FUNCTIONAL ANALYSIS OF RGD MOTIFS OF GOAT VITRONECTIN BY SITE-DIRECTED MUTAGENESIS

THESIS

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By

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Roll No. 1087

To

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Dated: 25/6/2011

Certificate

Certified that the research work embodied in this thesis entitled **"Functional analysis of RGD motifs of goat vitronectin by site-directed mutagenesis"** *submitted by* ***Dr. T. Lakshmi Prasanth, Roll No. 1087,*** *for the award of* ***Doctor of Philosophy Degree*** *in* ***Animal Biochemistry*** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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(Lakshmi Prasanth. T.)

Abbreviations

%	:	Percentage
α	:	Alpha
β	:	Beta
β-ME	:	Beta mercaptoethanol
@	:	at the rate of
~	:	Around/about
μg	:	Microgram
μl	:	Microlitre
μM	:	Micromolar
A_{260}	:	Absorbance at 260 nm
A_{280}	:	Absorbance at 280 nm
aa	:	amino acid
Ab	:	Antibody
AGD	:	Arginine-Glycine-Aspartate
BHI	:	Brain heart infusion broth
BLAST	:	Basic local alignment search tool
bp	:	base pair(s)
BSA	:	Bovine serum albumin
CBB	:	Coomassie brilliant blue
cDNA	:	Complementary deoxyribonucleic acid
CNBr	:	Cyanogen bromide
DAB	:	Diaminobenzidine
DEAE	:	Diethyl amino ethyl
DEPC	:	Diethyl pyrocarbonate
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxy nucleotide triphosphate
DTT	:	Dithiothreitol
E. coli	:	*Escherichia coli*
ECM	:	Extracellular matrix
EDTA	:	Ethylenediamine tetra acetic acid
FBS	:	Fetal bovine serum

Fig.	:	Figure
g	:	Gram(s)
GAGs	:	Glycosaminoglycans
GGD	:	Glycine-Glycine-Aspartate
h	:	Hour(s)
H_2O_2	:	Hydrogen peroxide
HBS	:	HEPES buffered saline
HCl	:	Hydrochloric acid
HRPO	:	Horse radish peroxidase
IgG	:	Immunoglobulin G
IPTG	:	Isopropyl ß-D-thiogalactoside
kb/kbp	:	Kilo base pair(s)
kDa	:	Kilodalton
LB	:	Luria-Bertani
M	:	Molar
MDI	:	Microdevices India
mg	:	Milligram(s)
min	:	Minute(s)
ml	:	Millilitre(s)
mM	:	Millimolar
mRNA	:	Messenger RNA
NaCl	:	Sodium chloride
$NaHCO_3$	:	Sodium bicarbonate
NaPi	:	Sodium phosphate
NCBI	:	National Center for Biotechnology Information
NCP	:	Nitrocellulose paper
ng	:	Nanogram
NiCl	:	Nickel chloride
nm	:	Nanometer
nt	:	Nucleotide
ºC	:	degree centigrade
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis
PAI-1	:	Plasminogen Activator Inhibitor-1
PBS	:	Phosphate buffered saline
PBST	:	Phosphate buffered saline with 0.5 % Tween 20

PCR	:	Polymerase chain reaction
pmoles	:	Picomoles
PMSF	:	Phenyl methyl sulfonyl fluoride
RE	:	Restriction enzyme
RGA	:	Arginine-Glycine-Alanine
RGD	:	Arginine-Glycine-Aspartate
RPMI	:	Roswell Park Memorial Institute
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
rVn	:	Recombinant vitronectin
S. aureus	:	*Staphylococcus aureus*
SDS	:	Sodium dodecyl sulphate
TAE	:	Tris acetic acid EDTA
TEMED	:	N,N,N',N' – tetramethyl ethylene diamine
Tm	:	Melting temperature
U	:	Unit(s)
USB	:	US biochemicals
UV	:	Ultraviolet
v/v	:	volume/volume
VN	:	Vitronectin
w/v	:	weight/volume
$ZnSO_4$	:	Zinc Sulphate

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Introduction

1 Introduction

Vitronectin (VN) is a major cell adhesion glycoprotein present in plasma and in the extracellular matrix (ECM). It is also known as serum spreading factor, complement S protein. The plasma VN is a folded monomer whereas the protein in ECM is aggregated into multimers (Schvartz *et al.*, 1999). VN is involved in diverse physiological activites such as coagulation, fibrinolysis, complement mediated immune response, cell adhesion and spreading. Mastitis is a major threat to the dairy industry. The common pathogens associated with mastitis include *Streptococcus agalactiae, Staphylococcus aureus, Streptococcus uberis, E.coli, Streptococcus dysagalactiae*. Among them *S. aureus* is the major pathogen. *S. aureus* colonization is an important feature of pathogen survival in the host. This bacterium binds to ECM components via specific cell surface receptors. VN along with other ECM proteins play an important role in bacterial adhesion to the host cells. VN has been characterized from many animal species and show significant structural similarities among them. However, bovine and goat VN are unique in having two Arg-Gly-Asp (RGD) motifs instead of one seen in other species (Mahawar and Joshi, 2008). This tripeptide sequence imparts adhesive characteristic to the protein (Cherny *et al*, 1993). The precise function(s) of the two RGD motifs in goat VN is not known. Understanding the structure-function relationship of proteins remain the biggest challenge

in proteomic research. Site-directed mutagenesis is an elegant technique to explore the function(s) of protein motifs. This technique can be performed by different ways, including PCR based alterations in the base sequence of the desired site(s) in the protein (Ling and Robinson, 1997). In the present study, the function(s) of both the RGD motifs in goat VN will be analyzed by site-directed mutagenesis. Considering the importance of VN in general physiology and its involvement in facilitating bacterial adhesion to host cells, the proposed study is an attempt in this direction with the following objectives.

1. **Site-Directed Mutagenesis of both the RGD motifs of goat VN**
2. **Expression of the mutants in prokaryotic expression system.**
3. **Functional studies of the mutated proteins.**

Review of Literature

2 Review of Literature

Extracellular matrix (ECM), the microenvironment on the periphery of cells is composed of a network of protein fibers,carbohydrates and a large amount of water (Alberts *et al*, 1994). Functionally,the macromolecules of ECM can be divided into i) structural and ii) adhesive types. Structural type consists of collagen and elastin whereas the adhesive type includes proteins such as fibronectin, laminin,VN and tenascin.

VN is a multifunctional glycoprotein of about 75 kDa synthesized to a large extent by the hepatocytes (Seiffert *et al*, 1991).It is present mainly in two forms.(i)A circulating,low molecular weight monomeric form and a bound,high molecular weight multimeric form. It is also present in amniotic fluid, cerebellar cortex, alpha granules of platelets and endometrium. VN facilitates adhesion of different cell types like fibroblasts, endothelium, platelets (Pytela *et al*, 1985) and binds to complement, beta endorphin, plasminogen activator inhibitor–1 (PAI-1), insulin-like growth factor-II (IGF-II). VN is also implicated in many pathological conditions like fibrosis, atherosclerotic plaques and tumors (Aaboe *et al*, 2003)

Animal Vitronectins

Kitagaki-Ogawa *et al* (1990) purified and partially characterized VN from 6 animal species including human, horse, porcine, bovine,

rabbit and chicken. In SDS-PAGE, human, horse, bovine and chicken VN showed two bands whereas a single band was observed in pig and rabbit VN. Subsequently, Nakashima *et al* (1992) purified VN from 13 animal species and showed two bands in human, dog, horse and bovine, one band in mouse and chicken, three bands in sheep and goats and one major band in pig, rabbit, rat, hamster, guinea pig and goose VN. The third band in sheep and goat VN could be a degradation product while in some species the minor band may escape detection.

Previous work done in our laboratory on goat plasma VN have shown that a significant part of the protein exists as high molecular weight species with 160 kDa and > 250 kDa forms constituting around 50% and the remaining is 81 kDa species.(Suchitra *et al*, 2003). Subsequently, the protein was expressed in *E. coli* and showed homology with VN from other species. The deduced amino acid sequence indicated two RGD triplets in the protein at positions 45 and 106, which is unique to this species. The lower size of recombinant VN (~.73 kDa) compared to the plasma VN (83 kDa) is due to the absence of carbohydrate moiety in the engineered protein**,** which was expressed in the prokaryotic expression system. Carbohydrates contribute to approx.10-12 kDa in the mature plasma VN. The additional 68 kDa band seen in the induced culture probably originated as a result of proteolysis (Mahawar & Joshi, 2008).

Domain Structure of VN

The structure of human VN has been studied extensively. The mature molecule is composed of 459 amino acids and consists of discrete domains (Fig. 2.1a).

The Somatomedin B domain

This domain represents the N-terminus of the protein and consists of 44 amino acids that are identical to the circulating protein,

Somatomedin B (SMB). Somatomedin B is a naturally occurring plasma protein (Wajchenberg *et al.*, 1980). The similarity between somatomedin B and the N-terminus of Vn (Fryklund and Sievertsson, 1978; Jenne and Stanely, 1985 and Suzuki *et al.*, 1985) and the finding that both Vn and a plasma protein identified by antibodies to somatomedin B are α globulins (Yalow *et al.*, 1975), suggested that the somatomedin B may arise from VN by limited proteolysis. This region contains 8 cysteine residues, which are strictly conserved in the VN isolated from different sources viz. rabbit (Deng *et al.*, 1996), mouse (Seiffert *et al.*, 1991) and in other somatomedin B-like proteins such as plasma membrane glycoprotein PC-1 (Buckley *et al.*, 1990) and autotaxin (Murata *et al.*, 1994). These cysteines are important for imparting compact structure to somatomedin B domain. Conversion of any cysteine residue to alanine destroyed PAI-1 binding activity (Deng *et al.*, 1996) of this domain (Seiffert and Loskutoff, 1991; Seiffert *et al.*, 1994b; Sigurdardottir and Wiman, 1994; Deng *et al.*, 1996 and Okumura *et al.*, 2002).

Eight cysteine residues are arranged in four disulfide bonds and connecting linkages are required for specific binding to PAI-1 (Seiffert and Wagner, 1997). Kamikubo *et al.* (2002) proposed arrangement of disulfide linkages in recombinant somatomedin B domain of Vn as cysteine5-9, cysteine19-21, cysteine25-31 and cysteine32-39.

Later on Horn *et al.* (2004) showed different arrangement of disulfide bond in plasma VN, cysteine5-9, cysteine19-31, cysteine21-32 and cysteine25-39. They also suggested that this arrangement of disulfide bonds in native SMB domain created a loop between cysteine25-39 similar to cystine-stabilized α-helical structures commonly observed in cystine knots. These features should involve this domain in binding to plasminogen activator inhibitor type-1 and the urokinase receptor. By small scattering measurement, Lynn *et al.*

(2005) suggested that this domain is well exposed to surrounding solvent making it accessible to various ligands, and the PAI-1 binding site in this domain is well separated from another binding site in the C-terminus allowing binding of two PAI-1 simultaneously (Fig. 2.1b).

Connecting Region

After the somatomedin B domain, there is a long connecting region starting from the RGD sequence (Suzuki *et al.,* 1984). Residues 54-130 form a linker region (Xu *et al.*, 2001). This region is important for embryonic morphogenesis (Boucaut *et al.,* 1984), platelet aggregation (Pytela *et al.,* 1986), cell attachment and spreading (Singer *et al.,* 1988) and contains acidic residues that interact with cationic cluster to stabilize VN conformation.

Hemopexin Type Domain

Hemopexin is a plasma glycoprotein, which can bind and transport heme to a cell surface receptor on liver parenchymal cells (Morgan *et al.*, 1976 and Smith and Morgan, 1984). The sequence of hemopexin consists almost entirely of two homologous domains joined by a short hinge region; each domain structure is derived from four tandem repeats (Hunt *et al.*, 1987). In VN, the first hemopexin domain is incomplete lacking the fourth copy of repeat. The second copy of the domain can be modeled as being complete although heparin-binding sequence is embedded in this. Putative hinge region connects the two regions. The 6 cysteine residues in the hemopexin-like domain are essential for maintaining structural and functional activity of the protein. It was suggested that the hemopexin-like region might be involved in the multimerization of VN (Paoli *et al.*, 1999) and residues 131-323 may provide binding sites for bacteria (Liang *et al.*, 1993 and 1997a).

Fig. 2.1a : Linear structure of human vitronectin

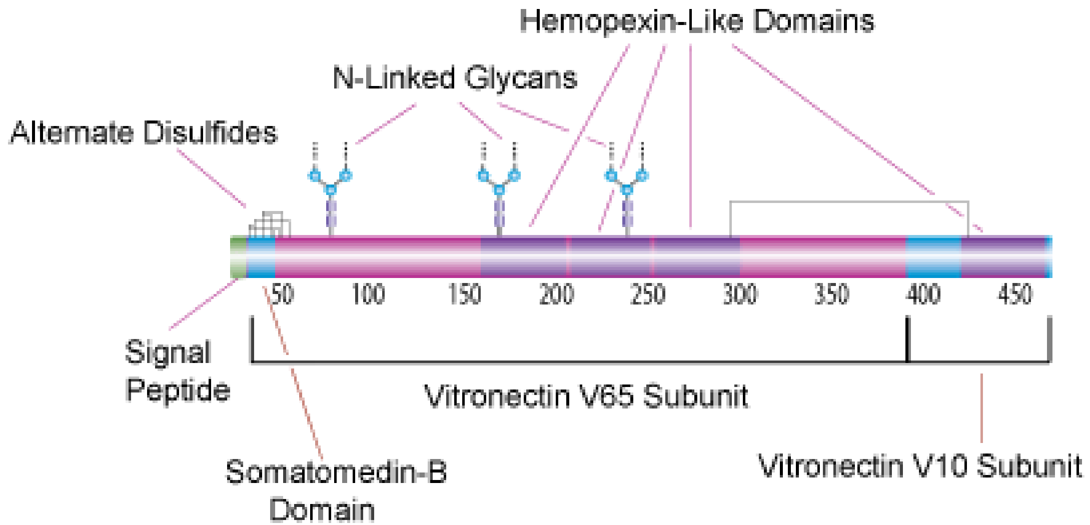
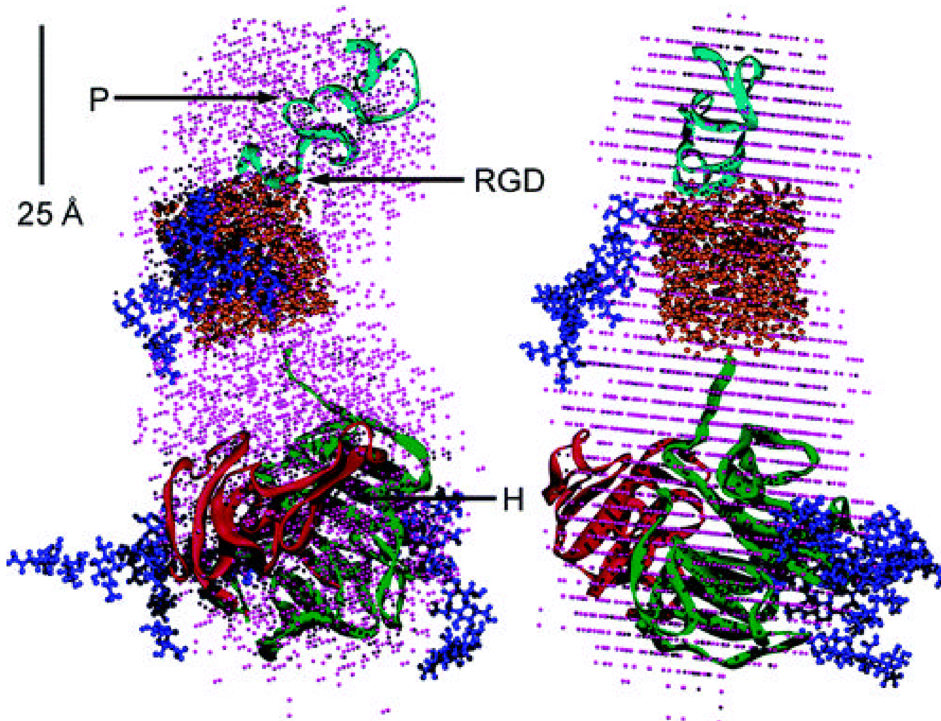


Fig. 2.1b : Two orthogonal views of the model produced by CONTRAST docked into the GA_STRUCT consensus envelope. The somatomedin B domain is colored cyan, the linker region amber, the central domain green, the heparin-binding domain red, and the carbohydrates blue. The consensus envelope is colored violet. Binding sites for ligands are indicated with arrows and labels : P for PAI-1, RGD for integrins, and H for heparin. (from : Lynn G. W. *et al.,* Biochemistry, 2005 Jan 18;44 (2): 565-74).



Heparin Binding Region

Within the second hemopexin-like domain of the VN, a highly cationic sequence representing residues 345-379 has been proposed as the primary binding site for heparin (Suzuki *et al.,* 1984; Tschopp *et al.,* 1988 and Kost *et al.,* 1992) as synthetic peptides derived from this region bound biotinylated heparin and diminished heparin-binding activity of the protein (Preissner and Muller-Berghaus, 1987 and Kost *et al.,* 1992). Also, the heparin binding activity of the VN was destroyed by proteolysis of this binding region (Sane *et al.,* 1991 and Gechtman *et al.,* 1997). This site was localized by proteolysis of the protein and using synthetic peptides (Preissner and Muller-Berghaus, 1987 and Kost *et al.,* 1992) corresponding to residues 341 to 380 including 18 residues (346- LAKKQRFRHRNRKGYSRQ-363) having alignment with consensus heparin-binding proteins like thrombin and anti-thrombin III (Cardin and Weintraub, 1989 and Sobel *et al.,* 1992). Involvement of arginine residues in the heparin binding was confirmed by labeling of 1-2 arginine residues that impaired heparin-binding activity of VN (Gibson *et al.,* 1999).

Initially, it was presumed that the heparin binding sequence was encrypted in native molecule as the untreated protein did not bind to heparin whereas the urea treated protein bound to immobilized heparin (Yatohgo *et al*., 1988 and Sobel *et al.,* 1992). Subsequently, it was shown that the heparin-binding site is fully exposed in native VN (Zhuang *et al.,* 1997) as this region is highly susceptible to proteolysis by thrombin (Gechtman *et al.,* 1997), plasmin (Sane *et al.,* 1991 and Gechtman *et al.,* 1997), and an endogenous protease that cleaved one allelic variant of VN between 379 & 380 (Tollefsen *et al.,* 1990) indicating surface orientation of the heparin-binding site in the native protein. Also, phosphorylation of serine at 378 (Chain *et al.,* 1991 and McGuire

et al., 1988) and the chemical modification of an arginine within the heparin binding sequence abolished heparin-binding activity (Gibson *et al.,* 1999) suggesting surface orientation of this region in native VN. In a separate study, the truncated form of recombinant VN, lacking the C-terminal 80 residues, showed comparable heparin-binding affinity as that of full-length VN suggesting that the C-terminal 80 residues were not involved in binding to heparin (Gibson and Peterson, 2001).

Using phage display technique, a secondary heparin-binding site was identified as a cationic cluster near the N-terminus (aspartate 82 to cysteine 137 and lysine 175 to aspartate 219) (Liang *et al.,* 1997b). Yoneda *et al.* (1998) reported that the first hemopexin repeat exhibited some heparin binding activity. The role of the secondary heparin-binding site was further investigated by producing recombinant VN containing 129 residues of the C-terminus. This fragment showed heparin-binding affinity comparable to full length VN (Gibson *et al.,* 1999). These results together with other study (Gibson and Peterson, 2001) confirm that the residues 341-380 represent the primary heparin-binding site and the role of secondary heparin-binding site(s) is still unknown.

Very recently,it was demonstrated that the heparin binding domain of VN was involved in the oligomerization process.This process(oligomerization)reinforces the the cell adhesion and spreading activities of VN as fragment of VN having RGD motif showed diminished cell adhesion and spreading function eventhough its binding affinity to $\alpha_v\beta_3$ integrin was similar to that of full VN (Chillakuri et al.,2010)

PAI – 1 and VN

Plasminogen Activator Inhibitor-1 (PAI-1) is the physiological inhibitor of tissue and urokinase type plasminogen activator (μPAR), the enzyme responsible for generating plasmin from its precursor

plasminogen and ultimately leading to clot lysis. In addition to balancing between coagulation and thrombolysis, PAI-1 plays very important role in regulating extra-cellular remodeling, angiogenesis, ovulation, embryogenesis and many pathological conditions like thrombotic and haemorrhagic disorders, connective tissue diseases, neoplasia and sepsis (Dano *et al.* 1985; Pollanen *et al.*, 1991 and Vassalli *et al.*, 1991). PAI-1 is a single chain glycoprotein and is a member of SERPIN superfamily (Carrell and Travis, 1985 and Loskutoff *et al.*, 1989). It lacks cysteine residues that may account, in part, for the spontaneous decay into an inactive "latent" form under physiological conditions.

VN binds to PAI-1 thus maintaining the inhibitor in its active conformation (Podor *et al.*, 2000) for a longer period of time with an increase in half-life to 2 to 4 fold (Declerck *et al.*, 1988). Indeed, most circulating PAI-1 is thought to be in a complex with VN, so that the complex serves as a reservoir of the physiologically active form of PAI-1 (Declerck *et al.*, 1988). VN in the extracellular matrix co-localizes with PAI-1 (Preissner, 1990; Seiffert *et al.*, 1990 and Podor *et al.*, 2002), thus increasing the half-life of the labile inhibitor to over 24 hours (Mimuro *et al.*, 1987).

VN residues 1-44 are responsible for PAI-1 binding and its stabilization and that all cysteine residues are crucial for binding (Deng *et al.*, 1996 and Mayasundari *et al.*,2004). This conclusion was drawn after extensive study involving proteolysis of VN, use of synthetic peptides and recombinant N-terminal fragment and site-directed mutagenesis (Seiffert and Loskutoff, 1991; Sigurdardottir and Wiman, 1994; Seiffert *et al.*, 1994b and Deng *et al.*, 1996). A second PAI-1 binding site has been identified in the C-terminal part of the molecule, residues 345-379 (Suzuki *et al.*, 1985; Preissner, 1990; Kost *et al.*, 1992; Gechtman *et al.*, 1993 and Gechtman *et al.*, 1997). Seiffert and Smith (1997)

demonstrated that the ligand binding to the C-terminal region regulates the binding of PAI-1 to the N-terminal somatomedin B region. Minor and Peterson (2002) showed that the binding of PAI-1 to VN induces its multimerization and subsequent change in the adhesive properties of the protein to cells, integrins and matrix proteins.

Using monoclonal antibodies to different epitopes and rigorous analytical ultra-centrifugation analysis, a 2:4 binding of VN-PAI-1, was observed indicating two PAI-1 binding sites in VN (Podor *et al.*, 2000). Later on, X-ray crystallography showed that the somatomedin B domain binds to PAI-1 in 1:1 stoichiometry and gives a tight complex (Zhou *et al.*, 2003). This binding subsequently blocks the interaction of VN with the cell surface integrins required for cellular motility (Stefansson and Lawrence, 1996) and with the receptor for cell bound urokinase-type plasminogen activator (Deng *et al.*, 1996). The complex of VN and PAI-1 leaves insufficient room for the integrin binding to RGD (Xiong *et al.*, 2002).

Lazar *et al.* (2004) observed that VN-PAI-1 complex impairs the alveolar epithelial repair. Takahashi *et al.* (2005) demonstrated that PAI-1 inhibited human fibrosarcoma cell (HT-1080) adhesion to VN involving $\alpha v \beta 3$ integrin, and stimulated cell migration from VN towards collagen type IV.

