75 grams. The density of this solution should be 1.30 g/mL. The solution was transferred into a Beckman polyallomer tube for the SW41 rotor and centrifuged for 48 hours at 37,000 RPM. The solution above the phage layer was carefully removed by aspiration and the phage recovered. The purified phage were then suspended in TBS and precipitated with PEG as described above.

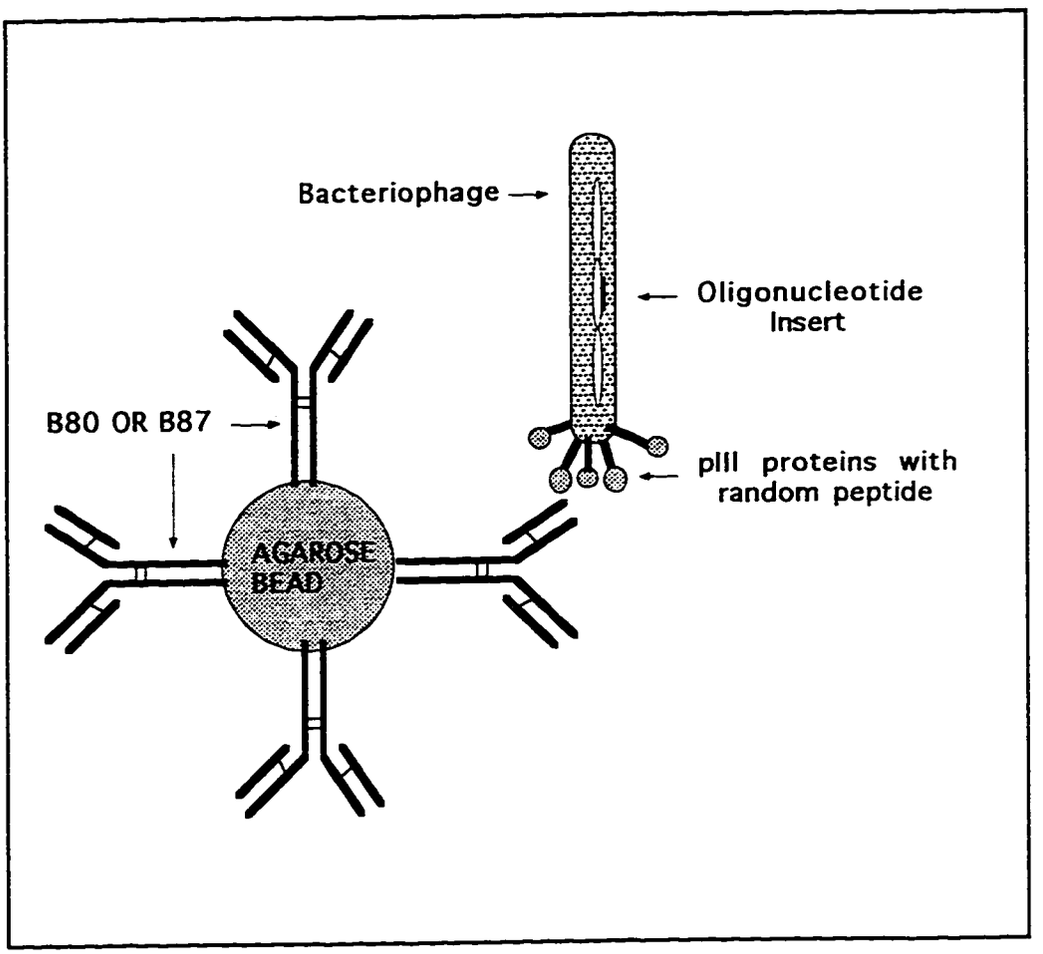


Figure 8: Affinity selection with a phage random peptide display library. Anti-PSA antibodies, B80 and B87 were immobilised on agarose beads. The beads were used to select phage particles which display a peptide on their pIII proteins which binds specifically to the anti-PSA antibodies. The affinity selected phage was amplified, and its DNA sequenced, to deduce the amino acid sequence of the peptide.

8.6 Affinity selection

An aliquot of bacteriophage peptide library displaying random decapeptides (16) (4.4×10^{11} colony forming units (cfu) in 40 μL of bovine serum albumin/Tris-buffered saline, pH 7.2 (BSA/TBS)) was added to 40 μg of immobilised B80 and B87 (10 μL of a 50% slurry of antibody linked Sepharose 4B, described above). The sample was gently mixed (room temperature, 1.5 hours) and the beads collected by centrifugation (30 s x4000 g). The supernatant was removed and the beads resuspended, washed with 500 μL of BSA/TBS, five minutes), and recovered by centrifugation. This wash was repeated two times with BSA/TBS, three times with 0.05% Tween 20 in TBS (TBS/TW), and once with TBS/BSA. The washed beads were then incubated with PSA (one hour, room temperature with shaking, three μg in 50 μL of BSA/TBS). The supernatant, designated as ligand eluate, was collected and the beads washed as before with 500 μL of citrate buffer (50 mM citric acid,150 mM NaCl, 1% BSA, pH 2.0). After five minutes, the supernatant, designated as the acid eluate, was collected. Both the ligand and acid eluates were amplified by infecting E. Coli cells with isolated phage following procedures described above. The second round of selection (or biopanning) was performed.

8.7 Immunological Screening of Affinity-Selected Phage.

Affinity-selected phage particles were screened using an <u>in situ</u> immunological assay with phage infected colonies (Christian et al., 1992). Phage clones from the eluates were used to infect fresh cells. The cells were then grown in selective media (15 mm x 150 mm plates, LB medium with 20 μg/mL tetracycline) at a density of 200 to 500 colonies/plate and transferred to nitrocellulose membrane filters. For screening with B80, the filters were washed (30 min, TBS/TW), blocked (30 min, 3% BSA/PBS) and then incubated with bs Mab B80 x HRPO (20 μg , with one mg of HRPO). The filters were washed again and incubated with TMB substrate for one hour. The filters were then washed with distilled water and allowed to air dry overnight. Alternatively, filters with colonies from a second set of plates were incubated with B87, (one mg in 100 mL BSA/PBS) for two hours at room temperature. After washing, the B87 filters were incubated with anti-mouse-HRPO (one to 1000 dilution in BSA/PBS, one hour, room temperature). The filters were washed three times and incubated with one step TMB

substrate. After one hour the filters were washed with distilled water and allowed to air dry overnight. Positive clones were picked from the plates and amplified for sequencing.

8.8 Purification of phage DNA

Two milliliters of a 20-to-24 hour phage-infected cell culture were placed in a microfuge tube and centrifuged (5 min, 15,000 RPM). The clear supernatant containing phage was heated (70 °C, 10 min) and recentrifuged to remove any residual cells. PEG solution was added to the supernatant (1/10 volume, 20% PEG in 2.5 M NaCl) and the phage allowed to precipitate (15 minutes, room temperature). The phage were pelleted by centrifuging (five min, 15,000 RPM) and the supernatant removed by aspiration. The pellet was recentrifuged for one minute and the remaining supernatant re-aspirated. The phage were then resuspended in 200 μL of TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0). The expected yield of phage in this preparation is 2×10^{12} phage particles.

The phage suspension was extracted with 200 μL of TE saturated phenol, centrifuged, and the aqueous layer transferred to a new tube. A second extraction was performed as above, using 180 μL of TE saturated phenol:chloroform (1:1) followed by a final extraction with 160 μL chloroform. The single-stranded phage DNA was precipitated with 0.1 volume of three M sodium acetate and two volumes of ethanol for 15 to 30 minutes at -70 °C. After centrifugation (30 min, 14,000 RPM, 4 °C) the pellet was washed with 70% ethanol and centrifuged, and the pellet dried in a Speed Vac for 15 to 30 minutes.

8.9 Sequencing of phage DNA

The single-stranded phage DNA was sequenced using the dideoxy method (Sanger et al., 1977), Sequenase 2.0, and dATP-P^{33} following the manufacturer's instructions. The sequencing primer (5'-CCC TCA TAG TTA GCG TAA CG-3') anneals 55 base pairs downstream of the cloning site. Approximately 50 fM of primer was used for a 2 mL phage DNA preparation. The M13 ssDNA supplied in the Sequenase 2.0 Kit was used as a control. The sequencing reactions were run on a 6% polyacrylamide/7 M urea/TBE (50 mM Trizma base, 100 mM Boric acid, 2.5 mM ETA) gel (38 x 50 cm) at 15 to 20 mA. The bands were visualised using autoradiography (Kodak XRP X-ray film,16 h to 5 d).

9.0 Synthesis and testing of peptides immobilised on cellulose

9.1 SPOTS Kit

A SPOTS (Simple Precise Original Test System) Kit was used to synthesise peptides identified in the phage selection procedure. The kit consists of a functionalized cellulose membrane, Fmoc-amino acid active esters, and Software. The Fmoc-amino acid active esters are identical to those used in the synthesis of peptides on pins.

The included software generates a synthesis schedule similar to the peptides-on-pins software. The options for synthesis are epitope scanning (SPOTscan), boundary residue analysis or size analysis (SPOTsize), analogue studies (SPOTsalogue), and multiple sequence synthesis (SPOTsalot). In this study the SPOTsalot option was used to synthese individual peptides in duplicate. SPOTsize was used for two peptides which showed specificity for B80 or B87 to determine the minimum size of the epitope. SPOTsalogue was used to identify critical residues by introducing alanine in each position of the peptide.

The cellulose membrane is derivatised to provide free amino groups in an 8 x 12 matrix of small circular spots. β-Alanine is first esterfied to the whole membrane via free hydroxyl groups on the cellulose. After Fmoc-deprotection, an even distribution of reactive amino groups is obtained. The positions for the Spots are marked before the initial coupling step and aliquots of reactive β-Alanine are dispensed onto each spot. After washing, all residual amino groups are capped by acetylation. Fmoc-deprotection generates free amino groups on the spots which are stained with bromophenol blue. The dipeptide on each is used as an anchor and spacer for the subsequently synthesised peptide.

9.2 Peptide synthesis

The DMF and NMP used for coupling was purified to remove amines by treating with activated 4 Å molecular sieves (20 g/litre, 48 hours). The purity of DMF and NMP was tested by adding 10μL of 1% bromophenol blue in DMF to 1 mL of solvent. A yellow colour indicated the absence of free amines. The solvents were repurified or discarded if the solution turned green or blue.

Once the synthesis schedule was created the first coupling reaction was begun by dispensing the first amino acid onto each spot. The scale of the synthesis was 50 nmol

with 0.9 ul of amino acid solution in NMP dispensed in each cycle. Dissolved amino acids were stored in aliquots at -70 °C. After 15 minutes a second aliquot of amino acid was dispensed. Individual couplings were monitored by the colour change on the bromophenol blue to green/yellow as the free acids reacted. After each coupling the membrane was washed (3 x 20 mL DMF) and free amine groups capped using 4% acetic anhydride in DMF (20 mL, 15 minutes). Again after washing, the Fmoc groups were removed using 20% piperidine in DMF (20 mL, 5 minutes). The membrane was washed and the spots stained using 1% bromophenol blue in DMF (20 mL, five min). After being washed in methanol (5 x 20 mL, two minutes) the membrane was air dried and a new coupling cycle begun. After the last coupling step the peptides were capped with 2% acetic anhydride in DMF as before. The side chain protecting groups were cleaved using 20 mL of TFA: methylene chloride:TIB Silane, 1:1:0.05. The membrane was extensively washed (3 x methylene chloride, 3 x DMF, 3 x methanol) and air dried. For the synthesis of peptides of varying lengths on the same membrane, the shorter peptides were capped using 0.4 M acetic anhydride in NMP in the coupling step.

9.3 Immunological screening of peptides

The membrane was washed with PBS (3 x 20 mL, two minutes), and blocked with BSA (3% BSA in PBS, overnight, room temperature). After washing (1x 20 mL PBS/Tw, two minutes) the membrane was incubated with the primary antibody, Bispecific B80 or B87 (ug/mL, in 20 mL 3% BSA in PBS,) for three to four hours. The membrane was washed and secondary antibody (anti mouse IgG-HRPO at 1/100 dilution in 3% BSA in PBS) incubated for two hours. After washing as before, one-step ABTS substrate solution was added and the colour of the spots noted. A photocopy of the membrane was made as a permanent record of the result.

The SPOTs membranes were regenerated by extensive washing with water (3 x 20 mL, 10 min), DMF (3 x 20 mL, 10 minutes), water (3 x 20 mL, 10 minutes), regeneration buffer A (8 M urea, 10% SDS w/v, 0.1% mercaptoethanol v/v, 3 x 20 mL,10 min), regeneration buffer B (water, ethanol, acetic acid, 4:5:1, 3 x 10 min) and methanol (2 x 10 min). The membrane was air dried and stored sealed in a plastic bag at -20 °C.

10.0 Modelling of PSA.

A model of PSA was generated by Dr. David Wishart of PENCE (Protein Engineering Centre of Excellence) using the INSIGHT II molecular modelling package (BIOSYM Technologies, San Diego, CA). The model was prepared by initially aligning the human PSA sequence against those of both porcine kallikrein (59% sequence identity) and rat tonin (54% sequence identity) using structural alignment routines in the SEQSEE software package (Wishart et al., 1994). Based on these two alignments and on the quality of the available structural data, the X-ray structure of rat tonin (Brookhaven Protein Databank accession code: ITON) was selected as the three-dimensional template for homology modelling the PSA structure. Using the sequence alignment produced by SEQSEE, the amino acid sequence of PSA was then substituted into that of tonin using the Biopolymer module of INSIGHT II. The model was subjected to a brief period of conjugate gradient energy minimisation to reduce unfavourable van der Waals contacts and torsional strain.

VIII. RESULTS

1.0 Purification and analysis of reagents.

PSA was eluted from the monoclonal affinity column in fractions 3 to 5, total of 1.37 mg of PSA was recovered from 1.5 litres of the LNcaP cell supernatant. The protein eluted from the column was measured at 280 nm and the PSA concentration of the pooled fractions determined using an ELISA. No remaining PSA was detected in the original supernatant after affinity purification.

The PSA-ACT complex was formed by incubating purified PSA with ACT using the conditions previously described (Lilja et al.,1990). Figure 9 shows the SDS-Page electrophoresis of purified PSA in lane 2, ACT in lane 3, PSA-ACT complex in lane 4, and molecular weight standards in lanes 1 and 5. Since PSA binds covalently to ACT the complex is not dissociated by treatment with SDS. The electrophoresis effectively resolved PSA at ~30 kD, ACT at ~60 kD and PSA-ACT complex at ~90 kD. A small amount of PSA-ACT complex was seen in the purified PSA (lane 2). Unreacted PSA and ACT were seen after complex formation (lane 4). The PSA-ACT complex immunoassay used in this study was designed to detect only PSA-ACT complex and not free PSA or free ACT.

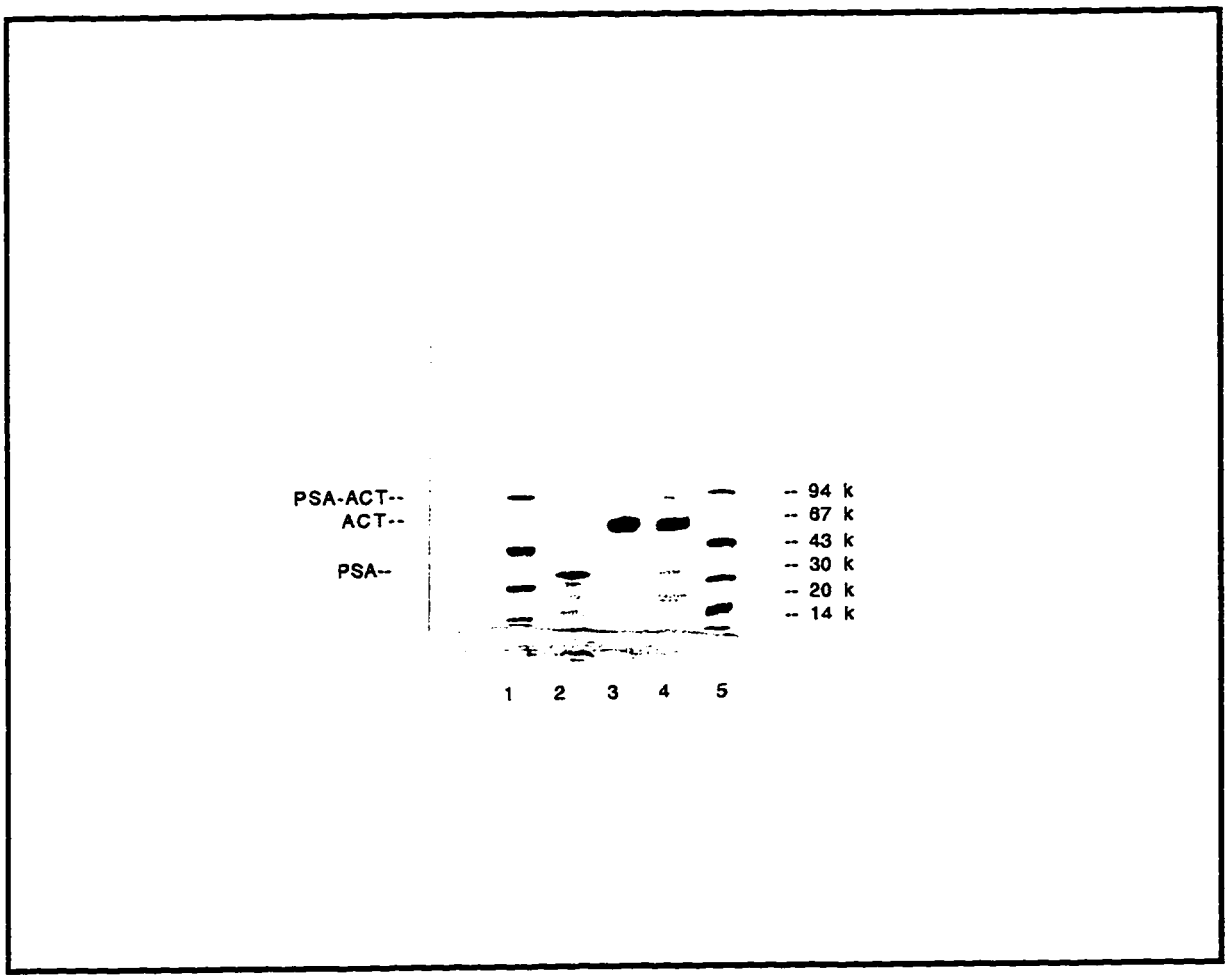


Figure 9: SDS-PAGE of PSA-ACT Complex.
Lanes 1 & 5 = Molecular Weight Standards
Lane 2 = purified PSA
Lane 3 = ACT
Lane 4 = PSA-ACT complex

2.0 Topographical mapping of PSA epitopes

2.1 Binding of B80 and B87 to PSA in a sandwich ELISA

A PSA sandwich ELISA was developed using monoclonal antibody B87 as the capture antibody, immobilised on plastic in a microtitre plate. Bispecific Mab B80 x HRPO was used as the tracer antibody. The assay was calibrated for PSA concentration using PSA obtained commercially. The manufacturer specified that the PSA was 99% pure and did not contain any PSA-ACT complex. The calibrators were diluted in BSA/PBS (and not serum). This assay was used to determine the PSA concentration in cell supernatant and PSA affinity-purified eluates. The dose response curve (Figure 10) is linear from 0 to 75 ng/mL of PSA and is not saturated even at 250 ng/mL of PSA. Each point is an average of duplicate measurements. B80 and B87 can be used to measure PSA in a sandwich immunoassay indicating that they recognise two distinct and non-overlapping epitopes. The ability of these antibodies to bind to PSA simultaneously indicates that the epitopes are non-overlapping and far enough apart to avoid steric hindrance. Both of these antibodies fail to form a homosandwich, indicating that each antibody recognises one unique and non-repeating epitope. This result is not unexpected considering the relatively small size of PSA.

