STUDIES ON THERMOSTABILIZATION OF PROTECTIVE ANTIGEN OF *B. anthracis*

Thesis submitted to the Jawaharlal Nehru University
in fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY
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CERTIFICATE

This is to certify that the work titled **'Studies On Thermostabilization of Protective Antigen of *B. anthracis*'** submitted to the Centre for Biotechnology, Jawaharlal Nehru University, New Delhi-110067, in fulfillment of the requirements for the award of the degree of Doctor of Philosophy, embodies faithful record of original research work carried out by **Samer Singh**. He has worked under our guidance and supervision. This work is original and has not been submitted so far in part or full for any other degree or diploma of any other university.

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Encouragement, Love & Support from my Mother, Father, Brother and beloved Family members can not be described by words……, I am their…

Samer Singh

ABBREVIATIONS

$A_{280/350/595/600}$	Absorbance at 280/350/595/600 nm
Ado.	Adonitol
ADP rib.	Adenosine Diphosphate ribosyltransferase
Amp	Ampicillin
APS	Ammonium persulfate
ATCC	American type culture collection
ATP	Adenosine Triphosphate
Bet.	Betaine
bp	Base pair
BSA	Bovine Serum Albumin
^{o}C	Degree celsius
C terminal	Carboxy terminal
DAB	Diamino Benzidine
ddH_2O	Double Distilled water
ddNTP	Di-Deoxyribose nucleotide
DMG	Dimethyl glycine
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
EF	Edema Factor
Ery.	Erythritol
FCS	Fetal Calf Serum
FPLC	Fast pressure Liquid Chromatography
g	Gravitational force
Gly.	Glycine
Glyl.	Glycerol
Gms	Grams
HRP	Horseradish peroxidase
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2 ethanesulfonic acid)

hr./hrs.	Hour/hours
IPTG	Isopropyl-β-D-thio-galactosidase
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
LF	Lethal Factor
LT	Lethal Toxin
M	Molarity
mg	Milligram
ml	Milliliter
min./mins	Minute/Minutes
mM	Milli Molar
MTT	3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide
N	Normailty
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
N terminal	Amino-Terminal
O/N	Over night
PA	Protective antigen
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PMSF	Phenyl Methyl Sulfonyl Fluoride
rpm	Revolutions per minute
RNase	Ribonuclease
RPMI	Roswell Park memorial Institute
Sar.	Sarcosine
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophorsis
Sec.	Second
Sor.	Sorbitol

$T_{1/2}$:	Temperature at which 50% activity is retained after 20 min. of incubation
TAE	Tris acetate EDTA
TEMED	N,N,N',N' tetramethyl ethylene diamine
Trh.	Trehalose
Tris.	Tris (hydroxymethyl) amino methane
TTBS	Tris buffered Saline with Tween 20
U	Unit
UV	Ultra Voliet
x g	Times gravity (centrifugal force)
Xyl.	Xylitol
β-ME	Beta mercaptoethanol
µg	Microgram
µl	Microliter
%	Percentage
~	Approximately

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CHAPTER 1: GENERAL INTRODUCTION

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1.1 ABOUT ANTHRAX

Anthrax has remained a disease of great concern to human beings since time immemorial. Though herbivores are on a direct threat, humans have faced the wrath of this disease both in ancient times and the present era. Anthrax derives its name from black scars or coal like (*Anthrakis* = Coal in Greek) lesions on skin in the case of cutaneous anthrax. Evidence dates back to the time of Moses. The fifth and sixth plagues of Egypt, described in the book of Genesis (Exodus 9), are widely believed to have been anthrax. Clinical cases are reported in ancient Roman and Hindu writings as well. Virgil (25 B.C.) described anthrax in his writings (Dirchx, 1981). In medieval times the "Black bane" that swept across the Europe and caused large number of human and animal deaths, is believed to be anthrax (Turnbull, 1991). Although it was the first disease for which etiological agent was determined (Koch, 1876; Jay, 2001) and based on those findings Koch gave his famous postulates, yet it still remains among the less studied diseases. Anthrax has the distinction of being the first bacterial disease against which vaccine was developed. Louis Pasteur (1881) and William Greenfield (1880) were the pioneers of anthrax vaccination (Zydowicz, 1998). Their work laid the foundation of experimental immunology.

Anthrax mainly infects wild and domestic herbivores e.g., Sheep, cattle, horse, pig, goat, camel, antelope, bison, elephant etc. (Koehler, 2000). Humans acquire disease from infected animals. It is not contagious in strict sense but can be acquired by ingesting contaminated meat or handling contaminated animal products such as meat, fur, hides, etc or even through body fluids. Therefore animals suspected of dying from anthrax must be handed carefully and livestock in endemic area must be vaccinated. The disease is initiated by entry of causative organism *B. anthracis*, a gram positive, spore forming bacteria into the host body via minor abrasions, an insect bite or by eating contaminated meat or inhaling air born spores. Disease is characterized by high fever, spleen enlargement and swollen neck and throat. Depending upon the route of entry and organs involved, it is of following four types:

1. **Cutaneous Anthrax:** Accounts for 90-95% cases. It has an incubation period of 1-10 days. It starts with lodging of spores in skin through abrasions or insect bite. Clinically it begins as small painless prutic papule that generally develops in to a

vesicle or ring of vesicle surrounded by ring of erythema. The fatality rate in case of cutaneous anthrax is 20% without antibiotic treatment and <1% with antibiotic treatment (Brachman and Kaufmann, 1998; Dixon, 1999; Ashford *et al.*, 2000).

2. **Pulmonary anthrax (Woolsorters disease)**: It is acquired by inhaling the spores. Most of the time it is an occupational hazard in wool industry dealing with the processing of wool and leather products. It is the most fatal form and mortality rate is more than 95% in untreated cases.
3. **Gastro-intestinal Anthrax**: Commonly occurs in animals grazing over contaminated pastures and in humans by ingesting inadequately cooked contaminated food products. It is rare in humans. The fatality rate of gastrointestinal anthrax is unknown but is estimated to be 25-60% (Brachman and Kaufmann, 1998; Jena, 1980; Ndyabahinduka, 1984). Its incubation period is 1-7 days.
4. **Anthrax Meningitis:** In about 5% cases of cutaneous anthrax, meningitis develops as a sequel (Whitford, 1987), but it can arise from any of the three forms of the disease described above. The clinical signs of meningitis and the appearance of blood in cerebrospinal fluid are followed rapidly by loss of consciousness and death (Drake and Blair, 1971; Koshi *et al.*, 1981; Levy *et al.*, 1981); the prognosis in this form of anthrax is grave.

Etiological agent of anthrax is *Bacillus anthracis* a gram positive, non-motile, spore forming, facultative anaerobic rod shaped bacteria, 1-1.5 μm X 4-10 μm (Watson *et al.*, 1994) in dimensions. It is the only known obligate bacillus pathogen of vertebrates. It can grow in single cells or short chains in the blood. It can turn even gram negative in old cultures (Turnbull, 1991). Under adverse growth conditions it sporulates (Leive and Davis, 1980). Spores are very hardy and can survive vagaries of nature for years (Brachman, 1986) or centuries altogether in environment, as corroborated by finding of these spores in mummies and the said curse associated with them (Kezwer, 1998). Recent studies of agricultural outbreaks of anthrax have suggested that conditions for multiplication are favourable when the soil has pH >6.0 and rich in organic matter.