Using different combinations of null mice for both the proteins (VN and PAI-1), Koschnick *et al.* (2005) observed that thrombotic phenotype of mice with a combined deficiency of PAI-1 and VN did not differ significantly from the phenotype of mice with deficiencies in only PAI-1 or VN. Thus, they concluded that both the proteins may influence thrombus stability by regulating a common pathway.

VN inhibits rapid inactivation of thrombin and factor Xa by anti-thrombin III in the presence of glycosaminogylcans (Preissner and

Muller-Berghaus, 1987). VN forms a tri-molecular complex with thrombin-antithrombin III complex, (Vn-T-ATIII) (Podack and Muller-Eberhard, 1979) thus protecting thrombin against heparin-induced potentiation of antithrombin activity. Binding of VN-T-AT III complex to human umbilical vein endothelial cells involves heparin-binding site of VN and proteoglycans (de Boer *et al.*, 1992).

VN & Cell adhesion

VN promotes attachment and spreading of a wide variety of both fibroblastic and epithelial cells *in vitro.* It also stimulates growth and differentiation of a number of cell types in culture in hormone supplemented serum free media (Barnes and Sato, 1979). The cell-binding site of VN is located near the amino terminus, immediately after the carboxy terminal side of the somatomedin-B sequence. Binding of VN to cells occurs via tripeptide Arg-Gly-Asp (RGD) sequence. Cells adhere to VN primarily by using VN receptors of integrin type. Integrins are cell surface glycoproteins involved in cell-cell and cell-matrix interaction. Integrins are heterodimeric complex composed of α and β-subunits linked non-covalently.

The functional diversity of integrins is dictated by the particular α/β subunit composition. Till date, VN is known to bind at least 5 different integrins, which contain either of the two distinct α subunits, α_v and α_{IIb}. The α_{IIb} subunit is expressed only on platelets and in combination with β_3, it binds VN (Bodary and McLean, 1990). The other four-VN receptors are expressed on a variety of cells and contain α_v and either β_1, β_2, β_5 or β_8. Both $\alpha_{IIb}\,\beta_3$ and $\alpha_v\,\beta_3$ acts as receptor for adhesive proteins viz. fibrinogen, VN, vonWillebrand factor, fibronectin, thrombospondin involved in platelets-subendothelium and platelet-platelet interaction. The multiple ligand binding capacity of these

receptors is due to their ability to recognize RGD sequence present in different proteins (Hynes, 1987). The major VN receptor appears to be $\alpha_v \beta_3$, which is expressed on mature osteoclast and this receptor is involved in the regulation of bone resorption (Preissner, 1991). Expression of integrin $\alpha_v \beta_3$ has also been linked to malignant progression of melanoma and metastatic melanoma cells (Albelda *et al.,* 1990). Recently, binding of VN to $\alpha_v\beta_6$ integrin on epithelial cells was demonstrated but the expression of this integrin occurs during tissue remodeling, wound healing and carcinogenesis (Thomas *et al.*, 2006).

VN & Bacterial interactions

Adhesion and colonization is the first step during bacterial infection. This is a complex mechanism involving multiple interactions between host tissue and bacterial surface components with an overall aim to evade host defense system and to establish an infection. A number of host tissue molecules - proteins as well as glycosaminogylcans, interact with bacteria.

Many bacteria bind to VN directly; these include *E. coli, S. aureus, S. epidermidis, Streptococcus species, Enterococcus species, Pneumocystis carinii, Helicobacter pylori* etc. (Chhatwal *et al.*, 1987; Liang *et al.*, 1993; Limper *et al.*, 1993; Zareba *et al.*, 1997; Hussain *et al.*, 2001b and Eberhard and Ullberg, 2002). This binding might contribute to the pathogenicity of these organisms by interfering with complement mediated bacterial killing and facilitating bacterial adherence to host tissues which posses surface receptors for VN. The bacterial binding to VN is mediated probably through two specific sites present in the protein (Chhatwal *et al.*, 1987).

The first site may involve cell-binding domain at the amino terminus, which contain the RGD sequence. The binding of Group-G streptococci, *S. aureus* and *E. coli* is proposed to be mediated through this site (Chhatwal *et al.*, 1987). The second site, involving the heparin-binding region of the VN, facilitates attachment of Group A and C streptococci (Limper *et al.*, 1993). In addition to initial attachment, VN also facilitates internalization of pathogens such as *Neisseria gonorrhoeae* by Chinese hamster ovary cells (Duensing and Van-Putten, 1997).

In many cases, bacterial colonization of mucosal surface and subsequent tissue invasion largely depended on recruitment of host derived sulfated polysaccharides. These host factors serve as a molecular bridge between bacterial surface receptors and mammalian matrix constituents such as VN and fibronectin. Duensing and Van-Putten (1998) reported that the sulfated polysaccharide interacts with both heparin-binding sites of VN and OpaA adhesin of *Neisseria gonorrhoeae* and thus forms a trimolecular complex. These observations are further supported by the presence of heparin-binding consensus sequence of various proteins present in both eukaryotes and prokaryotes (Cardin *et al.*, 1991). Heparin-binding consensus sites within various proteins are relatively ambiguous requiring only clusters of six to eight alternating basic and hydrophobic amino acid residues to interact with heparin (Cardin and Weintraub, 1989; Cardin *et al.*, 1991 and Jackson *et al.*, 1991).

Heparin has also been shown to interact with bacterial, viral and parasitic pathogens, as well as with numerous mammalian proteins, including cytokines, adhesive glycoproteins, growth factors, complement components, plasma lipoproteins (Jackson *et al.*, 1991). Thus, the binding of heparin and functionally related sulfated polysaccharides

may be an efficient strategy devised by these pathogens to recruit a diverse array of mammalian heparin-binding proteins onto their surfaces, bypassing the need to synthesize individual receptors for each of these proteins.

Duensing *et al.* (1999) reported that dextran sulfate caused strong binding of *Neisseria gonorrhoeae* to heparin-binding proteins like VN and fibronectin, which did not bind to this organism in the absence of dextran sulfate. Apart from this organism, they also observed that microbes like *Helicobacter pylori, Streptococcus pyogenes, Yersinia, S. aureus* did not bind significantly to the heparin-binding proteins including VN in the absence of dextran sulfate. Binding of bacteria to sulfated polysaccharide subsequently helped tissue invasion of *Neisseria gonorrhoeae* and *S. pyogenes* to Chinese hamster ovary (CHO) cells (Duensing *et al.*, 1999). Francois *et al.* (1999) identified residues 347-361 of VN, which facilitated binding of S. aureus but not *Staphylococcus epidermidis.*

Many bacteria prefer VN over other extracellular matrix protein such as fibronectin for attachment to host tissues. *Streptococcus dysgalactiae* can bind to fibronectin and this binding involves cell attachment domain of fibronectin. Because of the occupancy of cell binding domain, fibronectin cannot facilitate binding of this bacterium to bovine epithelial cells. *S. dysgalactiae* binds to bovine S protein (VN) that supports attachment to bovine epithelial cells possibly by region other than cell attachment site since no inhibition of VN mediated adhesion of bacteria was observed in the presence of Gly-Arg-Gly-Asp-Ser peptide (Filippsen *et al.*, 1990).

The mechanism of host-bacterial interactions has been studied to some extent. Chhatwal *et al.* (1987) observed inhibition of S protein

binding to group G streptococci and *E. coli* by fibronectin and heparin but in case of group A and C streptococci and *S. aureus* weak to moderate inhibition was observed suggesting different kind of interaction and/or involvement of different site(s). *Pneumocystis carinii* uses VN for attachment to lung cells (Limper *et al.*, 1993) whereas fibronectin did not support such adhesions due to the absence of receptors on mature bronchial epithelial cells (Albelda, 1991).

S .aureus adhesins

Gram positive bacteria display various molecules on their surface that are involved in recognition and binding to host extracellular matrix components. These bacterial molecules are called adhesins or microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Duensing, and Van-Putten, 1998). *S. aureus* is reported to have many different adhesins that facilitate binding to host molecules such as fibronectin, collagen and VN.

McGavin *et al.* (1993) reported a 72 kDa bone sialoprotein (BSP) binding protein having affinity for fibrinogen, VN, thrombospondin and to some extent collagen. This protein was preferentially expressed in LB broth compared to its low expression in tryptic soy broth. The protein was extracted from the bacterial cell surface by treatment with 1 M LiCl.

Liang *et al.* (1995) reported the presence of a 60 kDa protein in LiCl extract with high affinity for VN. They also showed that this protein interacted with the synthetic peptide representing sequence (Ala^{347}-Arg^{361}) comprising the heparin-binding consensus sequence of VN.

A 60 kDa *S. aureus* secreted protein called Eap/Map (extracellular adherence protein/major histocompatibility complex class II analog protein) has broad-spectrum binding characteristics (Hussain *et al.*,

2001a and 2002). *S. aureus* mutant lacking this protein adhered to a lesser extent to cultured fibroblasts than the wild type and the adherence was restored upon complementation (Hussain *et al.*, 2002). Hussain *et al.* (2001b) reported a 38.5 kDa extracellular matrix binding protein (Emp) with broad-spectrum affinity for fibronectin, fibrinogen, collagen and VN. This protein was expressed during the stationary growth phase, was closely associated with the cell surface and could be extracted by SDS. The largest (1.1 megaDalton) *Staphylococcal* adhesin reported so far is Ebh (ECM-binding protein homologue) from the strain COL that specifically binds to human fibronectin (Clarke *et al.*, 2002).

A new class of staphylococcal adhesins, the autolysins/adhesins (Aaa) has been described which are surface associated proteins having both enzymatic (amidase and glucosaminidase) and adhesive functions (Heilmann *et al.*, 1997 and 2003). Heilmann *et al.* (2005) reported a 35.8 kDa protein with bacteriolytic activity as well as adhesive properties to various extracellular matrix molecules like fibrinogen, fibronectin and VN. This protein binds to ECM molecules in a dose dependent and saturable manner. The knockout mutant showed reduced adherence to surface adsorbed fibrinogen/fibronectin suggesting a role for autolysins/adhesins (Aaa) in the colonization of host factor-coated polymer surfaces and/or host tissue.

Some adhesins are expressed under regulated conditions. In media containing iron limitations, an iron-regulated adhesin, IsdA , was expressed whose expression was not seen in standard laboratory media. This protein had affinity for fibrinogen and fibronectin (Clarke *et al.,* 2004). Another 140 kDa protein with haptoglobin-haemoglobin binding activity was also expressed in iron limited conditions (Dryla *et al.*, 2003).

The fibronectin binding protein A of *S. aureus* can interact with $\alpha_5\beta_1$ integrin via fibronectin bridge to mediate adhesion and co-stimulation of T lymphocytes (Miyamoto *et al.* 2001). Harraghy *et al.* (2003) described a 70 kDa Eap (Map) that also modulated immune response. Nandakumar *et al.* (2005) constructed a reference map for 36 membrane and cell wall associated proteins of *S. aureus*. By comparing different cell lytic and solubilization method, they concluded that the cell lysis with lysostaphin was the most effective, followed by solubilization with 8 M urea, 2 M thiourea, 14 % aminosulfobetaine and DTT.

Site -directed Mutagenesis of RGD motifs

Human VN possess only one RGD motif which is responsible for the adhesive nature of the protein (Suzuki *et al*., 1985). Subsequently, this observation was confirmed by site-directed mutagenesis of the RGD motif of human VN. The recombinant mutants, RAD-VN and RGE-VN showed complete loss of cell adhesion compared to wild type VN. This observation suggested that the RGD sequence in VN is essential for cell adhesion (Cherny *et al.* 1993). Interestingly, another study reported that the RGD motif in human VN is cryptic (Seiffert and Smith, 1997) raising doubts about the involvement of this triplet sequence in cell adhesion.

Previous work on goat VN from our laboratory has shown the presence of two RGD motifs in this protein (Mahawar and Joshi, 2008). Similar observation was made in the bovine protein. The function(s) of two RGD motifs in goat VN is not clear.

Materials and Methods

3 Materials and Methods

MATERIALS:

Bacterial strains and cell lines:

E. coli strain DH5α was used for protein expression. *S. aureus* strain 8325-4, a kind gift from Dr. V.K. Singh, Dept. of Microbiology and Immunology AT, Still University of Health Sciences, Kirksville College of Osteopathic Medicine, MO, USA. Vero cells and BHK-21 cells were kindly provided by Drs.Satish Kumar and PK Gupta of the Biotechnology division of this Institute.

Vectors and plasmids :

Prokaryotic expression vector, pPRO ExHT system (Life Technologies, USA) and plasmid pVTNFL (recombinant full length vitronectin) from laboratory repository were utilized.

Chemicals

Phenyl methyl sulfonyl fluride (PMSF), Heparin-sepharose, 3,3'-diaminobenzidine (DAB), Tween-20, Nickel chloride, Magnesium chloride, Calcium chloride, Potassium acetate, Potassium chloride, Magnesium sulfate, HEPES buffer, TEMED, APS were procured from Sigma-Aldrich (USA). EDTA, sodium azide, sodium dihydrogen phosphate, potassium dihydrogen phosphate, disodium hydrogen

phosphate, Urea (carbamide), Chloroform, 2-propanol were purchased from Merck India Ltd., Mumbai, India LB agar, BHI broth, Ethidium bromide were from Sisco Research Laboratories, Mumbai, India. LB broth (Miller) was procured from Amaresco (USA). SDS, Glycine, Tris-Base, Ammonium persulfate, protein molecular weight markers, TEMED were from Bio-Rad Laboratories, USA. Ampicillin, IPTG, X-gal, Agarose (Low EEO) were from Biomatik corporation, Biochem Life Sciences, India. Restriction Enzymes were from Bangalore Genei (India), Fermentas Life Sciences (USA) and New England Biolabs (USA).

Taq DNA polymerase and PR™ (proof reading) polymerase were from Bangalore Genei (India) RNAaseA and T4-DNA ligase, were purchased from Fermentas Life Sciences (USA). Goat anti-rabbit IgG-peroxidase conjugate, rabbit IgG HRPO, DMSO, phenol (pH 8.0) were from Bangalore Genei, India. dNTPs were from Biogene, USA. DNA Ladders were procured from Bangalore Genei India, Fermentas Life Sciences(USA) and New England Biolabs(USA). Nitrocellulose membranes and 0.2 μm syringe filters were from MDI (Ambala, India). Oligos utilized in this study were synthesized at Bioserve Technologies Pvt. Ltd., Hyderabad, India. PrepEase™ gel extraction kit, PrepEase™ plasmid purification kit were from USB (USA).

All other chemicals used in this study were of the highest grade available.

Media, buffers and reagents :

The details of the media, buffers and other reagents used in the study are given in appendix.

Plastic glassware and consumables:

Plasticware were purchased from Tarsons (India), Greiner-Bioone (Germany), Axygen(USA), Nunc (Denmark). Glasswares used were from Borosil (India) and Schott-Duran (Germany).

Equipments :

Thermal cycler-Mastercycler series (Eppendorf,Germany), Gel documentation system (Alpha Innotech, USA), Orbital Shaker Incubators – New Brunswick, USA and SEC, India), Centrifuges – Remi Cooling centrifuge (Remi,Mumbai, India), Sorvall RC-5 plus high speed centrifuge (Dupont, USA), Microplate reader (Model 680, Bio-Rad, USA), Electronic balance (Afcoset Licence, India), Inverted microscopes (Olympus, Japan) and Nikon Eclipse T21(Nikon,Japan), -20°C deep freezer (Vest frost, Blue Star, India). -40°C Biomedical freezer (Sanyo Ltd, Japan), Waterbath (Atto, Japan), uv-vis spectrophotometer (Varian Inc. USA) Ultra Klenz laminar flow (Klenzaids, India).

Horizontal, vertical gel electrophoresis apparatus (Bangalore Genei, India), Ice flaker machine (Harrison Scientific Instruments, Delhi, India), Variable volume pipettes-Labware(USA), Eppendorf (Germany) and Gilson (France) were used in this study.

METHODS

Generation of Mutant Virtonectins :

Large scale purification of pVTNFL:

The full length recombinant goat vitronectin (hereinafter referred to as wild vitronectin (or) wild VN was available in the laboratory(Mahawar and Joshi,2008) and labeled as pVTNFL and kept in repository as glycerol stocks. One of the aliquots was streaked onto an LB Ampicillin agar plate and kept overnight for incubation at 37°C. Five isolated colonies were selected and grown overnight in LB tubes (5ml) with ampicillin (100 μg/ml) final concentration.

Plasmid minipreps were done for all the five cultures using standard protocol and plasmids were resuspended in 20 μl of nuclease

free water with 0-5 µl of RNAse A solution (10 mg/ml). All the five plasmids were screened for the presence of full length vitronectin using restriction enzyme assay. The assay (30 µl) was performed as follows :

10 x Assay Buffer	3.0 µl
100 x BSA	0.3 µl
EcoRI (20 U/ µl)	0.5 µl
Hind III (10 U/µl)	0.5 µl
Plasmid	5.0 µl
Nuclease free H_2O	to make 30 µl

The tubes were incubated in water bath at 37°C for 3-4 hours and then checked for insert release in 1% agarose gel. The plasmids which had insert with a size of approximately 1330 base pairs were labeled as positive clones and selected for experiments. They were labeled as wild VN (or pVTNFL) and stored in aliquots in -20°C

Large scale preparation of plasmid,pVTNFL.

One of the positive cultures were inoculated into fresh 50 ml LB ampicillin broth and grown overnight at 37°C in a shaker incubator. The culture was used for plasmid isolation and the plasmid was stored at -20°C after confirming the presence of the insert. About 50 µl of the plasmid (approx. 1 µg) was sent for sequencing to Bioserve Technologies Ltd., Hyderabad. Once the full length sequence of the insert was available and analyzed for the presence of RGD sequences and their positions (Fig. 2.2), the plasmid was diluted 1:20 and used as template for generating mutant vitronectins.

Generation of recombinant, mutant vitronectins :

Mutant vitronectins were generated with the following features. There are two RGD motifs in the goat VN at positions 45-47 and 106-108(Mahawar and Joshi,2008). Herein after we refer to these two RGD motifs as RGD1(45-47) and RGD2 (106-108) (Fig. 2.2). Three mutants

Fig. 2.2 : Nucleotide and deduced amino acid sequence of Wild-VN

Nucleotide sequence of Wild-VN

```
gac caa gag tca tgc aag ggc cgc tgc acc gag ggc ttc aac gcc acc agg aag tgt cag 60
tgt gac gag ctc tgt tct tac tac cag agc tgc tgt gcc gac ttc atg gcc gag tgc aag 120
ccc caa gtg act cgt ggg gat gtg ttc cat ctg cca gaa gat gag tac ggc acc tat gac 180
tac ggc gag gtg cag acg gtc aac cgc agc ctg gaa gca cag ccc gag agc ccc acc ctg 240
gcc cct gtt ctg cag gcc gag att cct gtc cag gca ccg gtt ctc aac cct gag aaa gag 300
gcc cca tca cct ggg cgg gga gat tca gac cct ggg ctg ggg acc agt gac cta ggg acc 360
tct gag tcc cca gca gag gag gaa atg tgc agt ggg aaa ccc ttt gat gcc ttc acc gac 420
ctc aag aat ggt tcc ctc ttt gcc ttc cga ggg ctg tac tgc tat gag ctg gat gaa aag 480
gca gtg agg cct gga tac ccc aaa ctc atc cga gat gtc tgg ggc att gag ggg ccc att 540
gat gcc gcc ttc acc cgg ttc aac tgt cag gga aag acg tac ctc ttc aag ggt agt cag 600
tac tgg cgc ttt gag gat ggt gtc ctg gaa cct gac ttc ccc cga aac att tcg gat ggc 660
ttc aag ggt att ccg gat gac gtg gac gca gcc ttg gcc ctc ccc gct cac agc tac aac 720
ggc agg gag cga gtc tac ttc ttc aag ggc aac cac tac tgg gaa tat gtg ttc cag cag 780
cag ccc agt cga gag gag tgt gaa ggc agc tcc cag ccg gcc gca ttt aaa cac ttt gcc 840
ctg atg cag cgg gac agc tgg gag gac atc ttc cga ctt ctc ttc tgg ggc ggt tcc ttt 900
ggt ggt gct ggc cag ccc cag ctc atc agc cgc gac tgg ttt ggt ctg ccg gga aaa ctg 960
gat gcg gcc atg gcc ggc cac atc tac atc tca ggc tcc gct ccc agc tcc ccc cgg gcc 1020
aag atg act aag tct gcg cgg cgc cat cgc aaa cgt tac cgc tcg ctc cga agc cgt ggc 1080
cga ggc cgc ggc cgc gcc cgc agc cag aac ccc tac cgg cga ttt cgg tcc acc tgg ctg 1140
tcc tgg ttc tcc agc gag gag ctg ggc ctg gga gcc gac aac tat gat aac tac gag atg 1200
gac tgg ctt gtg cct gca acc tgt gag ccc atc cag agt gtc tac ttc ttc tca gaa gac 1260
aag tac tac cga gtg aac ctt cgc acg cgg cgg gtg gac act gtg atc cct ccc tac cca 1320
cgc tcc atc gct  1332
```

Deduced amino acid sequence of Wild-VN

```
DQESCKGRCT EGFNATRKCQ CDELCSYYQS CCADFMAECK PQVTRGDVFH LPEDEYGTYD 60
YGEVQTVNRS VEAQPESPTL APVLQAEIPV QAPVLNPEKE APSPGRGDSD PGLGTSDLGT 120
SESPAEEETC SGKPFDAFTD LKNGSLFAFR GLYCYELDEK AVRPGYPKLI RDVWGIEGPI 180
DAAFTRFNCQ GKTYLFKGSQ YWRFEDGVLE PDFPRNISDG FKGIPDDVDA ALALPAHSYN 240
GRERVYFFKG NHYWEYVFQQ QPSREECEGS SQPAAFKHFA LMQRDSWEDI FRLLFWGGSF 300
GGAGQPQLIS RDWFGLPGKL DAAMAGHIYI SGSAPSSPRA KMTKSARRHR KRYRSLRSRG 360
RGRGRARSQN PYRRFRSTWL SWFSSEELGL GADNYDNYEM DWLVPATCEP IQSVYFFSED 420
KYYRVNLRTR RVDSVIPPYP RSIA 444
```

were created in the present study, two for RGD2 and one mutant for RGD1. They were named as GGD1-VN(G in place of R), RGA2-VN(A in place of D) and AGD2-VN(A in place of R).

In RGD1 mutant, the RGD2 was kept intact and vice versa. In wild VN both the RGDs were conserved.