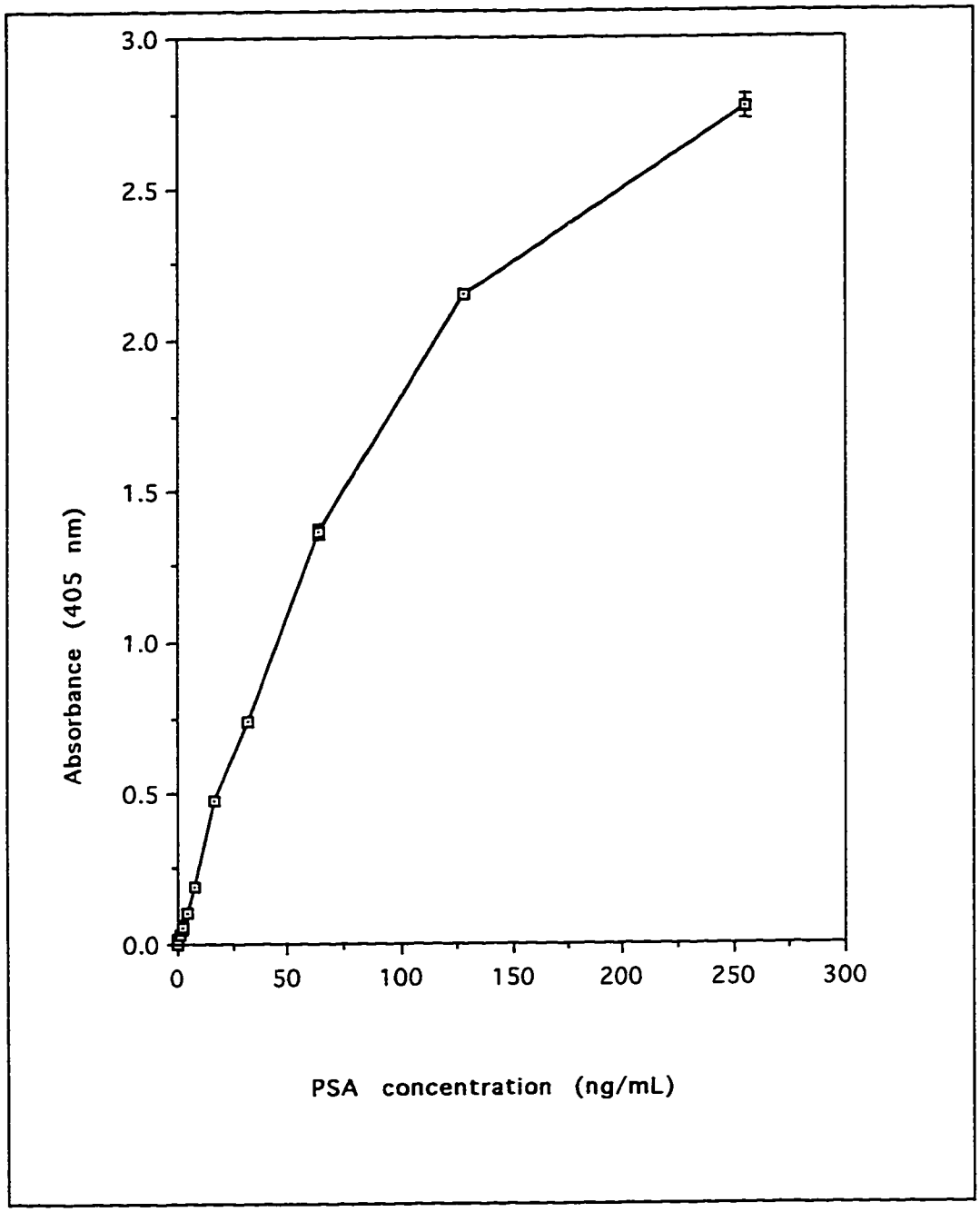


Figure 10: PSA sandwich assay using bispecific B80 and B87.

2.2 Cross-reactivity with kallikrein

Pancreatic porcine kallikrein was immobilised on plastic microtitre wells. B80 or B87 was incubated with the immobilised protein to evaluate its cross-reactivity. The binding of these antibodies to immobilised PSA, (Figure 11) reaches saturation in this assay at approximately 12 ng/mL for B80 and 50 ng/mL for B87. No binding to kallikrein was observed for either antibody up to a concentration of one μg/mL of antibody. Purified PSA was used as a positive control and BSA was used as a negative control. The fact that neither B80 or B87 cross-react with kallikrein which has a 57% amino acid sequence homology with PSA, indicates that both antibodies recognise sequences that are not common to both proteins. Since we can also assume that the epitope resides on an exposed surface structure and not in the interior of the molecule, the number of possible epitopes is further reduced.

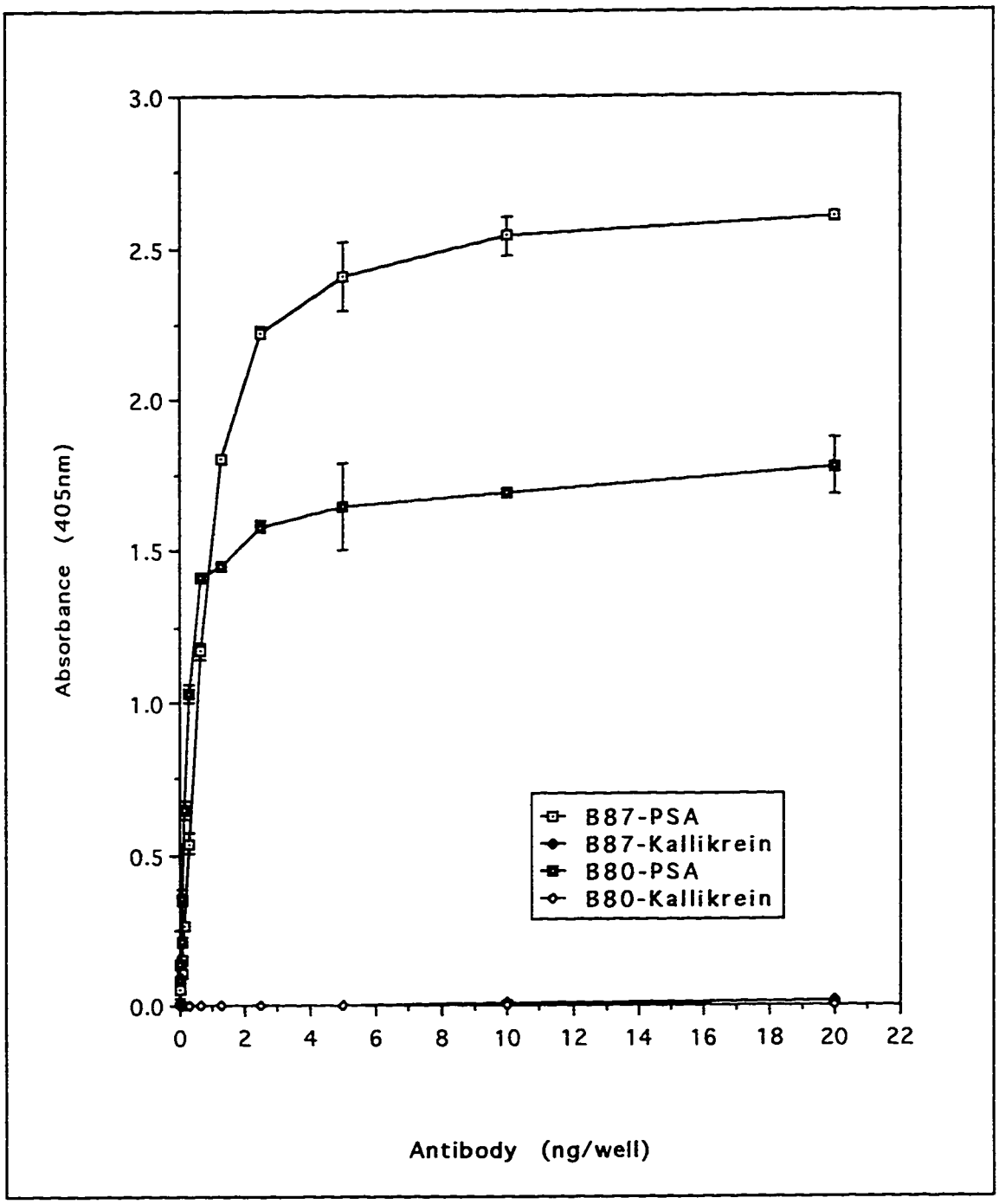


Figure 11: Cross-reactivity to Kallikrein

2.3 Analysis of PSA-ACT Complex.

An immunoassay specific for the PSA-ACT complex was developed using monoclonal antibodies B80 or B87 immobilised on microtitre plates and rabbit anti-ACT polyclonal antibody. Goat anti-rabbit antibody conjugated with HRPO was used as the signal generating antibody (Figure 5). The results are shown in Figure 12. Free PSA is bound by the anti-PSA capture antibody but not by the anti-ACT antibody and was not detected in this assay. Free ACT is not bound by the immobilised anti-PSA antibody and was not detected in this assay format. Goat anti-rabbit-HRPO conjugate and rabbit anti-ACT antibody did not show binding to immobilised monoclonal antibodies B80 or B87 (data not shown). Twofold dilutions of PSA-ACT were made from 500 ng/mL of PSA. The concentration of antigen is expressed in ng/mL of complexed PSA. Both B80 and B87 were able to effectively detect the PSA-ACT complex (Figure 12). The dose response curves were linear up to 50 ng/mL and saturated at about 500 ng/mL of PSA-ACT complex. As little as 20 ng/mL of complexed PSA could be detected in this assay. If the epitopes for B80 or B87 are masked by the binding of ACT, the PSA-ACT complex will not be detected in this assay. Since both antibodies recognised the complex, both epitopes must be located in a region that is neither on nor near the region that binds to ACT as the epitopes are available for binding to antibodies when PSA is complexed to ACT.

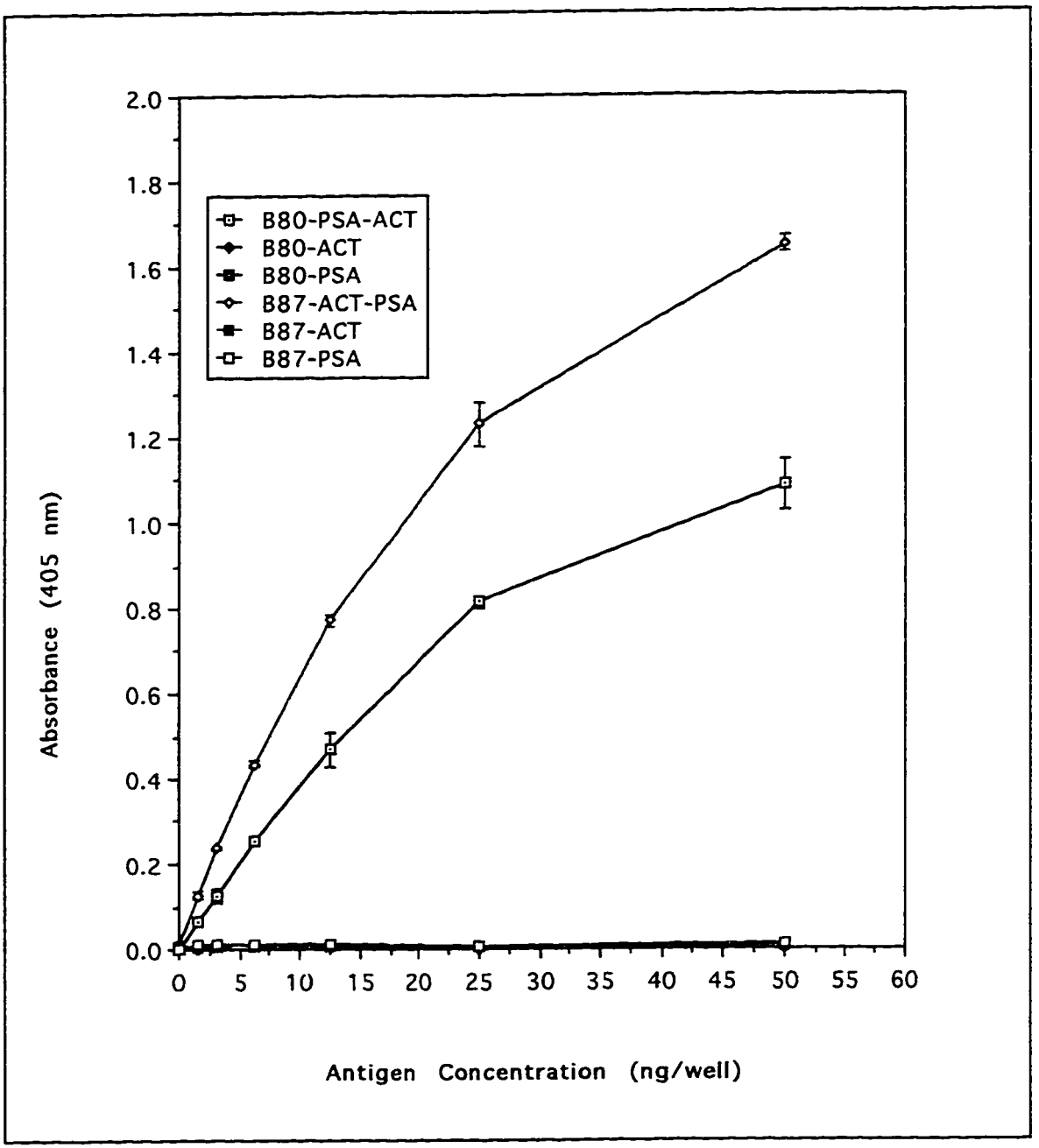


Figure 12: Binding of B80 and B87 to PSA-ACT complex

2.4 Discussion of results

PSA has a high degree of sequence homology with other serine proteases, especially kallikreins (Watt et al., 1986, Henttu et al., 1989, Vihinen et al., 1994). Figure 1 shows the amino acid sequence of PSA (hK3), human serum kallikrein (hK2), porcine pancreatic kallikrein (pK1), and human pancreatic kallikrein (hK1). This homology may result in cross reactivity of anti-PSA antibodies. Human pancreatic kallikrein mRNA has been found in the prostate although the protein itself has not been detected (Henttu et al., 1989).

PSA complexes have been identified in serum (Table 2), the most common being PSA-ACT and PSA-MG (Christensson et al.,1990). When PSA is complexed to MG is thought to be completely surrounded by the macro-globulin and unavailable for binding to antibodies (Scottrup-Jensen, 1989). In contrast, when PSA is complexed to ACT certain epitopes are exposed and recognisable by anti-PSA antibodies. Some antibodies may recognise epitopes that are masked by the binding of ACT (Lilja et al., 1991). These antibodies will not recognise the complexed form of PSA. An immunoassay using these antibodies will yield different results from an assay that uses antibodies that recognise both free and complexed PSA (Stenman et al., 1991).

The specificity of B80 and B87 was determined by measuring the degree of cross-reactivity to porcine pancreatic kallikrein. The amino acid sequence homology of kallikreins is given in Table 10.

Table 10 The amino acid sequence identity of tissue kallikreins (% homology) with PSA.

Protein	Designation	% Homology
porcine pancreatic kallikrein[a]	pK1	59.6
porcine pancreatic kallikrein[a]	pK1	(64.3 with hK2)
human pancreatic/renal kallikrein[b]	hK1	~62
human glandular/serum kallikrein	hK2[b]	~80

[a] Vihinen et al., 1994

[b] Lilja et al., 1993

Neither of the antibodies tested cross reacted with this kallikrein, indicating that the epitopes are in regions of PSA that are not shared by porcine pancreatic kallikrein. Figure 1 shows the amino acid sequence of PSA and several related proteases. Regions with similar sequences are shown in boxes.

Most of the regions that are unique to PSA and not shared by other kallikreins appear at the surface of the molecule as determined by homology computer modelling (Bridon and Dowel, 1995). One group (Bridon and Dowel, 1995) attempted to produce PSA-specific antibodies by synthesising a peptide representing two loops on PSA, residues 41 to 56, which differs between PSA and hK2. This peptide incidentally contains the B80 epitope, residues 50-56. Sheep antisera generated using this peptide were used in a competitive immunoassay and affinity chromatography. The exact specificity of this polyclonal antibody has not yet been determined (Bridon and Dowel, 1995).

It has been suggested that the ratio of complexed to free PSA increases with prostate cancer due to the fact that cancer cells secrete elevated levels of ACT (Lilja et al, 1991). This increase is not seen in BPH. Recently assays have been developed specifically to measure the PSA-ACT complex as this parameter may have clinical significance. It is important, therefore, to know if a PSA assay is measuring free PSA, complexed PSA or both. In order to determine if the epitopes are masked by the formation of the PSA-ACT complex, a two-step forward sandwich ELISA was developed to measure PSA-ACT using immobilised B80 or B87, purified PSA-ACT, and anti-ACT polyclonal antisera. In this assay PSA-ACT and free PSA were captured by the immobilised antibody. Anti-ACT is used to detect the PSA-ACT complex. Free PSA, bound by the capture antibody, was not detected by the anti-ACT polyclonal serum. Free ACT present in the sample was not immobilised or detected. Both B80 and B87 were able to recognise PSA when complexed to ACT, indicating that the epitopes are located in a region that is not masked by the binding of ACT. This result agrees with the results obtained by Wu *et al.* (1995) which showed that B80 was able to recognise PSA-ACT complex.

In conclusion, topographical mapping of the B80 and B87 epitopes revealed that the two epitopes are separated from each other and not masked by the binding of PSA to ACT.

3.0 Epitope scanning using PSA derived hexapeptides.

3.1 Synthesis and testing of PSA hexapeptides

In order to further identify the PSA epitopes recognised by B80 and B87, overlapping hexapeptides derived from the entire linear amino acid sequence on PSA were synthesised. The Mimotope Kit has positive and negative control pins and a control monoclonal antibody which recognises the peptide sequence PLAQ and not GLAQ. These two peptides were synthesised with each 96-pin block of hexapeptides as synthesis controls. The control pins provided in the kit were tested in each ELISA. The ELISA results are shown in Table 11.