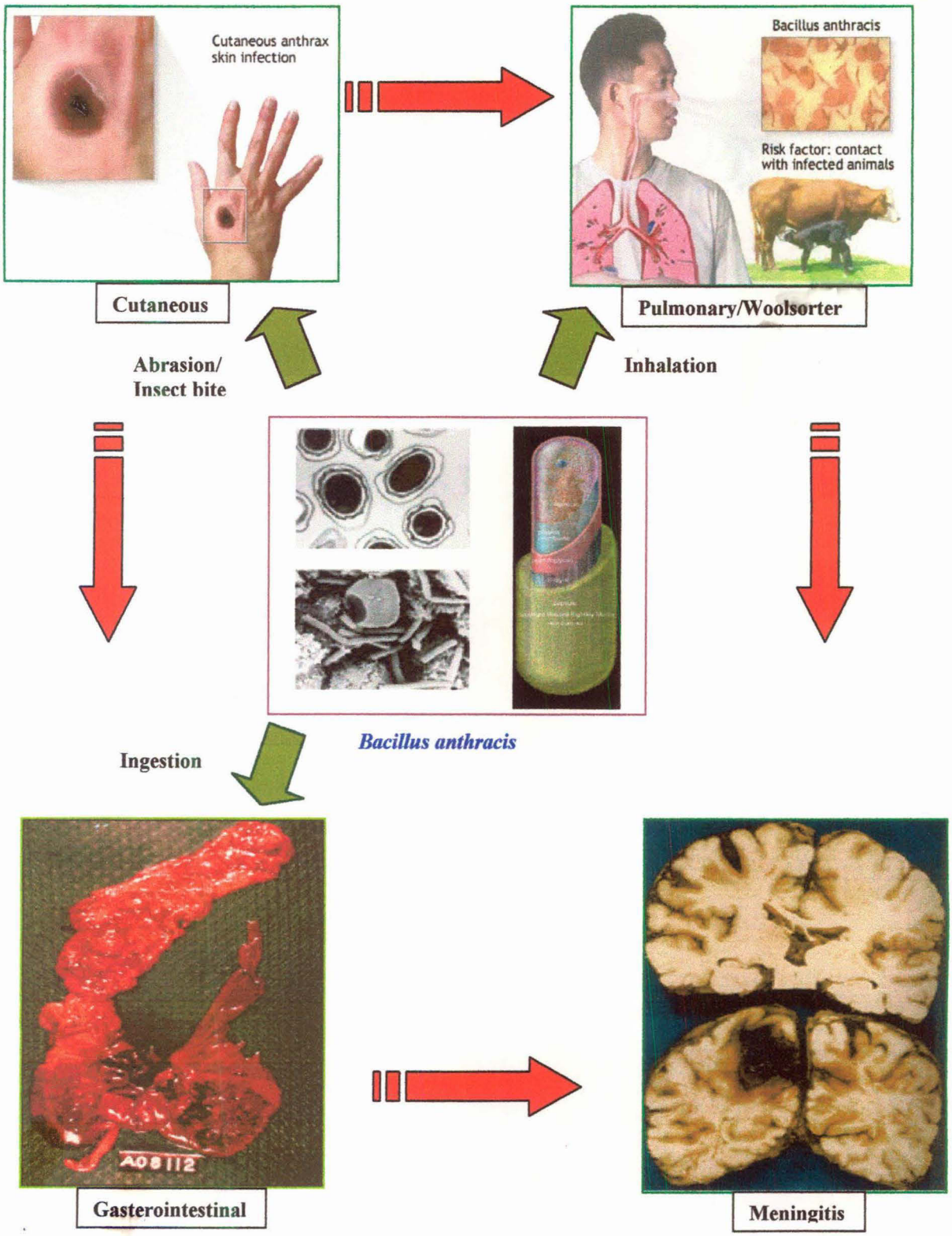
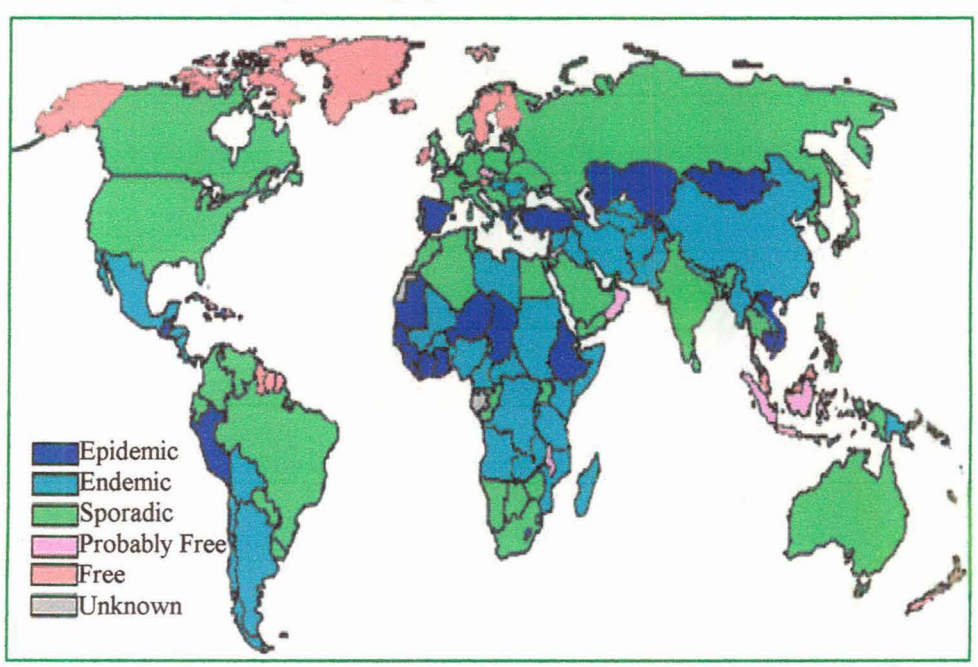


Fig 1.1 Types of Anthrax depending upon route/site of infection and the organ involved. Arrows indicate transfer/inoculation of *Bacillus anthracis* at indicated site. Red arrows indicate transfer through body fluids

1.1.1 WORLD WIDE INCIDENCES AND GEOGRAPHICAL DISTRIBUTION

With the improvement of sanitation and public health services the incidences of human anthrax saw a declining trend in last century all over the world. However, it is still prevalent and enzootic in various countries of Europe, Asia, Africa, and South America with sporadic incidences in developed nations such as USA, Canada, Australia, UK etc. In 1958, Glassman estimated approximately 20,000–1,00,000 cases worldwide which decreased up to 200 annually by an estimate in 1980. There have been some sporadic extraordinary epidemics reported from some part of the world in last quarter century as a result of agricultural and military disruptions. The largest human epidemic occurred in Zimbabwe, during Rhodesian civil war. After that failure of veterinary vaccination programs led to an anthrax epidemic infecting approximately 10,000 persons with more than 200 fatalities between 1979-1985 (Turner, 1980; Davies, 1982, 83, 85). An accidental explosion in military facility in Sverdlovsk (Ekateringburg), Russia in 1979, exposed the city population to aerosol of anthrax spores resulting in man made epidemic which caused 66 reported human deaths of inhalation anthrax (Meselson *et al.*, 1994). Human anthrax is still prevalent in most part of the world and numbers of cases vary depending upon quarantine practices, public health services and living standards. In United States and other developed nations it is the disease of wild animals with a few sporadic human and wild animal cases.