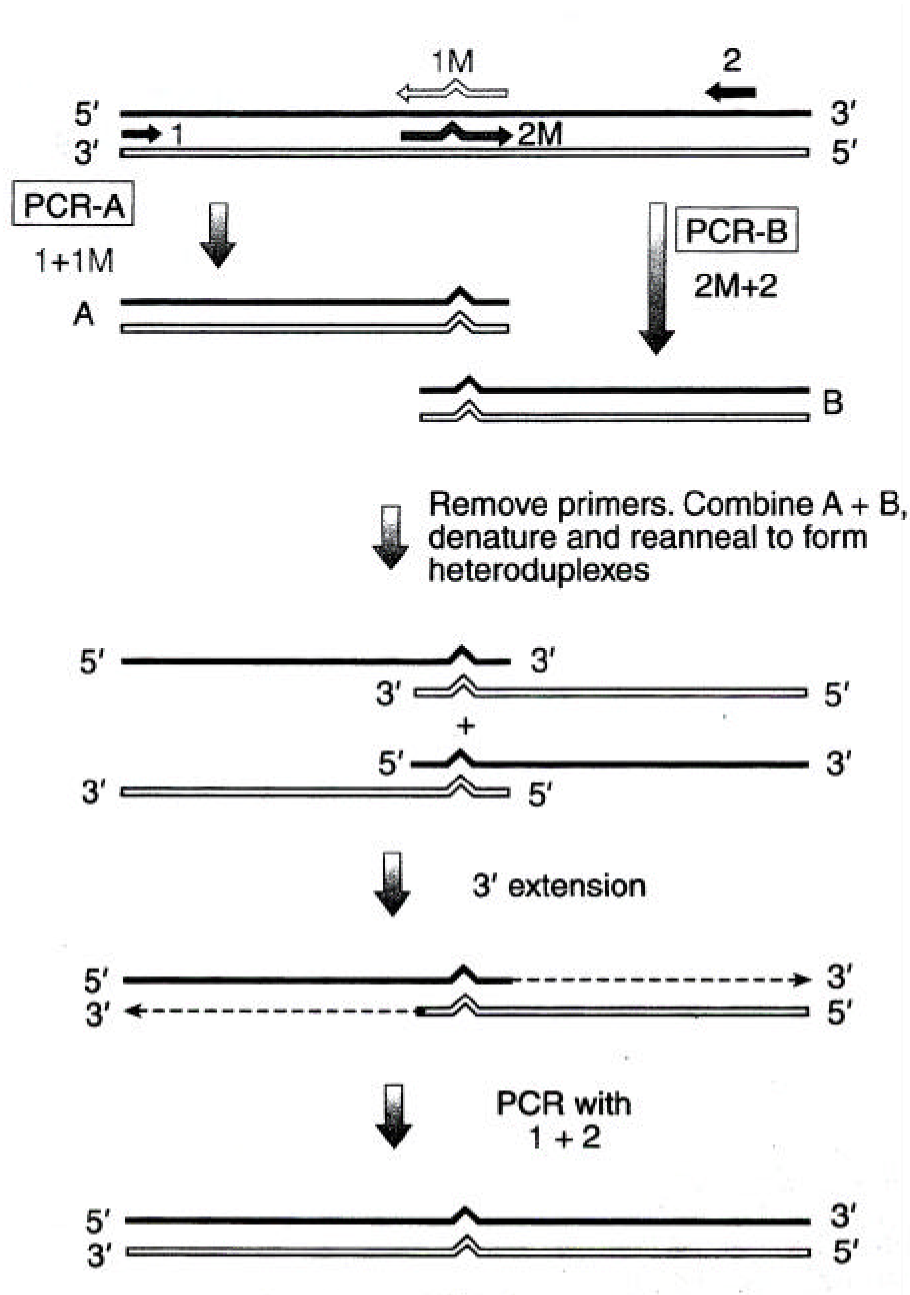
Initially megaprimer based method and various modifications of the method were tried for PCR based site-directed mutagenesis with little (or) no success. Subsequently,overlap extension PCR based site-directed mutagenesis technique was tried which worked quite efficiently. The scheme is shown in Fig. 3.1.

For each mutant to be generated there will essentially be a pair of overlapping, complementary primers with the mutated nucleotide in the middle of the primers. In addition to this, there will be a pair of flanking primers, the normal forward and reverse primers for the wild VN (or) pVTNFL (Table 1).

By convention, the mutagenic primers will be labeled as M_F (mutagenic forward) and M_R (mutagenic reverse). The normal flanking primers will be labeled as N_F (normal forward) and N_R (normal reverse). So, four primers M_F, M_R, N_F, N_R are required for the generation of each mutant.

There will be three PCR reactions/assays per mutant. In the first PCR reaction, primers M_F and N_R and wild template will be utilized to generate an amplicon (labeled as amplicon 1). During the second PCR reaction, primers M_R and N_F and wild template will be used to generate amplicon 2. Both the amplicons will be gel purified. In the third PCR reaction, the extracted amplicons 1 and 2 will be used as template without addition of any primers and after few cycles of extension, the normal primers (N_F and N_R) will be added to the reaction tubes and the

Fig. 3.1 : Schematic diagram of overlap extension PCR strategy for site - directed mutagenesis



1 and 2 refers to normal flanking primers and 1M and 2M refers to mutagenic primers

PCR reaction will be continued. The full length amplicon (amplicon 3) generated will be analyzed for the presence of the desired mutations after cloning and sequencing.

Primer Designing for Mutant Vitronectins :

The mutagenic primers utilized for generation of mutant vitronectins are given in table 1.

Generation of recombinant, mutant full length amplicon GGD1-VN

As described earlier, three PCR assays were performed. The assay conditions were as follows :

PCR 1 : The first PCR assay was performed as follows :

10x Assay buffer	2.5 µl	
GGD1-F, (M_F)	1.0 µl	(10 pm)
Wild VN-R, (N_R)	1.0 µl	(10 pm)
dNTP mix	1.0 µl	(2.5 mM each)
Template (wild VN)	1.5 µl	
Enzyme (PR Polymerse)	0.5 µl	(1.5 U)
Nuclease free H_2O	to make 25 µl	

The programme used for amplification is as follows :

Step 1	:	94°C for 5 minutes
Step 2	:	94°C for 1 minutes
Step 3	:	62°C for 30 secs.
Step 4	:	72°C for 1 min 30 sec
Step 5	:	Go to 2 repeat 29 cycles
Step 6	:	72°C for 10 minutes

The resulting amplicon was analyzed on a 1% agarose gel with low range DNA ladder.

Table 1 : Primers for amplification of the mutant vitronectins

Mutant name	Mutagenic primer	Sequence (5' - 3')
GGD1 – VN		
	(a) GGDI – F (M_F)	C AAG CCC CAA GTG ACT gGc GGG GAT GTG TTC CAT C
	(b) GGDI – R (M_R)	G ATG GAA CAC ATC CCC gCc AGT CAC TTG GGG CTT G
RGA2 – VN		
	(a) RGA2 – F (M_F)	CA CCT GGG CGG GGA GcC TCA GAC CCT GGG CTG G
	(b) RGA2 – R (M_R)	C CAG CCC AGG GTC TGA GgC TCC CCG CCC AGG TG
AGD2 – VN		
	(a) AGD2 – F (M_F)	GCC CCA TCA CCT GGG gCG GGA GAC TCA GAC CCT GGG CTG
	(b) AGD2 – R (M_R)	CAG CCC AGG GTC TGA GTC TCC Cgc CCC AGG TGA TGG GGC

Note : The letters in lower case represent the substituted nucleotides. (F), (R) represent forward and reverse and M_F, M_R represent mutagenic foward, mutagenic reverse.

Primer for amplification of wild vitronectin

	Primer	Sequence
	(a) Wild VN – F (N_F)	CCC GAA TTC GAC CAA GAG TCA TGC AAG GG
	(b) Wild VN – R (N_R)	CCC AAG CTT CTA AGC GAT GGA GCG TGG GTA GG

Note : *Eco*RI and *Hind*III sites have been appended to the forward (N_F) and reverse primers (N_R) respectively and have been underlined.

The size of the amplicon was approx 1.2 kb. The amplicon was gel extracted, labeled as GGDI-VN amplicon 1 and stored in a microfuge tube at -20°C.

PCR 2 : The second PCR assay was performed with the following conditions:

10x Assay buffer	2.5 µl	
GGD1-R, (M_R)	1.0 µl	(10 pm)
Wild VN-F, ($N_{F)}$	1.0 µl	(10 pm)
dNTP mix	1.0 µl	
Template (wild VN)	1.5 µl	(~10 mg)
PR Polymerase	0.5 µl	(1.5 U)

The programme used for amplification is as follows :

Step 1	:	94°C for 5 minutes
Step 2	:	94°C for 1 minutes
Step 3	:	62°C for 30 secs.
Step 4	:	72°C for 1 min 30 sec
Step 5	:	Go to 2 repeat 29 cycles
Step 6	:	72°C for 10 minutes

The resulting amplicon was analyzed on a 1.5% agarose gel with 100 bp DNA ladder.

The amplicon size was approximately 160 bp.

The amplicon was gel purified, labeled as GGD1-VN amplicon 2 and stored at -20°C.

PCR 3 : The third PCR assay (Overlap extension PCR) was performed as follows:

10x Assay buffer	2.5 µl	
GGD1-VN amplicon 1	1.5 µl	(5 ng approx.)

GGD1-VN amplicon 2	1.5 µl	(5 ng approx.)
dNTP mix	1.0 µl	(2.5 mM each)
PR Polymerase	0.5 µl	(1.5 U)
Nuclease free water	To make 25 µl	

The programme used for amplification is as follows :

Step 1	:	94°C for 5 min
Step 2	:	94°C for 1 min
Step 3	:	55°C for 2 min
Step 4	:	72°C for 2 min
Step 5	:	Go to 2 repeat 9 cycles
Step 6	:	72°C for 10 min
Step 7	:	Pause Press Enter

After step 6, the flanking primers (N_F and N_R) were added 1 µl(10pm) each to the tubes and then the following programme was continued.

Step 8	:	94°C for 5 min
Step 9	:	94°C for 1 min
Step 10	:	61°C for 45 secs.
Step 11	:	72°C for 1 min 30 secs
Step 12	:	Go to 9 repeat 20 cycles
Step 13	:	72°C for 10 min

The resulting amplicon was analyzed on a 0.8% agarose gel with low range DNA ladder. The amplicon size was approx. 1.3 kb.

The amplicon was gel extracted, labeled as GGD1-VN full length and stored at -20°C.

Generation of recombinant, mutant RGA2-VN:

Similar to the previous mutant three PCR assays were performed with the following conditions.

PCR 1 : The first PCR assay was performed as follows :

10x Assay buffer	2.5 µl	
RGA2-F, (M_F)	1.0 µl	(10 pm)
Wild VN-R, (N_R)	1.0 µl	(10 pm)
dNTP mix	1.0 µl	
Template (wild VN)	1.5 µl	
PR Polymerase	0.5 µl	(1.5 U)
Nuclease free water	to make 25 µl	

The programme used for amplification was as follows :

Step 1	:	94°C for 5 min
Step 2	:	94°C for 1 min
Step 3	:	64°C for 30 secs.
Step 4	:	72°C for 1 min 30 secs.
Step 5	:	Go to step 2 repeat 30 cycles
Step 6	:	72°C - 10 minutes

The resulting amplicon was analyzed on an 1% agarose gel with low range DNA ladder.

The expected size of the amplicon was approximately 1 kb.

The amplicon was gel purified, labeled as RGA2-VN amplicon 1 and stored at -20°C.

PCR 2 : The second PCR assay was performed as follows :

10x Assay buffer	2.5 µl	
Wild VN-F,(N_F)	1.0 µl	(10 pm)
RGA2-R,(M_R)	1.0 µl	(10 pm)

dNTP mix	1.0 µl	
Template	1.5 µl	
PR Polymerase	0.5 µl	(1.5 U)
Nuclease free water	to make 25 µl	

The programme used for amplification was as follows :

Step 1	:	94°C for 5 min
Step 2	:	94°C for 1 min
Step 3	:	64°C for 30 secs.
Step 4	:	72°C for 45 secs.
Step 5	:	Go to step 2 repeat 29 cycles
Step 6	:	72°C for 10 minutes

The resulting amplicon was analyzed on a 1.5% agarose gel with 100 bp DNA ladder.

The expected size of the amplicon was approximately 340 bp.

The amplicon was gel purified, labeled as RGA2-VN amplicon 2 and stored at -20°C.

PCR 3 : The third PCR assay (Overlap extension PCR) was performed as follows.For the first 10 cycles of the PCR, the reaction mixture did not contain any primers :

10x Assay buffer	2.5 µl	
RGA2-VN amplicon 1	1.5 µl	(~5 ng)
RGA2-VN amplicon 2	1.5 µl	(~5 ng)
dNTPs	1.0 µl	
Enzyme (PR Polymerase)	0.5 µl	(1.50)
Nuclease free water	To make 25 µl	

The programme used for amplification is as follows :

Step 1	:	94°C for 5 min
Step 2	:	94°C for 1 min
Step 3	:	55°C for 2 min
Step 4	:	72°C for 2 min
Step 5	:	Go to 2 repeat 9 cycles
Step 6	:	72°C for 10 min
Step 7	:	Pause Press Enter

After step 6, the flanking primers N_F and N_R were added 1 μl (10 pm) to each tube and the PCR was continued as described below.

Step 8	:	94°C for 5 min
Step 9	:	94°C for 1 min
Step 10	:	61°C for 45 secs.
Step 11	:	72°C for 1 min 30 secs
Step 12	:	Go to 9 repeat 20 cycles
Step 13	:	72°C for 10 min

The amplicon generated was analyzed on a 0.8% agarose gel with low range DNA ladder.

The size of the amplicon was approximately 1.3 kb.

The amplicon was gel purified, labeled as RGA2-VN full length and stored at -20°C.

Generation of recombinant, mutant AGD2-VN:

Similar to the previous mutant three PCR assays were performed with the following conditions.

PCR 1 : The first PCR assay was performed as follows :

10x Assay buffer	2.5 µl	
AGD2-F, (M_F)	1.0 µl	(10 pm)
Wild VN-R, (N_R)	1.0 µl	(10 pm)
dNTP mix	1.0 µl	
Template (wild VN)	1.5 µl	
PR Polymerase	0.5 µl	(1.5 U)
Nuclease free water	to make 25 µl	

The programme used for amplification was as follows :

Step 1	:	94°C for 5 min
Step 2	:	94°C for 1 min
Step 3	:	64°C for 30 secs.
Step 4	:	72°C for 1 min 30 secs.
Step 5	:	Go to step 2 repeat 30 cycles
Step 6	:	72°C - 10 minutes

The resulting amplicon was analyzed on an 1% agarose gel with low range DNA ladder.

The expected size of the amplicon was approximately 1 kb.

The amplicon was gel purified, labeled as AGD2-VN amplicon 1 and stored at -20°C.

PCR 2 : The second PCR assay was performed as follows :

10x Assay buffer	2.5 µl	
Wild VN-F,(N_F)	1.0 µl	(10 pm)
AGD2-R,(M_R)	1.0 µl	(10 pm)
dNTP mix	1.0 µl	
Template	1.5 µl	

PR Polymerase	0.5 µl	(1.5 U)
Nuclease free water	to make 25 µl	

The programme used for amplification was as follows :

Step 1	:	94°C for 5 min
Step 2	:	94°C for 1 min
Step 3	:	64°C for 30 secs.
Step 4	:	72°C for 45 secs.
Step 5	:	Go to step 2 repeat 29 cycles
Step 6	:	72°C for 10 minutes

The resulting amplicon was analyzed on a 1.5% agarose gel with 100 bp DNA ladder.

The expected size of the amplicon was approximately 340 bp.

The amplicon was gel purified, labeled as AGD2-VN amplicon 2 and stored at -20°C.

PCR 3 : The third PCR assay (Overlap extension PCR) was performed as follows.For the first 10 cycles of the PCR, the reaction mixture did not contain any primers :

10x Assay buffer	2.5 µl	
AGD2-VN amplicon 1	1.5 µl	(~5 ng)
AGD2-VN amplicon 2	1.5 µl	(~5 ng)
dNTPs	1.0 µl	
Enzyme (PR Polymerase)	0.5 µl	(1.50)
Nuclease free water	To make 25 µl	

The programme used for amplification is as follows :

Step 1	:	94°C for 5 min
Step 2	:	94°C for 1 min

Step 3	:	55°C for 2 min
Step 4	:	72°C for 2 min
Step 5	:	Go to 2 repeat 9 cycles
Step 6	:	72°C for 10 min
Step 7	:	Pause Press Enter

After step 6, the flanking primers N_F and N_R were added 1 µl (10 pm) to each tube and the PCR was continued as described below.

Step 8	:	94°C for 5 min
Step 9	:	94°C for 1 min
Step 10	:	61°C for 45 secs.
Step 11	:	72°C for 1 min 30 secs
Step 12	:	Go to 9 repeat 20 cycles
Step 13	:	72°C for 10 min

The amplicon generated was analyzed on a 0.8% agarose gel with low range DNA ladder.

The size of the amplicon was approximately 1.3 kb.

The amplicon was gel purified, labeled as AGD2-VN full length and stored at -20°C.

Directional cloning of GGD1-VN, RGA2-VN, AGD2-VN full length amplicons in a prokaryotic expression vector:

Briefly about 1 µg of the full length amplicons GGD1-VN, RGA2-VN and AGD2-VN and approximately 2 µg of the vector, pPROExHTa were digested separately at 37°C for 6-8 hours in a circulating water bath with *Eco*RI and *Hind*III (20 units of each) in a total reaction volume of 100 µl. The reaction was stopped by incubating the mixture at 65°C for 20 minutes. Both the vector and the amplicons were gel purified using PrepEase™ gel purification kit (Fig.). The eluted products were

quantitated in 1% analytical agarose gel. Then on the same day approx 30 ng of insert was ligated to 50 ng of vector using 2:1 molar ratio of insert and vector. The ligation reaction was carried out as follows :

10x Assay buffer	2 µl	
Insert	3 µl	(30 ng)
Vector	2 µl	(50 ng)
T4 DNA ligase	1 µl	(2000)
Enzyme (PR Polymerase)	0.5 µl	(1.50)
Nuclease free water	To make 20 µl	

The ligation mixture was kept at 16°C overnight and then stored at +8°C in refrigerator for another 8 hours.

Approximately 5 µl of the ligation mixture was used to transform competent DH5α cells and plated onto LB agar plates with ampicillin (100 µg/ml). The plates were incubated at 37°C overnight. The next day, the plates were checked for colonies and the isolated colonies (around 15 nos.) were picked and grown in 5 ml of LB broth in the presence of ampicillin (100 µg/ml) (Fig. 3.2).

The cultures were grown overnight at 37°C in a shaker incubator and the plasmids were isolated and screened for insert release using *Eco*RI and *Hind*III enzymes. Cultures which released ~1.33 kb fragment were labeled as positive clones and plasmids were saved at -20°C. Around 6 positive clones were selected for small scale induction for each mutant.

Small scale induction of recombinant, GGDI-VN, RGA2-VN & AGD2-VN:

Positive clones were subcultured in LB broth in the presence of ampicllin for 15 hours at 37°C. This was again subcultured in fresh LB broth (10 ml) and grown to 0.5-0.6 absorbance at 600 nm. About 1 ml

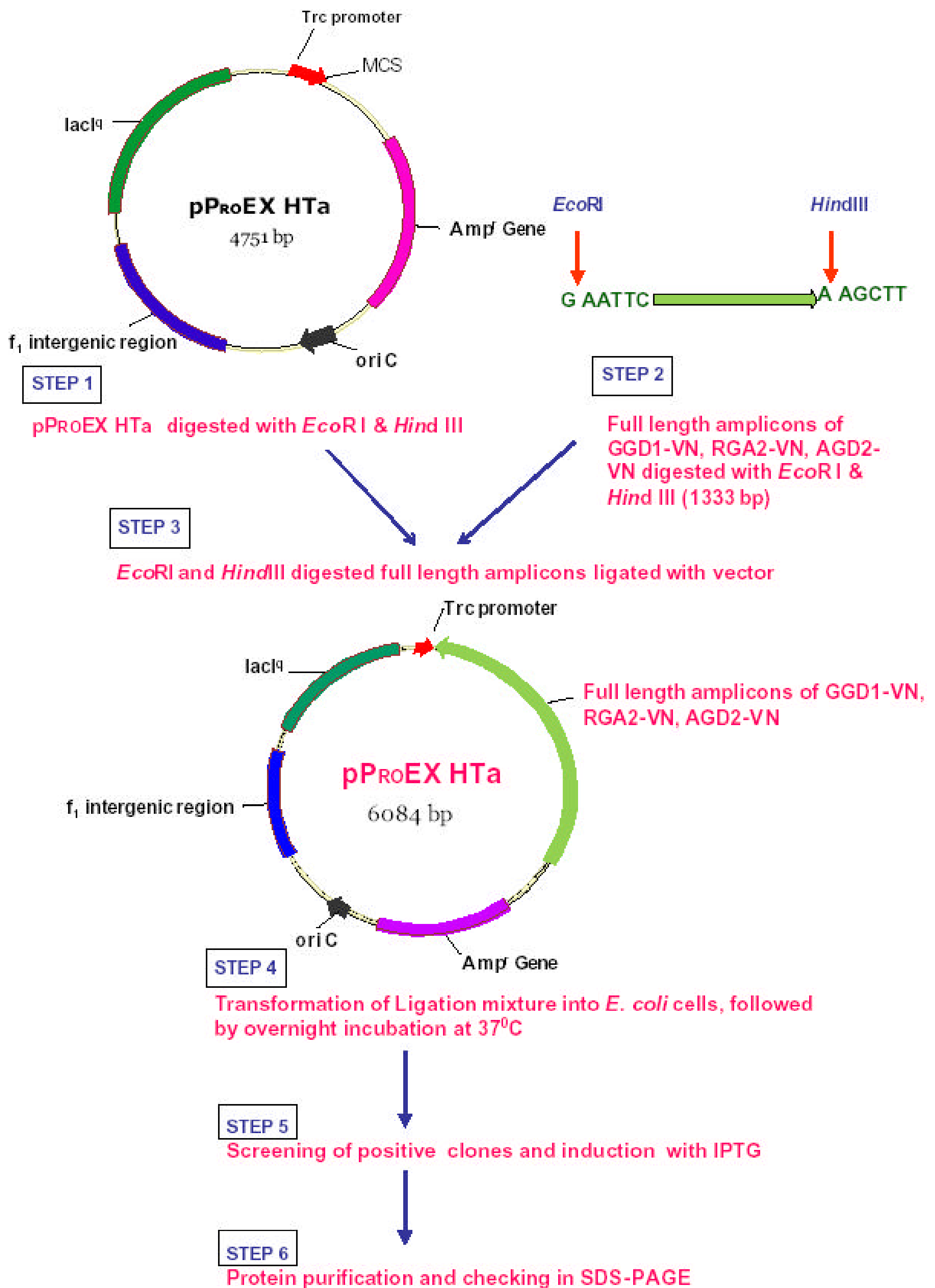


Fig. 3.2 : Cloning and Expression strategy of Recombinant, mutant vitronectins in pPROEx HTa expression system

of this culture was removed and the remaining culture was induced by adding IPTG to 1mM final concentration. After about 6 hours of induction (post-induction) the bacteria were pelleted by centrifugation and stored at -20°C.

DH5α cells harbouring pPROExHTa vector alone induced in the same way served as negative control. The bacterial pellet obtained from 1 ml of the culture was resuspended in 20 μl of water and 20 μl of 5X gel loading SDS sample buffer, boiled at 95°C for five minutes and subjected to SDS-PAGE.

The clones which showed sufficiently high expression, were selected and sent for sequencing to Bioserve Technologies Ltd., Hyderabad by primer walking. The sequence was analyzed for the desired mutation and other features with DNA Star(Lasergene,USA) and NCBI online bioinformatics tools.

Once the sequencing results were obtained, the clone with the desired mutation and good expression level was selected for performing large scale protein expression and purification.

Large scale induction, protein expression and purification of recombinant, mutant proteins GGD1-VN, RGA2-VN, AGD2-VN:

Plasmids of the corresponding positive clones of GGDI-VN, RGA2-VN, AGD2-VN which were analyzed by sequencing(Fig.) and expression profile in SDS-PAGE were freshly transformed into competent DH5α cells. The transformants were picked up from LB agar plates with ampicllin and grown in 5 ml LB broth with ampicillin for overnight. The culture was reinoculated into 4 tubes of 5 ml LB with ampicillin and grown at 37°C in a shaker incubator for 10-12 hours. Then it was inoculated into 2L of LB broth with ampicillin. It was kept at 37°C for 3-

5 hours until OD_{600} reached 0.6 (approximately). Then IPTG was added to a final concentration of 1 mM. After 4-6 hours, the culture was centrifuged at 6000 rpm for 20 min at 4°C. The pellet was suspended in PBS and transferred into 50 ml tubes, centrifuged at 10000 rpm for 15 min at 4°C.The supernatant was discarded and the pellet was treated with about 3-4 volumes of lysis buffer (approx. 10 ml) with the following composition; {20 mM NaPi(pH 7.4)/100 mM NaCl/10 mM EDTA(pH 7.5) and 1mM PMSF} and kept in ice for 2-3 hours. Intermittent homogenization was done with glass stirrer.The suspension was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was saved as soluble extract .The pellet was extracted with 8 M urea buffer {8M urea in 20 mM NaPi(pH7.4)/100 M NaCl/10 mM EDTA(pH 7.5)/10 mM DTT}at 4°C for 2 hours. Then centrifuged at 10,000 rpm for 20 min at 4°C(SS-34,RC 5C Sorvall centrifuge). The supernatant was saved as urea extract.This extract was used for subsequent purification.