The control pins supplied with the kit had higher absorbance values than the synthesised pins in all cases, indicating that the yield of peptide on the synthesised pins is lower than on the control pins. The control antibody was able to distinguish PLAQ form GLAQ with both the kit pins and the synthesised pins, indicating that the synthesis had been successful. A limitation of this system is that it is not feasible to sequence all of the peptides individually. The control pins only indicate that the synthetic chemistry was working.

Table 11: Binding of control antibody to synthesis control pins.

CONTROL PIN	SEQUENCE	OD (405 nm)
BLOCK 1	PLAG	0.541
BLOCK 1	GLAQ	0.087
KIT	PLAQ	0.775
KIT	GLAQ	0.313
BLOCK 2	PLAQ	0.358
BLOCK 2	GLAQ	0.097
KIT	PLAQ	0.519
KIT	GLAQ	0.131
BLOCK 3	PLAQ	0.453
BLOCK 3	GLAQ	0.144
KIT	PLAQ	0.751
KIT	GLAQ	0.110
ALL BLOCKS	PLAQ	0.420 ± 0.05
ALL BLOCKS	GLAQ	0.109 ± 0.03
ALL KIT PINS	PLAQ	0.682 ± 0.14
ALL KIT PINS	GLAQ	0.185 ± 0.11

3.2 Hexapeptide scanning with B80 and anti mouse IgG-HRPO conjugate

Figure 13 shows the results of a two-step ELISA performed on the PSA hexapeptides using B80 and anti-mouse IgG-HRPO conjugate. The peptide number represents the position on the PSA sequence occupied by the first amino acid of the synthesised hexapeptide. The two gaps seen between the peptides represent the boundary between individual blocks of pins. A large number of peptide sequences show greater than 30% of maximum binding in this assay. Some of the positive peptide sequences may be cross-reacting with the polyclonal anti-mouse IgG antibody-HRPO conjugate.

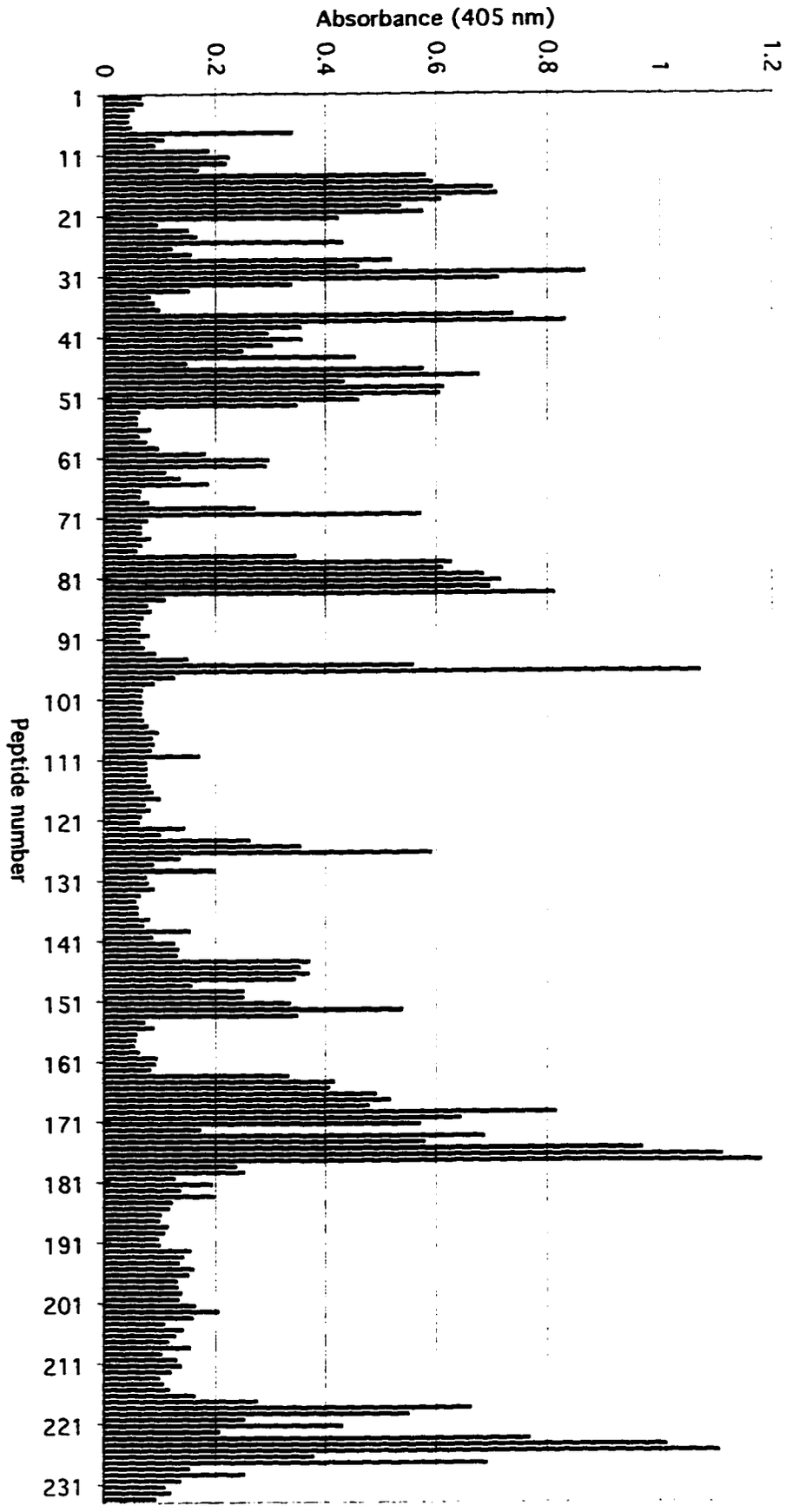


Figure 13: B80 binding to PSA hexapeptides using anti-mouse-IgG-HRPO conjugate

Figure 14 shows the results of an ELISA using just the anti-mouse conjugate. This reagent recognises at least 12 different peptide sequences and is, therefore, not useful for determining the location of PSA epitopes. No sequences which bind only to B80 were identified in this assay.

3.3 Hexapeptide scanning with biotinylated antibodies

B80, B87, and Mab170, a non-specific control monoclonal antibody, were biotinylated and tested for binding to PSA in a sandwich assay using streptavidin-HRPO conjugate as the secondary reagent. The results are shown in Figure 15. Both biotinylated B80 and biotinylated B87 are able to recognise PSA in this assay and reach saturation at 160 ng/mL of PSA. This compares favourably to the results obtained in a sandwich ELISA with bispecific B80 and immobilised B87 (Figure 8). The control antibody was tested by coating the biotinylated antibody directly on a microtitre plate. Streptavidin-HRPO was used to detect the presence of bound biotin (data not shown). Based on these results the biotinylated antibodies were found to be suitable for use in binding to PSA hexapeptides.

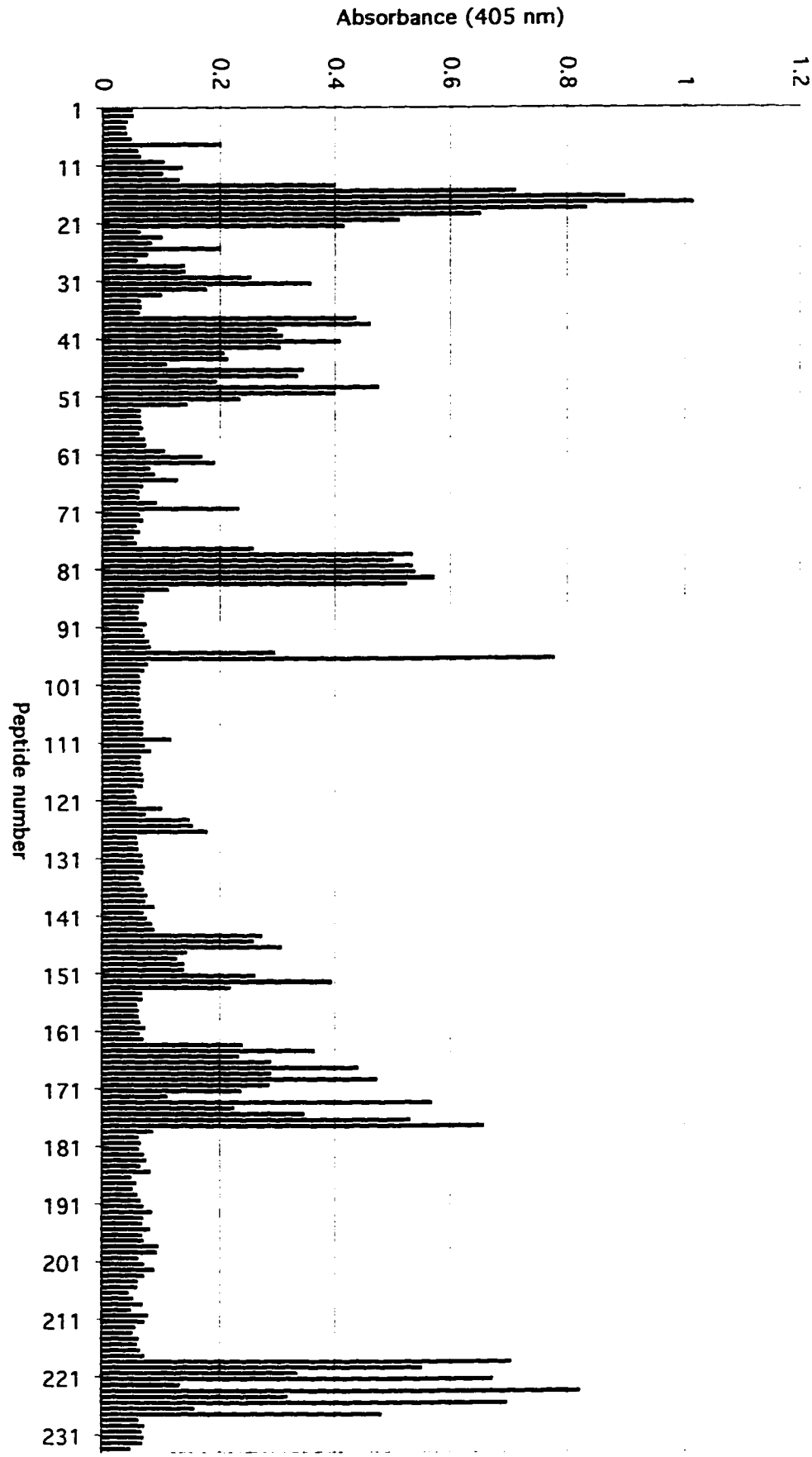


Figure 14: Binding of Anti mouse IgG-HRPO conjugate to PSA hexapeptides (non-specific binding)

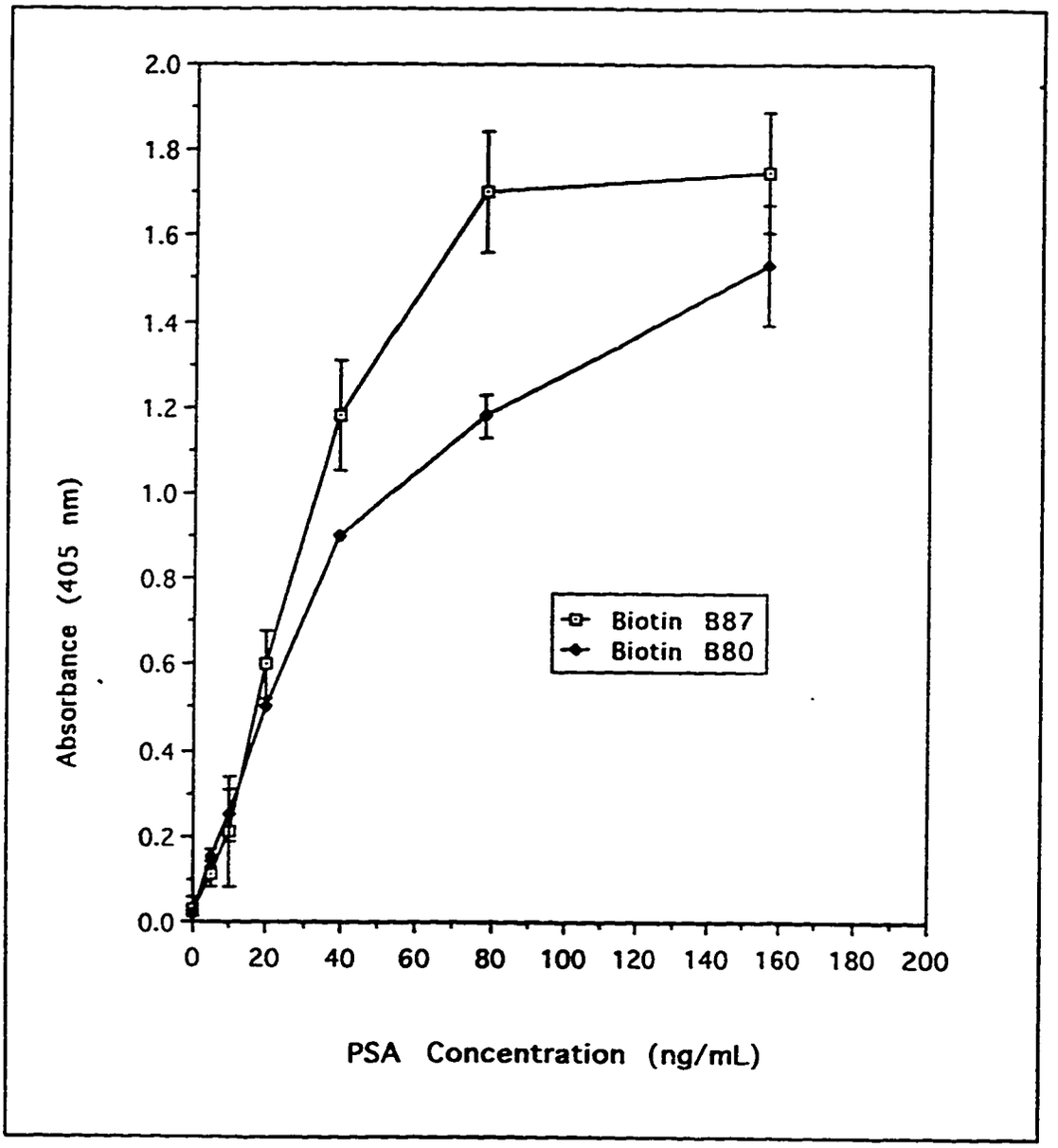


Figure 15: Testing of Biotin-Mabs in a sandwich PSA assay

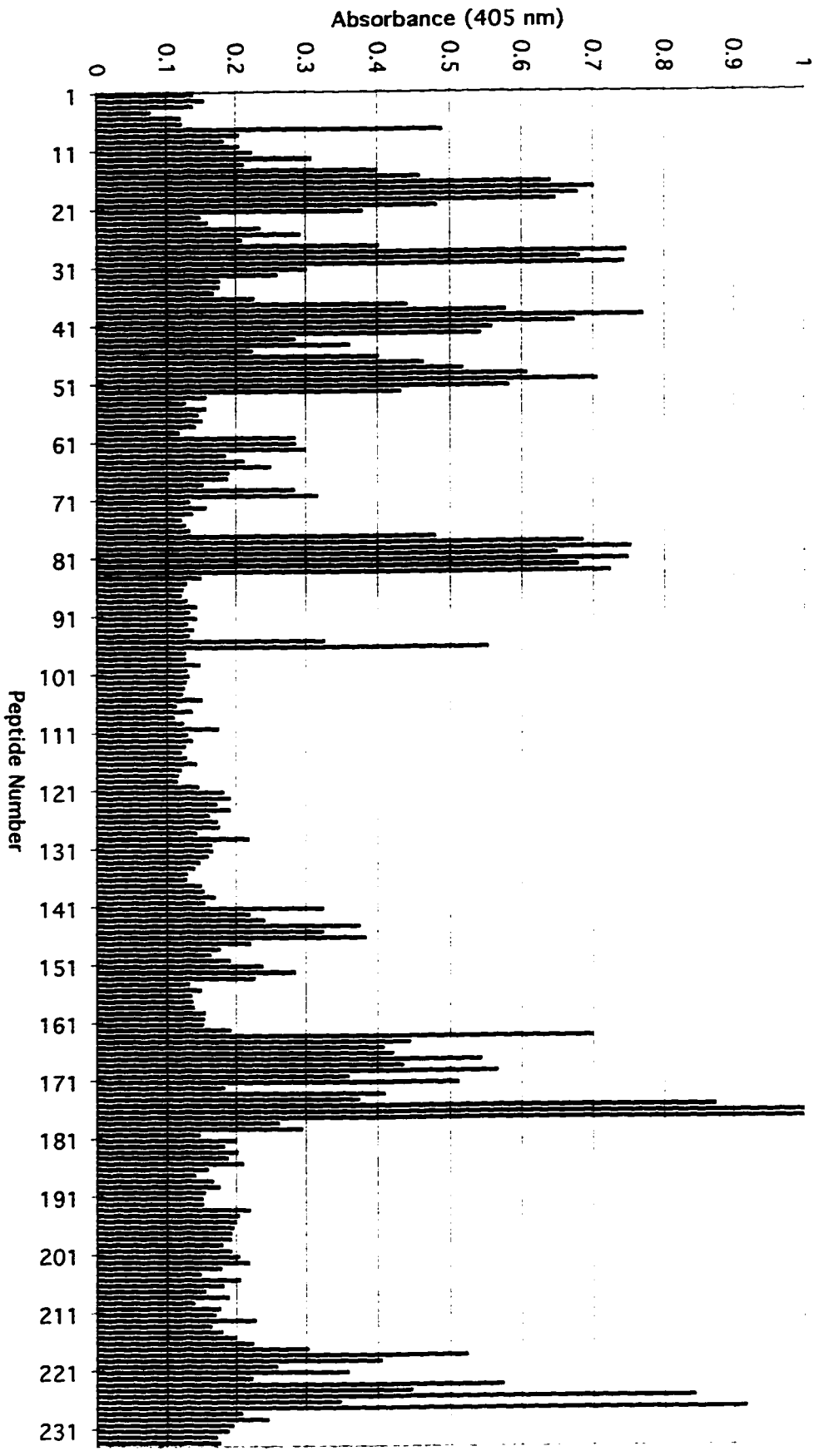


Figure 16: Biotin B80 binding to PSA hexapeptides

Figure 16 shows the hexapeptide epitope scan by ELISA using biotinylated B80 and streptavidin-HRPO conjugate. In this assay a number of potential B80 epitope sequences were identified. A biotinylated antibody, Mab 170H, not specific for PSA, was used as a negative control. The results, shown in Figure 17, show a number of sequences which cross-react to mouse IgG or the streptavidin-HRPO conjugate. When these sequences with a greater than 30% maximum binding were eliminated, the following peptide sequences remained as potential PSA epitopes for B80: 26 to 35 (GVLVHPQWVL), 38 to 48 (AHCIRNKSVIL), 46 to 58 (VILLGHRSLFHPE), 97 to 104 (LLRLSEPA), 146 to 153 (KKLQCVQL), 167 to 177 (QKVTKFMLCAG), and 221 to 229 (TKVVHYRKW). While this scan is an improvement over the scan using anti-mouse HRPO conjugate, the epitope has not been clearly identified.