Fig 1.2 Geographical Distribution of Anthrax



In Europe, the eastern parts are the most affected one. Turkey and Greece top the list of incidences. It is regularly reported in Spain, Albania, Italy and Romania. Central Spain suffers quite an extraordinary number of human cases each year from 152 in 1990 to 50 in 1996.

The western Africa, which is hyper-endemic for anthrax has recurring occurrence of anthrax with maximum number of reported cases.

1.1.2 ANTHRAX IN INDIA

Although anthrax is endemic and widespread in India outbreaks and occurrence of anthrax in animals and man are not well documented. It is endemic in Tamil Nadu, Andhra Pradesh and Karnataka, but sporadic occurrence from Orissa, J&K, West Bengal and Pondicherry are also reported. There have been some clinical cases as well. Anthrax outbreaks have occurred in pockets of the southern states of Andhra Pradesh, Tamil Nadu, Karnataka (1999), West Bengal (2000) and the Union territory of Pondicherry (1999) in recent years. Recently 23 cases of cutaneous anthrax has been reported due to an endemic outbreak in south India supposedly because of vector borne

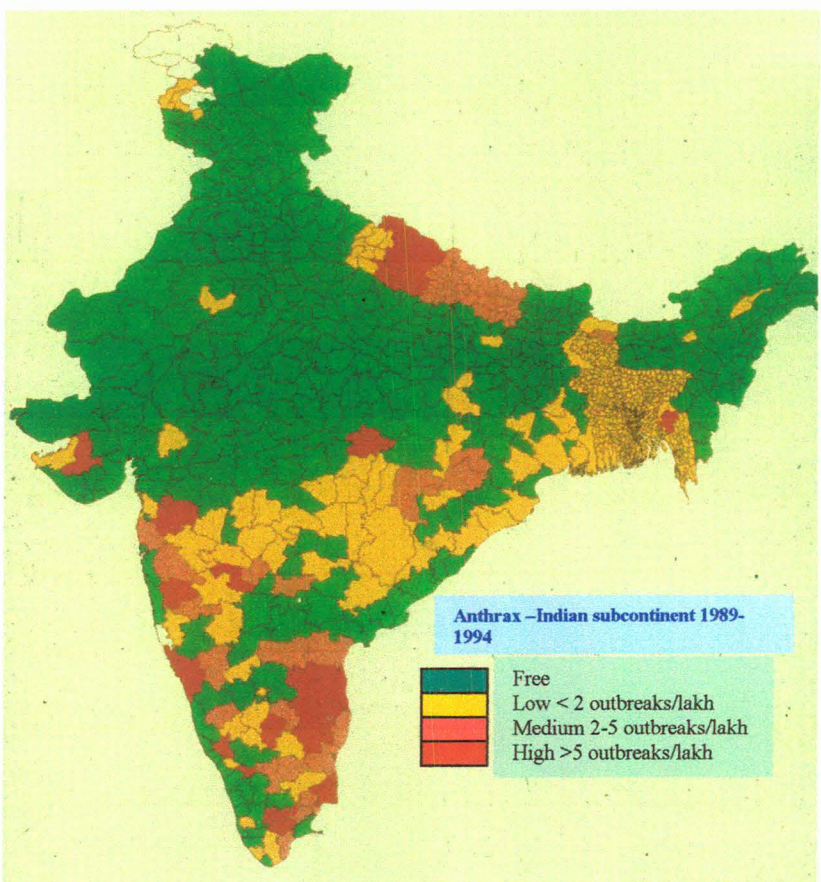


Fig 1.3 Anthrax in Indian sub continent

transmission as there was no evidence of direct human contact with infected animals (Vijaikumar *et al.*, 2002).

Animal anthrax is also endemic in south India. According to one estimate on an average 300 outbreaks/year occurred in nine years (1974-1983). Transmission of anthrax occurs through grazing on contaminated pastures, blood sucking flies and ticks, dirty stagnant pools of water and movement of nomadic herds. During 1991-96, 1613 outbreaks occurred in animals with 62.5% mortality rate of which 20% was in cattle and buffalo and 80 % in sheep and goat (Repot on Incidence of anthrax in India during 1991-1996 by Disease Surveillance Unit of the Department of Animal Husbandry and Dairying, Ministry of Agriculture, Government of India). The disease is more prevalent in coastal areas of West Bengal, Orissa and Tamil Nadu. Sporadic reports of animal anthrax keep on coming, from south India, from time to time.

1.1.3 MAJOR VIRULENCE DETERMINANTS

1. **Poly D-glutamic acid capsule:** It inhibits the phagocytosis (Zwartouw and Smith, 1956) and interferes with complement system, thus allowing colonization and establishment of infection.

2. **Tripartite protein exotoxin** (Stanley and Smith, 1961) It consists of protective antigen (PA, 735 amino acid, 83 kDa) a cell surface binding moiety which acts as piggy back for translocation of two other molecules of the complex i.e. lethal factor (LF) and edema factor (EF). LF (776 amino acid, 90 kDa) is a Zinc metalloprotease (Klimpel *et al.*, 1994), which cleaves MAPKKs (Vitale *et al.*, 1998; Duesbery *et al.*, 1998) and causes death of sensitive macrophages (Friedlander, 1986). EF (89 kDa, 767 amino acids) is a calmodulin dependant

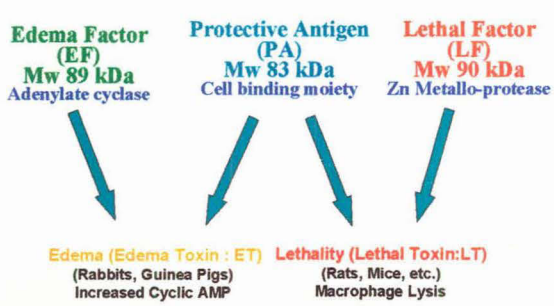


Fig 1.4 ANTHRAX TOXIN COMPONENTS

adenylate cyclase (Leppla *et al.*, 1982; Hoover *et al.*, 1994) that causes Edema.

Genes for these virulence determinants are plasmid borne. pXO2 harbors genes for poly D-glutamic acid capsule while pXO1 harbors the gene encoding for PA, LF and EF. The first conclusive evidence of involvement of protein exotoxins in virulence came from work of Smith *et al.*, (1955) when he showed that plasma from dying guinea pigs of anthrax could transfer the associated symptoms like edema in skin and death of guinea pigs when injected in normal animals.

Independent of the site of primary inoculation, *B. anthracis* resists phagocytosis and proliferate at site of inoculation and eventually reach lymphatics and spleen, where they multiply and are released in sudden bursts into circulatory system causing bacteremia and sudden increase in toxin level that results in fever, comma, cardiac failure, depression of central nervous system anaphylactic shock, respiratory distress and death.

1.1.4 VIRULENCE PLASMIDS

1.1.4.1 pXO1

It has been completely sequenced (Okinaka *et. al.*, 1999). It is ~182 kb plasmid that codes for protein exotoxins and some other virulence factors. (Mikesall *et al.*, 1983; Okinaka *et al.*, 1990, 1999). Genes for PA (*pagA*), LF (*lef*) and EF (*cya*) are located contiguously within a 30 kb region of pXO1 (Vodkin, 1983; Robertson *et al.*, 1986, 1988; Mock *et al.*, 1988; Uchida, 1993; Koehler *et al.*, 1994) which is part of a 44.8 Kb pathogenicity island bordered by IS1627 elements (Okinaka *et al.*, 1999). pXO1 encoded genes are not essential for bacterial survival at all and strains lacking them give no protection on immunization (Mikesall *et al.*, 1983).