The purification protocol in brief is as follows:

A 3 ml heparin-sepharose column (prepacked) was washed with approx. 10 volumes (30 ml) of 2M NaCl,then equilibriated with 8 M Urea extraction buffer containing 10 mM DTT. After the urea extract was loaded, the column was washed extensively with 10-15 volumes of extraction buffer.The bound protein was eluted with 150 mM and 500 mM NaCl in a stepwise manner. The fraction size was 3 ml and the fractions were analyzed on 5-15% linear gradient SDS-PAGE.(Fig.)

Refolding of denatured VNs :

The fractions which contained VNs (after SDS-PAGE) were slowly dialyzed with many changes of PBS to remove urea.The fact that no precipitation occurred during dialysis suggests correct protein folding.

Dot Blot Assay :

Briefly, approx. 10 µl of the recombinant proteins viz. wild VN, GGD1-VN, RGA2-VN, AGD2-VN (both denatured and refolded) were loaded onto the nitrocellulose paper strips. The paper was air dried, blocked overnight with 5% fat free milk powder in PBS-Tween.The strip was incubated with rabbit anti-goat VN antiserum(1:500 dilution in PBS-T)(Mahawar and Joshi,2008) for 2 hours.The strip was washed several times with PBS-Tween and then incubated with goat anti-rabbit HRPO conjugate (1:500 dilution in PBS-T) for 1 hour. The enzyme reaction was initiated by adding DAB.

***S.aureus* binding to Vitronectins:**

S.aureus strain 8325-4 preserved as glycerol stock was revived and used for binding studies.Two methods were followed to assess *S.aureus* binding to vitronectins.

In the solution phase about 200µl *S.aureus* suspension in HBS was mixed with chilled vitronectin (200µl, ~5 µg protein) and kept in ice for 30 min (The 200µl *S.aureus* suspension represented 500µl of freshly grown bacterial culture with A_{600} =0.6).

The *S.aureus* –VN suspension was then centrifuged at 6000 rpm(10 min/4°C)in a Remi microfuge.The pellet was washed twice with chilled PBS.The presence of bound VN in the bacterial pellet was identified by Western blot.

In the second method,VN was immobilized onto the wells of a microtitre plate.In brief,wells were coated with 100µl/well of VN solutions (10µg/ml)in 0.1M NaPi(pH 7.4) overnight at 4°C. After two washes, the free sites on the wells were blocked by adding denatured BSA(10mg/ml,denatured at 70 °C for 10 min). After 1.5 hour, the wells were washed

twice with PBS and 500 µl *S.aureus* suspension in HBS was added and the plate was kept at room temperature (22-25 °C) for an hour.The 100 µl suspension was equivalent to 400µl of freshly grown bacteria with A_{600} = 0.6.The unadhered bacteria were removed by careful suction and the wells were washed gently twice with PBS.This was followed by adding 100µl/well of rabbit IgG-HRPO conjugate(1:3000 dilution in PBS-T)The plate was incubated at room temperature for an hour.This step was included to enhance the signal of *S.aureus* bound to VN as this strain (8325-4)is positive for protein A,a ligand for IgG.

After several washes,the peroxidase activity was measured by adding o-phenylenediamine(OPD),and the intensity of colour produced was read at 490nm.

Wells coated with BSA served as control and the mean absorbance of these wells was subtracted from the VN coated wells to assess S.aureus binding.The effect of EDTA,heparin and the RGD tripeptide on *S.aureus*-VN interactions was studied as follows.The EDTA was included at the time of addition of *S.aureus* to the wells.Heparin was added to the VN coated wells and incubated at 4 °C for 30 min.before adding bacteria .The RGD tripeptide was preincubated with *S.aureus* at 4 °C for 30 min and the mixture was added to the wells.

Cell Adhesion Assay:

VN binding to vero cells and BHK21 cells were performed by coating proteins onto the plastic surface.(Joshi et al.,1993 & Mahawar and Joshi,2008).In brief,protein solution(100 µl/well) in PBS(~10µg/ml) was added to wells of a microtitre plate and kept at 4°C for overnight. Next day, the wells were emptied,washed twice with PBS and the free sites on the plastic were blocked by adding 100 µl/well BSA(denatured at 70°C for 10 min). After 60 – 90 min,wells were emptied,washed with

PBS and 100 µl cells(35,000-50,000) in RPMI medium without serum were added to each well and the plate was incubated at 37°C for an hour. The unbound cells were removed by gentle suction and the wells washed with PBS. The bound cells were fixed with 3% formaldehyde in saline for an hour and stained with 0.05% amido black for 60-90 min.After several washes with saline,the bound cells were viewed under a light microscope for rough quantitation and morphological features.The cell binding to VN substrata was quantitated by lysing the cells with 2% SDS(100 µl/well) and measuring the absorbance of the blue color at 595nm.

The effect of heparin,EDTA and the RGD peptide was studied as follows.EDTA(5mM,final concentration)was added at the time of addition of cells to the wells. For heparin, it was added to VN coated wells at 200µg/ml (final concentration) in 50 µl PBS and incubated at room temperature for 30 min before adding cells.The RGD peptide (500µg/ml final concentration) was added to cells and kept at 4°C for 30 minutes.This mixture was added to VN coated wells.

SDS-PAGE and Western Blot :

SDS-PAGE was performed in 5-15% linear gradient gels in a discontinuous buffer system (Laemmli, 1970). Samples for electrophoresis were mixed with the sample buffer and incubated at 37°C for 15 min prior to electrophoresis. Protein bands in the gel were visualized by staining with Coomassie Brilliant Blue R-250.

For Western blotting, protein bands were transferred from the gel onto the nitrocellulose membrane at 200 mA for 1.5-2 hour at 20-25°C and then blocked with 5% skim milk powder for an hour. The membrane was then incubated in a solution of primary antibody (1:500 dilution) for 3 hours at room temperature, washed with several changes

of PBS Tween 20 and incubated with secondary antibody conjugated to HRPO (1:500 dilution) for 2 hours. The membrane was washed thoroughly with PBS-Tween and developed with DAB. (Towbin *et al.,* 1992).

Agarose gel electrophoresis:

Agarose gel electrophoresis of DNA samples was performed in 0.6-1.5% horizontal gels with 1X TAE buffer as gel and tank buffer (Sambrook and Russell, 2001). Samples were mixed with gel loading buffer and electrophoresis was carried out at a constant voltage (50 V) till the tracking dye reached the other end of the gel. Subsequently, the gels were examined under UV-light in a gel documentation system.

Results

4 Results

Generation of recombinant, mutant vitronectins :

Large scale preparation of plasmid template, wild vitronectin (Wild VN (or) pVTNFL)

The recombinant full length vitronectin plasmid, pVTNFL was prepared by inoculation of 50 ml LB ampicillin culture and isolation of plasmid. Upon digestion with EcoRI and HindIII, an insert relase of ~1.3 kb was seen (Fig. 4.4). The plasmid yield was ~ 2 µg/ml. The sequencing results revealed 2 RGD motifs at position 45-47 and 106-108. The plasmid was used as template for generation of mutant vitronectins.

Generation of recombinant mutant vitronectin GGD1-VN :

The PCR generated full length amplicon, GGD1-VN (Figs 4.1a,b,c,d and 4.3a,b) upon subsequent cloning and expression showed a band of ~68 kDa in SDS-PAGE after induction. This clone was sent for sequencing and the results showed desired mutation i.e R at position 45 was replaced with G.In addition there were two other undesired alterations :W in place of C at position 267 and T replaced M at position 400 (Fig. 4.5a).

The expressed protein was purified on a heparin-Sepharose column in the presence of 8 M urea and 10 mM DTT. The bulk of

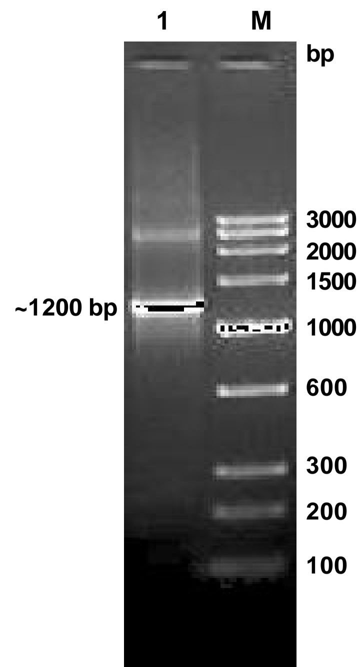


Fig. 4.1a : GGD1-VN amplicon generated by PCR 1
Lane 1 : GGD1-VN amplicon 1
Lane M : Low range DNA ladder

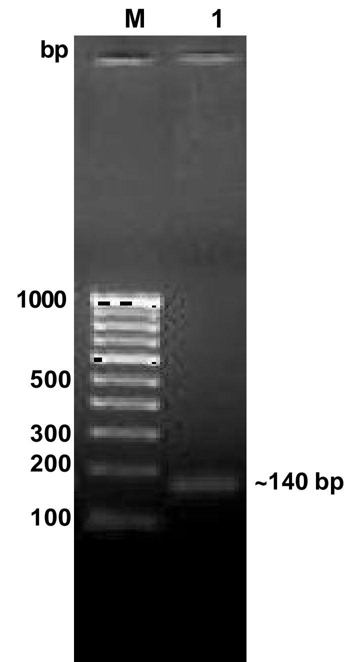


Fig. 4.1b : GGD1-VN amplicon generated by PCR 2
Lane 1 : GGD1-VN amplicon 2
Lane M : 100 bp DNA ladder

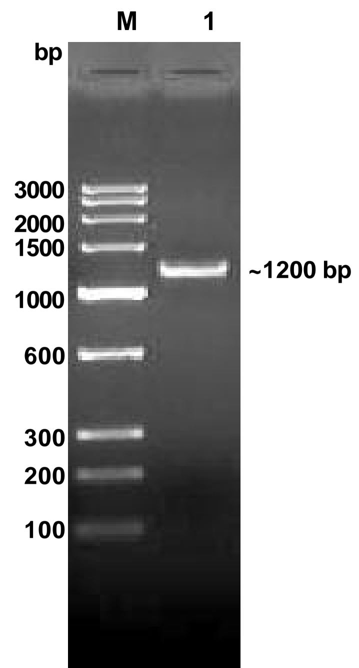


Fig. 4.1c : Gel purified GGD1-VN amplicon 1
Lane 1 : GGD1-VN amplicon 1
Lane M : Low range DNA ladder

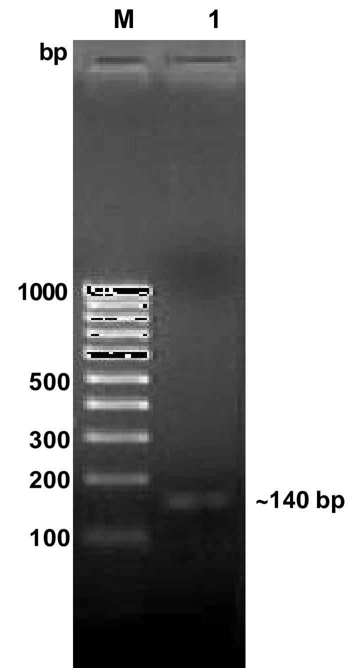


Fig. 4.1d : Gel purified GGD1-VN amplicon 2
Lane 1 : GGD1-VN amplicon 2
Lane M : 100 bp DNA ladder

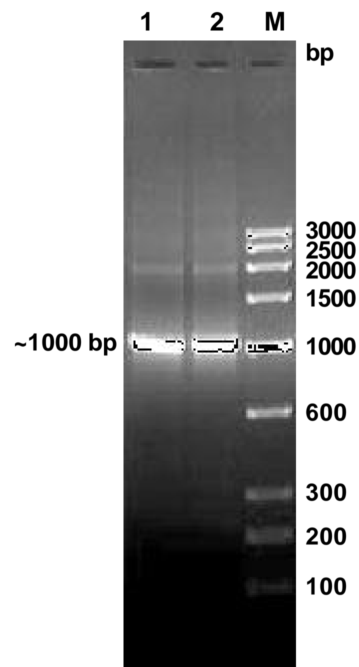


Fig. 4.2a : RGA2-VN, AGD2-VN amplicons generated by PCR 1
Lane 1 : RGA2-VN amplicon 1
Lane 2 : AGD2-VN amplicon 1
Lane M : Low range DNA ladder

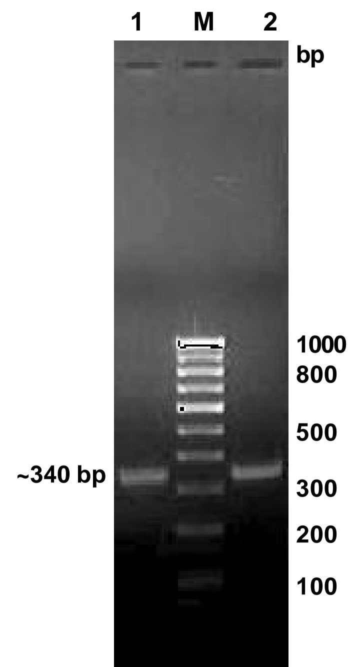


Fig. 4.2b : RGA2-VN, AGD2-VN amplicons generated by PCR 2
Lane 1 : RGA2-VN amplicon 2
Lane 2 : AGD2-VN amplicon 2
Lane M : 100 bp DNA ladder

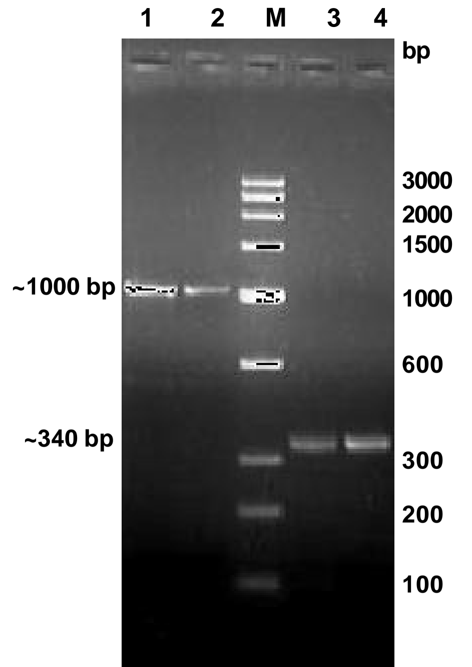


Fig. 4.2c : Gel purified amplicons of RGA2-VN, AGD2-VN
Lane 1 : RGA2-VN amplicon 1
Lane 2 : AGD2-VN amplicon 1
Lane 3 : RGA2-VN amplicon 2
Lane 4 : AGD2-VN amplicon 2
Lane M : Low range DNA ladder

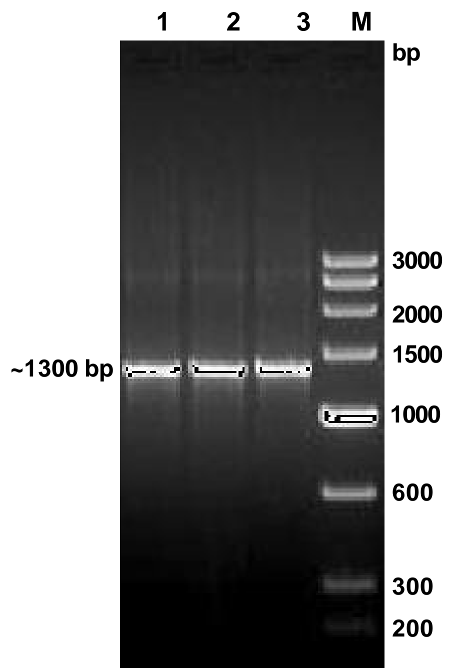


Fig. 4.3a : Full length amplicons of GGD1-VN, RGA2-VN and AGD2-VN generated by PCR 3

Lane 1 : GGD1-VN full length amplicon
Lane 2 : RGA2-VN full length amplicon
Lane 3 : AGD2-VN full length amplicon
Lane M : Low range DNA ladder

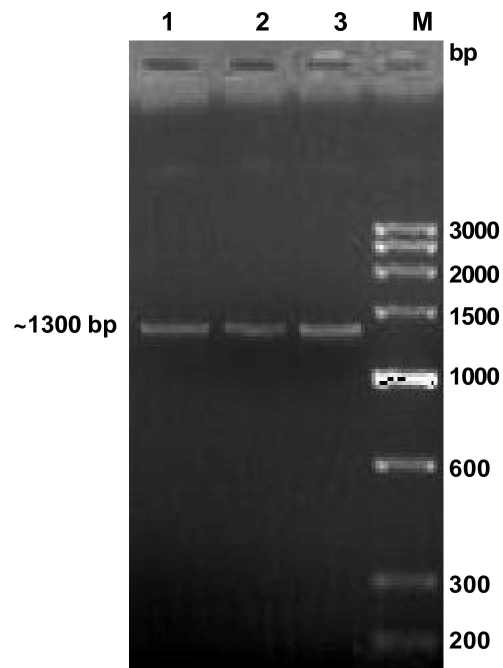


Fig. 4.3b : Gel purified full length amplicons of GGD1-VN, RGA2-VN and AGD2-VN

Lane 1 : Gel purified GGD1-VN
Lane 2 : Gel purified RGA2-VN
Lane 3 : Gel purified AGD2-VN
Lane M : Low range DNA ladder

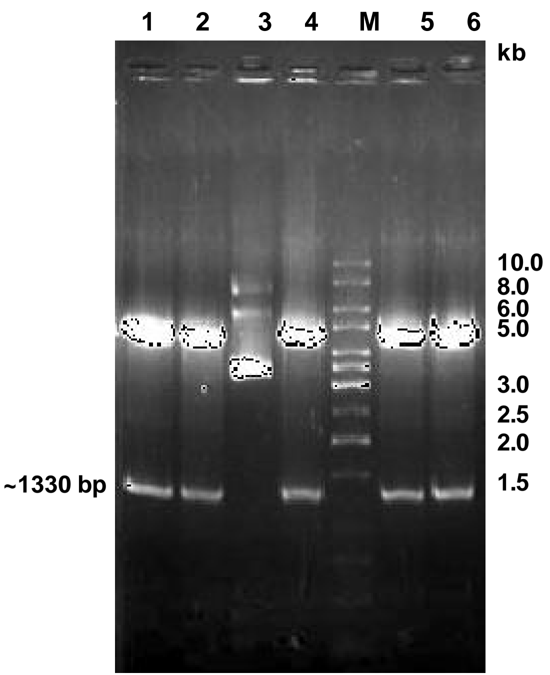


Fig. 4.4 : Recombinant clones of GGD1-VN, RGA2-VN, AGD2-VN and Wild VN showing insert release of ~1.33 kb after digestion with *Eco*RI and *Hind*III

Lane 1 : GGD1-VN recombinant clone
Lane 2 : RGA2-VN recombinant clone
Lane 3 : Undigested clone
Lane 4 : GGD1-VN recombinant clone
Lane 5 : AGD2-VN recombinant clone
Lane 6 : Wild VN recombinant clone
Lane M : 1 kb DNA ladder

protein eluted at 0.5M NaCl which had >95% purity (Fig. 4.6a). About 1.5-2.0 mg protein could be recovered from a liter of bacterial culture.

Generation of recombinant mutant RGA2-VN

The PCR generated full length amplicon, RGA2-VN (Fig. 4.2a,b,c and 4.3a,b) upon subsequent cloning, screening (Fig. 4.4) and expression (Fig. 4.5b) showed a band of ~ 68 kDa in SDS-PAGE after induction. The clone expressing desired protein upon sequencing showed expected mutation where D108 was replaced with A (Fig.). There was no other alteration.The protein was purified employing heparin-sepharose where bulk of the protein (~85%) eluted at 500 mM NaCl.The remaining eluted at physiological salt concentration(150 mM NaCl) (Fig. 4.6a) The recovery of protein was ~ 2.0 mg from a liter of bacterial culture.

Generation of recombinant mutant AGD2-VN:

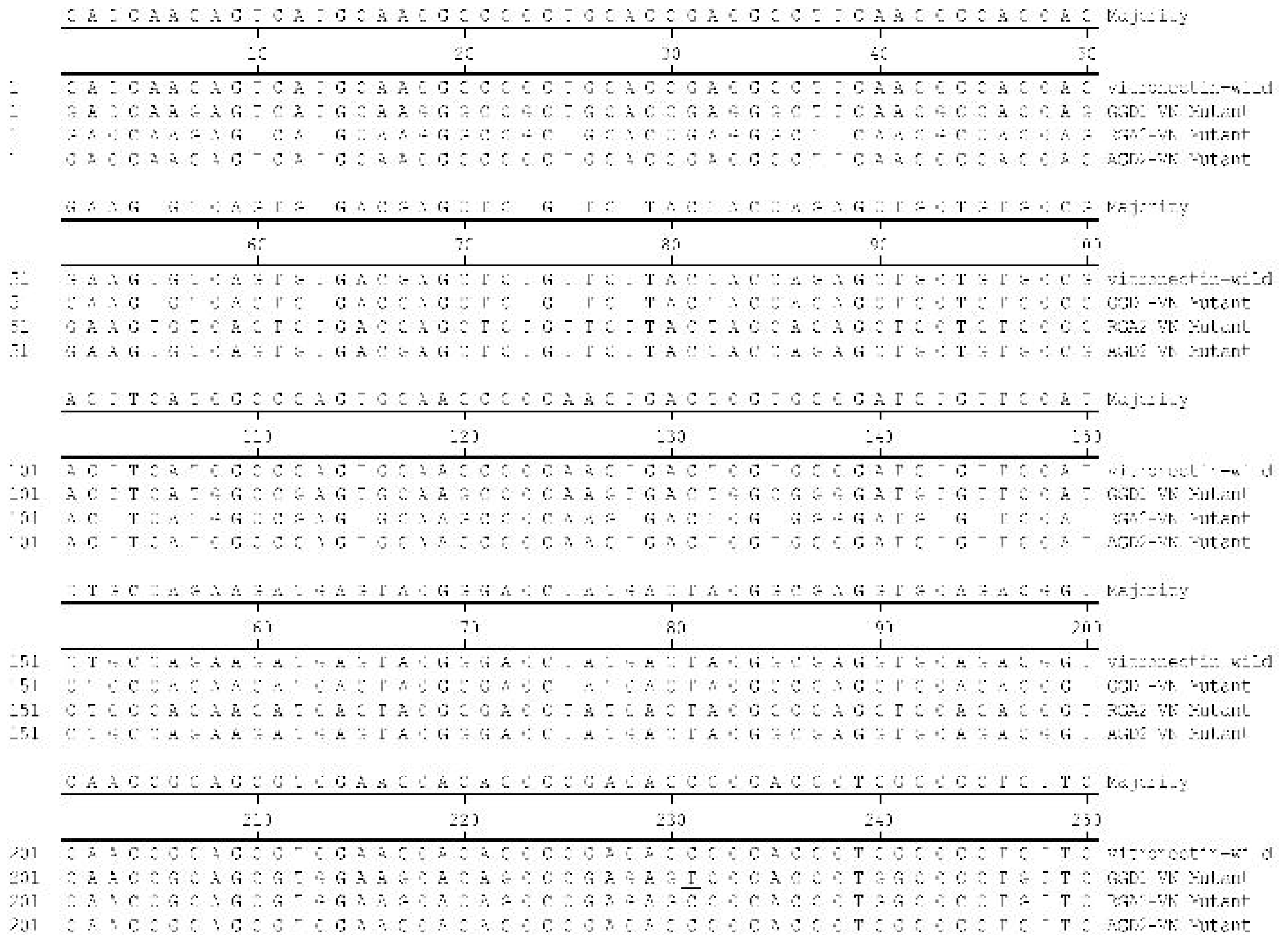
The PCR generated full length amplicon, AGD2-VN (Figs. 4.2a,b,c and Fig. 4.3a,b) upon subsequent cloning (Fig. 4.4) and expression showed a band of ~ 68 kDa in SDS-PAGE after induction.Sequence analysis of the clone expressing the VN indicated desired mutation, R106 was replaced by A. In addition P 440 was changed to L 440 (Fig. 4.5b). The bulk of induced protein eluted at 0.5M NaCl (Fig. 4.6a). The recovery of purified protein was ~2 mg/ liter of bacterial culture.