Figure 18 shows the hexapeptide epitope scan by an ELISA using biotinylated B87 on the PSA hexapeptides. The maximum absorbance was less than 0.6 in this assay. Two peptides which are potential B87 epitopes identified in this assay are 132 (GWGSIE) and 148 (KKLQCV). These peptides were found to be in an internal region of the PSA and are not likely candidates for the B87 epitope. This technique, which can identify linear epitopes that can be defined by a short linear peptide, failed to locate the B87 epitope. Conformational epitopes, made up of non-adjacent residues brought together by the folding of the molecule, are obviously not identified by this method. It is likely that B87 recognises a conformational epitope that cannot be mimicked by a short peptide based on the linear sequence of PSA. The peptides identified by this method can be considered as potential mimotopes.

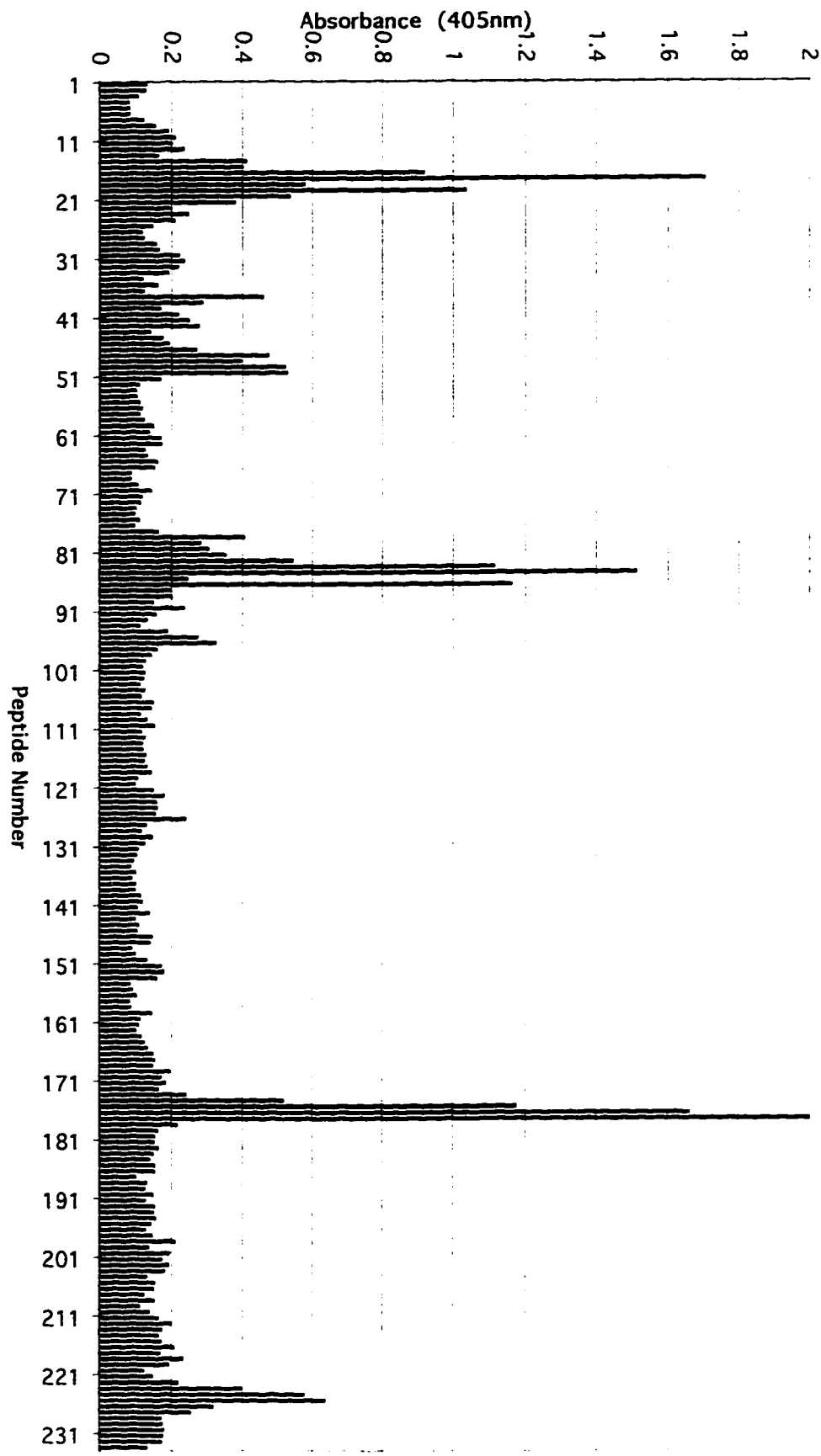


Figure 17: Biotin Mab 170 binding to PSA hexapeptides (non-specific binding)

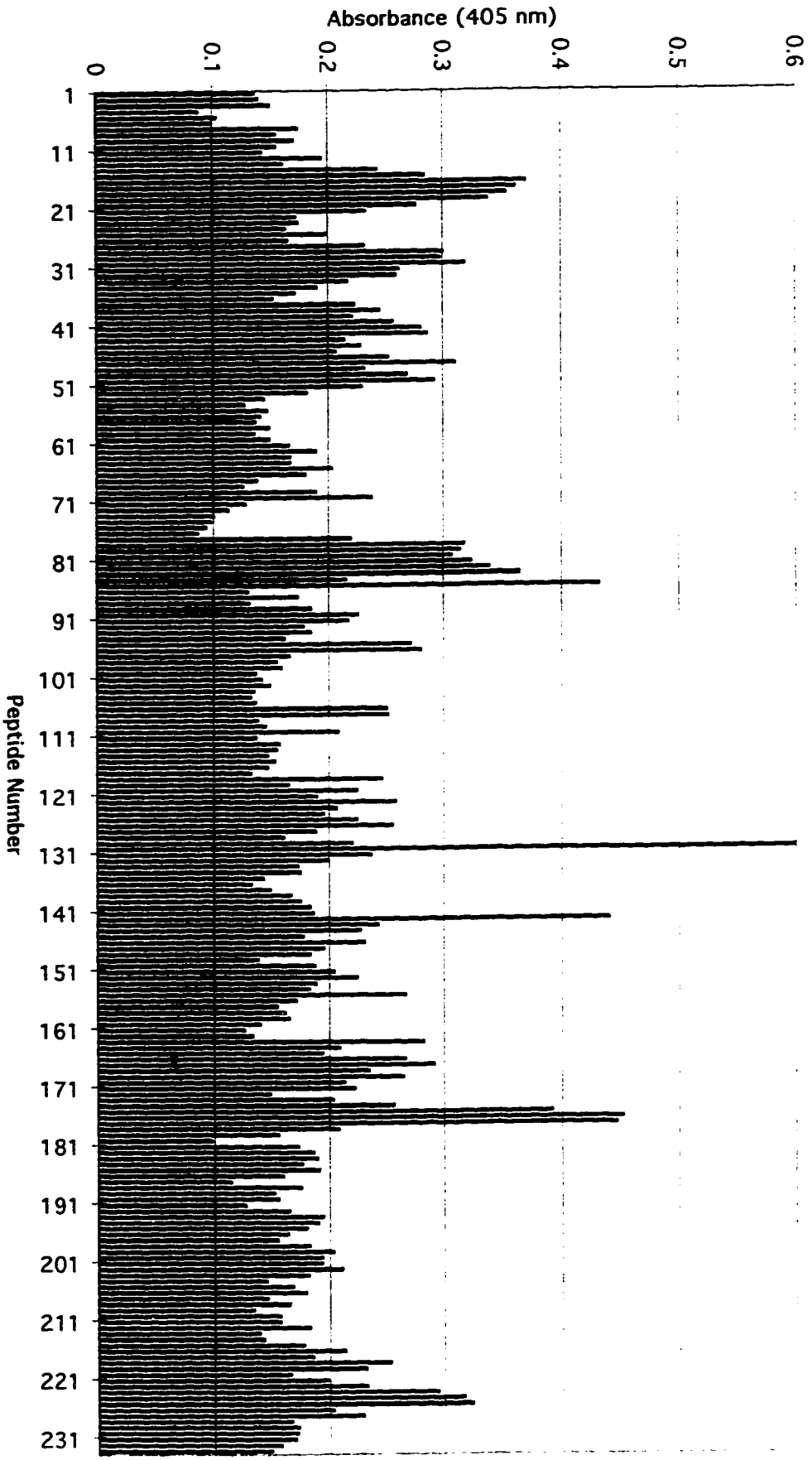


Figure 18: Biotin B87 binding to PSA hexapeptides

3.4 Hexapeptide scanning with bispecific B80-HRPO

Figure 19 shows the results of an ELISA using bispecific B80 which eliminates the need for a secondary HRPO conjugated reagent (see Methods; Figure 3). In this assay the pins were incubated with the antibody for 30 minutes. It is to be emphasised that the data shown was not adjusted for non-specific binding and illustrates the remarkably low degree of non-specific binding to the hexapeptides that are not recognised by the bispecific monoclonal antibody. A smaller number of peptide sequences show binding to the antibody in this assay due to the elimination of cross reactivity with the secondary reagent and binding to low affinity peptides. Four potential epitopes, exhibiting strong binding to B80, were identified in this assay: peptide 19-30 (RGRAVCGGVLVH), peptide 28-41 (VLVHPQWVLTAAHC), peptide 51-62 (RHSLFHPEDTGQ), and peptide 193-204 (PLVCNGVLQGIT). A few other peptides show moderate reactivity. This hexapeptide scan identified four possible locations of the B80 epitope, two of which (residues 28-41 and 193-204) occur in a non-exposed region in PSA and were eliminated. The remaining two (residues 19-30 and 51-62) were found to be adjacent to one another on PSA. Each may represent one portion of a larger conformational epitope that is formed by the folding of the polypeptide chain. The location, consistent with the epitope mapping data, is on a highly exposed region not masked by ACT.

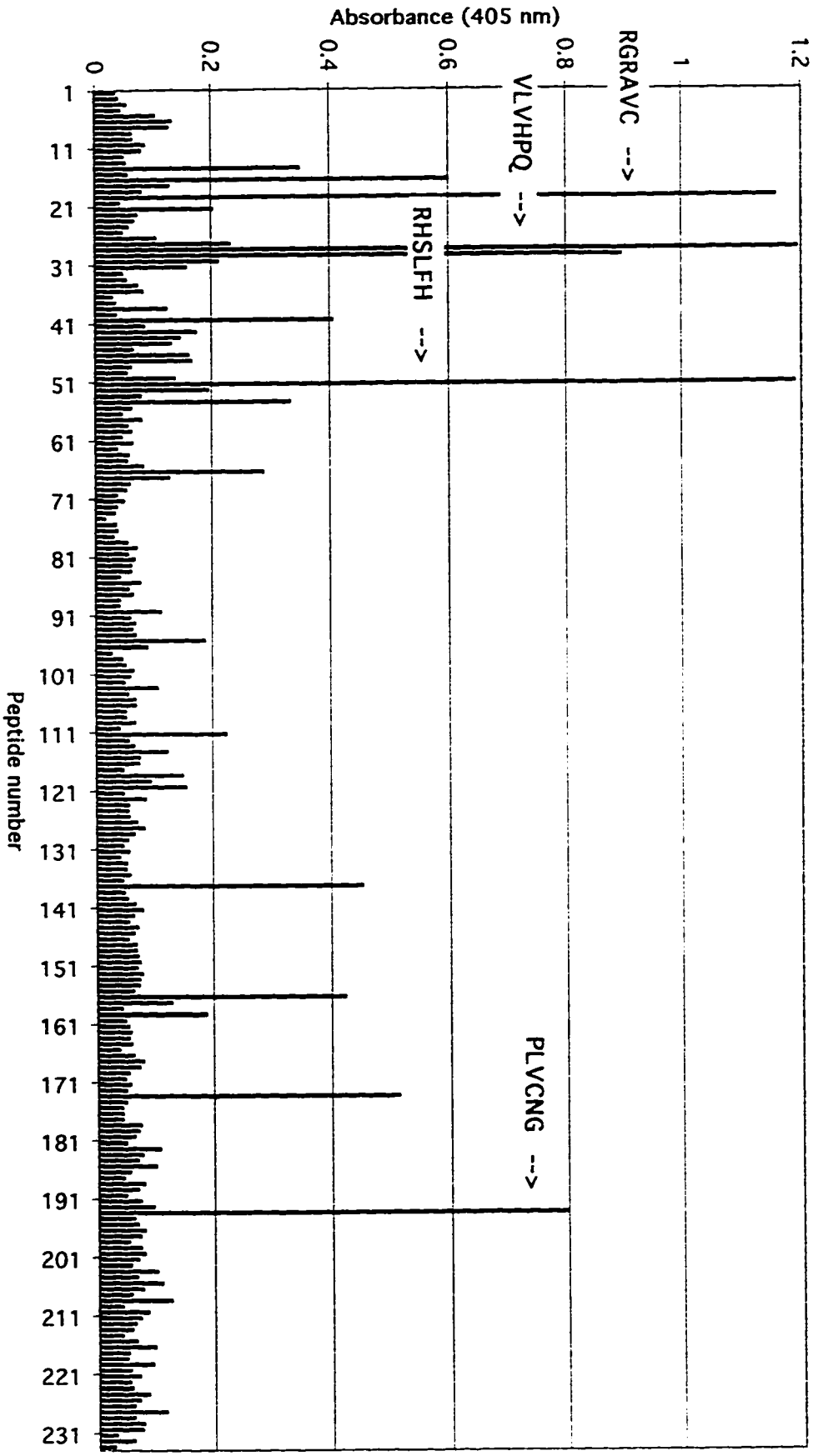


Figure 19: Bi-specific B80 binding to PSA hexapeptides

3.5 Discussion of results

Linear protein epitopes can be identified and located by epitope scanning, a technique first introduced by Geysen (Geyson et al., 1984, 1986). It is based on the principle that linear epitopes can be mimicked by peptides with as few as six amino acid residues. Some of these peptides represent actual sequences found on the antigen and indicate the location of the epitope on the protein molecule. Others mimic the conformation and binding characteristics of the epitope and can bind specifically to the antibody. These peptides are referred to as mimotopes. A list of peptide mimotopes identified using synthetic peptide libraries is given in Table 9.

There are a number of problems associated with this technique, the most serious being the non-specific binding of secondary polyclonal antibodies to the peptides. Hence, anti-mouse-IgG-HRPO conjugate was not useful for this study due to its recognition of a whole range of hexapeptides (Figure 14). Biotinylated antibody used with streptavidin-HRPO (Figure 16) produced better results but the bispecific B80 antibody used with a short incubation time (30 minutes) yielded the best results with minimal non-specific binding (Figure 19).

The number of peptides that can be synthesised at one time is limited by practical considerations. The peptides were not synthesised in duplicate and only two control pins were made for each block of pins. the efficiency of the synthesis could not be verified directly because it is not practical to send all 240 pins for amino acid analysis. Instead the control pins and antibody supplied by the manufacturer were used and found to be acceptable as positive controls. The stability of the peptides on pins after repeated use is unknown. It was observed that after more than 10 ELISAs the absorbance values obtained were substantially reduced. This made direct comparison of results between assays difficult.

The size of the peptide will determine how many epitopes are discovered. If the peptide mimic is too small (less than six residues) a larger linear epitope may be missed. Only one peptide in the epitope for B80, residues 53-59, showed significant binding (Figure 19). The use of longer peptides may have produced a number of adjacent peptides which would have shown binding to B80. The size of the epitope may be deduced by the number of positive peptides (Geyson et al., 1984, 1986: Tribick et al., 1998).

Peptides synthesised on pins may have a different conformation and density than the equivalent peptide in PSA (van Regenmortel, 1992). Only linear epitopes are identified by this technique and there is evidence to suggest that most protein epitopes

are conformational and non-linear. Linear epitopes that are sensitive to conformational changes will also be missed. Further, a potential peptide mimotope may not bind when in solution phase, due to the lack of conformational constraints on the untethered peptide (van Regenmortel, 1992).

In conclusion, four potential linear epitopes, peptide 21-32 (RGRAVCGGVLVH), peptide 30-43 (VLVHPQWVLTAAHC), peptide 53-64 (RHSLFHPEDTGQ), and peptide 195-206 (PLVCNGVLQGIT), were identified for B80 with very clean background with the bispecific monoclonal antibody. The location of the B87 epitope was not identified by this method.

4. Phage peptide display library

4.1 Affinity selection from a phage display library

A phage decapeptide display library (Christian et al., 1992) was used to identify peptides which would specifically bind to B80 or B87 and mimic PSA epitopes. Figure 20 shows a DNA sequencing gel of the random oligonucleotide insert of the phage peptide display library. The random oligonucleotide insert is shown in duplicate as ..... GAC GTG GCC (ALL, ALL, G & T)$_{10}$ GCG GCC TCT GGG GCC ..... The third sequence shown on the gel is the M13 ssDNA control provided in the Sequenase sequencing kit. The library was used to affinity select peptide sequences which bind specifically to B80 or B87. This process is also referred to as biopanning.

The Sepharose beads with immobilised antibody were used for the affinity selection and the elution conditions were the same as those used for the affinity purification of PSA. This column, with 133 pmoles of immobilised antibody, was able to bind 40 pmoles of PSA without saturating (molar ratio of antibody to PSA of 3.3 to 1). The molar ratio of antibody to phage was approximately 70 to 1. The phage were eluted by incubating resin-bound phage with PSA or acid. Unbound phage was separated by centrifugation. The molar ratio of immobilized antibody to the PSA used to elute the phage was approximately 2.7 to 1. The molar ratio of PSA to phage particles was 25 to 1. The eluted phage were then amplified and reselected with ligand and acid to generate four final phage fractions. Table 12 shows the yields of phage particles in each eluate for the two rounds of selection. After the second round, the yield increased by 140 fold for the ligand elution and a threefold increase was seen with the acid elution.