1.1.4.2 pXO2

It is a 97 kb plasmid responsible for capsule formation (Green *et al.*, 1985). Genes responsible for capsule formation (*capC, capB, capA*) (Makino *et al.*, 1989) and modulation by depolymerization (*dep*) constitute an operon and are transcribed together as a single messenger RNA (Uchida *et al.*, 1993). Cap gene products are membrane associated and

mediate synthesis of capsular polypeptides while that of *dep* gene modulate the structure of capsule.

1.1.5 STRUCTURE OF ANTHRAX TOXIN

1.1.5.1 PROTECTIVE ANTIGEN

Crystal structure of PA was elucidated in 1997 by Petosa *et al.*. The PA monomer molecule is ~100 Å in length, 50-70 Å in width and 30-40 Å deep. The 735 residues of PA fold into four domains of predominantly β-sheet structure. The first three domains are in extensive contact with each other, but the fourth domain is loosely associated with them. The PA heptamer is a mushroom shaped ring of 160 Å in diameter and 85 Å in height with the central pore with an average diameter of 35 Å and a minimum diameter of 20 Å at some places. The lining of the pore is made up of residues from domain 1b and domain 2 and consists of polar and negatively charged residues. No major conformational changes occur during the assembly of the heptamer from the monomers. The PA heptamer can be changed to a membrane insertion competent, SDS-insoluble form by lowering pH. Crystal forms of PA obtained at pH values 7.5 and 6.0 show the largest structural difference at the tip of a small pH sensitive loop of domain 2 (342-355), which includes three hydrophobic residues Trp346, Met350 and Leu352. At pH 7.5 these residues are buried in the domain core but at pH 6.0 they become disordered and exposed to solvent. Out of the above three residues Trp346 and Leu352 have been implicated in pH dependent membrane insertion (Batra *et al.*, 2001).

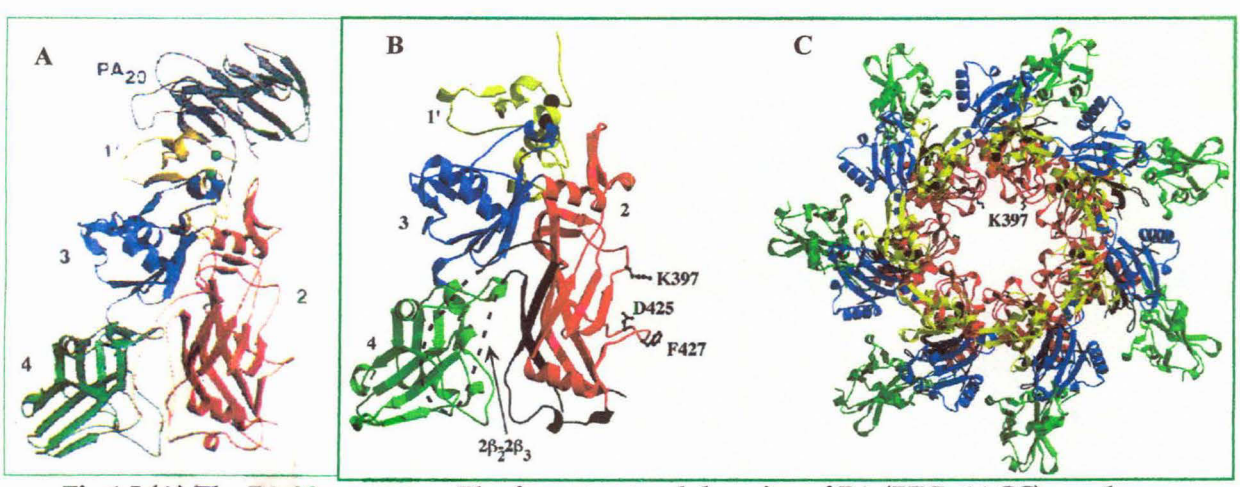


Fig 1.5 (A) The PA 83 monomer: The four structural domains of PA (PDB: 1ACC) are shown as 1, 2, 3, and 4. (B) Proteolytically activated PA or PA63 cut out from PA heptamer (C)[taken from Petosa *et al.*, 1997 and Mourez *et al.*, 2002]

There are sequence homologies between PA and the *Clostridium perfringens* iota – toxin Ib (32% identity) and the Vegetative Insecticidal Protein (VIPI) from *Bacillus cereus* (27% identity) (Perelle *et. al.*, 1993; Warren, 1996; Petosa and Liddington, 1997). Both iota-Ib and VIP-1 are binary toxins, bind to host cell receptors, are activated by proteolytic nicking and are responsible for delivering toxic enzymatic counterparts to the cytoplasm of host's cells. C-terminal domains do not show significant homology, which is consistent with their cell receptor binding function.

Domain 1

The domain 1 (residues 1-258) comprises a β-sandwich with a jelly-roll topology and several small helices. It is involved in the binding of LF/EF to PA. The trypsin cleavage site (164RKKR167) is located on an exposed loop between strands 1β11 and 1β12. Proteolysis and the release of 20 kDa fragment i.e., PA20, from N-terminal, cause a large structural change, exposing a large hydrophobic surface on the rest of the domain 1b by rupture of a B-sheet between 1β2 and 1β13. This surface exposes a large, flat, hydrophobic patch on the top of the heptamer in which hydrophobic residues Pro184, Leu187, Phe202, Leu203, Pro205, Ile207, Ile210, Trp226 and Phe236 become exposed to solvent, which are buried in PA. Residues Phe202, Leu203, Pro205 and Ile207 have been shown to play important role in the interaction with LF (Chauhan and Bhatnagar 2002).

The 1b domain contains two Calcium ions coordinated in the form of a variant of the EF hand motif, which are supposed to be stabilizing PA63 after the loss of PA20. Extensive side chain interactions and hydrogen bonding interactions with domain 2 are also thought to aid stabilization of 1b. Due to this extensive interaction with domain 2, it is possible to 'nick' PA without dissociating the PA20 fragment.

Domain 2

Domain 2 (residues 259-487) is involved in oligomerization and also insertion into the membrane and translocation of LF/EF. It has a beta barrel core with modified Greek key topology and excursions, which includes the loop between the strands 2β2 and 2β3 implicated in membrane insertion. A long loop between residues 302-325 has been

implicated to be present in the channel lumen on the basis of various mutagenesis studies (Benson *et. al.*, 1998; Sellman *et. al.*, 2001). This loop contains alternating hydrophilic and hydrophobic residues. It is speculated that this loop peels away from the rest of the molecule in the form of a hairpin and interaction of seven such hairpins in the heptamer leads to the formation of a 14 -stranded β-barrel like structure similar to that of α-hemolysin (Song *et. al.*, 1996) and other bacterial toxins. This hairpin contains a number of histidine residues. It is thought that at low pH, protonation of these residues occurs which is energetically unfavourable and the stress is relieved by the opening up of the hairpins.