Refolding of denatured vitronectins and dot blot analysis:

The slow dialysis of column eluted VN, to remove urea and DTT, probably facilitated correct folding as no precipitation occurred during this process.

The reactivity of refolded VN and the parent denatured VN were tested by dot blot assay.All the refolded proteins reacted with anti-VN

Fig. 4.5a : Multiple alignment of nucleotide sequences of Wild-VN and mutant vitronectins by clustal W (DNA star)



Contd.....

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     GCAGGCCGAGATTCCTG CCAGGCACCGGTTCTCAACCCTGAGAAAGAG  Majority
                260       270       280       290       300
251  TGCAGGCCGAGATTCCTGTCCAGGCACCGGTTCTCAACCCTGAGAAAGAG  vitronectin wild
251  TGCAGGCCGAGATTCCTGTCCAGGCACCGGTTCTCAACCCTGAGAAAGAG  GG1-VN Mutant
251  TGCAGGCCGAGATTCCTG CCAGGCACCGGTTCTCAACCCTGAGAAAGAG  GG2-VN Mutant
251  TGCAGCCCCCAGATTCCTGTCCACCCACCCCTTCTCAACCCTGACAAAGAC  AG2-VN Mutant

     GCCCCATCACCTGGCCGGGGAGACTCAGACCCTGGGCTGGGGACCAGTGA  Majority
                310       320       330       340       350
301  CCCCCATCACCTCGCCCCGCCAGACTCAGACCCTCGCCCTGCGGACCACTGA  vitronectin-wild
301  CCCCCATCACCTCGCCCCGCCAGACTCAGACCCTCGCCCTGCGGACCACTGA  GG1 VN Mutant
301  GCCCCATCACCTGGCCGGGGAG|CCTCAGACCCTGGGCTGGGGACCAGTGA  GG2 VN Mutant
301  GCCCCATCACCTGGGACGGGAGACTCAGACCCTGGGCTGGGGACCAGTGA  AG2-VN Mutant

     CCTAGCCCACCTCTGACTCCCCACCACAGGAGGAAACCTGCAGTCGCAAAC  Majority
                360       370       380       390       400
351  CCTAGGGACCTCTGAGCCCCCAGCAGAGGAGGAAACGTGCAGTGGGAAAC  vitronectin-wild
351  CCTAGGGACCTCTGAGCCCCCAGCAGAGGAGGAAACGTGCAG GGGAAAC  GG1-VN Mutant
351  CCTAGCCCACCTCTGACTCCCCACCACAGGAGGAAACCTGCAGTCGCAAAC  GG2-VN Mutant
351  CCTAGCCCACCTCTGACTCCCCACCACAGGAGGAAACCTGCAGTCGCAAAC  AG2 VN Mutant

     CCITTGAIGCCITCACCGACCTCAAGAAIGGTICCCICIITGCCTICCGA  Majority
                410       420       430       440       450
401  CCTTTCATCCCTTCACCGACCTCAACAATCGTTCCCTCTTTGCCTTCCGA  vitronectin wild
401  CCTTTGATGCCTTCACCGACCTCAAGAATGGTTCCCTCTTTGCCTTCCGA  GG1 VN Mutant
401  CCTTTGATGCCTTCACCGACCTCAAGAATGGTTCCCTCTTTGCCTTCCGA  GG2-VN Mutant
401  CCTTTGATGCCTTCACCGACCTCAAGAATGGTTCCC C  TGCC  CCGA  AG2-VN Mutant

     GGTCTCTACTGCTATCAGCTGGATCAAAACGCACTCAGGCCTCCATACCC  Majority
                460       470       480       490       500
451  GGTCTCTACTGCTATCAGC GGATCAAAAGGCAGTGAGGCCTGGA ACCC  vitronectin-wild
451  CGTCTCTACTGCTATCAGCTGGATCAAAACGCACTCAGGCCTCCATACCC  GG1-VN Mutant
451  GGTCTCTACTGCTATCAGCTGGATCAAAACGCACTCAGGCCTCCATACCC  GG2 VN Mutant
451  GGTCTGTACTGCTATGAGCTGGATGAAAAGGCAGTGAGGCCTGGATACCC  AG2 VN Mutant
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Contd.....

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     CAAACTCATCCGAGATGTCTGGGGCATTGAGGGGCCCATTGATGCCGGCC Majority
              510       520       530       540       550
501  CAAACTCATCCGAGATGTCTGGGGCATTGAGGGGCCCATTGATGCCGGCC vitronectin-wild
501  CAAACTCATCCGAGATGTCTGGGGCATTGAGGGGCCCATTGATGCCGGCC AGI1-VN Mutant
501  CAAACTCATCCGAGATGTCTGGGGCATTGAGGGGCCCATTGATGCCGGCC AGA2-VN Mutant
501  CAAACTCATCCGAGATCTCTGGCCCATTGAGGCCCCCATTGATCCCCCCT AGI2-VN Mutant

     TCACCCCGTTCAACTGTCAGGGAAAGACGTACCTCTTCAAGGGTAGTCAG Majority
              560       570       580       590       600
551  TCACCCCGTTCAACTCTCACGGAAACACGTACCTCTTCAAGGCTACTCAC vitronectin-wild
551  TCACCCCGTTCAACTCTCACGGAAACACGTACCTCTTCAAGGCTACTCAC AGI1-VN Mutant
551  TCACCCCGTTCAACTGTCAGGGAAAGACGTACCTCTTCAAGGGTAGTCAG AGA2-VN Mutant
551  TCACCCCGTTCAACTGTCAGGGAAAGACGTACCTCTTCAAGGGTAGTCAG AGI2-VN Mutant

     TATTGCCCGCTTCAGCATCCTGTCCCTCGAACCTCACTTCTCCCAAACAT Majority
              610       620       630       640       650
601  TATTGGCGCTTTGAGGATGGTGTCCTGGATCCTGACTTCTCCCGAAACAT vitronectin-wild
601  TATTGGCGCTTTGAGGATGGTGTCCTGGATCCTGACTTCTCCCGAAACAT AGI1-VN Mutant
601  TATTGCCCGCTTCAGCATCCTGTCCCTCGAACCTCACTTCTCCCAAACAT AGA2-VN Mutant
601  TATTGCCCGCTTCAGCATCCTGTCCCTCGAACCTCACTTCTCCCAAACAT AGI2-VN Mutant

     TTTGGATGGCTTCAAGGGTATTCCCGATGACGTGGACGGCAGCCTTGGCC Majority
              660       670       680       690       700
651  TTTGGATCCCTTCAACCGTATTCCCCATGACGTCGACGGCAGCCTTCCCC vitronectin-wild
651  TTTGGATGGCTTCAAGGGTATTCCCGATGACGTGGACGGCAGCCTTGGCC AGI1-VN Mutant
651  TTTGGATGGCTTCAAGGGTATTCCCGATGACGTGGACGGCAGCCTTGGCC AGA2-VN Mutant
651  TTTGGATGGCTTCAAGGGTATTCCCGATGACGTGGACGGCAGCCTTGGCC AGI2-VN Mutant

     TCTCCCCTCACACCTACAACGGCACCCAGCGACTCTACTTCTTCAAGGGC Majority
              710       720       730       740       750
701  TCTCCGGCTCACAGGCTACAACGGCACGGAGTGAGTCTACTTCTTCAAGGGC vitronectin-wild
701  TCTCCCCTCACACCTACAACGGCACCCAGCGACTCTACTTCTTCAAGGGC AGI1-VN Mutant
701  TCTCCCCTCACACCTACAACGGCACCCAGCGACTCTACTTCTTCAAGGGC AGA2-VN Mutant
701  TCTCCGGCTCACAGGCTACAACGGCACGGAGTGAGTCTACTTCTTCAAGGGC AGI2-VN Mutant
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Contd.....

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    AACCACTACTGGGAATATCTGTTCCAGCAGCAGCCCAGTCGAGAGGAGTG Majority
             790       790       780       790       800
751 AACCACTACTGGGAATATCTGTTCCAGCAGCAGCCCAGTCGAGAGGAGTG vitronectin-wild
751 AACCACTACTCGGAATATCTCTTCCAGCACCACCCCAGTCGACAGCAGTC GOD1 VX Mutant
751 AACCACTACTGGGAATATCTGTTCCAGCAGCAGCCCAGTCGAGAGGAGTG RGA2 VX Mutant
751 AACCACTACTGGGAATATCTGTTCCAGCAGCAGCCCAGTCGAGAGGAGTG AGD2-VX Mutant

    TGAAGGCAGCTCCCAGCCGGCCGCATTTAAACACTTTGCCCTGATGCAGC Majority
             810       820       830       840       850
801 TGAAGGCAGCTCCCAGCCGGCCGCATTTAAACACTTTGCCCTGATGCAGC vitronectin-wild
801 TGAAGGCAGCTCCCAGCCGGCCGCATTTAAACACTTTGCCCTGATGCAGC GOD1 VX Mutant
801 TGAAGGCAGCTCCCAGCCGGCCGCATTTAAACACTTTGCCCTGATGCAGC RGA2-VX Mutant
801 TGAAGGCAGCTCCCAGCCGGCCGCATTTAAACACTTTGCCCTGATGCAGC AGD2 VX Mutant

    CGGACACCTCCGACCACATCTTCCCACTTCTCTTCTGGCCCCCCTTCCTT Majority
             860       870       880       890       900
851 CGGACACCTCCGACCACATCTTCCCACTTCTCTTCTGGCCCCCCTTCCTT vitronectin-wild
851 CGGACACCTCCGACCACATCTTCCCACTTCTCTTCTGGCCCCCCTTCCTT GOD1-VX Mutant
851 GGGACAGCTGGGAGGACATCTTCCGACTTCTCTTCTGGGGCGGTTCCTTT RGA2 VX Mutant
851 GGGACAGCTGGGAGGACATCTTCCGACTTCTCTTCTGGGGCGGTTCCTTT AGD2-VX Mutant

    GGTGGTGCTGGACAGCCCCCAGCTCATCAGCCGCGACTGGTTTGGTCTGCC Majority
             910       920       930       940       950
901 CGTCGTCCTCCACACCCCCCACCTCATCACCCCCGACTGCTTTCGTCTGCC vitronectin-wild
901 GGTGGTGCTGGACAGCCCCCAGCTCATCAGCCGCGACTGGTTTGGTCTGCC GOD1-VX Mutant
901 CGTCGTCCTCCACACCCCCCACCTCATCACCCCCGACTGCTTTCGTCTGCC RGA2-VX Mutant
901 CGTCGTCCTCCACACCCCCCACCTCATCACCCCCGACTGCTTTCGTCTGCC AGD2 VX Mutant

    GGGAAAACTGGATGCGGCCATGGCCAGCCACATCTACATCTCAGGCTCAA Majority
             960       970       980       990      1000
951 GGGAAAACTGGATGCGGCCA GGCCAGCCACATCTACATCTCAGGCTCAA vitronectin-wild
951 CCGAAAACTCCATCCGCCCATCCCCCGCCACATCTACATCTCAGGCTCAC GOD1 VX Mutant
951 CCGAAAACTCCATCCGCCCATCCCCCGCCACATCTACATCTCAGGCTCAC RGA2 VX Mutant
951 GGGAAAACTGGATGCGGCCA GGCCAGCCACATCTACATCTCAGGCTCAA AGD2-VX Mutant
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Contd.....

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     C T C C C A G C T C C C C C C C G C C C A A C A T C A C T A A C T C T C C G C C G C C C C A T C C C  Majority
                       1010                1020                1030                1040                1050
1001 C T C C C A G C T C C C C C C C G C C C A A C A T C A C T A A C T C T C C G C C G C C C C A T C C C  vitronectin-wild
1001 C T G C C A G C T C C C C C C G G C C A A G A T G A C T A A G T C T G C G C G G C G C C A T C G C  GGD1-VX Mutant
1001 C T C C C A G C T C C C C C C G G C C A A G A T G A C T A A G T C T G C G C G G C G C C A T C G C  RGD2-VX Mutant
1001 C T C C C A G C T C C C C C C C G C C C A A C A T C A C T A A C T C T C C G C C G C C C C A T C C C  AGD2-VX Mutant

     A A A C G T T A C C G G C T C G C T C C G A A G C C G T G G C C G A G G C C G C G G C C G C G C C C  Majority
                       1060                1070                1080                1090                1100
1051 A A A C G T T A C C G G C T C G C T C C G A A G C C G T G G C C G A G G C C G C G G C C G C G C C C  vitronectin-wild
1051 A A A C G T T A C C G G C T C G C T C C G A A G C C G T G G C C G A G G C C G C G G C C G C G C C C  GGD1-VX Mutant
1051 A A A C C T T A C C C C C T C C C T C C G A A C C C C T G C C C C A G C C C G C C G C C C G C C C C  RGD2-VX Mutant
1051 A A A C G T T A C C G G C T C G C T C C G A A G C C G T G G C C G A G G C C G C G G C C G C G C C C  AGD2-VX Mutant

     C A G C C A G A A C C C C T A C C G G C G A T T T C G G T C C A C C T G G C T G T C C T G G T T C T  Majority
                       1110                1120                1130                1140                1150
1101 C A G C C A G A A C C C C T A C C G G C G A T T T C G G T C C A C C T G G C T G T C C T G G T T C T  vitronectin-wild
1101 C A G C C A G A A C C C C T A C C C C C G A T T T C G G T C C A C C T C G C T C T C C T G C T T C T  GGD1-VX Mutant
1101 C A G C C A G A A C C C C T A C C G G C G A T T T C G G T C C A C C T G G C T G T C C T G G T T C T  RGD2-VX Mutant
1101 C A G C C A G A A C C C C T A C C G G C G A T T T C G G T C C A C C T G G C T G T C C T G G T T C T  AGD2-VX Mutant

     C C A G C G A G G A G C T G G C C C T G G C A G C C G A C A A C T A T G A T A A C T A C G A G A T C  Majority
                       1160                1170                1180                1190                1200
1151 C C A G C G A T C A C C T G C C C C T G G C A G C C G A C A A C T A T C A T A A C T A C G A G A T C  vitronectin-wild
1151 C C A G C G A G G A G C T G G C C C T G G G A G C C G A C A A C T A T G A T A A C T A C G A G A T C  GGD1-VX Mutant
1151 C C A G C G A G G A G C T G G C C C T G G G A G C C G A C A A C T A T G A T A A C T A C G A G A T C  RGD2-VX Mutant
1151 C C A G C G A G G A G C T G G C C C T G G G A G C C G A C A A C T A T G A T A A C T A C G A G A T C  AGD2-VX Mutant

     G A C T G G C T T G T G C C T G C A A C C T G T G A G C C C A T C C A G A G T G T C T A C T T C T T  Majority
                       1210                1220                1230                1240                1250
1201 G A C T G G C T T G T G C C T G C A A C C T G T G A G C C C A T C C A G A G T G T C T A C T T C T T  vitronectin-wild
1201 G A C T G G C T T G T G C C T G C A A C C T G T G A G C C C A T C C A G A G T G T C T A C T T C T T  GGD1-VX Mutant
1201 G A C T G G C T T G T G C C T G C A A C C T G T G A G C C C A T C C A G A G T G T C T A C T T C T T  RGD2-VX Mutant
1201 G A C T G G C T T G T G C C T G C A A C C T G T G A G C C C A T C C A G A G T G T C T A C T T C T T  AGD2-VX Mutant
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Contd.....

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    C T C A G A A G A C A A G   A C   A C C G A G   G A A T C     C G C A C G T G G C G G G   G G A C   Majority
                        260                 270                 280                 290               300
251 C T C A G A A G A C A A G T A C T A C C G A G T G A A T C T T C G C A C G T G G C G G G T G G A C T Vitronectin-wild
251 C T C A G A A G A C A A G T A C T A C C G A G T G A A T C T T C G C A C G T G G C G G G T G G A C T GGD1-VN Mutant
251 C T C A G A A G A C A A G T A C T A C C G A G T G A A T C T T C G C A C G T G G C G G G T G G A C T RGA2-VN Mutant
251 C T C A G A A G A C A A G T A C T A C C G A G T G A A T C T T C G C A C G T G G C G G G T G G A C T ACD2-VN Mutant

    C   G   G A   T C C   C C C   A C T C A C G C   C C A   C G C                                     Majority
                        310                 320                 330
301 C T G T G A T T C C T C C C T A C T C A C G C T C C A T C G C T                                     Vitronectin-wild
301 C T G T G A T T C C T C C C T A C T C A C G C T C C A T C G C T                                     CCD2-VN Mutant
301 C T G T G A T T C C T C C C T A C T C A C G C T C C A T C G C T                                     RGA2-VN Mutant
301 C T G T G A T T C C T C C C T A C T _ A C G C T C C A T C G C T                                     AHD2-VN Mutant
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ecoration 'Decoration #1': Box residues that differ from the Consensus.

Fig. 4.5b : Multiple alignment of deduced amino acid sequences of Wild-VN and mutant vitronectins by clustal W (DNA star)

	Sequence	
	Asp Gln Glu Ser Cys Lys Gly Arg Cys Thr Gln Gly Phe Asn Ala Thr Arg Lys Cys Gln Cys Asp Gln Leu Cys	Majority
	10 20	
1	Asp Gln Glu Ser Cys Lys Gly Arg Cys Thr Gln Gly Phe Asn Ala Thr Arg Lys Cys Gln Cys Asp Gln Leu Cys	vitronectin-wild
1	Asp Gln Glu Ser Cys Lys Gly Arg Cys Thr Gln Gly Phe Asn Ala Thr Arg Lys Cys Gln Cys Asp Gln Leu Cys	GGD1-VN Mutant
1	Asp Gln Glu Ser Cys Lys Gly Arg Cys Thr Gln Gly Phe Asn Ala Thr Arg Lys Cys Gln Cys Asp Gln Leu Cys	RGA2-VN Mutant
1	Asp Gln Glu Ser Cys Lys Gly Arg Cys Thr Gln Gly Phe Asn Ala Thr Arg Lys Cys Gln Cys Asp Gln Leu Cys	AGD2-VN Mutant
	Ser Tyr Tyr Gln Ser Cys Cys Ala Asp Phe Met Ala Glu Cys Lys Pro Gln Val Thr Arg Gly Asp Val Phe His	Majority
	30 40 50	
76	Ser Tyr Tyr Gln Ser Cys Cys Ala Asp Phe Met Ala Glu Cys Lys Pro Gln Val Thr Arg Gly Asp Val Phe His	vitronectin-wild
76	Ser Tyr Tyr Gln Ser Cys Cys Ala Asp Phe Met Ala Glu Cys Lys Pro Gln Val Thr [Gly] Gly Asp Val Phe His	GGD1-VN Mutant
76	Ser Tyr Tyr Gln Ser Cys Cys Ala Asp Phe Met Ala Glu Cys Lys Pro Gln Val Thr Arg Gly Asp Val Phe His	RGA2-VN Mutant
76	Ser Tyr Tyr Gln Ser Cys Cys Ala Asp Phe Met Ala Glu Cys Lys Pro Gln Val Thr Arg Gly Asp Val Phe His	AGD2-VN Mutant
	Leu Pro Glu Asp Glu Tyr Gly Thr Tyr Asp Tyr Gly Glu Val Gln Thr Val Asn Arg Ser Val Glu Ala Gln Pro	Majority
	60 70	
151	Leu Pro Glu Asp Glu Tyr Gly Thr Tyr Asp Tyr Gly Glu Val Gln Thr Val Asn Arg Ser Val Glu Ala Gln Pro	vitronectin-wild
151	Leu Pro Glu Asp Glu Tyr Gly Thr Tyr Asp Tyr Gly Glu Val Gln Thr Val Asn Arg Ser Val Glu Ala Gln Pro	GGD1-VN Mutant
151	Leu Pro Glu Asp Glu Tyr Gly Thr Tyr Asp Tyr Gly Glu Val Gln Thr Val Asn Arg Ser Val Glu Ala Gln Pro	RGA2-VN Mutant
151	Leu Pro Glu Asp Glu Tyr Gly Thr Tyr Asp Tyr Gly Glu Val Gln Thr Val Asn Arg Ser Val Glu Ala Gln Pro	AGD2-VN Mutant
	Gln Ser Pro Thr Leu Ala Pro Val Leu Gln Ala Glu Ile Pro Val Gln Ala Pro Val Leu Asn Pro Glu Lys Gln	Majority
	80 90 100	
226	Gln Ser Pro Thr Leu Ala Pro Val Leu Gln Ala Glu Ile Pro Val Gln Ala Pro Val Leu Asn Pro Glu Lys Gln	vitronectin-wild
226	Gln Ser Pro Thr Leu Ala Pro Val Leu Gln Ala Glu Ile Pro Val Gln Ala Pro Val Leu Asn Pro Glu Lys Gln	GGD1-VN Mutant
226	Gln Ser Pro Thr Leu Ala Pro Val Leu Gln Ala Glu Ile Pro Val Gln Ala Pro Val Leu Asn Pro Glu Lys Gln	RGA2-VN Mutant
226	Gln Ser Pro Thr Leu Ala Pro Val Leu Gln Ala Glu Ile Pro Val Gln Ala Pro Val Leu Asn Pro Glu Lys Gln	AGD2-VN Mutant
	Ala Pro Ser Pro Gly Arg Gly Asp Ser Asp Pro Gly Leu Gly Thr Ser Asp Leu Gly Thr Ser Glu Ser Pro Ala	Majority
	110 120	
301	Ala Pro Ser Pro Gly Arg Gly Asp Ser Asp Pro Gly Leu Gly Thr Ser Asp Leu Gly Thr Ser Glu Ser Pro Ala	vitronectin-wild
301	Ala Pro Ser Pro Gly Arg Gly Asp Ser Asp Pro Gly Leu Gly Thr Ser Asp Leu Gly Thr Ser Glu Ser Pro Ala	GGD1-VN Mutant
301	Ala Pro Ser Pro Gly Arg Gly [Ala] Ser Asp Pro Gly Leu Gly Thr Ser Asp Leu Gly Thr Ser Glu Ser Pro Ala	RGA2-VN Mutant
301	Ala Pro Ser Pro Gly [Ala] Gly Asp Ser Asp Pro Gly Leu Gly Thr Ser Asp Leu Gly Thr Ser Glu Ser Pro Ala	AGD2-VN Mutant

Contd.....