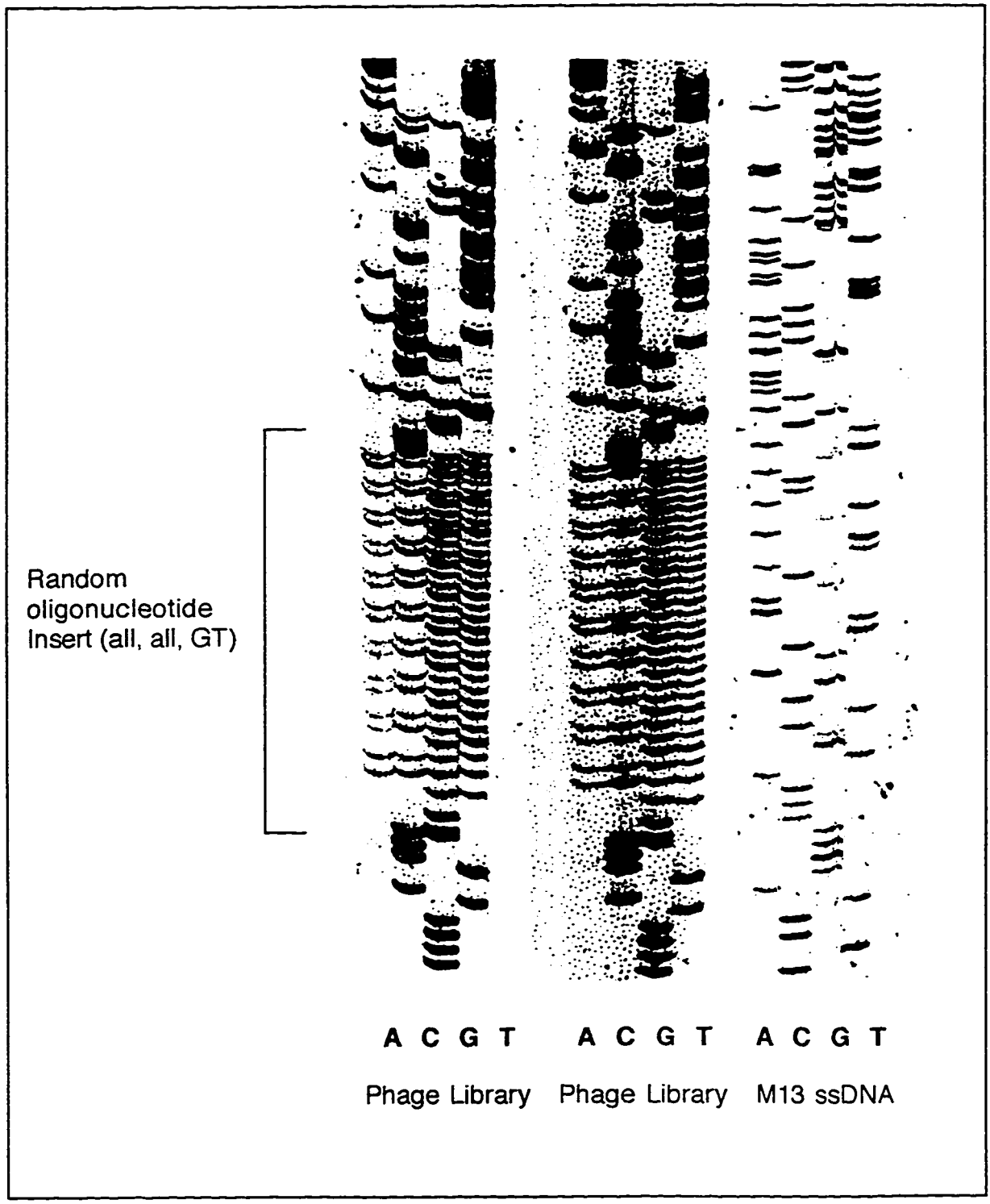


Figure 20: DNA sequence electrophoresis gel of the phage decapeptide display library.

Table 12: Yields of bacteriophage from affinity selection with B80 and B87 anti-PSA antibodies

Eluate	Round of panning	Phage Yield (% of Input)	Enrichment (round 2/round 1)
Ligand (PSA)	1	0.7×10^{-6}	
	2	1×10^{-3}	140
Acid	1	1×10^{-5}	
	2	3.5×10^{-4}	3

4.2 Immunoscreening for phage displaying B80 and B87 specific peptides

In order to identify the phage specifically binding to B80 and to B87, an *in situ* immunoassay was performed. Phage eluted from the affinity beads were grown in E. coli. as individual colonies on agar plates. A nitrocellulose filter was used to lift the phage-infected colonies onto a solid support. After washing, phage particles bound to the membrane were screened for binding to the two Mabs in a standard immunoblot assay. Two set of plates and filters were prepared. One set was screened with B80 and the other with B87. Table 13 shows the number of positive clones for each elution. The results are reported as the average of four plates ± one standard deviation. Screening with B87 revealed 22% positive colonies with the phage that were eluted first with ligand and second with acid. The other phage elutions did not show binding to B87. The fraction obtained by eluting with acid in both rounds of selection did not yield any specific phage clones. B80 positive clones were seen for both eluants using ligand in the first round. B87 positive clones were only found in the elution which was eluted with acid in the first round and ligand in the second round.

Table 13: Number of positive clones identified by immunoblotting of phage-infected colonies

Eluate	Selection procedure round 1	round 2	B87 % positive	B80 % positive
1	ligand	ligand	0	2.9 ± 2.3
2	ligand	acid	0	1.2 ± 0.6
3	acid	ligand	22.3 ± 8.3	2.0 ± 1.4
4	acid	acid	0	0

4.3 Sequences of peptides identified by immunoselection and immunoblotting

Once identified by immunoblotting, individual colonies were selected and amplified for DNA sequencing using the dideoxynuceotide sequencing method. The resulting sequences specific for B80 are shown in Figure 21. Sequences for B87 are shown in Figure 22. In all, over 60 clones were sequenced. Many gave the identical DNA sequence indicating that they were copies of the same clone. One consensus sequence, subsequently identified as a non-specific sequence, had an altered sequence directly following the insert region. The origin of this mutation is unknown. Another investigator identified this sequence from affinity selection using non-related antibodies with this library (H. Parker, personal communication). The library was originally evaluated by biopanning with an antibody which recognised the sequence RAFHTTGRII (Christian et al., 1992).

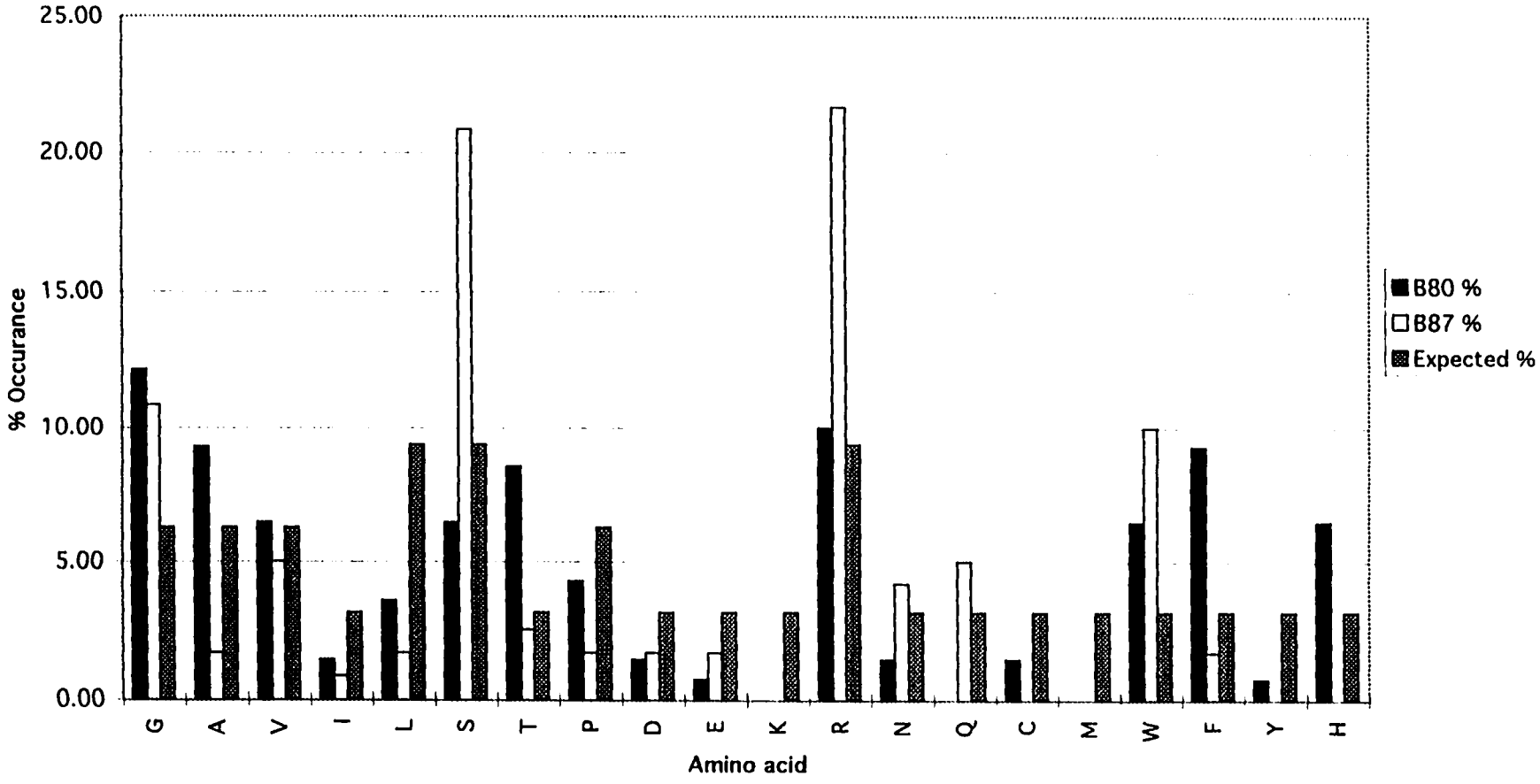
GCG	TTT	CAT	ACT	ACG	GGT	CGT	ATT	GCT	GGG
CGC	AAA	GTA	TGA	TGC	CCA	GCA	TAA	CGA	CCC
A	F	H	T	T	G	R	I	A	A
GCC	GAC	GAG	GCC	TTT	CAT	ACT	ACG	GGT	CGT
CGG	CTG	CAC	CGG	AAA	GTA	TGA	TGC	CCA	GCA
A	D	E	A	F	H	T	T	G	R
CCT	CGA	CGT	GCG	TTT	CAT	ACT	ACG	GGT	CGT
GGA	GCA	GCA	CGC	AAA	GTA	TGA	TGC	CCA	GCA
P	R	R	R	F	H	T	T	G	A
CCT	CGA	CGT	GCG	TTT	CAT	ACT	ACG	GGT	CGT
GGA	GCA	GCA	CGC	AAA	GTA	TGA	TGC	CCA	GCA
P	R	R	R	F	H	T	T	G	A
AAT	CAC	TGG	TCT	CTT	CGT	GGT	AAT	GGT	CAG
TTA	GTG	ACC	AGA	GAA	GCA	CCA	TTA	CCA	GTC
A	N	H	T	R	G	R	P	L	V
TGG	AAG	GGT	CGG	CCT	TCT	GGT	CTG	GTG	GGT
ACC	TTC	CCA	GCC	GGA	AGA	CCA	GAC	CAC	CCA
T	L	G	R	P	S	G	L	V	G
AGT	CAG	TGG	GTT	CGG	CGG	AGT	AGT	GGT	TGG
TCA	GTC	ACC	CAA	GCC	GCC	TCA	TCA	CCA	ACC
S	V	W	G	A	A	S	S	P	W
GAC	GTG	CCG	TCG	TTT	CAT	ACG	ACG	GGT	CGT
CTG	CAC	GGC	AGC	AAA	GTA	TGC	TGC	CCA	GCA
D	V	P	S	F	H	W	W	G	R
ACT	CCG	TCC	TTT	CAT	ACT	ACG	GGT	CGT	?
TGA	GGC	AGC	AAA	GTA	TGA	TGC	CCA	GCA	?
I	P	S	F	H	N	W	G	R	?
AGT	TGT	CTG	TTT	TAT	AAT	ACT	GTG	TGT	TGT
TCA	ACA	GAC	AAA	ATA	TTA	TGA	CAC	ACA	ACA
S	C	L	F	Y	N	T	V	C	V
GAC	GTC	CAT	GCG	TTT	CAT	ACT	ACG	GGT	CCT
CTG	CAG	GTA	CGC	AAA	GTA	TGA	TGC	CCA	GCA
L	V	H	A	F	H	T	T	G	A

Figure 21: DNA and amino acid sequences of B80 affinity selected peptides

AGT	CAG	TGG	GTT	CGG	CGG	AGT	AGT	GGT	TGG
TCA	GTC	ACC	CAA	GCC	GCC	TCA	TCA	CCA	ACC
S	R	W	V	R	R	S	S	G	W
AGT	CAG	TGG	TCG	CGT	TCT	CGG	AAT	CCT	CGG
TCA	GTC	ACC	AGC	GCA	AGA	GCC	TTA	GGA	GCC
S	R	W	S	R	S	R	N	P	R
AGT	CAG	TGG	GTT	CGG	CGG	AGT	AGT	GGT	TGG
TCA	GTC	ACC	CAA	GCC	GCC	TCA	TCA	CCA	ACC
S	Q	W	V	R	R	S	S	G	T
TGG	AAG	GGT	CGG	CCT	TCT	CGG	TCT	GGT	GGT
ACC	TTC	CCA	GCC	GGA	AGA	GCC	AGA	CCA	CCA
W	L	G	R	P	S	R	S	G	G
TGG	AAG	GGT	CGG	CGT	TCT	CGG	CCT	GGT	GGT
ACC	TTC	CCA	GCC	GCA	AGA	GCC	GGA	CCA	CCA
T	L	G	R	R	S	R	P	G	G
AGG	CAG	TGG	TTT	CGG	CGG	AGT	AGT	GGT	TGG
TCC	GTC	ACC	AAA	GCC	GCC	TCA	TCA	CCA	ACC
R	P	W	P	R	R	S	S	G	W
AGT	CAG	GTG	GTT	AGG	CGG	AGT	AGT	CCG	TGG
TCA	GTC	CAC	CAA	TCC	GCC	TCA	YCA	GGA	ACC
S	Q	V	V	R	R	S	S	P	W
AGG	TTT	GGC	AAT	CGT	GCG	ACG	ATT	GCT	TTT
TCC	AAA	CCA	TTA	GCA	CGC	TGC	TAA	CGA	AAA
R	F	A	Q	R	A	T	I	A	F
AGT	CAG	TGG	GTT	CGG	CGG	AGT	AGT	GGC	CGG
TCA	GTC	ACC	CAA	GCC	GCC	TCA	TGA	CCG	GCC
S	Q	W	V	R	R	S	S	G	R
AAT	GAG	TGG	TCG	GAT	CGT	CGG	AAT	GGT	CAG
TTA	CTC	ACC	AGC	CTA	GCA	GCC	TTA	CCA	GTC
N	E	W	S	D	R	R	D	G	Q

Figure 22: DNA and amino acid sequences of B87 affinity selected peptides

Figure 23: Frequency of occurence of an amino acid in a peptide sequence obtained from a phage display library.



This sequence is found among the phage affinity selected in this study (Figure 22). Control bacteriophages displaying this peptide sequence were also used. It is possible that the aliquot of library used in this study was contaminated with control phage bearing this sequence or with phage affinity selected by the library evaluation process.

The design of the random oligonucleotide insert (all, all, G or T) used to create the library used in this study resulted in a bias towards some residues due to the nature of the genetic code. Figure 23 shows the probability of any one amino acid occurring at any position in the library and the frequency of occurrence of residues that were affinity selected. A statistical analysis of the frequences of occurrence of amino acids in the affinity selected peptides was made using a Chi squared test. The difference between the expected frequency and the observed frequencies seen with B80 and B87 was found to be statistically significant ($P = 0.001$). The sequences identified in this study, therefore, are not distributed as is expected from the inherent bias of the library indicating that selection of specific sequences has occurred.

Five unique sequences were identified for B80 and nine for B87. Consensus sequences, shown in Table 14, are underlined. The most interesting finding was that one consensus sequence was found to be homologous to a linear sequence on PSA, peptide 51-55 (-LGRHS-). This sequence closely matches the B80 specific peptide sequence 53-59 (-GRHSLFH-) identified by epitope scanning (Figure 21). The fact that two different techniques independently identified the same region on PSA as the B80 epitope is strong evidence that the epitope is located on or near PSA residues 50-58. The other B80-specific sequences, WGFDFGFGSS and SCLFYNTVCV, may represent B80 mimotopes, peptides that mimic the conformation of the epitope. The peptide mimotope SCLFYNTVCV has a cystein residue on either end and may occur as a loop when displayed on the phage pIII protein.

As none of the B87-specific sequences are found in PSA, the epitope for B87 was not identified by this method, possibly because the B87 epitope is non-linear. The sequence WGRRSS and NEWSDRR appeared a number of times in the affinity selection, suggesting that these sequences are a possible mimotope for the nonlinear epitope of B87.

Table 14: B80- and B87-specific peptide insert sequences identified by affinity selection of a bacteriophage peptide display library

B80 sequences	B87 sequences	non-specific sequences
WLGRPSRSGG[a]	SVWGAASSPW	AFHTTGRIAA
TLGRRSRPGG	SRWVRRSSGW	DVRAFHWWGR
TLGRPSGLVG	SRWSRSRNPR	ADEAFHTTGR
ANHTRGRPLV	SQWVRRSSGT	PRRRFHTTGA
WGFDFGFGSS	RPWPRRSSGW	DVPSFHWWGR
SCLFYNTVCV	SQVVRRSSPW	LVHAFHTTGA
	SQWVRRSSGR	
	RFRQRATIAF	
	NEWSDRRNGQ	
	NEWSQRRDGQ	

PSA sequence 45-60 = -NKSVILLGRHSLFHPE-
Sequence identified by epitope scanning = -NKSVILLGRHSLFHP E -

4.4 Discussion of results

Bacteriophage peptide display libraries can be used to affinity select peptides which specifically bind to a given antigen or antibody. When constructed, the decapeptide library used in this study contained 4×10^8 individual phage clones (Christian et al., 1992). Since there are five hexapeptide sequences in each decapeptide, the library size was equivalent to 2×10^9 hexapeptides. In this study an aliquot containing 4×10^{11} phage clones was used (~200 library equivalents) for affinity selection so that each peptide sequence in the library was represented in the affinity selection using B80 and B87. There is an inherent bias in libraries (shown in Figure 25) as a result of the genetic code for amino acids. The design of this library (NNK) used an equal mixture of 32 triplets encoding all 20 amino acids and a stop codon. To represent all possible decapeptides, 31^{10} or 2.5×10^{16} different clones would be required.

Affinity selection of a phage library identified peptide sequences that bind specifically to B80 or B87. The presence of consensus sequences, sequences which occur in different positions in the decapeptide or which have different DNA coding, indicates that these peptides are not randomly occurring but are selected by the affinity selection process. Only two rounds of selection were performed in this study. More rounds of

biopanning, with PSA as ligand, would have selected fewer sequences but may have identified peptides with higher affinity.