Domain 3

The domain 3 (residues 488-595) has a four stranded mixed β-sheet, two smaller sheets and four helices, which adopts a fold seen in ferredoxins and toxic shock syndrome toxin-1 (Prasad *et al.*, 1993). One helix of this domain is a part of a triangular strand-helix-loop structure that permits five residues to form a flat surface exposed to the solvent, constituting a 'hyrophobic patch'. In crystals of PA, Phe427 of a neighbouring molecule occupies this patch. This patch may be involved in a protein-protein interaction during host cell intoxication. Although the complete role of this domain is not yet known, mutagenesis studies from our lab (Ahuja *et al.*, 2001) and others (Mogridge *et. al.*, 2001) have demonstrated its involvement in oligomerization. It is also supposed to play a role in binding of LF/EF to PA (Little *et. al.*, 1996).

Domain 4

Domain 4 (residues 596-735) has an initial hairpin and helix followed by a β-sandwich with an immunoglobulin like fold. Within this fold, a 19 residue highly accessible loop lies that is analogous to the antigen binding CDR3 loop of antibodies and the receptor binding loop of diphtheria toxin. A part of this loop is involved in the interaction of domain 4 with the receptor (Brossier *et al.*, 1999, Varughese *et al.*, 1999). It has been recently shown that domain 4 alone is able to provide protective immunity against anthrax (Flick-Smith et al., 2002).

1.1.5.2 LETHAL FACTOR

Pannifer *et al.*, (2001) have elucidated the crystal structure of LF. It is a 100Å tall and 70 Å wide at its base consisting of 4 domains, with domain 1 perched on top of other three domains which are intimately connected and probably comprise a single folding unit. Domain 1 consists of 12 helix bundles that pack against one face of a mixed four stranded β-sheet, with a large (30 residue) ordered loop, L1, between the second and third β–strands forming a flap over the distal face of the sheet. Domain I bind to PA. Domain II, III and IV create a long deep groove that holds the 16th residues of N-terminal tail of MAPKK before cleavage. Domain II (residues 263-297 and 385-550) resembles the ADP-ribosylation toxin from *B. cereus*, but the active site appears to be mutated to help in substrate recognition. Domain III is a small helical bundle with a hydrophobic core (residues 303-382), inserted at a turn between the second and third helices of Domain II. It seems to have arisen from a repeated duplication (four times) of a structural element of Domain II. Domain III is required for LF activity as insertion and point mutations of buried residues in this domain, done in our laboratory, abrogated its function (Singh *et al.*, 2002). Domain IV (residues552-776) consist of nine-helix bundle packed against a four-stranded β-sheet. It is distantly related to the Zinc metallo-protease family, and contains the catalytic center; it also resembles Domain I.

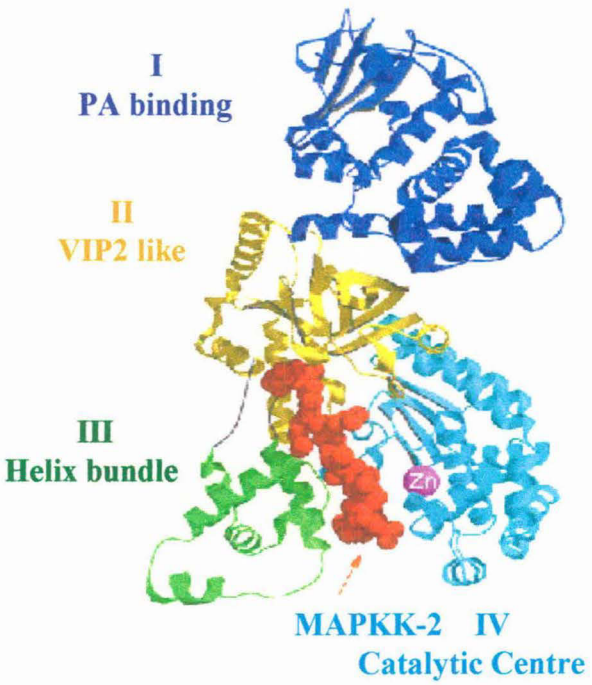


Fig 1.6 Structure of LF (taken from Pannifer *et al.*, 2001)

1.1.5.3 EDEMA FACTOR

Crystal structure of catalytic portion of EF alone, EF with calmodulin, and EF complexed with calmodulin and a non-cyclizable nucleotide analogue, 3′-deoxy-ATP has been solved by Drum *et. al.,* in 2002. It does not show any structural homology with mammalian adenylyl cyclases or any other protein. The N-terminal PA binding portion of EF shares extensive homology with the N-terminal 1-254 amino acids of LF and supposed to share structural similarity to LF as well. Conserved N-terminal domains of EF and LF are mostly hydrophilic, probably because they are located on the surface where they could interact with PA. The catalytic portion of EF comprises three globular domains. The active site lies at the interface of two domains, C_A (amino acids 249-349 and 490-622) and C_B (350-489), which together constitute the catalytic core. A third, helical domain (660-800) is connected to C_A by a linker (623-659). Calmodulin activates EF by inserting itself between C_B and helical domain in an extended conformation that causes large-scale relative movement among individual domains. Calmodulin binding site extends over a large surface area of EF involving extensive interaction with four discrete regions i.e., 510-540, 615-634, 647-672 and 695-721. Calmodulin binding causes formation of high affinity binding site for ATP.

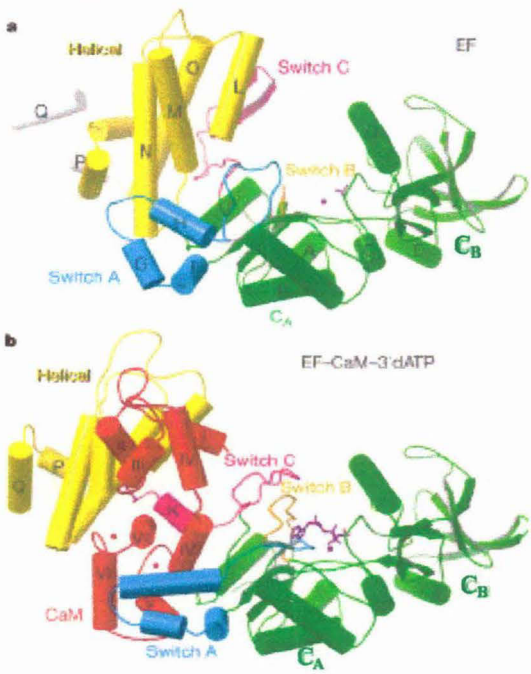


Fig 1.7 Structure of EF: Secondary structures of EF. (a) EF alone; (b) The EF-CaM-39-deoxy-ATP complex. CaM (red) is held on one side by a fingerlike projection of CA (green), and on the other side by switch C (magenta) and the helical domain (yellow). Switch A and switch B are in cyan and orange, respectively. The 3′-deoxy-ATP analogue (purple) and metal (purple) are bound between the CA and CB domains (green), which form the active site. In the EF-alone structure, the last two helices, P and Q (amino acids 771-799, white), which are not part of the CaM interface or the active site, are swapped between two EF molecules. This swap is unlikely to be physiologically relevant because EF is a monomer on the basis of its gel-filtration properties. (taken from Drum *et al.*, 2002)

1.1.6 MECHANISM OF ACTION

Anthrax toxin complex belongs to AB type of bacterial toxin (Batra and Bhatnagar 2001) where A is the catalytic moiety whose entry is dependant or mediated by another component B. In case of anthrax toxin complex PA is the binding component (B) while LF and EF are alternate catalytic moiety (A). Once inside the cell they show their detrimental effects.