	Sequence	
	Gln Ala Glu Thr Cys Ser Gly Lys Pro Phe Asp Ala Phe Thr Asp Leu Lys Asn Gly Ser Leu Phe Ala Phe Arg	Majority
	130 140 150	
376	Gln Ala Glu Thr Cys Ser Gly Lys Pro Phe Asp Ala Phe Thr Asp Leu Lys Asn Gly Ser Leu Phe Ala Phe Arg	vitronectin-wild
376	Gln Ala Glu Thr Cys Ser Gly Lys Pro Phe Asp Ala Phe Thr Asp Leu Lys Asn Gly Ser Leu Phe Ala Phe Arg	SGD1-VN Mutant
376	Gln Ala Glu Thr Cys Ser Gly Lys Pro Phe Asp Ala Phe Thr Asp Leu Lys Asn Gly Ser Leu Phe Ala Phe Arg	RGA2-VN Mutant
376	Gln Ala Glu Thr Cys Ser Gly Lys Pro Phe Asp Ala Phe Thr Asp Leu Lys Asn Gly Ser Leu Phe Ala Phe Arg	AGD2-VN Mutant

	Sequence	
	Gly Leu Tyr Cys Tyr Glu Leu Asp Glu Lys Ala Val Arg Pro Gly Tyr Pro Lys Leu Ile Arg Asp Val Trp Gly	Majority
	160 170	
451	Gly Leu Tyr Cys Tyr Glu Leu Asp Glu Lys Ala Val Arg Pro Gly Tyr Pro Lys Leu Ile Arg Asp Val Trp Gly	vitronectin-wild
451	Gly Leu Tyr Cys Tyr Glu Leu Asp Glu Lys Ala Val Arg Pro Gly Tyr Pro Lys Leu Ile Arg Asp Val Trp Gly	SGD1-VN Mutant
451	Gly Leu Tyr Cys Tyr Glu Leu Asp Glu Lys Ala Val Arg Pro Gly Tyr Pro Lys Leu Ile Arg Asp Val Trp Gly	RGA2-VN Mutant
451	Gly Leu Tyr Cys Tyr Glu Leu Asp Glu Lys Ala Val Arg Pro Gly Tyr Pro Lys Leu Ile Arg Asp Val Trp Gly	AGD2-VN Mutant

	Sequence	
	Ile Glu Gly Pro Ile Asp Ala Ala Phe Thr Arg Phe Asn Cys Gln Gly Lys Thr Tyr Leu Phe Lys Gly Ser Gln	Majority
	180 190 200	
526	Ile Glu Gly Pro Ile Asp Ala Ala Phe Thr Arg Phe Asn Cys Gln Gly Lys Thr Tyr Leu Phe Lys Gly Ser Gln	vitronectin-wild
526	Ile Glu Gly Pro Ile Asp Ala Ala Phe Thr Arg Phe Asn Cys Gln Gly Lys Thr Tyr Leu Phe Lys Gly Ser Gln	SGD1-VN Mutant
526	Ile Glu Gly Pro Ile Asp Ala Ala Phe Thr Arg Phe Asn Cys Gln Gly Lys Thr Tyr Leu Phe Lys Gly Ser Gln	RGA2-VN Mutant
526	Ile Glu Gly Pro Ile Asp Ala Ala Phe Thr Arg Phe Asn Cys Gln Gly Lys Thr Tyr Leu Phe Lys Gly Ser Gln	AGD2-VN Mutant

	Sequence	
	Tyr Trp Arg Phe Glu Asp Gly Val Leu Glu Pro Asp Phe Pro Arg Asn Ile Ser Asp Gly Phe Lys Gly Ile Pro	Majority
	210 220	
601	Tyr Trp Arg Phe Glu Asp Gly Val Leu Glu Pro Asp Phe Pro Arg Asn Ile Ser Asp Gly Phe Lys Gly Ile Pro	vitronectin-wild
601	Tyr Trp Arg Phe Glu Asp Gly Val Leu Glu Pro Asp Phe Pro Arg Asn Ile Ser Asp Gly Phe Lys Gly Ile Pro	SGD1-VN Mutant
601	Tyr Trp Arg Phe Glu Asp Gly Val Leu Glu Pro Asp Phe Pro Arg Asn Ile Ser Asp Gly Phe Lys Gly Ile Pro	RGA2-VN Mutant
601	Tyr Trp Arg Phe Glu Asp Gly Val Leu Glu Pro Asp Phe Pro Arg Asn Ile Ser Asp Gly Phe Lys Gly Ile Pro	AGD2-VN Mutant

	Sequence	
	Asp Asp Val Asp Ala Ala Leu Ala Leu Pro Ala His Ser Tyr Asn Gly Arg Glu Arg Val Tyr Phe Phe Lys Gly	Majority
	230 240 250	
676	Asp Asp Val Asp Ala Ala Leu Ala Leu Pro Ala His Ser Tyr Asn Gly Arg Glu Arg Val Tyr Phe Phe Lys Gly	vitronectin-wild
676	Asp Asp Val Asp Ala Ala Leu Ala Leu Pro Ala His Ser Tyr Asn Gly Arg Glu Arg Val Tyr Phe Phe Lys Gly	SGD1-VN Mutant
676	Asp Asp Val Asp Ala Ala Leu Ala Leu Pro Ala His Ser Tyr Asn Gly Arg Glu Arg Val Tyr Phe Phe Lys Gly	RGA2-VN Mutant
676	Asp Asp Val Asp Ala Ala Leu Ala Leu Pro Ala His Ser Tyr Asn Gly Arg Glu Arg Val Tyr Phe Phe Lys Gly	AGD2-VN Mutant

Contd.....

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      Asn His Tyr Trp Glu Tyr Val Phe Gln Gln Gln Pro Ser Arg Glu Glu Cys Glu Gly Ser Ser Gln Pro Ala Ala  Majority
                                            260                                 270
751   Asn His Tyr Trp Glu Tyr Val Phe Gln Gln Gln Pro Ser Arg Glu Glu Cys Glu Gly Ser Ser Gln Pro Ala Ala  vitronectin-wild
751   Asn His Tyr Trp Glu Tyr Val Phe Gln Gln Gln Pro Ser Arg Glu Glu Trp Glu Gly Ser Ser Gln Pro Ala Ala  GG1 VN Mutant
751   Asn His Tyr Trp Glu Tyr Val Phe Gln Gln Gln Pro Ser Arg Glu Glu Cys Glu Gly Ser Ser Gln Pro Ala Ala  RGA2-VN Mutant
751   Asn His Tyr Trp Glu Tyr Val Phe Gln Gln Gln Pro Ser Arg Glu Glu Cys Glu Gly Ser Ser Gln Pro Ala Ala  AGI2 VN Mutant

      Phe Lys His Phe Ala Leu Met Gln Arg Asp Ser Trp Glu Asp Ile Phe Arg Leu Leu Phe Trp Gly Gly Ser Phe  Majority
                    280                                 290                                 300
826   Phe Lys His Phe Ala Leu Met Gln Arg Asp Ser Trp Glu Asp Ile Phe Arg Leu Leu Phe Trp Gly Gly Ser Phe  vitronectin wild
826   Phe Lys His Phe Ala Leu Met Gln Arg Asp Ser Trp Glu Asp Ile Phe Arg Leu Leu Phe Trp Gly Gly Ser Phe  GG1-VN Mutant
826   Phe Lys His Phe Ala Leu Met Gln Arg Asp Ser Trp Glu Asp Ile Phe Arg Leu Leu Phe Trp Gly Gly Ser Phe  RGA2 VN Mutant
826   Phe Lys His Phe Ala Leu Met Gln Arg Asp Ser Trp Glu Asp Ile Phe Arg Leu Leu Phe Trp Gly Gly Ser Phe  AGI2 VN Mutant

      Gly Gly Ala Gly Gln Pro Gln Leu Ile Ser Arg Asp Trp Phe Gly Leu Pro Gly Lys Leu Asp Ala Ala Met Ala  Majority
                                            310                                 320
901   Gly Gly Ala Gly Gln Pro Gln Leu Ile Ser Arg Asp Trp Phe Gly Leu Pro Gly Lys Leu Asp Ala Ala Met Ala  vitronectin-wild
901   Gly Gly Ala Gly Gln Pro Gln Leu Ile Ser Arg Asp Trp Phe Gly Leu Pro Gly Lys Leu Asp Ala Ala Met Ala  GG1 VN Mutant
901   Gly Gly Ala Gly Gln Pro Gln Leu Ile Ser Arg Asp Trp Phe Gly Leu Pro Gly Lys Leu Asp Ala Ala Met Ala  RGA2 VN Mutant
901   Gly Gly Ala Gly Gln Pro Gln Leu Ile Ser Arg Asp Trp Phe Gly Leu Pro Gly Lys Leu Asp Ala Ala Met Ala  AGI2-VN Mutant

      Gly His Ile Tyr Ile Ser Gly Ser Ala Pro Ser Ser Pro Arg Ala Lys Met Thr Lys Ser Ala Arg Arg His Arg  Majority
                    330                                 340                                 350
976   Gly His Ile Tyr Ile Ser Gly Ser Ala Pro Ser Ser Pro Arg Ala Lys Met Thr Lys Ser Ala Arg Arg His Arg  vitronectin wild
976   Gly His Ile Tyr Ile Ser Gly Ser Ala Pro Ser Ser Pro Arg Ala Lys Met Thr Lys Ser Ala Arg Arg His Arg  GG1 VN Mutant
976   Gly His Ile Tyr Ile Ser Gly Ser Ala Pro Ser Ser Pro Arg Ala Lys Met Thr Lys Ser Ala Arg Arg His Arg  RGA2-VN Mutant
976   Gly His Ile Tyr Ile Ser Gly Ser Ala Pro Ser Ser Pro Arg Ala Lys Met Thr Lys Ser Ala Arg Arg His Arg  AGI2 VN Mutant

      Lys Arg Tyr Arg Ser Leu Arg Ser Arg Gly Arg Gly Arg Gly Arg Ala Arg Ser Gln Asn Pro Tyr Arg Arg Phe  Majority
                                            360                                 370
1051  Lys Arg Tyr Arg Ser Leu Arg Ser Arg Gly Arg Gly Arg Gly Arg Ala Arg Ser Gln Asn Pro Tyr Arg Arg Phe  vitronectin wild
1051  Lys Arg Tyr Arg Ser Leu Arg Ser Arg Gly Arg Gly Arg Gly Arg Ala Arg Ser Gln Asn Pro Tyr Arg Arg Phe  GG1-VN Mutant
1051  Lys Arg Tyr Arg Ser Leu Arg Ser Arg Gly Arg Gly Arg Gly Arg Ala Arg Ser Gln Asn Pro Tyr Arg Arg Phe  RGA2 VN Mutant
1051  Lys Arg Tyr Arg Ser Leu Arg Ser Arg Gly Arg Gly Arg Gly Arg Ala Arg Ser Gln Asn Pro Tyr Arg Arg Phe  AGI2-VN Mutant
```

Contd.....

```
     Arg Ser Thr Trp Leu Ser Trp Phe Ser Ser Glu Gln Leu Gly Leu Gly Ala Asp Asn Tyr Asp Asn Tyr Glu Met  Majority
                         380                                     390                                     400
1128 Arg Ser Thr Trp Leu Ser Trp Phe Ser Ser Glu Gln Leu Gly Leu Gly Ala Asp Asn Tyr Asp Asn Tyr Glu Met  vitronectin-wild
1128 Arg Ser Thr Trp Leu Ser Trp Phe Ser Ser Glu Gln Leu Gly Leu Gly Ala Asp Asn Tyr Asp Asn Tyr Glu Met  CGD1-VN Mutant
1128 Arg Ser Thr Trp Leu Ser Trp Phe Ser Ser Glu Gln Leu Gly Leu Gly Ala Asp Asn Tyr Asp Asn Tyr Glu Met  RGA2-VN Mutant
1128 Arg Ser Thr Trp Leu Ser Trp Phe Ser Ser Glu Gln Leu Gly Leu Gly Ala Asp Asn Tyr Asp Asn Tyr Glu Met  AGD2 VN Mutant

     Asp Trp Leu Val Pro Ala Thr Cys Glu Pro Ile Gln Ser Val Tyr Phe Phe Ser Glu Asp Lys Tyr Tyr Arg Val  Majority
                                             410                                     420
1201 Asp Trp Leu Val Pro Ala Thr Cys Glu Pro Ile Gln Ser Val Tyr Phe Phe Ser Glu Asp Lys Tyr Tyr Arg Val  vitronectin-wild
1201 Asp Trp Leu Val Pro Ala Thr Cys Glu Pro Ile Gln Ser Val Tyr Phe Phe Ser Glu Asp Lys Tyr Tyr Arg Val  CGD1-VN Mutant
1201 Asp Trp Leu Val Pro Ala Thr Cys Glu Pro Ile Gln Ser Val Tyr Phe Phe Ser Glu Asp Lys Tyr Tyr Arg Val  RGA2 VN Mutant
1201 Asp Trp Leu Val Pro Ala Thr Cys Glu Pro Ile Gln Ser Val Tyr Phe Phe Ser Glu Asp Lys Tyr Tyr Arg Val  AGD2 VN Mutant