The affinity selection identified a peptide sequence found in PSA (-LGRHS-). This peptide sequence was also identified in epitope scanning as a possible epitope for B80. Since two different techniques independently identified the same sequence it is highly likely that this is the epitope for B80. This sequence occurs in a region that is not identical to porcine pancreatic kallikrein but is unique to PSA. This is consistant with the fact that the antibody does not recognise porcine pancreatic kallikrein. No such sequence was identified for B87. This may be due to the fact that the epitope for B87 may be a conformational epitope that cannot be mimicked by a linear peptide fragment. The other sequences identified for B80 and B87 represent mimotopes, peptides that mimic the conformational structure of the epitope and are able to bind specifically to the antibodies. Two potential mimotopes were identified for B80: WGFDFGFGSS and SCLFYNTVCV. Several potential mimotopes with the consensus sequences WGRRSS or NEWSDRR were identified for B87. These sequences are lead compounds from which peptides (including constrained derivatives) of high specificity and affinity can be developed.

In conclusion, a possible location of the B80 epitope was identified by phage peptide library affinity selection. This peptide sequence was also identified by epitope scanning. A number of other peptide sequences were identified which may represent peptide mimotopes for B80 and B87.

5.0 Peptide mimotopes

5.1 Analysis of peptides on cellulose

Representative peptide sequences identified in Table 12 were synthesised in duplicate on a cellulose membrane and tested for binding to B80 or B87 (Figure 24, 25). The peptides were synthesised from the C terminus and acetylated at the N-terminus as described in Methods, section 9.2. Peptide SRLKNFVREP (#37, 36) was used as a negative control. This peptide sequence was identified from affinity selection using an antibody that is not specific to PSA. Peptides #35,36, #39,40, #41,42, and #43,44 are specific to B87 (by affinity selection) and contain the consensus sequence W(S/P)RRSS. Peptides #45,46, #47,48, #49,50, and #51,52 contain the consensus sequence found on PSA, LGRXS, which is believed to be the epitope for B80. Peptide

#59,60 is a potential mimotope for B80. The remaining peptides #53,54, #55,56, and #57,58 are believed to be non-specific peptides.

The results of an immunoassay of the immobilised peptides using bispecific B80 are given in Figure 24. The negative control peptide (#37,38) did not bind to B80 in this assay. Peptide #59,60 (WGFDFGFGSS) showed strong relative binding to B80. The other peptides, including the peptides that contain the consensus sequence believed to be the epitope for B80, showed little or no binding in this assay. The reason may be that these peptide have low affinity to B80 or that the conformation of the peptides is altered when they are immobilised on cellulose. The cellulose was regenerated as previously described and the assay repeated three times with identical results. The result of the first assay is shown in Figure 24.

Figure 25 shows the binding of B87 and the anti-mouse HRPO conjugate to the immobilised peptides described above. The secondary reagent used in this assay cross-reacts with a number of peptides (#35,36, #39,40, #41,42, #45,46, and #46,47). Peptide #57, 58 (DVRAFWWGR) binds to B87 but not to the anti-mouse HRPO conjugate in this assay. This sequence was identified as one of the non-specific consensus sequences. Significantly, peptide #59,60, which showed strong binding to B80 in the previous assay, did not show binding to B87 or the anti-mouse HRPO conjugate, indicating that this peptide is specific for B80.

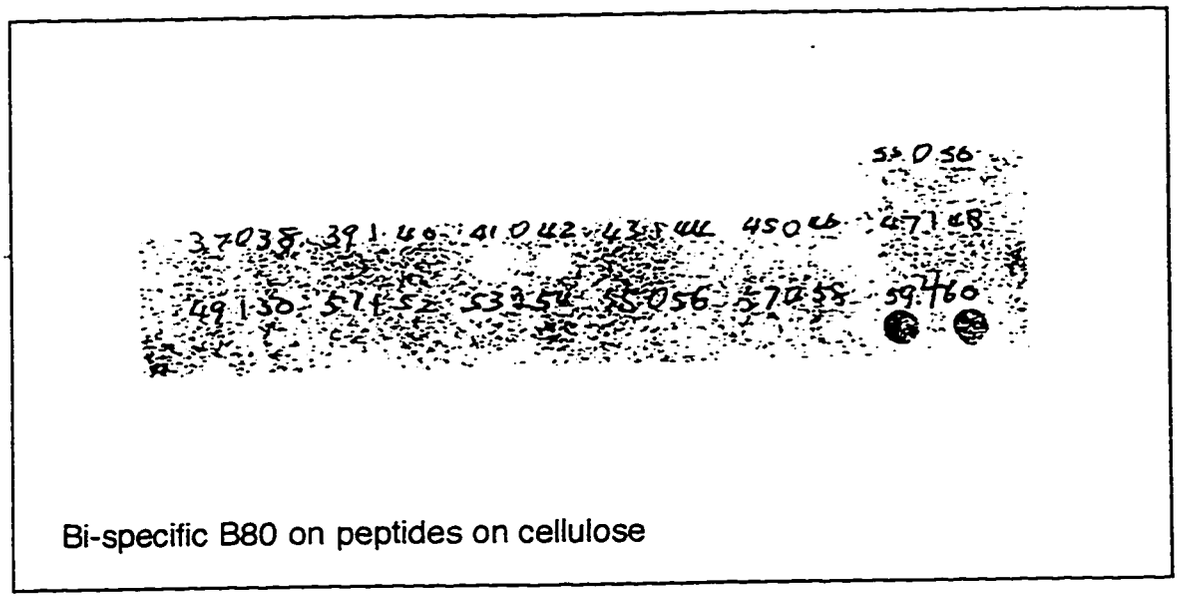
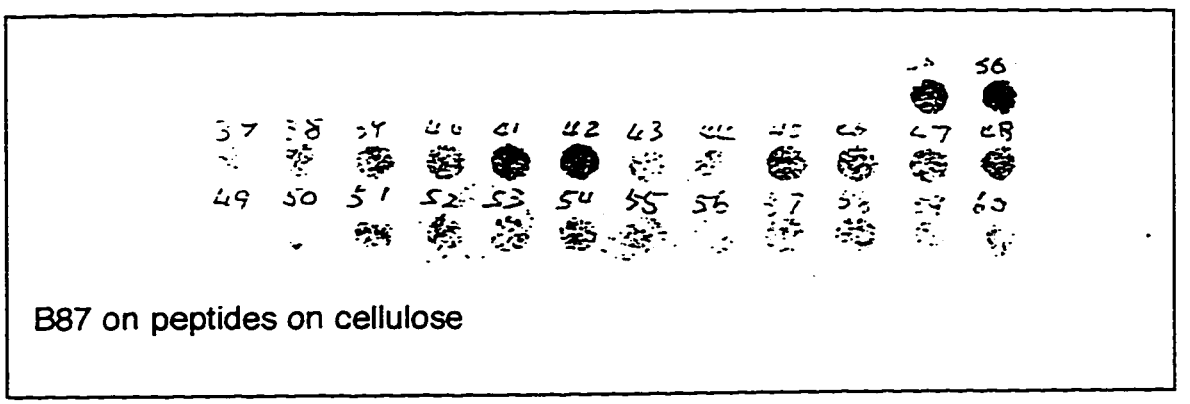
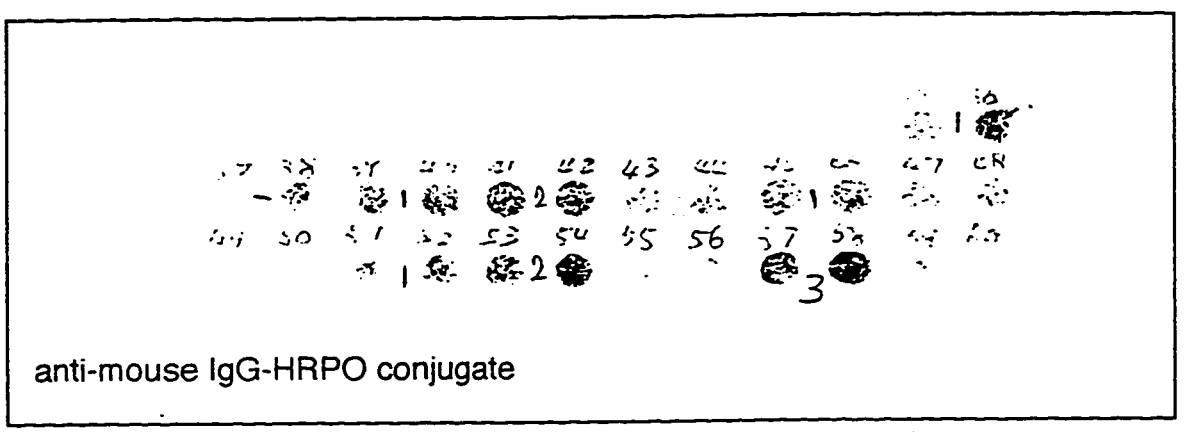


Figure 24: Binding of bispecific antibody B80 on peptides synthesised on cellulose

Peptide number	Sequence
35-36	SRWVRRSSGW
37-38	SRLKNFVREP (NON-SPECIFIC SEQUENCE)
39-40	SRWSRSRNPR
41-42	RPWPRRSSGW
43-44	NEWSDRRNGQ
45-46	WLGRPSRSGG
47-48	TLGRRSRPGG
49-50	TLGRPSGLVG
51-52	ANHTRGRPLV
53-54	AFHTTGRIAA
55-56	ADEAFHTTGR
57-58	DVRAFWWGR
59-60	WGFDFGFGSS



B87 on peptides on cellulose



anti-mouse IgG-HRPO conjugate

Figure 25: Binding of bispecific antibody B80 on peptides synthesized on cellulose

Peptide number	Sequence
35-36	SRWVRRSSGW
37-38	SRLKNFVREP (non-specific sequence)
39-40	SRWSRSRNPR
41-42	RPWPRRSSGW
43-44	NEWSDRRNGQ
45-46	WLGRPSRSGG
47-48	TLGRRSRPGG
49-50	TLGRPSGLVG
51-52	ANHTRGRPLV
53-54	AFHTTGRIAA
55-56	ADEAFHTTGR
57-58	DVRAFWWGR
59-60	WGFDFGFGSS

5.2 Size Analysis and Alanine Replacement Analysis.

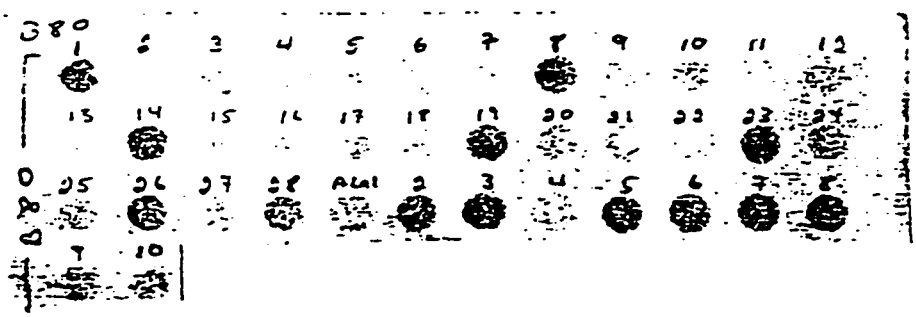
Figure 26 and 27 shows the results of size analysis and alanine replacement analysis with peptide WGFDFGFGSS and DVRAFHWWGA, identified by the previous assays as specific to B80 and B87 respectively. In these assays a set of peptides was synthesised on two cellulose membranes which represent every possible 4-mer (four residue peptide), 5-mer, 6-mer, 7-mer, 8-mer, 9-mer, and 10-mer peptide derived from the mimotope peptide sequences WGFDFGFGSS or DVRAFHWWGA. These peptides are numbered one to 28 and are listed in Figures 28 and 29. An immunoassay to determine the binding of B80 or B87 to these sets of peptides will identify the minimum size of the mimotope and help locate the residues that are critical to binding to a maximum resolution of four amino acids.

An alanine replacement analysis was performed by synthesising a set of peptides where each position in the mimotope peptides was replaced in turn by alanine. If the residue that is replaced is critical to binding, the alanine-containing analogue will not bind to the antibody. The alanine analogs, listed in Figure 28 and Figure 29, are numbered one to 10 and appear as the last 10 peptides on the cellulose membrane. The first peptide in the set is labelled as Ala1.

The size analysis, shown in Figure 26, shows that B80 recognises peptides which contain the sequence WGFD. They are: 1 (WGFD), 8 (WGFDF), 14 (WGFDFG), 19 (WGFDFGF), 23 (WGFDFGFG), 26 (WGFDFGFGS), and 28 (WGFDFGFGSS). This result clearly indicates that the minimum size of the mimotope required for binding is four amino acids and all the critical residues are contained in the peptide WGFD.

The alanine replacement analysis (Figure 26) shows that B80 does not recognise the mimotope analogs 1 (AGFDFGFGSS) and 4 (WGFAFGFGSS), indicating that residues 1 (W) and 4 (D) are critical to binding. This result is consistent with the size analysis that identified the critical residues as being contained in the peptide WGFD. In conclusion, the B80 mimotope can be defined as WGFD.

Figure 27 shows the results of size analysis and alanine replacement analysis for the peptide DVRAFHWWGR. No distinct pattern of binding is seen in this assay, indicating that this peptide may be binding non-specifically with B87 or the anti-mouse IgG HRPO-conjugate.



Bi-specific B80 on peptides on cellulose

Figure 26: Size analysis and alanine replacement analysis of a potential B80 mimotope

SIZE ANALYSIS				ALANINE REPLACEMENT	
Peptide	Sequence	Peptide	Sequence	Peptide	Sequence
1	WGFD	16	FDFGFG	1	AGFDFGFGSS
2	GFDF	17	DFGFGS	2	WAFDFGFGSS
3	FDFG	18	FGFGSS	3	WGADFGFGSS
4	DFGF	19	WGFDFGF	4	WGFAFGFGSS
5	FGFG	20	GFDFGFG	5	WGFDAGFGSS
6	GFGS	21	FDFGFGS	6	WGFDFAFGSS
7	FGSS	22	DFGFGSS	7	WGFDFGAGSS
8	WGFDF	23	WGFDFGFG	8	WGFDFGFASS
9	GFDFG	24	GFDFGFGS	9	WGFDFGFGAS
10	FDFGF	25	FDFGFGSS	10	WGFDFGFGSA
11	DFGFG	26	WGFDFGFGS		
12	FGFGS	27	GFDFGFGSS		
13	GFGSS	28	WGFDFGFGSS		
14	WGFDFG				
15	GFDFGF				

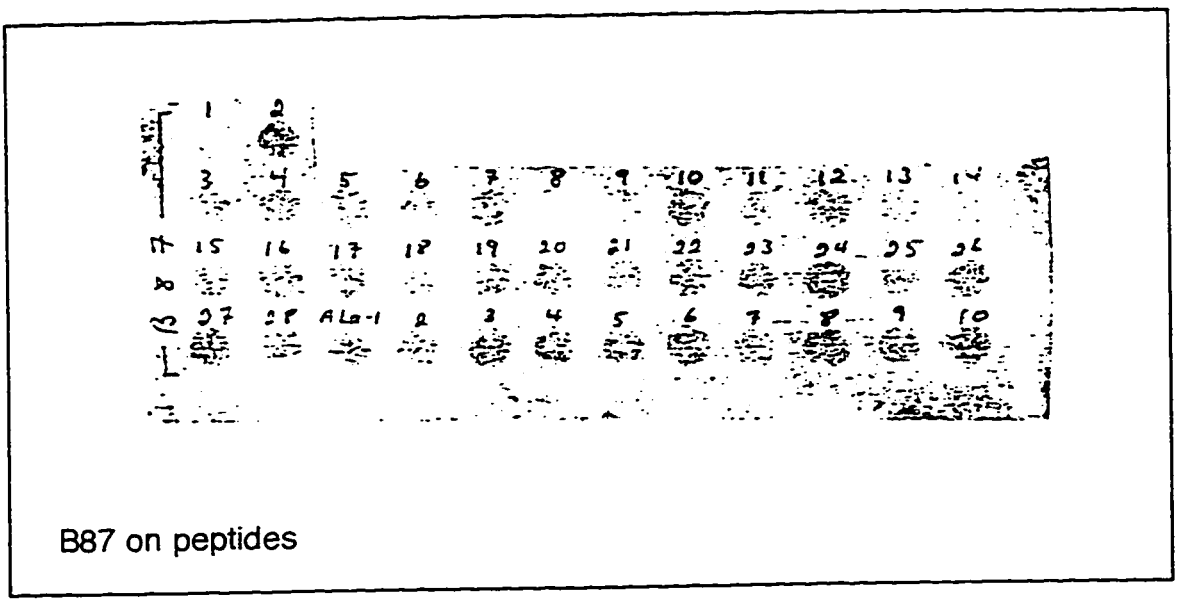


Figure 27: Size analysis and alanine replacement analysis of a potential B87 mimotope

SIZE ANALYSIS				ALANINE REPLACEMENT	
Peptide	Sequence	Peptide	Sequence	Peptide	Sequence
1	DVRA	16	RAFHWW	1	AVRAFHWWGR
2	VRAF	17	AFHWWG	2	DARAFHWWGR
3	RAFH	18	FHWWGR	3	DVAAFHWWGR
4	AFHW	19	DVRAFHW	4	DVRAFHWWGR
5	FHWW	20	VRAFHWW	5	DVRAAHWWGR
6	HWWG	21	RAFHWWG	6	DVRAFAWWGR
7	WWGR	22	AFHWWGR	7	DVRAFHAWGR
8	DVRAF	23	DVRAFHWW	8	DVRAFHWAGR
9	VRAFH	24	VRAFHWWG	9	DVRAFHWWAR
10	RAFHW	25	RAFHWWGR	10	DVRAFHWWGA
11	AFHWW	26	DVRAFHWWG		
12	FHWWG	27	VRAFHWWGR		
13	HWWGR	28	DVRAFHWWGR		
14	DVRAFH				
15	VRAFHW				

5.3 Discussion of results

The peptide sequences identified by affinity selection were synthesised on a derivatised cellulose membrane. Peptide synthesis on this matrix was found to be more convenient than the pin method. Less solvent is used for each step and less time is required to complete each cycle. Four coupling cycles can be completed in one day as opposed to one cycle a day for the pin method. A colour reagent is used to monitor the deblocking and coupling steps to ensure that the reactions are working. The ELISA result obtained with this method is not quantitative and the membrane can only be reused a few times.

The peptides which contained sequences found on PSA (LGRHS) did not show significant binding in this assay, indicating that the affinity of B80 to these peptides is low. One peptide sequence (WGFDFGFGSS) was seen to bind specifically to bispecific B80 (Figure 24). The specificity of this peptide was confirmed by repeated testing during size analysis and replacement set analysis. Figure 26 shows that the minimum mimotope is clearly WGFD. All of the positive peptides contain this sequence and peptides that do not contain this sequence were not recognised by B80. Alanine replacement showed that residues 1 (W) and 4 (D) are critical to binding. The other residues, G and F, may act as spacers to stabilise the conformation of the peptide.

It appeared that two peptide sequences were recognised by B87 (shown in Table 14). Both of these consensus sequences were later found to be non-specific. Size analysis and alanine replacement analysis of the peptide DVRAFHWWGR failed to identify the epitope for B87. Binding to this peptide appears to be non-specific.

In conclusion, a possible peptide mimotope was identified for B80 as being WGFD. However, no mimotope was identified for B87 using this technique.