Mechanism of anthrax toxin action follows some discrete steps, which are as under:

1.1.6.1 Binding of PA to cell membrane: Binding of PA to recently identified, 368 amino acid long, type 1 integral membrane protein named Anthrax Toxin Receptor (ATR) (Bradley *et al.*, 2001) is essential for toxicity. It has been proved with the help of anti-PA antibodies that only after binding of PA to cell surface LF/EF, the enzymatic moieties, could compete for binding to PA for cellular uptake (Little *et al.*, 1988, 1991). Singh *et al.*, 1991 identified that last 12 amino acids of C-terminal of domain 4 of PA are essential for receptor binding. Deletion and substitution mutation in domain 4 (Brossier *et al.*, 1999; Varughese *et al.*, 1999) have shown that small solvent exposed loop comprising of residues 679-693 of domain 4 is indispensable in receptor binding while another loop encompassing residues 703-723 have conformation stabilizing role. Addition of extra 16 amino acids to C-terminal does not affect the toxicity, which reinforces the assumption that these residues are not positionally but conformationally important. PA with domain 4 deletions was unable to generate a strong immune response (Brossier *et al.*, 2000) insinuating it as the most immunogenic portion of PA.

1.1.6.2 Proteolytic Activation of PA: It is achieved by cleavage at a contiguous stretch of four basic amino acids 164-167 (RKKR) specifically on C-terminal of Arg167 (Novak *et al.*, 1992) by furin (Singh *et al.*, 1989; Klimpel *et al.*, 1992; Molloy *et al.*, 1992) and furin like proteases such as PC1, PC2, PACE4 (Friedman *et al.*, 1995; Gordon *et al.*, 1997; Sucic *et al.*, 1999) etc. on cell surface, resulting into a N-terminal 20 kDa and C-terminal cell bound 63 kDa fragment referred to as PA20 and PA63 respectively. In the blood samples of anthrax infected animals, PA exists primarily as PA63, complexed with LF or EF and this

conversion of PA83 to PA63 is known to be catalyzed by calcium dependent, heat-labile serum protease (Ezzell *et al.*, 1992).

1.1.6.3 Oligomerization of PA: Removal of PA20 allows PA63 to oligomerize into a ring shaped heptamer (Petosa *et al.*, 1997) with a central pH dependent voltage gated channel (Blaustein *et al.*, 1989, 1990). The morphology and characteristic of PA heptamer is pH dependent. Heptamers formed at pH 9.0 are diffuse and not resistant to SDS while those formed at pH 5.0 are compact and resistant to boiling in the presence of SDS.

Although PA itself does not have any catalytic activity of its own, the formation of said channels would have probably caused the observed transient alterations in neurological and cardiovascular functions in monkeys and chimpanzees (Vick *et al.*, 1968). This ability to form ion conductive channels by PA can have potentially deleterious effects in the presence of impurities in vaccine preparations.

Work from our lab gave credence to the belief that residues of hydrophobic patch i.e. Phe552, Phe554, Ile562, Leu566, and Ile574, are involved in oligomerization (Ahuja *et al.*, 2001). The chymotrypsin sensitive site FFD (313-315) of PA has also been implicated in oligomerization (Singh *et al.*, 1994). Any mutation in these sequences renders PA inactive in its ability to form oligomers.

1.1.6.4 Binding of LF/PA to PA63: LF/EF both compete for binding to proteolyticaly activated PA (Little *et al.*, 1988). Heptamerization of PA63 is not a prerequisite for binding of LF/EF to PA63 (Ezzel *et al.*, 1992; Zhao *et al.*, 1995) as observed by others (Singh *et al.*, 1994; Mogridge *et al.*, 2002; Cunningham *et al.*, 2002). The observed inability of binding of LF/EF to oligomerization defective PA could be a result of change in conformation of PA mutants which would be otherwise playing critical role in interaction with LF/EF rather than prerequisite of PA63 heptamerization. Our lab has shown that residues Phe202, Leu203, Pro 205 and Ile207 of PA are important in binding to LF (Chauhan and Bhatnagar, 2002)

The ability to bind PA63 resides in N terminal region of LF (Arora *et al.*, 1992; Quinn *et al.*, 1991) and EF (Little *et al.*, 1994; Kumar *et al.*, 2001) while catalytic activity is borne by C-terminus (Quinn *et al.*, 1991; Little *et al.*, 1994; Kumar *et al.*, 2001). Among the

nine His residues of binding domain of LF, His35 is important for binding to PA63, while His42 is important for maintaining proper conformation of LF (Arora *et al.*, 1997). Our laboratory demonstrated that residues Tyr148, Tyr149, Ile151 and Lys153 of LF are involved in binding to PA on the basis of site directed mutagenesis studies (Gupta *et al.*, 2001), later the residues at the same position in EF have also been shown to be involved in binding to PA (Kumar *et al.*, 2001).

1.1.6.4 Insertion of the PA into membrane and translocation of LF/EF to the cytosol: Heptamerization or clustering of PA63 on cell surface is needed for fast endocytosis as furin resistant PA or mutants which cannot be processed by furin remain at cell surface for a long time (Beauregard *et al.*, 2001), while heptamerized PA63 or antibody driven cluster of furin resistant PA can be readily internalized within 5 minutes. Heptamerization of PA63 forces the association of its receptor, which is normally in glycerophospholipidic, non-raft region of the membrane, to noncaveolar cholestrol and sphingolipid rich region of plasma membrane or rafts. It is followed by dynamin and Eps15 dependent clathrin mediated endocytosis (Abrami *et al.*, 2003). Inside endosome the lowering of pH is required for translocation of LF/EF to cytosol. Inhibition of lowering of lumenal acidic pH either by blocking activity of vacuolar ATPase proton pump using macrolide antibiotics bafilomycins and concanamycin A that specifically inhibits the activity of the pump (Menard *et al.*, 1996) or incubation with agents which prevent lowering of lysosomal pH such as lysosomotrophic amines, ammonium chloride, chloroquine, ionophore monensin etc., have been shown to protect the macrophage cells against LT mediated death (Friedlander, *et al.*, 1986).

Interaction of PA/LF/EF to lipid membranes is presumed to be driven by electrostatic interactions as there is no significant membrane spanning domains in either of them (Kochi et. al., 1994). The binding to membrane is pH dependent and unique in itself for each anthrax toxin component. Binding of PA is irreversible to membrane while that of LF is reversible and in case of EF it varies with pH. In each case they insert inside lipid membrane and undergo conformational change with pH (Wang *et al.*, 1996). The interaction with membrane is maximal near the pH close to their pI (Leppla *et al.*, 1991). The alternating residues of stretches 302-311 and 316-325 within D2L2 have been

implicated as ion conductive channel facing residues of PA heptamer as treatment of their cysteine mutants with cysteine specific membrane impenetrable reagent, which imparts positive charge to cysteine residue, have been found to decrease the conductance of the channel formed by mutant PA63 heptamer (Benson *et al.*, 1998). This finding corroborated the speculation that heptamer prepore gets converted to a 14-strand β-barrel pore made up by tearing away of D2L2 loop from the base of PA heptamer following a low pH trigger and each monomer provides the two β-strands for pore formation. The D2L2 deletion mutants have been shown to be able to form SDS resistant oligomers but devoid of ability to form the pore in membrane and translocate LF/EF to cytosol, implying that conformational changes that make heptamer SDS resistant occur in 1 and 2 or 3 and 4 domain following lowering of the pH. Batra *et al.*, 2001 have shown that Trp346 and Leu352 play important role in membrane insertion and translocation of LF/EF to cytosol. A single mutation in D2L1 loop of domain 2 (P260A) has been shown to decrease membrane insertion and translocation of LF into the cytosol (Khanna *et al.*, 2001).