     Asn Leu Arg Thr Arg Arg Val Asp Ser Val Ile Pro Pro Tyr Pro Arg Ser Ile Ala                          Majority
                         430                                     440
1276 Asn Leu Arg Thr Arg Arg Val Asp Ser Val Ile Pro Pro Tyr Pro Arg Ser Ile Ala                          vitronectin-wild
1276 Asn Leu Arg Thr Arg Arg Val Asp Ser Val Ile Pro Pro Tyr Pro Arg Ser Ile Ala                          CGD1 VN Mutant
1276 Asn Leu Arg Thr Arg Arg Val Asp Ser Val Ile Pro Pro Tyr Pro Arg Ser Ile Ala                          RGA2 VN Mutant
1276 Asn Leu Arg Thr Arg Arg Val Asp Ser Val Ile Pro Pro Tyr[Leu]Arg Ser Ile Ala                          AGD2 VN Mutant
```

Decoration 'Decoration #1': Box residues that differ from the Consensus.

antiserum but the intensity of color produced was higher in the denatured species (Fig. 4.6b) BSA showed no reaction.

***S.aureus* binding to Vitronectin:**

Solution phase Assay:

The Western blot analysis of refolded VN indicated that wild,GGD1-VN and RGA2-VN predominantly existed as monomer (~ 68 kDa) whereas AGD2-VN as dimer (Fig. 4.7a). *S.aureus* bound to vitronectin as indicated by the presence of VN in *S.aureus* pellet, recovered after incubation with VN (Fig. 4.7b). *S.aureus* control (with no VN added) also reacted with anti-VN antiserum. However, the bands that reacted with antibody had lower size compared to the VN band. This reaction was probably contributed by Protein A on the surface of 8325-4 strain of the organism which has affinity for IgG (Mahawar and Joshi, 2008).

Immobilized Vitronectin Assay:

As the solution phase binding assay is qualitative in nature, *S. aureus*-VN interaction was quantitated by immobilizing VN on to the plastic surface.This assay also facilitated in evaluating the effect of various substances on *S.aureus* binding to VN.There was no significant difference in *S.aureus* binding to wild and mutated RGA2-VN and GGD1-VN whereas *S.aureus* binding to AGD2-VN was slightly less (Fig. 4.7c).

The effect of heparin, EDTA and the RGD peptide is shown in (Fig. 4.7d). Heparin showed no inhibition as the binding was almost similar to that of control (with no heparin added) in all VN preparations. However, the presence of EDTA and the RGD peptide showed varying degree of inhibition.The most pronounced effect was observed with AGD2-VN where the presence of RGD peptide inhibited ~85% of

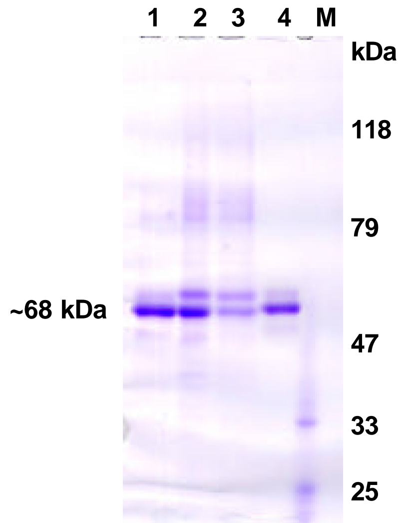


Fig. 4.6a : SDS-PAGE analysis of heparin sepharose purified recombinant vitronectins
Lane 1 : Wild VN**; Lane 2 :** GGD1-VN**; Lane 3 :** RGA2-VN**; Lane 4 :** AGD2-VN**; Lane M :** Pre-stained molecular weight marker

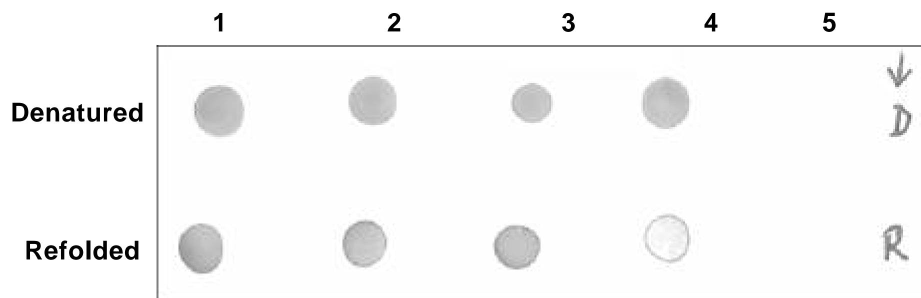


Fig. 4.6b : Dot-blot analysis of denatured and refolded recombinant vitronectins
Lane 1 : Wild VN**; Lane 2 :** GGD1-VN**; Lane 3 :** RGA2-VN**; Lane 4 :** AGD2-VN**; Lane 5 :** BSA control

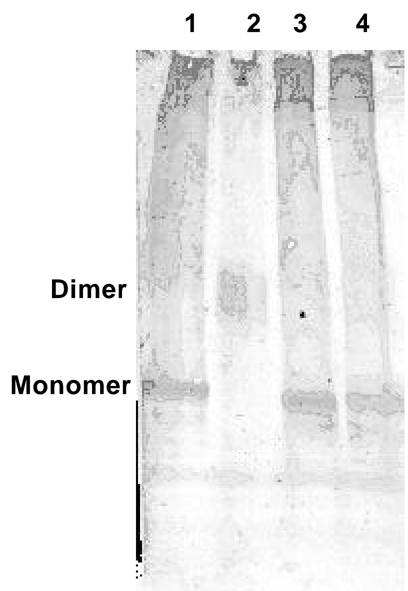


Fig. 4.7a : Western-blot analysis of refolded vitronectins
Lane 1 : RGA2-VN**; Lane 2 :** AGD2-VN**; Lane 3 :** GGD1-VN**; Lane 4 :** Wild VN

Fig. 4.7b : Western blot analysis of vitronectin incubated with *S. aureus*
Lane 1 : *S. aureus* control**; Lane 2 :** *S. aureus* + AGD2-VN**; Lane 3 :** *S. aureus* + Wild-VN**; Lane 4 :** *S. aureus* + RGA2-VN; **Lane 5 :** *S. aureus* + GGD1-VN

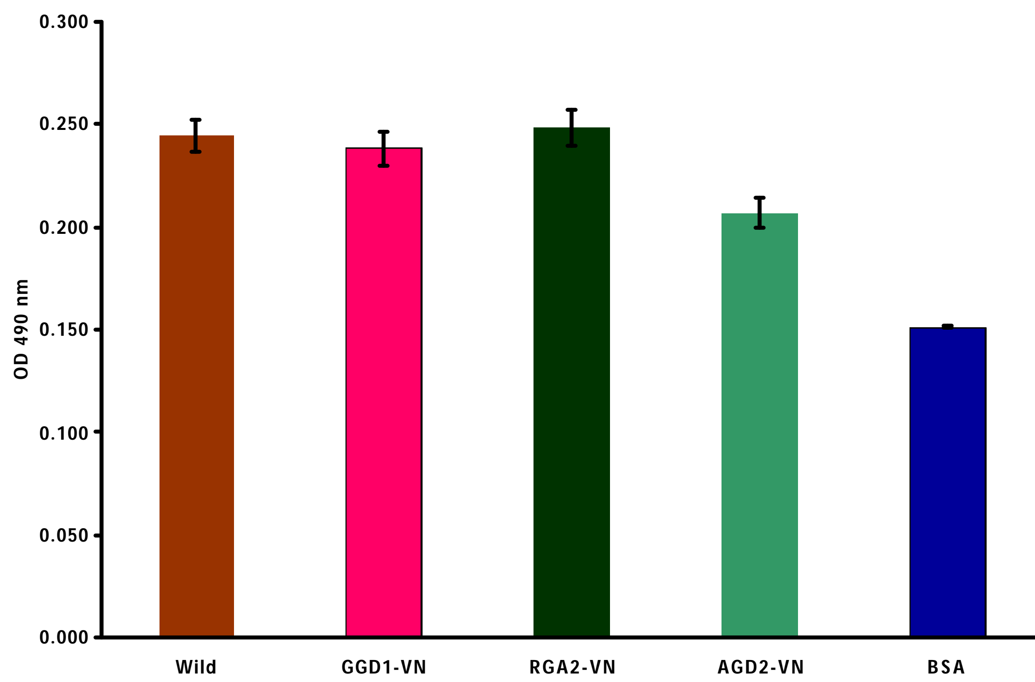


Fig. 4.7c : *S. aureus* binding to different VN substrata

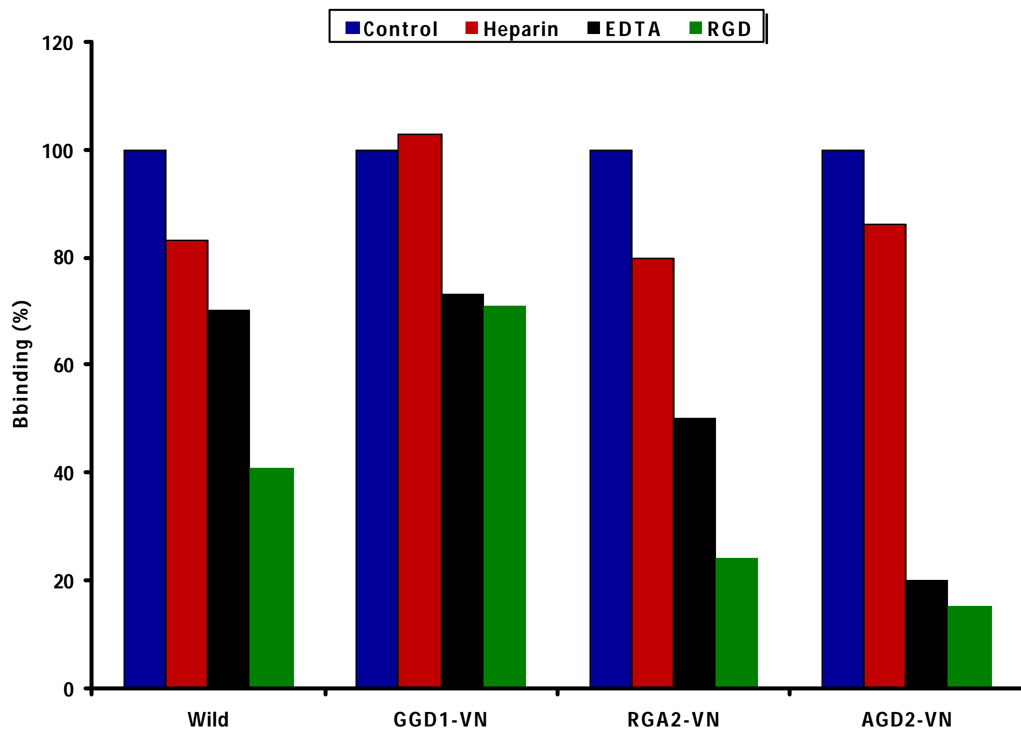


Fig. 4.7d : Effect of heparin, EDTA and RGD peptide on *S. aureus* binding to vitronectin

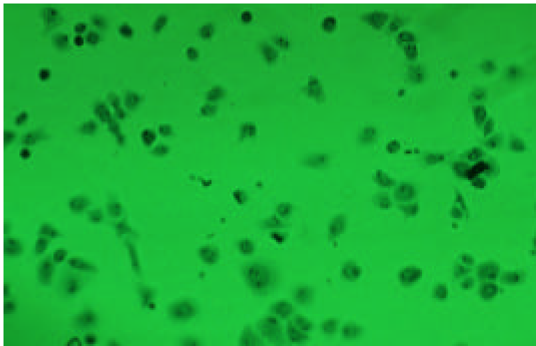
S.aureus binding.The presence of EDTA inhibited ~80% of *S. aureus* binding to AGD2-VN. Similarly, RGD peptide and EDTA inhibited ~76% and 50% binding respectively of *S.aureus* to RGA2-VN.With other mutated protein,GGD1-VN, RGD mediated inhibition was ~29% and EDTA inhibited to ~27%. Infact, *S.aureus* binding to wild VN was ~49% that of the control in the presence of the RGD peptide and 70% when EDTA was included (Fig. 4.7d).

Cell Adhesion Assay:

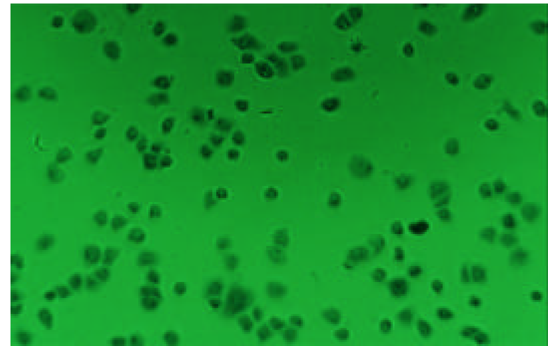
Vero cells and BHK-21 cells adhered and spread on wild VN (Fig. 4.8a and 4.9a). The number of Vero cells adhered was far more than the BHK-21 cells though the concentration of protein used for coating the wells was same in both the cases. Also the protein batch/lot was same in both the cell types. Both the cell types adhered and spread on mutant VN (Fig. 4.8b,c and d). EDTA significantly affected adhesion of both the cell types and the inhibition was more pronounced with Vero cells (50-70% inhibition) (Fig. 4.8e) and the adhered cells were round whereas the BHK-21 cells (Figs. 4.9b,c and d) showed moderate spreading. Cell attachment and the morphology of adhered cells were not affected by external heparin except for minor inhibition of BHK cells with AGD2-VN and GGD1-VN without noticeable change of adhered cell morphology (Fig. 4.9b and 4.9d). Presence of soluble RGD caused low to moderate inhibition of Vero cell binding to different VN (Fig. 4.8e). With BHK cells, RGD mediated inhibition was observed only with AGD2-VN and GGD1-VN mutants (Fig. 4.9e).

Fig. 4.8 : Adhesion and spreading of Vero cells on different VN substrata (10x)

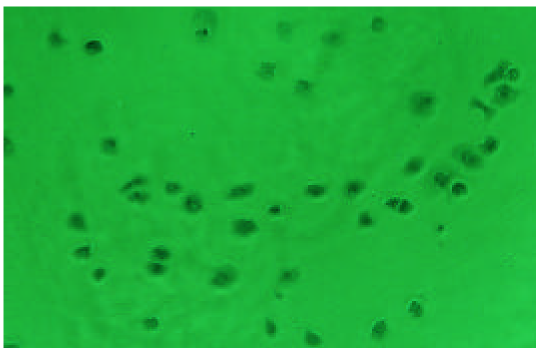
(A) Wild -VN



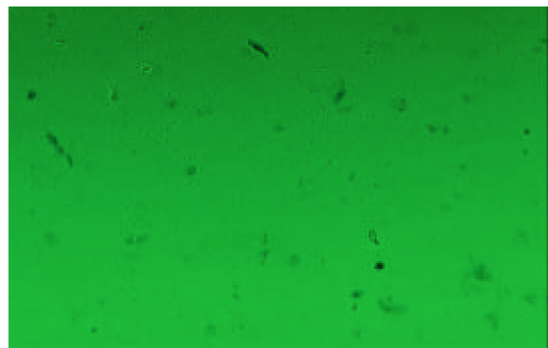
Cells only



Cells + heparin

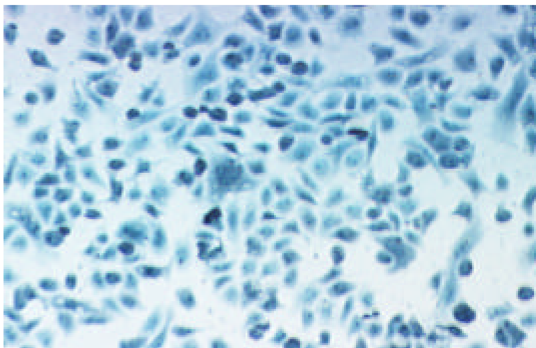


Cells + RGD peptide

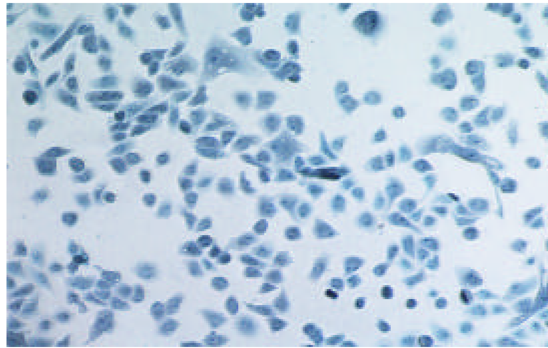


Cells + EDTA

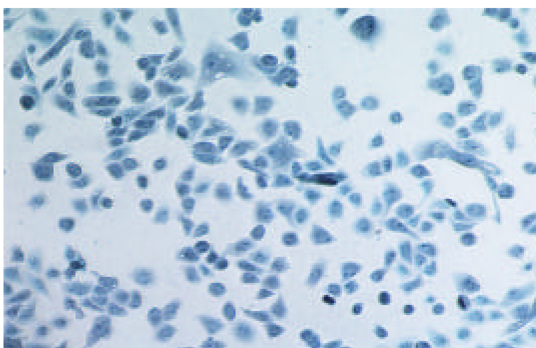
(B) GGD1 -VN



Cells only



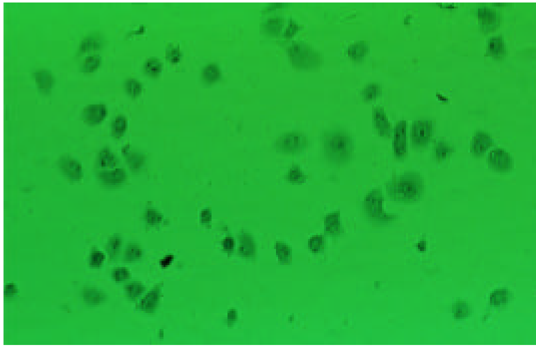
Cells + heparin



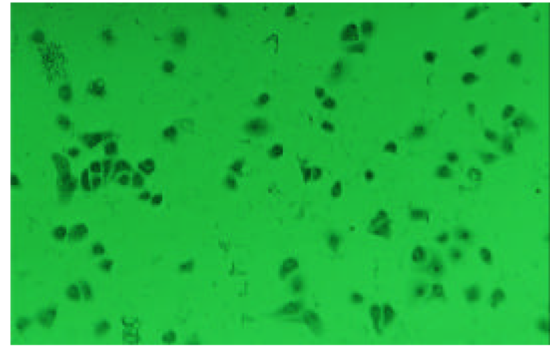
Cells + RGD peptide

Cells + EDTA

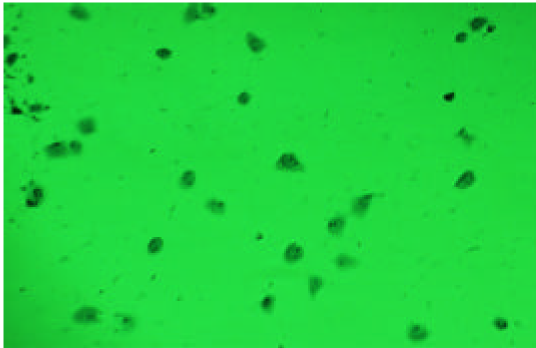
(C) RGA2 -VN



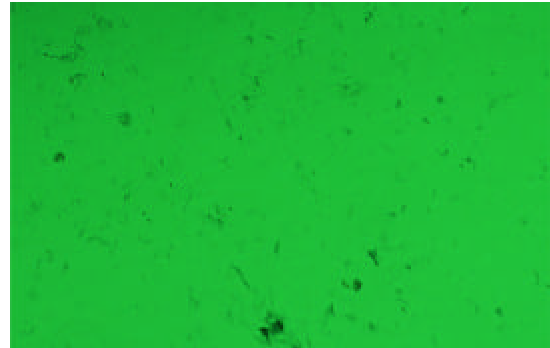
Cells only



Cells + heparin

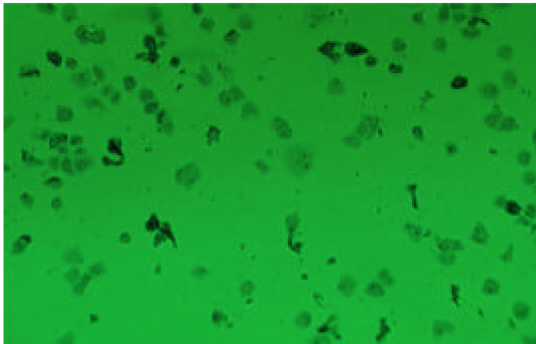


Cells + RGD peptide

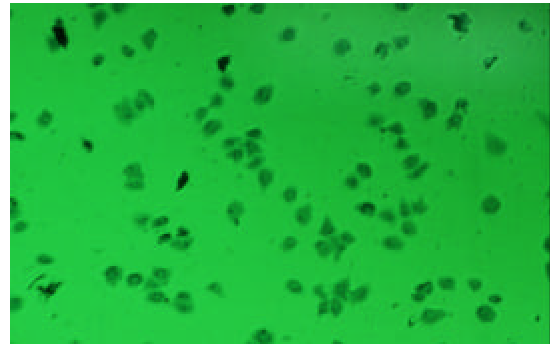


Cells + EDTA

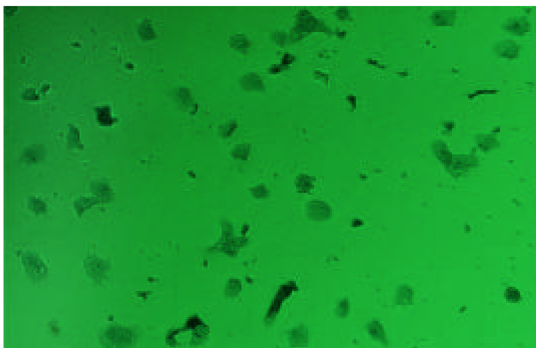
(D) AGD2 -VN



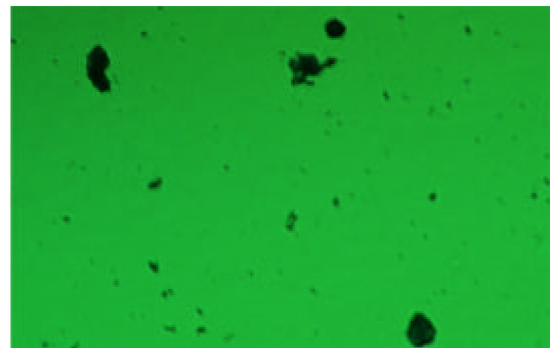
Cells only



Cells + heparin



Cells + RGD



Cells + EDTA

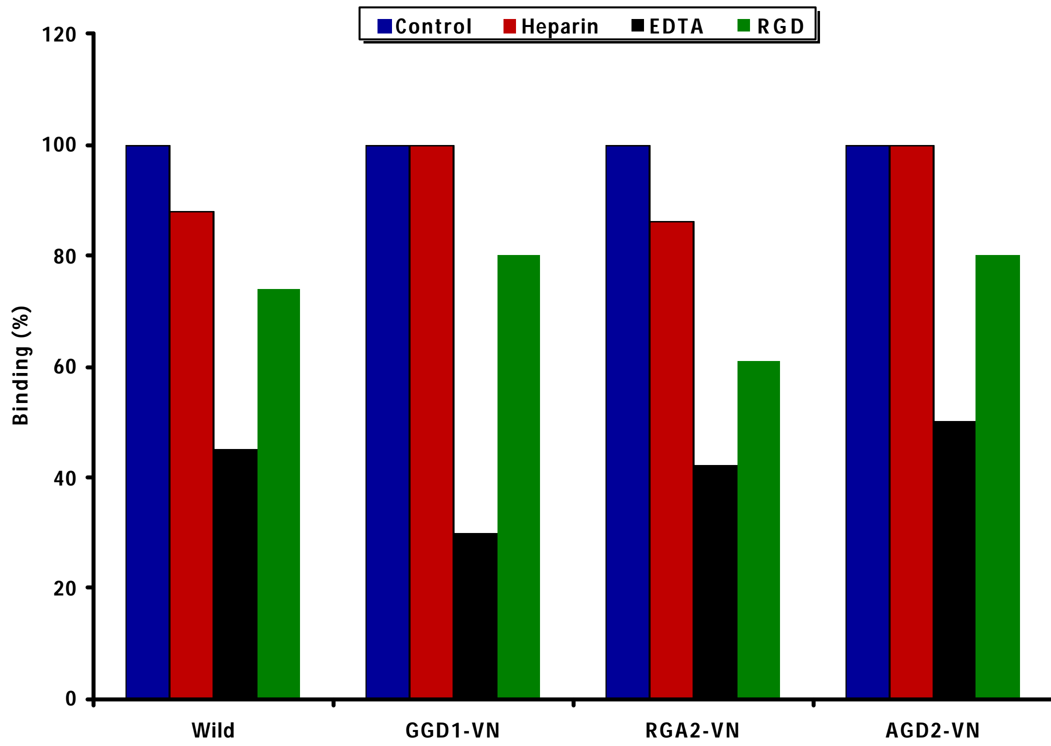
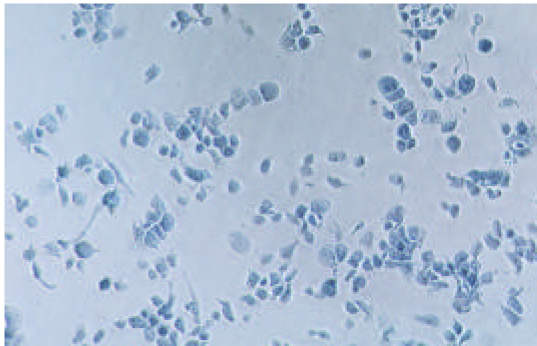


Fig. 4.8e : Effect of heparin, EDTA and RGD peptide on Vero cell attachement to VN

Fig. 4.9 : Adhesion and spreading of BHK-21 cells on different VN substrata (10x)

(A) Wild -VN



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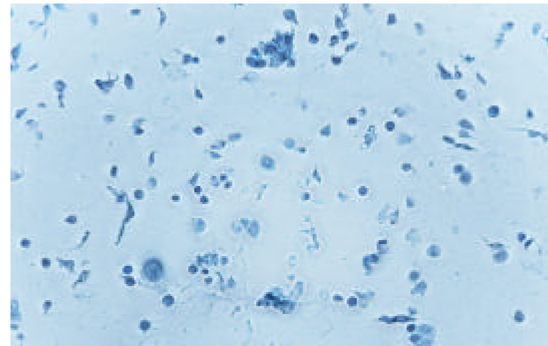
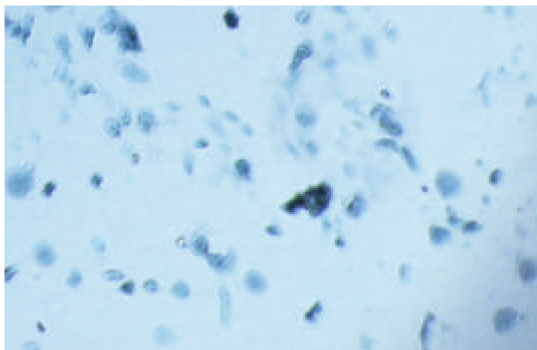
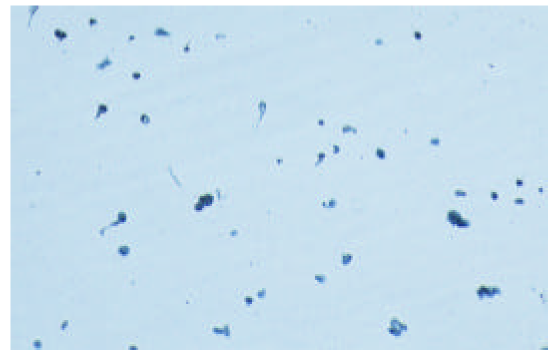


Cells + heparin

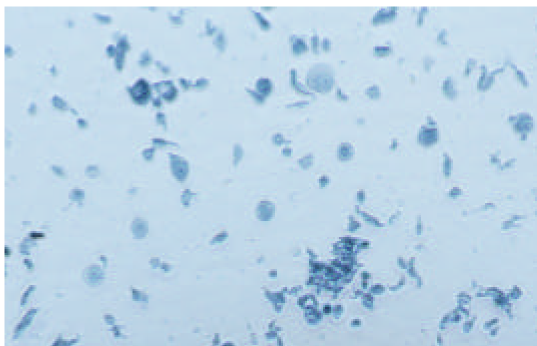


Cells + RGD peptide

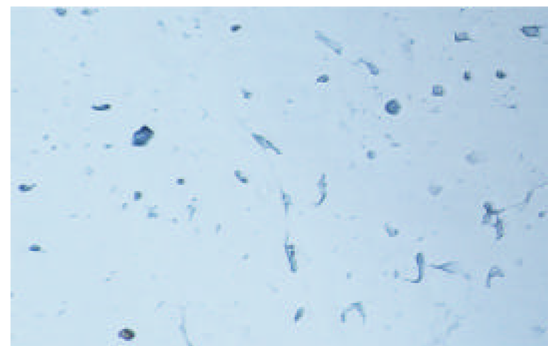


Cells + EDTA

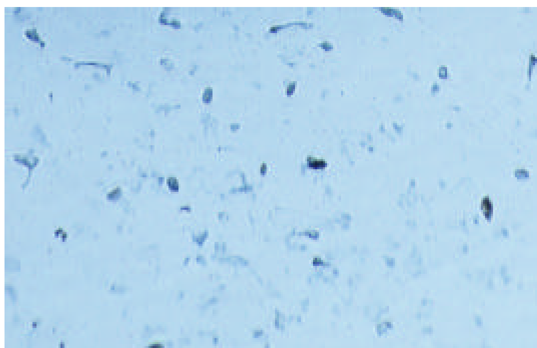
(B) GGD1 -VN



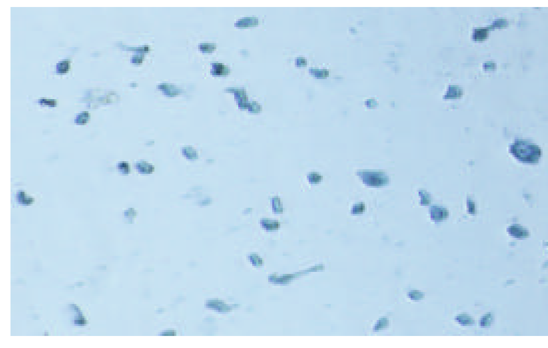
Cells only



Cells + heparin

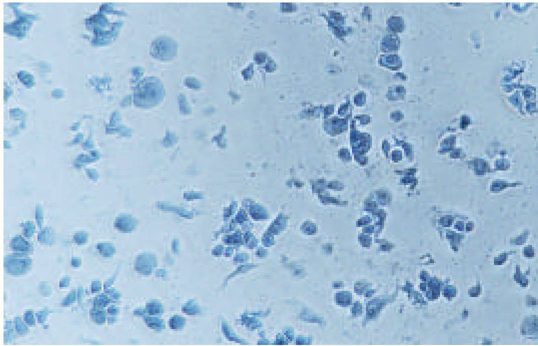


Cells + RGD

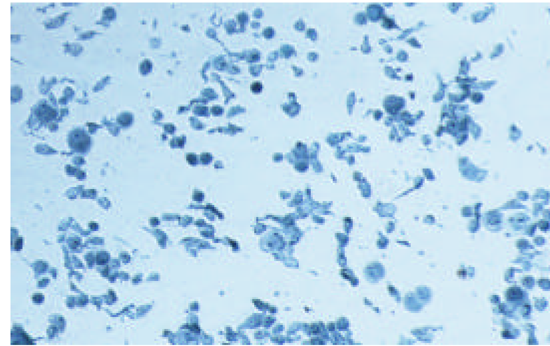


Cells + EDTA

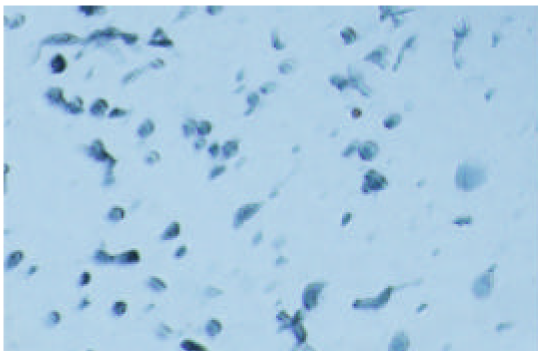
(C) RGA2 -VN



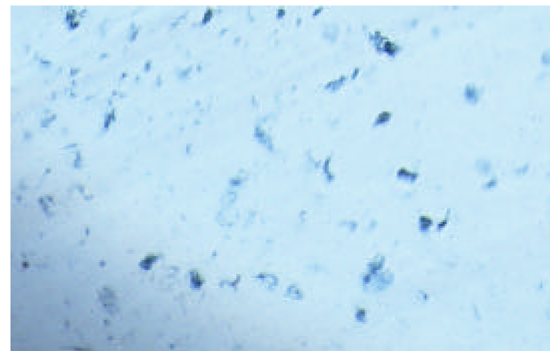
Cells only



Cells + heparin

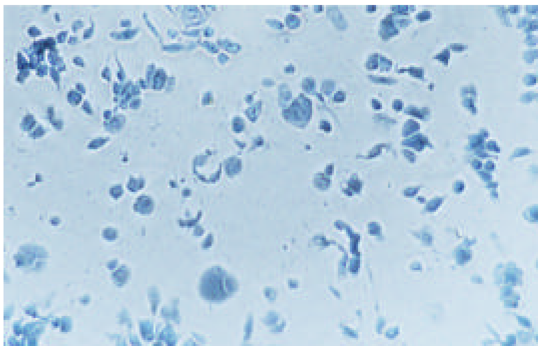


Cells + RGD peptide

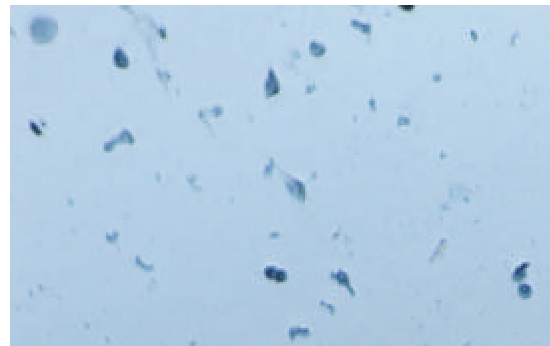


Cells + EDTA

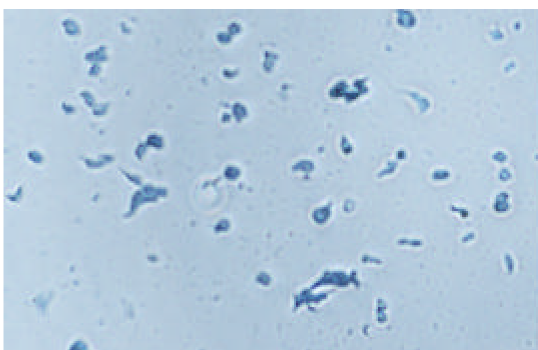
(D) AGD2 -VN



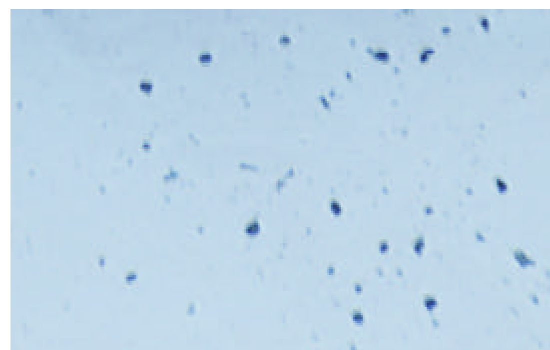
Cells only



Cells + heparin



Cells + RGD



Cells + EDTA

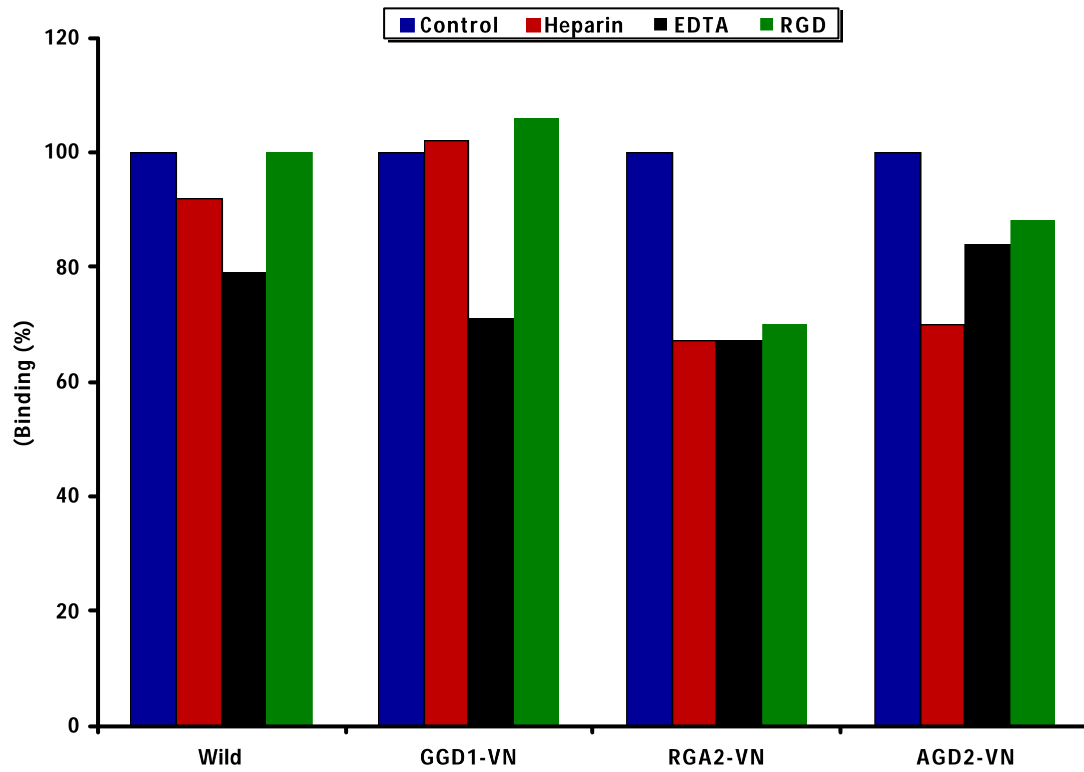


Fig. 4.9e : Effect of heparin, EDTA and RGD peptide on BHK-21 cell attachement to VN

Discussion

5 Discussion

This study was primarily directed to analyze structure-function relationship of RGD motifs in goat VN. Goat and bovine VN are unique in possessing two RGD motifs (Mahawar and Joshi, 2008 and bovine Accession BC103132), whereas VN from all other species studied so far have only one RGD motif (Kitagaki-Ogawa *et al.*,1990 and Nakashima *et al.*,1992).

The overlap extension PCR approach for generating RGD mutants of goat VN was highly successful. The targeted residue was replaced with one having a smaller side chain to ensure no structural alterations. The first RGD of goat VN, occupying the same position as that of human VN (residues 45-47), was mutated to GGD. The mutated protein had two other alterations at positions 267 and 400 where the C was replaced with W and T with M respectively. These unwanted changes in the protein moiety account for < 0.5% alterations (the mature goat VN has 444 residues). The W has slightly bulkier side chain compared to C whereas T has a smaller size than M. Therefore, these replacements should nullify each other's any adverse effect on the structural integrity of the molecule. Further, refolding of denatured VN to soluble form with no precipitation further suggest correct protein folding.

Another unwanted change was observed in the second RGD

(positions 106-108), which was mutated to AGD. The undesired alteration took place at residue 440, P replaced with L. This change is very close to the C-terminus of the protein, far away from the RGD motifs, and unlikely to cause any structural alteration as the replacement is with a moiety of about similar size.

S. aureus bound to VN in solution phase as well as solid phase assay in agreement with previous observation (Mahawar and Joshi, 2008). No significant difference in S. *aureus* binding to wild and GGD-1 and RGA-2 mutant VN were observed. The AGD-2 showed somewhat less *S. aureus* binding. The reason for this is not clear though this mutant VN existed predominantly in the dimer state (~90-95% as judged by SDS-PAGE and Western blot) after refolding. *S. aureus* binding to AGD-2 mutant VN was greatly inhibited (~85%) in the presence of RGD peptide suggests involvement of this residue in *S. aureus* binding and that this region is exposed on the surface of the protein. Similarly, mutation of D in RGD-2 to RGA-2 also caused ~75% reduction in *S. aureus* binding when soluble RGD peptide was included in the assay. Thus, both the charged residues of RGD-2 are involved in supporting *S. aureus* binding. The noticeable inhibition of *S. aureus* binding to AGD-2 and RGA-2 by EDTA suggests involvement of divalent cations in RGD dependent *S. aureus* binding. Two important points emerged from the above observations. First, in the absence of one charged residue of the RGD-2 motif, the second charged moiety could support *S. aureus* binding. However, this binding crumbles in the presence of excess soluble RGD peptide. Second, why RGD-1 did not overcome the effect of added RGD peptide during *S. aureus* binding to AGD-2 or RGA-2 since only 20% reduction in *S. aureus* binding to mutated RGD-1 i.e GGD-1 was observed? This suggests that the RGD-1 is not involved in *S. aureus* binding and the observed bacterial binding with GGD-1

mutant VN was probably mediated by RGD-2 motif. It is, therefore, likely that the RGD-1 may be cryptic in goat VN similar to its human homologue (Seiffert and Smith,1997). In goat VN, the RGD-2 is flanked by residues (GRGDS) that are known to enhance adhesive character of the protein (Pierschbacher and Ruoslahti,1984 andWegener *et al.,*1998). In addition, the flanking proline residues in goat VN (PGRGDSDP) should favour β-turn loop thus exposing the second RGD region onto the surface where it can interact with other ligands. Sequence analysis on PROTEAN programme of DNA STAR (Emini *et al.,* 1985) suggests surface orientation of this region whereas the first RGD motif as cryptic. However, the precise orientation of both the RGD motifs would be known once the crystal structure of goat VN is deduced.

Heparin caused negligible to no inhibition of *S. aureus* binding to wild or mutant VN. This is in contrast to an earlier observation which suggested involvement of residues 347-361 of human VN in facilitating *S. aureus* binding. These residues are part of the heparin-binding region of the protein (Francois *et al.,* 1999). Previous study from this laboratory has demonstrated that a 22 kDa C-terminal fragment of goat VN with complete heparin-binding site supported *S. aureus* binding whereas a smaller fragment (14 kDa) lacking residues 323-362 (truncated heparin-binding site) did not support *S. aureus* binding (Mahawar and Joshi,2008). In the present study, the amount of heparin added was 20 times the amount of VN used for coating the wells and it remained in the wells during *S. aureus*-VN interactions. The orientation of the heparin-binding site in human VN is still not clear. Some authors have demonstrated surface orientation of this site (Zhuang *et al.*, 1997, Preissner, 1990, Sobel *et al.,* 1992 and Stockmann *et al.*,1993) whereas other studies have shown this site as cryptic (Barnes *et al.*,1985 and Hayashi *et al.*,1985). It is noteworthy that very little (<5%) plasma VN

bind to heparin-Sepharose under physiological conditions, and this amount can be increased significantly after denaturation of plasma with urea and thus exposing the buried heparin binding site (Yatohgo *et al.,* 1988). The conformation of the heparin binding site in the intact protein may differ significantly compared to smaller fragments because of folding constraints and thus the results of binding experiments with fragments can not be extrapolated to VN molecule.