6.0 Computer model of PSA

A structural model of the molecule, if not available from X-ray crystallography, can be generated using proteins of known structure that have a high degree of homology with the antigen. The model can assist in locating highly exposed surface regions and illustrate the spatial relationships between various binding or catalytic sites and antibody epitopes.

Figure 28 shows the computer-generated homology model of PSA. The catalytic triad for this serine protease-like molecule (His-41, Asp-96, and Ser-71) is shown

in white. The location of the ACT binding site, based on what is known about the binding of ACT to chymotrypsin, is indicated by the shaded residues. The B80 epitope identified by hexapeptide scanning and phage affinity selection, residues 53 to 60, is indicated in yellow. It is seen to be on an exposed region of the molecule well away from the ACT binding site. An epitope for B87 was not identified in this study but can be assumed to be in an exposed region that is not masked by the binding of ACT or B80 to PSA.

Computer models of PSA have been recently described by Vihinen (Vihinen et al., (1994) and Bridon and Dowell (Bridon and Dowell, 1995). The three dimensional structures of PSA and glandular kallikrein were modelled based on porcine pancreatic kallikrein. Loops unique to PSA were modelled by searching from a database. The structure was refined by energy minimisation and molecular dynamics. The resultant model closely resembles the PSA model generated in this study (Figure 28). From the location of the binding site of ACT it is apparent that most of the molecule is still exposed and available for binding to antibodies when ACT is complexed to PSA. The B80 epitope, (shown in yellow in Figure 28) located in this study is on a highly exposed region near the N-terminus, well away from the ACT binding site. The exact location of the B87 epitope was not determined but the possible epitopes could be limited to exposed regions that are unique to PSA and do not overlap with the B80 epitope or the ACT binding site.

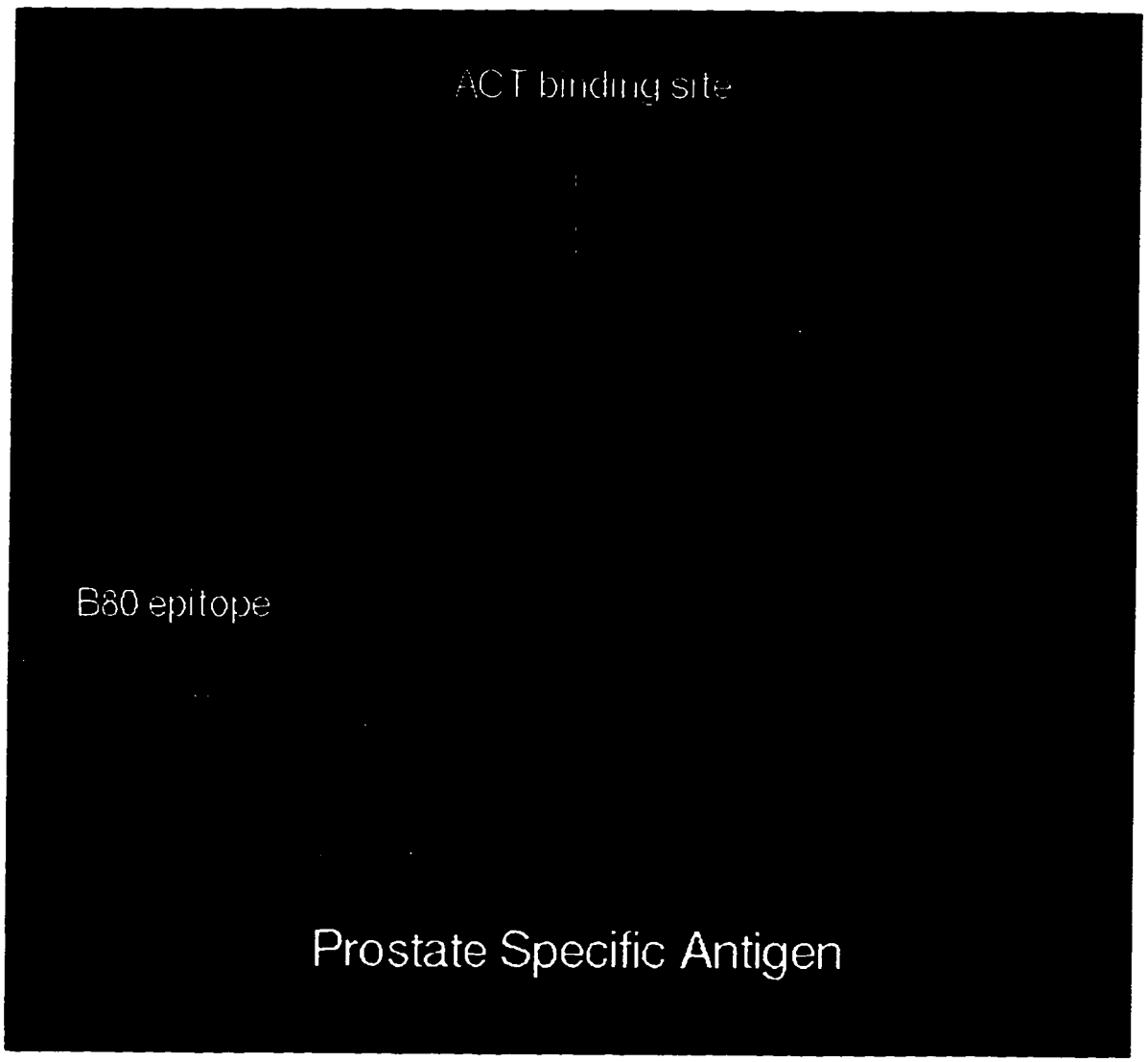


Figure 28: Computer model of PSA showing the location of the B80 epitope and ACT binding site.

IX GENERAL DISCUSSION

Several commercial assays have been developed to detect PSA (Table 4) and to datefour have received FDA approval. Different assays give different PSA values with the same sample (Table 5). This is believed to be due to a number of causes. PSA can occur in a number of different isoforms. It is a serine protease with chymotrypsin-like activity and can occur as an enzyme precursor, an active enzyme, a zymogen, or an internally clipped enzyme. Further, free and complexed forms of PSA exist in serum, adding to the complexity. Differences in PSA assay results obtained with different commercial tests may be explained by identifying and characterising the epitopes that are recognised by the anti-PSA antibodies used in these kits as well as the cross-reactivity to related kallikreins. Characterization of the epitopes on PSA may help to understand and standardise the assays now in use, and assist in the development of new assays specific to one or more forms of PSA in clinical samples.

Recently, the need to standardise PSA assays has been emphasized (Graves et al., 1990, 1993; Stamey et al., 1994; Vessella et al., 1993). In order to create an international PSA standard, a well defined method of purification and characterization of native PSA must be established. The molecular weight of PSA was determined by mass-spectrometry (Belanger et al., 1995). Primary standards can be used to assign mass values to the calibrators used in PSA assays. The fact that PSA forms complexes in serum makes standardisation even more difficult. Graves et al. (1993) tested four assays that measure PSA and PSA-ACT in different molar ratios. This group concluded that the optimal assay configuration is an assay that measures free and complexed PSA in equal molar ratios and this type of assay is referred to as an equimolar assay. Since this type of assay is not affected by complexation of PSA it will give the true estimate of PSA in serum regardless of the ratio of free-to-complexed PSA that is present in the sample. In this situation, free PSA or complexed PSA can be used as a standard.

A non-equimolar or skewed assay may occur for a number of reasons. In assays that use polyclonal antibodies or monoclonal/ polyclonal antibodies, the poly-mono assay may show a loss of signal with PSA-ACT due to the masking of some epitopes by the binding of ACT. Some of the antibodies in the polyclonal antibody population will not recognise complexed PSA. In a comparison of a polyclonal (Proscheck, Yang laboratories) and a monoclonal immunoassay (Tandem R, Hybritech) for PSA, differences were seen that could not be corrected with a simple conversion factor (Table 5). A series of factors along a sliding scale dependent on PSA concentration was needed

(Graves et al.,1990). When PSA-ACT was used as a standard, the differences between these two assays disappeared. The polyclonal-based assays measured free PSA in excess. When free PSA was used as a calibrator, the assay underestimated PSA values from 5% to 27% resulting in underdiagnosis, especially in the early stages of prostate cancer. When complexed PSA was used, the assay overestimates the true molar PSA by 5% to 22% resulting in overdiagnosis (Graves, et al.,1990).

Assay kinetics may also result in skewed assays. Assays employing short incubation times, which do not allow the binding of antibody to antigen to reach equilibrium, may capture free PSA in a higher proportion than the higher molecular weight PSA-ACT complex. Differential rates of binding have been observed when assays with short incubation times are compared to assays with longer incubation times (Graves et al., 1993).

Steric hindrance of binding by a monoclonal antibody to PSA may occur when the epitope recognised by the antibody is near the ACT binding site. In this case the binding of ACT to PSA may not completely obscure the epitope but may interfere with antibody binding resulting in lower affinity of the antibody to PSA. The result is a loss of signal with complexed PSA and a skewed assay (Graves et al., 1993).

The homology of PSA with hK2 and hK1 may also contribute to differences between assays (Vessella and Lange, 1993). It is possible that purified PSA preparations may contain small amounts of these related serine proteases. Cross-reactivity of antibodies to these contaminants could adversely affect the results of a PSA assay. Human serum kallikrein (hK2) may be important as a new marker for the detection of prostate cancer. Like PSA, it is specific to prostate tissue and may be released into the serum of patients with prostate cancer. It has been suggested that some uncomplexed PSA seen in serum may in fact be hK2 since, unlike PSA, hK2 is believed to have trypsin-like specificity and does not form a complex with ACT (reviewed by McCormack et al., 1995). If this is true, the differences between immunoassays for PSA may reflect differences in the degree of cross-reactivity between PSA and hK2 by the antibodies used in the assays. In a preliminary study as part of an international workshop on PSA antibodies, a few antibodies were found to cross react with kallikreins (see Appendix). Producing an antibody specific for hK2 that does not cross-react with PSA may be difficult, however, due to the high degree of homology (80%) between these two molecules (Lilja et al., 1993; Schedlich et al., 1987). The recent availability of recombinant hK2 and hK1 (Rahn et al.,1992; Saedi et al., 1995) may help to better define the specificities of existing PSA assays and aid in the development of assays

specific for other kallikreins (Piironen, 1996).

A number of solutions have been proposed to the problem of assay standardisation:

i) Use only equimolar assays to determine PSA values (Graves et al., 1990).

ii) Use PSA-ACT as an international standard since most of the PSA found in serum is complexed (Stamey et al., 1994).

iii) Use an international mass standard consisting of a mixture of bound and unbound PSA to assign a unit value (units/mL instead of ng/mL) to an international assay standard (Vessella and Lange, 1993).

iv) And lastly, develop assays that measure both PSA and PSA-ACT separately (Graves, 1993).

Another solution suggested by the work presented in this thesis is to locate and characterise specific epitopes on PSA that are recognised by anti-PSA antibodies. Using topographical mapping, the anti-PSA antibodies could be grouped into epitope families. By knowing the location and characteristics of the epitopes on PSA, the problems associated with PSA assays using a particular set of antibodies could be predicted in advance. Antibodies that recognise epitopes not common to other kallikreins and not masked by the binding of ACT to PSA could be selected for assay development.

Two anti-PSA monoclonal antibodies, B80 and B87, were used in this study to investigate the nature of the epitope recognised on PSA. In addition, a recently developed unique bispecific monoclonal antibody was used (Kreutz and Suresh, 1995). The hybidoma cell line producing the B80 antibody was fused with an antihorseradish peroxidase (HRPO) cell line to produce a hybrid-hybidoma or quadroma. The antibody that was created by this method has one binding site that recognises PSA and one binding site that recognises HRPO. Using this reagent the need for a biotinylated or secondary anti-mouse HRPO-conjugated antibody is eliminated. High backgrounds typically seen with conventional secondary polyclonal antibody-enzyme conjugates are not seen when a bispecific antibody is used. This low background greatly reduced the number of peptides that were identified as potential PSA epitopes in the hexapeptide scan and was critical in identifying a sequence that was also identified with affinity selection of the bacteriophage peptide display library.

The fact that B80 and B87 were able to form a sandwich immunoassay indicates that these antibodies recognise two distinct and non-overlapping epitopes that are far enough apart to avoid steric hindrance. The fact that both antibodies failed to form a homosandwich is evidence that each antibody recognises a unique and non-repeating

epitope.

Both B80 and B87 were able to recognise the PSA-ACT complex. This result indicates that both epitopes are located far enough away from the ACT binding site to avoid steric hinderance.

Neither B80 nor B87 cross-reacted with porcine pancreatic kallikrein (pK1), which shows a 59.6 % amino acid sequence homology with PSA. This result indicates that both antibodies recognise sequences on PSA that are not common to pK1 and PSA. Since we can also assume that the epitope is on the surface of the molecule and away from the ACT binding site, the number of possible sites of the two epitopes are greatly reduced.

Hexapeptides representing all possible overlapping amino acid sequences of PSA were synthesised and tested for binding to B80 and B87. These hexapeptide fragments represent an entire set of potential epitopes or partial epitopes on PSA. A considerable number of these are likely buried in the center of the molecule. Many others are surface epitopes and are likely structures interacting with antibodies selected to measure the native molecule in serum. In this study, four possible locations of the B80 epitope were identified using this technique. Two of the peptides, at residues 30 to 36 and 195 to 201, were found to be in the interior of the molecule using a computer generated model of PSA, and are not likely candidates for the B80 epitope. The two remaining sequences at residue 21 to 27 and 53 to 59 were found to be adjacent to one another on the surface of the molecule.

No epitope was identified for B87 using this technique. It is possible that B87 recognises a conformational epitope that cannot be mimicked by a short peptide or that B87 recognises a linear epitope that is longer than six reisdues. Also, neighbouring residues present in PSA but not present in the synthetic hexapeptide may cause important conformational changes in the epitope that are critical to binding. Paratopes directed against proteins have a different overall structure than paratopes that bind to peptides. Protein paratopes tend to be shallow and cover a relatively large surface area (Wilson, 1993). The epitopes recognised by this type of paratope is usually discontinuous and cannot be represented by a linear peptide. It may be difficult to find a peptide that can specifically bind to an antibody directed against a protein antigenthat exhibits this paratope structure (Wilson, 1993,1994). Peptide paratopes, on the other hand, tend to be grooved and the peptide antigen is often partially buried in the structure of the paratope (Wilson, 1993). The ability to find linear epitopes and peptide mimotopes for a given antibody may depend on the characteristics of the paratope rather than on the structure of the peptide (Wilson, 1993).

It is also possible that B87 recognises a carbohydrate epitope. PSA has a N-linked oligosaccharide at asparagine-45. The carbohydrate group is a N-acetyllactosamine type with a sialic acid group at the end of each of the two branches (Belanger et al., 1995). Seventy percent of the PSA molecules purified from seminal plasma contain a fucose group in the core chitobiose moiety. The calculated molecular weight of the carbohydrate group is Mr 2,351.8.

Antibodies can recognise both linear and branched oligosaccharides (reviewed in Cygler, 1994). The binding sites of anti-carbohydrate antibodies in general show stacking interactions between aromatic residues, usually tryptophan, and hydrophobic pactches on the sugars. Specific binding to carbohydrate epitopes is characterised by the formation of hydrogen bonds to charged or amide side chains and the involvement of ordered water molecules in the formation of a hydrogen-bonding network. (Vyas et al., 1993; Bundle and Young, 1992). The antibody-binding site typically contains aromatic residues such as tyrosine in the CDR. Histidine, which is rarely seen in framework regions, is also often present along with less charged or polar residues (Padlan, 1990). There is usually good complementarity of fit with the carbohydrate chain and van der Waals interactions are important to the overall binding energy (Cygler et al., 1991).

The ability of B87 to bind to a de-gycosolated form of PSA would indicate that the antibody does not recognise a carbohydrate epitope and that the absence of the carbohydrate chain does not significantly alter the conformation of the epitope. Since PSA has been cloned (Lundwall and Lilja, 1987), a source of PSA that does not contain a carbohydrate side chain is available, and could be used to determine if any of the anti-PSA antibodies recognise oligosaccharide epitopes. Alternativly, chemical deglycosolation may be carried out using reagents such as anhydrous trifluoromethanesulfonic acid which can cleave N- and O-linked glycans non-selectively from glycoproteins, leaving the primary structure of the protein intact (Sojar and Bahl, 1987). Glycosidases can also be used to enzymatically cleave carbohydrate chains from proteins. These can be exoglycosodases, which remove a terminal saccharide unit, endoglycosidases, which cleave between within the carbohydrate chain; or glycoamidases, which specifically cleave between an oligosaccharide unit and its N-linkage to the protein. Since the carbohydrate chain on PSA is known to contain sialic acid, sialidases can also be used to release sialic acid from the terminal position of the chain (Freeze, 1993; Powell and Hart, 1993; Varki et al., 1993).

In a second strategy adopted in this study, affinity selection with B80 was employed to screen and select a random decapeptide. The bacteriophage display library

identified one concensus sequence homologous to a linear sequence seen on PSA and identified as a possible B80 epitope by hexapeptide scanning as discribed above. The fact that two different techniques identified the same peptide sequence is strong evidence that the epitope for B80 involves residues 51 to 54. This sequence is located on an exposed surface loop well away from the ACT binding site.

There have been a number of reports of antibody epitopes which have been identified using phage display technology (Dower, 1992; Bottger and Lane, 1994; Lane and Stephen, 1993; Stephen and Lane, 1992). All of these studies identified a five-amino acid consensus sequence for each antibody necessary for binding. Bottger and Lane (1994) screened seven anti-human keratin antibodies with a random hexapeptide library. No hexapeptide motifs were identified for six of the seven antibodies. Other studies showed similarly low success rates (Lane and Stephen, 1993). This fact illustrates the limitations of this method for the identification of antibody epitopes. It is possible that some epitopes may not be present in the library used for screening since not all possible peptide sequences are found in an individual library. The hexapeptide library produced by Scott and Smith, for example, contains about 70% of all possible hexapeptide motifs (Scott and Smith, 1990). As discussed earlier, some antibodies may recognise conformational or discontinuous epitopes which cannot be mimicked by a phage display library (Jin et al., 1992; Laver et al., 1990). Continuous epitopes comprising more than six amino acids would not be represented in a hexapeptide library (Purkis et al., 1990). It is also possible peptides which bind with low affinity may have been missed because highly stringent biopanning procedures were used (Barrett et al., 1992). Using PSA to elute phage from the B80-, B87-affinity column was more successful in selecting possible epitopes than the more stringent acid elution. The epitope sequence may be present in the library but in a different conformation. The use of a variety of constrained libraries may help overcome this problem. The use of longer libraries and modified biopanning techniques may increase the success rate of these studies. It should be noted that the discovery of the B80 epitope was facilitated by the use of two different methods; affinity selection with a phage library and hexapeptide screening with an immobilized synthetic library.