Translocation of LF and EF probably requires large conformational changes as introduction of artificial disulfides in LF have been shown to block its translocation into cytosol (Wesche *et. al.*, 1998). Data presented by Wang *et al.*, 1997 suggests, that after translocation to cytosol, LF is released free into the cytosol while EF remains bound to membrane like other adenylate cyclases.

Decrease of conductance on binding of first 30 amino acids of LF/EF to PA heptamer (Finkelstein *et al.*, 1994) and inhibition of PA63 mediated release of Rb^{86} from CHO K1 cells under acidic conditions on binding of LF (Zhao *et al.*, 1995) or LFn (residues 1-254 of LF: fragment of LF needed for binding and translocation of LF to cytosol (Arora *et al.*, 1992)), suggest that PA63 can undergo heptamerization even without binding to LF and LF binding site remains exposed and accessible even after oligomerization of PA63 while binding of LF to the site blocks the channel and ion conductance through it.

1.1.6.5 LF/EF action in cytosol: Once inside the cytosol LF and EF exert their deleterious effects. Although all macrophage like cells are able to proteolytically activate PA and

cause translocation of LF/EF into cytosol, yet many others such as IC-21 are not sensitive to anthrax (Singh *et al.*, 1989). This deviation points to absence of some cellular target or cofactor needed for activity of LF or inactivation of LF inside cell or presence of some repair mechanism in resistant cells (Friedlander *et al.*, 1993). Dietrich and coworkers have shown that susceptibility to anthrax LT is mediated by Kif1C, a kinesin-like motor protein, in mouse macrophages that has been mapped to locus Ltx1 at chromosome 11 (Watters *et al.*, 2001a, b; Roberts *et al.*, 1998). Protein synthesis has been shown to be required for killing of LT sensitive macrophage like cell line J774A.1 (Bhatnagar *et al.*, 1994). The protein synthesis is probably required for formation of a protein that directly or indirectly mediates influx of Ca^{2+} ions from outer medium, a prerequisite for cell death following exposure to LT (Bhatnagar *et al.*, 1989) or ET induced toxicity (Kumar *et al.*, 2002).

Hanna *et al.*, 1993 have shown that death of the animals infected with anthrax is mediated by macrophages as depletion of macrophages by silica was able to protect animals against LT induced death following infection of anthrax while reintroduction of macrophages from anthrax sensitive mice was able to make the mice again susceptible to LT.

LF is a Zn metalloprotease. It behaves like Leukotriene A4 hydrolase and also prone to inhibition by its inhibitors (Menard *et al.*, 1996; Klimpel *et al.*, 1994). LF preferentially hydrolyzes proline-containing substrates (Hammond *et al.*, 1998) and targets all MAPK Kinases (Duesbery, 1998; Vitale *et al.*, 1998) with the exception of MEK5 (Vitale *et al.*, 2000) and is responsible for cell death (Chaudry *et al.*, 2002; Mourez *et al.*, 2002). Cleavage of MAPKKs is accompanied by phosphorylation of ERK. Transient production of phosphorylated ERK probably induces a pathway that causes sustained production of cytokines and oxygen radicals resulting in death of macrophages. LT also induce IL-1 and TNF expression in macrophages, which cause systemic shock and death of animal from anthrax (Shin *et al.*, 2000).

Sublytic dose of LT has been shown to suppress the NO and TNF formation although MAPKK cleavage remained unaffected (Pellizzari *et. al.*, 1999). Cytokine production was also inhibited by inhibition of transcription of cytokine mRNA (Erwin *et al.*, 2001). These result in suppression of inflammatory response and helps in survival of

B. anthracis in initial phase of infection. However, there exists no direct correlation of MAPKK cleavage and death of macrophage. Death caused by LT is too rapid to be attributed to the disruption of MAPKK pathway alone. It has been shown that MAPKKs disruption occur even in anthrax toxin resistant macrophages, implying involvement of some more yet unidentified factors. Our laboratory (Bhatnagar *et al.*, 1999) has shown that activation of phospholipase C and protein kinase C is required for LT action. Increase in level of IP3 is also observed during anthrax intoxication. Work by Swain *et al.*, 1997 established that LF is not degraded in cytoplasm even after 2 hrs of incubation and its activity does not require any processing by Golgi-network as it was active even in the presence of Brefaldin-A, a Golgi-network disrupting drug.

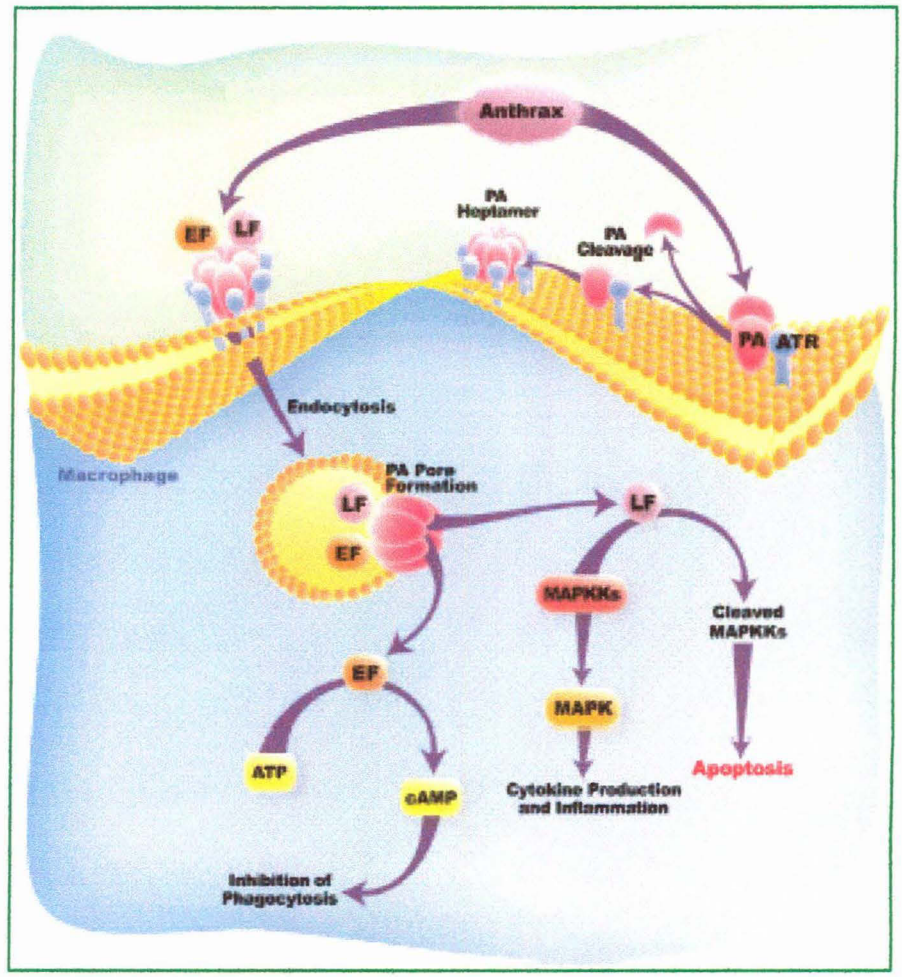


Fig 1.8 Overview of action of anthrax toxin components

Tang *et al.*, 1999 found that proteosome is also involved in LT mediated killing of macrophage, downstream to MAPKK cleavage by degrading yet unidentified molecule essential for macrophage survival.