It is well established that the attachment of different cell types to extracellular matrix proteins is mediated by cellular receptors, integrins, through RGD motif present in the protein (Schvartz *et al.*,1999;Hess *et al.*,1995 and Huang *et al.*,1998). Attachment of Vero cells and BHK21 cells on mutant VN with spreading comparable to that seen with wild VN suggest that change of one charged residue of the RGD motif is not sufficient to disrupt cell binding. The EDTA mediated inhibition of cell-VN interaction reflects involvement of integrin as cellular receptor. The identity of the integrin(s) involved is yet to be established. Surprisingly, moderate to low inhibition of Vero cell binding was observed in the presence of RGD peptide. The inhibition was still low with BHK cells. The concentration of RGD used was sufficiently high (500ug/ml). This situation is not unique. Inhibition (~70%) of cell attachment to fibronectin was achieved at mg/ml concentration of RGD peptide (Pierschbacher and Ruoslahti,1984). It is not clear why no inhibition of BHK cell binding to wild VN or RGA-2 mutant with RGD peptide was evident. Probably, a different kind of integrin than that involved in Vero cell binding may be involved with a stronger affinity for wild VN and RGA-2 mutant. This needs further investigation/experimentation.

The present study has relevance in bacterial pathogenesis. The identification of *S. aureus* binding site in goat VN may stimulate further research to elucidate the mechanism of *S. aureus* colonization which

has wider application in human and animal diseases. Based on the results, a scheme is proposed for *S. aureus* colonization in the tissues according to which the initial binding of the bacteria to VN takes place via the RGD-2 motif. This binding may cause structural changes in the VN molecule exposing the RGD-1 which may subsequently facilitate binding to epithelial or other cell types (Seiffert and Smith,1997). The role of heparin binding region seems unclear in this process as the external added heparin showed no inhibitory effect either on *S. aureus* or cell binding. The above proposal is being tested experimentally.

Summary and Conclusions

6 Summary and Conclusions

Vitronectin is a multifunctional, adhesive glycoprotein present in blood and extracellular matrix. It promotes cell adhesion, spreading and migration by interaction with specific integrins as well as with the urokinase receptor (μPAR). VN along with other extracellular matrix proteins play an important role in bacterial adhesion to the host cells. Among the animal vitronectins, bovine and goat are unique in having two Arg-Gly-Asp (RGD) motifs instead of one seen in other species. The present study was primarily directed to decipher the exact roles of the 2 RGD motifs in cell spreading and *S. aureus* binding. The structure-function relationships of this protein was elucidated by site-directed mutagenesis of the 2 RGD motifs by *in vitro* overlap extension PCR strategy. One mutant was generated for the first RGD motif where R was replaced with G (Glycine), GGD1-VN and two mutants were generated for the second RGD motif viz. RGA2-VN and AGD2-VN. The mutants were obtained after PCR amplification and cloning in pPROExHTa (prokaryotic expression system) and expressed in *E. coli*, (DH5α) cells. The expressed mutants were purified by heparin-sepharose chromatography using established protocols. Experiments were conducted with *S. aureus* strain, 8325-4 in both solution and solid phase to assess the interaction with different VN mutants.

Though no significant difference was observed in *S. aureus* binding to with wild, GGD1 and RGA-2 mutants, less binding was observed with mutant, AGD2-VN. Inhibition in the presence of RGD peptide with respect to AGD2-VN, suggests role for the second RGD motif in *S. aureus* binding. Moreover, both the charged residues (R&D) are implicated in *S. aureus* binding. Inhibition in the presence of EDTA suggests involvement of divalent cations in the RGD dependent binding of *S. aureus.* Moreover the experiments demonstrated that RGD2 is involved in *S. aureus* binding. It is likely that RGD-1 may be cryptic in goat vitronectin. Clearly further experiments are warranted to define the precise orientation of the RGD motifs. No inhibition of *S. aureus* binding to VN in the presence of added heparin suggests that the heparin binding site of VN may not be involved in *S. aureus* binding or is not accessible.

Experiments were performed to asses vero and BHK-21 cells adherence to different VN substrata. Results suggested that change in only one charged residue of the RGD was insufficient to abolish cell binding as the mutant VN showed equal cell adhesive characteristic. The EDTA mediated inhibition indicates involvement of integrins in cell adhesion. Moderate to low inhibition of cell adhesion was observed with the inclusion of RGD peptides on few VN substrata.

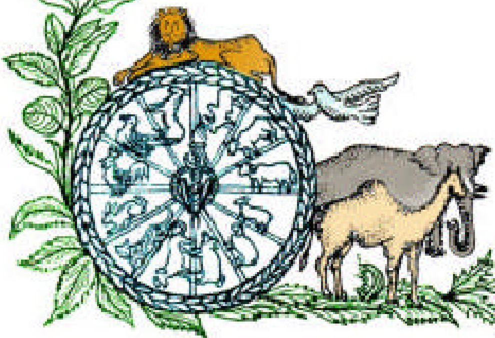
The present study has evolved a scheme for *S. aureus* colonization in the tissues. Accordingly, the initial binding of *S. aureus* to VN takes place via the RGD-2 motif and this causes a structural change in the VN molecule exposing the RGD1 motif, which may facilitate binding to cells. Further experiments to deduce the crystal structure of vitronectin may throw more light and corroborate the above results.

Mini Abstract

7 Mini Abstract

Vitronectin is a multifunctional adhesive glycoprotein present in blood and extracellular matrix. Goat vitronectin characterized previously in our laboratory is unique having 2 RGD motifs. The present study was directed at deciphering the roles of the RGD motifs by employing the technique of PCR based site directed mutagenesis. Three mutants, one for the first RGD motifs and two for the second RGD motifs were generated namely GGD1-VN, RGA2-VN and AGD2-VN. The mutants were analyzed for their ability to bind *S. aureus* and cell attachment property. Based on the results of our experiments, we conclude that the mutant AGD-2 showed less binding to *S. aureus* compared to the other 2 mutants probably due to its dimeric state. Divalent cations are essential in the RGD dependent binding and the second RGD motif is involved in *S. aureus* binding. The cell attachment of Vero and BHK-21 cells on the mutant vitronectin substrata showed that a single residue change in the RGD motifs did not abolish cell attachment. Further the role of integrins is implicated in cell adhesion. The present study has evolved a scheme for *S. aureus* colonization, where RGD2 motif is involved in initial binding to *S. aureus* and structural alterations of the protein leads to exposure of the RGD1 motif, which enhances the cell attachment property. Further experiments to deduce the crystal structure of wild and mutant vitronectins may throw more light and corroborate the above results.

लघु सारांश



8 लघु सारांश

विट्रोनेक्टीन एक बहु कार्यालीन आसंजक ग्लैको प्रोटिन है जो रक्त एवं बाह्य कोशिकीय मैट्रीक्स में मौजूद है पहले हमारे प्रयोगशाला में विश्लेषित बकरी विट्रोनेक्टीन में 2 आरजीडी स्पांकन अनूठा है। वर्तमान अध्ययन पीसीआर आधारित उतपरिवतीय निर्देशित स्थित तकनीक को नियोजीत करने से आरजीडी स्पांकनों की भूमिकाओं का गुढ़ रहस्य को खोलने के लिए निर्देश दिया गया। तीन उत्परिवर्ती, प्रथम आरजीडी स्पांकन के लिए एक तथा द्वितीय आरजीडी स्पांकन के लिए दूसरा उत्पन्न किया गया जो यथा जीजीडी1 वीएन, आरजीए–2 वीएन एवं एजीडी2 वीएन थे। विश्लेषित उत्परिवर्तित से एसआरएस एवं कोशिका संलग्न गुणस्वामान के बधन क्ष्ज्ञमता का विश्लेषण किया गया। हमारे प्रयोग के आधार पर निष्कर्ष निकाला गया कि उत्परिवर्ती एजीडी–2 की बंधन क्षमता एस आरयस के साथ अन्य 2 उत्परिवर्ती के तुलना में कम देखा गया, डाइमेटीक अवस्था के कारण 1 आरजीडी निर्भर बंधन में द्विसंयोजक कटायनों का होना आवश्यक है, एवं दूसरे आरजीडी स्पांकन एस आरयस बंधन में शामिल है। तेरो और उत्परिवर्ती विट्रोनेकटिन अधः स्तर पर बीएच के–21 कोशिकाओं में कोशिका लगात देखा गया जोकि आर जीडी स्पांकनों में एक भी छाछ परिवर्तन कोशिका लगान समाप्त नहीं हुआ था। इसके अलावा इन्टीगरीन की भूमिका कोशिका आसंजन में फंसा है। वर्तमान अध्ययन एस ओरिएस के उपनिवेश को विकसित करता है, जहाँ आरजीडी2 रूपांकन एस आरिएस के साथ शुरूआती बंधन में शामिल है और प्रोटिन के संरचनात्मक प्रत्यावर्तन आगे बढ़कर आरजीडी1 की पुष्टि करता है, जो कोशिकीय लगाव गुणात्मकता को बढ़ावा देता है। इसके अतिरिक्त अगली प्रयोग से जंगली एवं उत्परिवर्ती विट्रोनेक्टीन के स्फटीक संरचना को प्रदर्शित करता है, जो शायद उपर के परिणाम को ज्यादा प्रकाश एवं प्रबल पुष्टि करता है।

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Appendix

Appendix

SOLUTIONS FOR AGAROSE GEL ELECTROPHORESIS

Tris-acetate-EDTA (TAE) Buffer (50 X)

Tris base	24.2 g
Glacial acetic acid	5.71 ml
0.5 M EDTA	10.0 ml

Volume made up to 100 ml with double distilled water, sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle and stored at room temperature.

Ethidium Bromide solution (10 mg/ml)

10 mg of Ethidium bromide was dissolved in one ml of autoclaved double distilled water, wrapped in aluminium foil and stored at 4ºC.

Gel loading dye (6 X)

Bromophenol blue	0.25%
Sucrose in water	40.0%

Filtered through 0.22 µm filter and stored at 4ºC

SOLUTIONS (MEDIA FOR BACTERIAL CULTURE)

LB (Luria-Bertani) Broth

Tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	10.0 g

Dissolved in 950 ml of distilled water, pH was adjusted to 7.0 using 5 N NaOH; volume was made to one liter and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle

LB Agar

1.5 % Bacto Agar was added in LB broth and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle

BHI (Brain-Heart Infusion) Broth

37.0 g of BHI broth was dissolved in 950 ml of water. pH was adjusted to 7. After making the volume 1 liter, media was sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle.

SOB medium

Tryptone	2.0 g
Yeast extract	0.5 g
NaCl	0.05 g
250 mM KCl	1 ml
Distilled water	90 ml

Adjust the pH to 7.0 with 5 N NaOH. Volume made to 100 ml and sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle.

SOC medium

Sterile SOB	800 µl
2 M $MgCl_2$ (filter sterilized)	4 µl
1 M Glucose (filter sterilized)	16 µl

2 M Mg^{++} Solution

2 M $MgCl_2$ and 2 M $MgSO_4$ were prepared separately and mixed in equal volume and filter sterilized through 0.22 µm filter.

TSS

2 X LB medium	20 ml
30 % w/v PEG-8000	20 ml
2 M Mg^{++} solution	0.6 ml (filter sterilized)

Autoclaved distilled water and DMSO 17 ml + 3 ml

LB medium and PEG-8000 autoclaved at 15 psi for 20 minutes.

IPTG (100 mM)

IPTG	23.83 mg
Distilled water	1.0 ml

Sterilized by filtration through 0.22 mm filter and stored at –20°C.

SOLUTIONS FOR PLASMID ISOLATION

P_1 (Resuspension buffer)

Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM
Glucose	50 mM

Sterilized by autoclaving and stored at 4°C

P_2 (Lysis buffer)

NaOH	0.2 N
SDS	1 %

Sterilized by autoclaving and stored at room temperature.

P_3 (Neutralization buffer)

5 M Potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
Double distilled water	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. The solution was sterilized by autoclaving and stored at 4°C.

SOLUTIONS FOR SDS-PAGE

Acrylamide-bisacrylamide solution (30%)

Acrylamide	30 g
Bisacrylamide	0.8 g

Volume made to 100 ml with distilled water, stirred to mix well, filtered and stored at 4°C in brown coloured bottle.

8 X Separating gel buffer (pH 8.6)

1 N HCl	96 ml
Tris	73.2 g
TEMED	0.46 ml
SDS	1.6 g

Volume made to 200 ml with distilled water, filtered and stored at 4°C.

Stacking gel buffer (pH 6.8)

Tris	12.1 g
TEMED	232 ml
SDS	3.2 0g

Adjust the pH and then make up the volume to 100 ml with distilled water, filtered and stored at 4°C.

5 X Sample-loading buffer (pH 6.8)

Tris	1.89 g
SDS	5 g
Glycerol	25 ml
Bromophenol blue	
Few crystals	

Dissolve the Tris base in small amount of water and adjust the pH, then add SDS and glycerol finally make up the volume to 50 ml with distilled water.

Electrode buffer (running buffer)

Tris	1.5 g
Glycine	7.2 g
SDS	0.5 g

Volume made to 500 ml with distilled water.

Staining solution

Acetic acid	10 %
Methanol	50 %
Coomassie brilliant blue	0.10 %

Mix the dye on magnetic stirrer for 4-6 hours filtered and volume was made with distilled water and stored in brown bottle.

Destaining solution

Acetic acid	5 %

Ammonium per sulfate

20 % solution in distilled water

SOLUTIONS FOR WESTERN BLOT

Transfer / Electrode buffer

Tris	3.0 g
Glycine	14.4 g
Methanol	10 % (v/v)

Volume made to 1 liter with distilled water.

DAB substrate solution

2.0 M Tris (pH 7.4)	1.25 ml
DAB	20 mg
8 % nickel chloride	150 ml

Mix and make up to 25 ml with distilled water, filter the solution, then add 7.5 ml of H_2O_2 just before use.

PBS-Tween 20

0.05 % Tween-20 in PBS

Amido black stain

0.05 % amido black in 5 % acetic acid and 5 % methanol. Filtered and stored at room temperature in brown coloured bottle.

MISCELLANEOUS BUFFERS AND SOLUTIONS

Phosphate buffered saline (pH 7.4)

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
Na_2HPO_4 (anhydrous)	1.14 g

Dissolved and volume made to 1000 ml with distilled water.

PMSF

100 mM stock was prepared in 95 % Ethanol and 5 % Isopropanol. Stored at -20ºC in aliquates.

HEPES Buffered Saline (pH 7.2-7.4)

HEPES Sodium salt (10 mM), NaCl (145 mM), KCl (5 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM) and Glucose (5 mM). All salts were autoclaved, while glucose was filter sterilized.

OPD Substrate solution

Citric acid	85 mg
Na_2HPO_4	195 mg
Distilled Water	10 ml
OPD	4 mg
H_2O_2	10 ml

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