In this study, possible B80 and B87 mimotopes were also identified using affinity selection of a phage display library. The fact that only one of these sequences, WGFDFGFGSS, was found to bind when the peptides were synthetically made indicates that the binding affinity of these mimotopes may be low. Other groups have reported mimitopes for biotin, sugars, and other small peptides (see Tables 7 and 8). Peptide

mimotopes for larger proteins have, so far, been shown to have low affinity and have been used as lead structures for the development of higher affinity mimics (Wells, 1996). Recently, Wrighton et al. (1996) identified peptide sequences that bind to and activate the cytokine erythropoietin. This was acomplished by first screening with a number of different libraries containing linear peptides of up to 20 amino acids or disulfide-constrained peptides. The first round of selection used libraries where the random peptide sequences were displayed on the pVIII protein. An average of 100 to 200 copies of the peptide was displayed per phage particle. This multivalent display allowed for better capture of low affinity sequences. In addition, a thrombin cleavage site was introduced at the base of the antigen so that the ligand-phage complex could be cleaved from the plate. Higher affinity peptides were then identified using more stringent elution conditions and a sub-library where the sequences identified in the first round were randomized using mutagenesis, then displayed on the pIII protein of the phage. The result was the identification of a high affinity hormone mimic that was able to activate the cytokine. Following this example, the utility of phage display technology for the discovery of high affinity mimotopes could be improved by using multivalent libraries with constrained and longer linear peptides, and using sub-libraries screened using more stringent elution conditions.

The epitope located by epitope scanning and affinity selection was synthesised and found to bind, albeit weakly, to B80. The low binding affinity of synthetic peptides based on the structure of protein epitopes has been noted (Davies, 1990: Wilson, 1993, 1994). This may be due to the flexibility of the synthetic peptide and to the fact that a linear peptide may be binding to fewer contact points than the original protein epitope. The conformational protein epitope may have a more rigid structure and consequently more contacts, resulting in an increase in affinity (Davies, 1990; Wilson, 1993; Van Regenmortel, 1992). Several other peptides were identified in this study by affinity panning of the bacteriophage random display peptide library (Table 14). These mimotopes did not bear any homology to the sequence of PSA.

The mimotope structure (-WGHD-) identified by affinity selection, size analysis and alanine replacement analysis may mimic the conformational structure of the protein epitope. An alignment of the critical residues (W and D) can be made to a linear fragment on PSA adjacent to the epitope identified by epitope scanning and affinity selection. Since peptide antigens cover a smaller surface area of the paratope it is possible that different peptides can bind to different contact residues within the same paratope (Davies, 1990; Wilson, 1993). It is also possible that the mimotope is mimicking the structure of a

larger discontinuous epitope that cannot be represented by a linear peptide fragment of PSA (Van Regenmortel, 1992: Greenspan, 1992). The affinity of this peptide may be improved by synthesizing cyclic constrained peptides.

Another approach to epitope mapping is site-directed mutagenesis (Smith, 1991; reviewed in Van Regenmortel, 1992) Using gene cloning techniques, multiple substitutions can be made for any given amino acid. A contact residue is identified if substitution with an amino acid with approximately the same side-chain volume results in loss of binding. This restriction is needed because changes to the volume of a side chain may result in conformational changes which would alter the normal bond distances with other contact residues and the antibody (Van Regenmortel, 1992). PSA has been expressed on the the minor coat protein pIII of the filamentous phage (Eerola, et al., 1994). The authors suggest that these phage particles could be used to create a PSA phage display library that would be useful for epitope mapping.

Epitopes on PSA may be identified by co-crystallizing PSA and an Fab fragment of the B80 anti-PSA antibody for eventual three-dimentional structure determinations by X-ray diffraction methods. As discussed in the introduction section, this technique identifies structural (discontinuous) epitopes and contact residues by their close proximity within the epitope-paratope interface.

A series of competitive inhibition experiments using B80, B87 and other anti-PSA antibodies would identify overlapping epitopes. It would be interesting to see if the B80 epitope is common to other antibodies and if antibodies that inhibit the binding of B80 to PSA also recognise the B80 mimotope peptide WGFD. Cross-reactivity to the mimotope would suggest that the paratopes of these antibodies were similar in shape and conformation. Optimisation of the binding of this peptide may be achieved by amino acid replacement analysis. In this study only alanine was used. Other amino acids, natural and unnatural, could be substituted to identify peptides with improved binding affinity for PSA.

The immobilised hexapeptides derived from the PSA sequence can be used to epitope map other anti-PSA antibodies since the pins are reusable. Other anti-PSA antibodies which inhibit the binding of B80 to PSA could also be tested to see if they recognised the same peptide sequences as B80, indicating that the B80 epitope is an immunodominant antigenic determinant recognised by other antibodies.

Antibodies raised against the B80 mimotope (WGFD) conjugated to a protein could be tested for cross-reactivity to PSA. Testing the ability of this peptide to elicit antibodies that recognise PSA is further indication that this peptide is mimicking the

structure of an epitope on PSA. Similarly, antibodies could be raised against the phage clones identified by affinity selection. Cross-reactivity of these antibodies to PSA would indicate that the peptide structures displayed by the phage particles are mimicking an epitope on PSA. Constrained peptide derivatives of the various peptide epitopes and mimotopes could provide improved affinity to bind the B80 antibody. Further, N^{15}-labelled or fluorescent labelled peptides could also be used to study their interaction with anti-PSA antibodies by spectroscopic methods.

Another potential use of the PSA mimotope peptide (WGFD) is to purify recombinant B80 or B80Fv single chain antibodies from cell media. This could be achieved by conjugating the peptide to a CNBr activated Sepharose resin to produce an affinity column specific to the B80 paratope. The use of affinity chromatography can greatly simplify the inherently difficult purification of recombinant proteins from cell supernatent.

There has been a great deal of recent interest in developing antibodies that are specific to PSA or hK2 (Piironen et al., 1996). Piironen et al., (1996) generated 23 anti-PSA monoclonal antibodies and tested them for cross-reactivity to hK2. Seven of the antibodies recognised an epitope shared by PSA and hK2. These antibodies were used to measure PSA and hK2 in serum. Piironen et al., (1996) also tested a number of commercial assays for cross-reactivity and found that the ACS™ PSA assay from Ciba measured PSA and hK2 while the Hybritech assay did not. This result may explain the differences in results between these two assays. So far no hK2 monospecific antibody has been generated. Klee et al., (1995) produced sheep anti-peptide antisera to the 41-56 peptide region of hK2. This is a region on hK2 that is not homologous to PSA and is the region on PSA recognised by B80. Although this group was able to show specific binding to hK2 the limit of detection of the polyclonal-based assay was too low to be useful. An hK2 or PSA-specific monoclonal antibody could be developed using a synthetic peptide epitope or mimotope. Using this antibody with B80 (which is specific for PSA) and an antibody that recognised an epitope shared by PSA and hK2, an assay to measure PSA, hK2 and PSA + hK2 could be developed. The results of this new assay may prove to be of clinical significance and may improve the sensitivity and specificity of the current PSA assays.

In conclusion, I have demonstrated the utility and limitations of epitope mapping, epitope scanning, and affinity selection for the identification of protein epitopes and

mimotopes. Future work suggested by this study includes:

i) Confirmation of the location of the B80 and B87 epitopes by structural studies of the Fab-PSA complex by NMR and X-ray crystallography.

ii) The investigation of carbohydrate epitopes using de-glycosolated PSA.

iii) The development of peptide epitopes and mimotopes with higher affinity using a sub-library of longer constrained synthetic peptides.

iv) The use of the B80 PSA mimotopes and epitope peptides to purify B80 and recombinant B80Fv from cell supernatent.

v) The use of peptide mimotopes and epitope peptides to generate antibodies specific for PSA and hK2.

vi) The use of epitope mapping to determine the specificity of other anti-PSA antibodies, especially those used in commercial PSA assays.

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XI APPENDIX

Relative affinity and specificity of the ISOBM PSA workshop antibodies*

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Abstract

The relative affinities of the ISOBM anti-PSA antibodies were determined in a direct binding ELISA. The antibodies were ranked in order of highest to lowest affinity. In order to determine the specificity of binding to PSA the degree of cross-reactivity of the ISOBM PSA workshop antibodies to homologous proteins was determined. One antibody (ISOBM antibody 28) was found to cross-react with human plasma kallikrein and two others (ISOBM antibodies 29 and 56) with porcine pancreatic kallikrein. The ability of the ISOBM PSA workshop antibodies to recognise PSA when complexed to α1-antichymotrypsin was determined in an assay specific to the PSA-ACT complex. Twelve antibodies (ISOBM antibodies 25, 26, 27, 37, 40, 51, 68, 70, 73, 78, 83 and 89) failed to recognise PSA when complexed to ACT and one antibody (ISOBM antibody 69) was found to recognise an epitope on ACT.

*Presented at the first International Workshop on PSA Antibodies. ISOBM (International Society of Oncology, Biology and Medicine) 1995, Montreal, September 1995.

Methods

The relative affinities of the anti-PSA antibodies were determined using the concentration required to achieve 50% of maximum binding to imobilized PSA. The assays used to determine the ability of the antibodies to recognise kallikreins and ACT-PSA are described below.

1. Direct Binding ELISA. In this assay the antigen, PSA, ACT, or kallikrein, was immobilised on a microtitre plate. The ISOBM monoclonal antibodies were incubated on the plate and after washing, anti-mouse IgG conjugated with HRPO was used to detect bound antibody. For the rat and goat antibodies the appropriate secondary antibody was used.

2. ELISA of PSA-ACT Complex. Anti-PSA monoclonal antibodies were used as the capture antibody and immobilized on a microtire plate. PSA-ACT complex was incubated with the antibody and anti-ACT antibody was used to detect bound PSA-ACT complex. Anti-rabbit IgG conjugated with HRPO was used to detect the presence of the rabbit anti-ACT antibody.

Results: The results are shown in the following graphs and table:

Table 1: RELATIVE AFFINITIES OF ANTI-PSA ANTIBODIES

ISOBM NUMBER	R	C x e-10	ISOBM NUMBER	R	C x e-10
24	0.936	0.97	66	0.962	11.80
25-i	0.815	27.27	67	0.977	12.33
26	0.971	21.47	68	0.922	10.80
27-i	0.912	2.93	69	-	-
28	0.832	14.20	70	0.538	40.93
29	0.885	18.33	71	0.948	19.73
30	0.968	17.07	72	0.959	16.67
31	0.999	0.72	73-i	0.904	16.07
32	0.853	3.30	74	0.977	10.80
33	0.969	1.24	75	0.957	9.47
34	0.98	1.05	76	0.976	7.60
35	0.939	0.82	77	-	-
36	0.95	1.60	78	0.98	34.60
37	0.87	3.90	79	0.95	34.20
38	0.862	4.34	80	0.91	13.60
40-i	0.911	7.40	81	0.944	14.07
41-i	0.874	4.53	82	0.787	16.13
50	0.931	0.91	83	0.989	5.87
51	0.936	1.00	84-i	0.721	5.66
54	0.984	0.53	85-i	0.67	25.67
55	0.975	0.79	86-i	0.84	28.80
56-i	0.834	1.40	87	0.834	28.00
57-i	0.771	9.53	88	0.91	52.93
62	-	-	89	0.82	51.07
63	0.966	8.33	90	0.777	31.80
64	0.944	7.27	91	0.903	15.73
65	0.989	1.31			

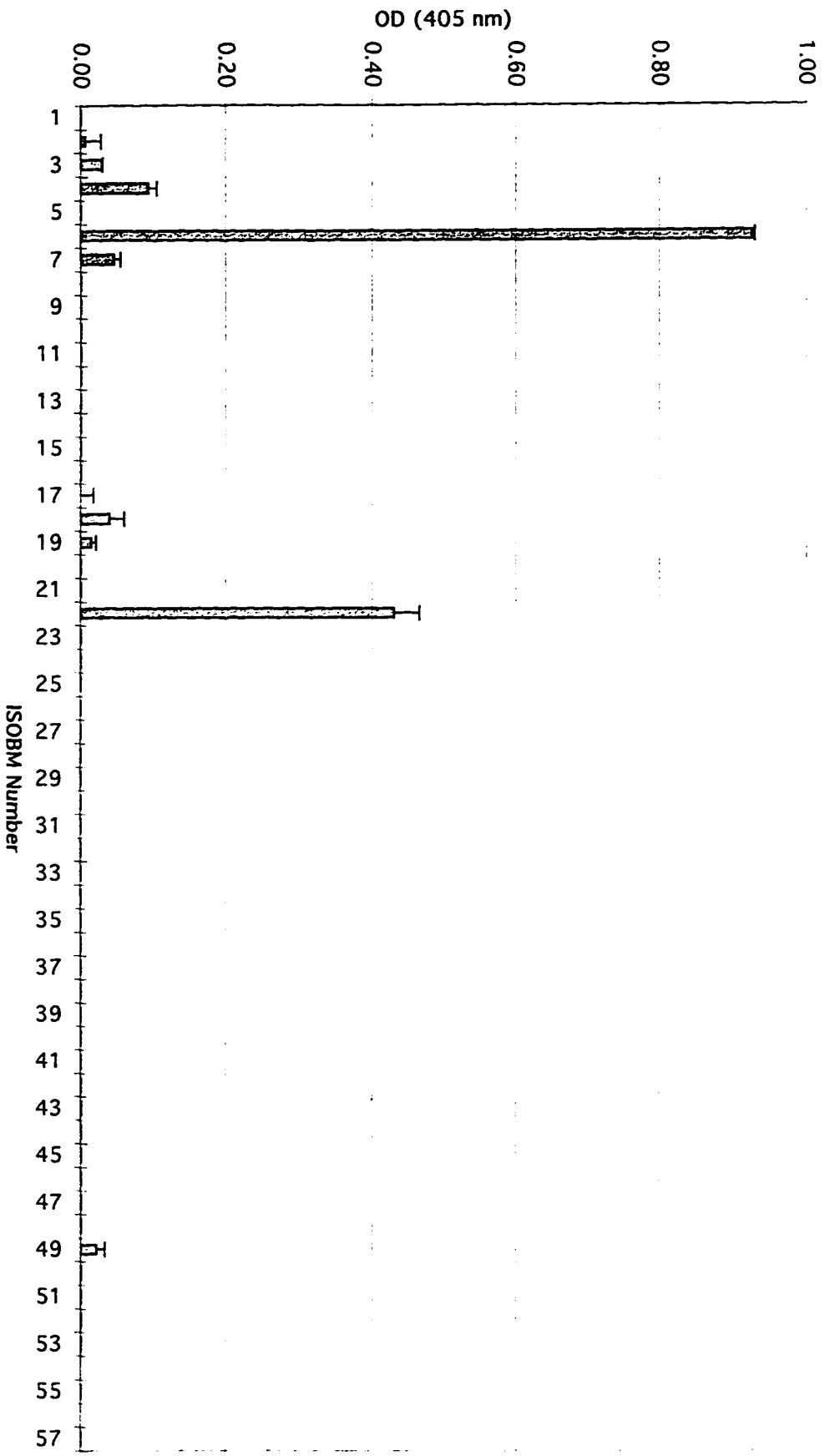

Figure 1: Cross-reactivity of anti-PSA antibodies to porcine pancreatic kallikrein

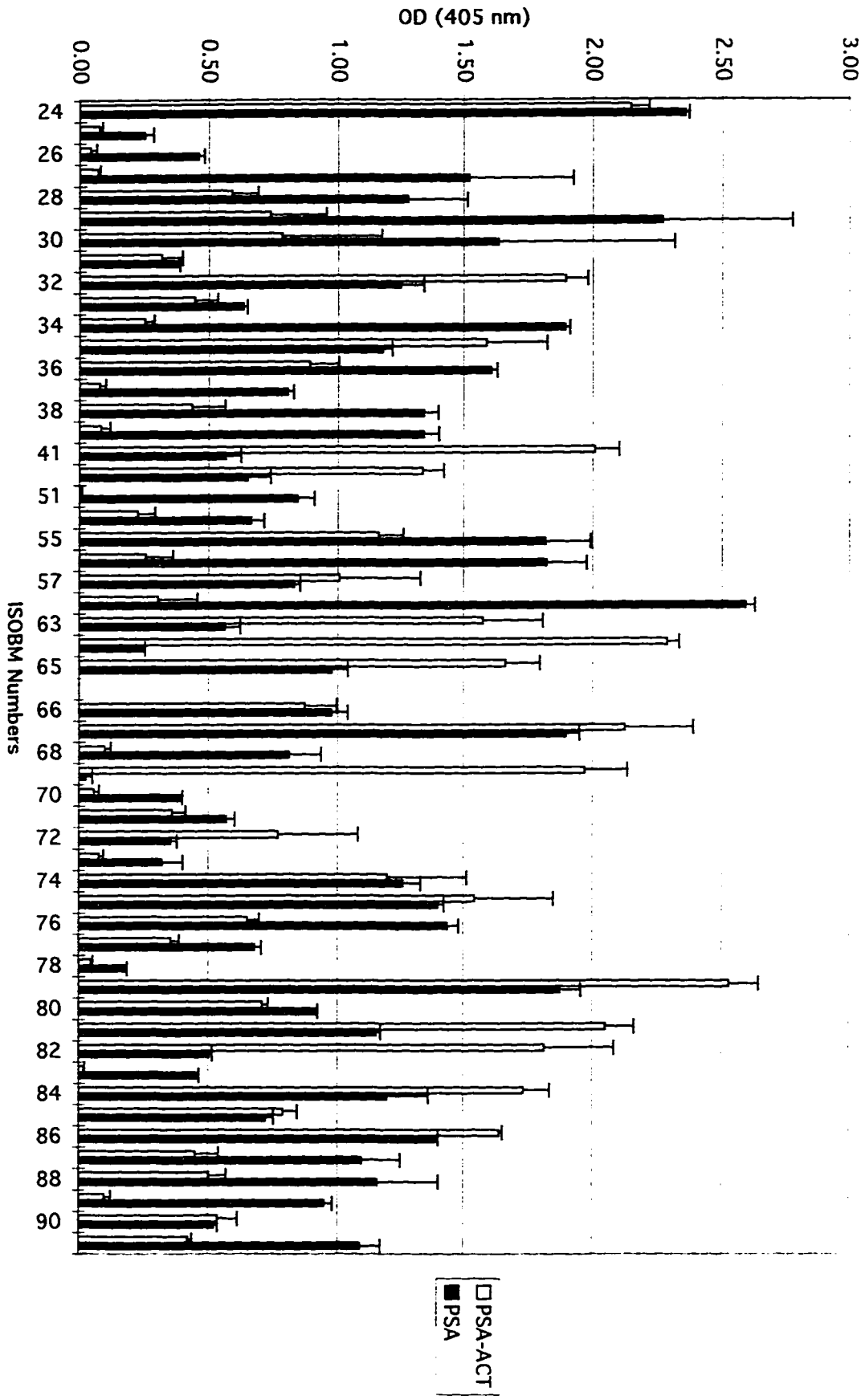

Figure 2: Binding to PSA and PSA-ACT by anti-PSA antibodies