EF is a calmodulin dependant adenylate cyclase (Leppla, 1982) that causes upto 200 fold increase in cytoplasmic cAMP level in CHO cells without a lag period. An increased cAMP level in polymorphonuclear neutrophils (PMNs) is associated with inhibition of phogocytosis (Ignarro and Chech, 1976) and increased host susceptibility to infection (O' Brien *et al.*, 1985). EF induces monocytes to secrete IL-6 and produce TNF-α but decreases LPS induced TNF-α synthesis, implying that cAMP accumulation impairs anti-microbial response of human monocytes and contribute to clinical signs and symptoms of the disease (Hoover *et al.*, 1994)

1.1.7 ANTHRAX AS A BIOLOGICAL WEAPON

Use of anthrax as a biological weapon dates back to the time of Moses. In the recorded history, Moses was the first person to use this as a biological weapon. Anthrax spores are one of the best choices in biological weapons for germ warfare (Turnbull, 1991). As per WHO estimate, 50 kg of *B. anthracis* released upwind of a population center of 500,000 could result in 95,000 deaths and 125,000 hospitalizations. During World War I and II both warring factions experimented extensively with anthrax (Bryskier, 2002). USA and Russia still have active biological warfare program. In recent times a Japanese terrorist group (Aum Shirikyo) tried to induce anthrax in population by dispersing anthrax in the form of aerosols in Tokyo subway stations (1995) but did not succeed because they were using avirulent non-encapsulated strain (Sterne strain) (Hawley, 2000). In aftermath of September 11 terrorist attacks in the USA, anthrax scare gripped the world in the form of anthrax spore laden postal envelops originating from anonymous location. These incidences have increased the interest in research on anthrax.

1.1.8 PROPHYLAXIS AND VACCINES

Vaccination remains the most effective prophylactic measure against anthrax as once disease appears (especially in case of inhalational and meningitis) it is highly fatal. Pasteur developed the first ever anthrax vaccine by growing *B. anthracis* at higher temperature (42-45°C). It suffered from variable and low potency (Sterne, 1937). An improved vaccine based on an avirulent non-capsulogenic variant of *B. anthracis* was

developed by Sterne, which is known as Sterne strain after him. It still remains the main veterinary vaccine. Success of Sterne strain lies in the fact that it carries pXO1 plasmid but not the pXO2 (Welkos *et al.*, 1988) while Pasteur's vaccine strain carries pXO2 but not the pXO1 plasmid (Mikesell *et al.*, 1983). Presence of pXO1 in Sterne strain cause production of major virulence factors such as PA, LF and EF and hence provides better immunity as compared to Pasteur's vaccine, which lacks pXO1. The residual protection offered by Pasteur's vaccine could be attributed to presence of some contaminating cells with pXO1 plasmid.

Alum precipitated toxin proteins secreted by Sterne strain (Belton *et al.*, 1956) and aluminium hydroxide gel adsorbed culture supernatants of V770-NPI-R strain with Benzethonium chloride as preservative and formaldehyde as stabilizer (Wright *et al.*, 1962; Puziss and Wright, 1963) had been introduced by Britain and USA respectively for human use. These are the sterile preparations and are still in use.

The spore vaccine offer better protection as compared to adsorbed human vaccine supposedly because of variation in epitopes expressed by PA when bound to adjuvants (Ezzell, 1992) or complexed with LF/EF (Pezard *et al.*, 1995). Presence of LF/EF probably acts synergistically with PA to generate the immune response. The protection offered by PA alone is low as compared to live spore vaccine so various adjuvants have been tried to increase the protective immunity generated by PA based vaccines. It has been found that adjuvants comprising of whole bacterial cells considerably increase the immunity, some times even more than live spore vaccine (Ivins *et al.*, 1992; Jones *et al.*, 1995).

Carbosap, the vaccine strain of *B. anthracis* that is in use in Italy has been shown to contain both virulence plasmids pXO1 and pXO2, carrying all major virulence factor genes and their regulators, but still attenuated. It casts doubt on ability of known major virulence factors in pathogenicity of anthrax and implies that some more unidentified virulence factors are also involved in anthrax pathogenicity (Adone *et al.*, 2002).

1.1.9 POTENTIAL ANTHRAX VACCINE CANDIDATES

1. Recombinant live vaccine strains of *B. anthracis* (Ivins *et al.*, 1988; Pezard *et al.*, 1993), *B. subtilis* (Ivins *et al.*, 1986, 1990), *Salmonella typhimurium* (Coulson *et al.*, 1994),

Lactobacillus (Zegers *et al.*, 1999), *Baculovirus and Vaccinia* virus (Iacono-Connors *et al.*, 1990) that produce PA but not LF or EF. Sterne strain derivatives, which have point or deletion mutation in functional domain of PA, LF or EF or in all of them, known as Sterne-Brossier strains (Brossier *et al.*, 1999). Strains detoxified in EF or PA are safer as they do not invoke edema or death in mice even at higher doses.

2. Biologically inactive mutant PA, particularly trypsin and chymotrypsin site deletion mutants, in combination with LF and EF generate better immuno-protection response (Bhatnagar *et al.*, 1998; Singh *et al.*, 1998). Other biologically inactive mutants defective in binding to LF/EF (Chauhan and Bhatnagar, 2002), oligomerization (Batra *et al.*, 2001; Ahuja *et al.*, 2001) or translocation of LF (Batra *et al.*, 2001) made in our laboratory can also be good candidates along with inactive LF (Gupta *et al.*, 2001; Singh *et al.*, 2002) and EF mutants (Kumar *et al.*, 2001).
3. Overexpression of recombinant PA (Gupta *et al.*, 1999; Chauhan *et al.*, 2001) and LF (Gupta *et al.*, 1998; Gupta *et al.*, 2001; Singh *et al.*, 2002) from our lab revolutionized the production of rPA and rLF in pure form for vaccination purpose as these are devoid of other contaminating cellular proteins and hence the associated side effects with currently used anthrax vaccine. The technology of rPA production and purification has already been transferred to Panacea Biotech. Ltd., India for commercialization.
4. Thermostabilized PA and LF preparations, as they are highly thermolabile (Radha *et al.*, 1996, Gupta, 1999). PA, the main immunogenic component of all available vaccines, is very thermolabile and loses activity within 48 hrs at 37°C and a few weeks at 4°C (Radha *et al.*, 1996). Although co-solvents such as $MgSO_4$ and trehalose have been shown to increase stability yet more work needs to be done in this direction to increase the thermostability of PA (Singh *et al.*, 2002).
5. DNA vaccines based on PA have been found to elicit and enhance both cell mediated and humoral response (Gu *et al.*, 1999). Combination of both PA and LF based DNA vaccines have been found to induce 4-5 times higher antibody titer as compared to when PA is given alone (Price *et al.*, 2001).
6. Edible PA vaccine is presently in the experimental stage. Once available it will provide mucosal immunity also, the primary defense against invading pathogens,

which is lacking in all other vaccines. It would be cheap and probably not require cold chain for preservation of efficacy, unlike currently available vaccines. PA was cloned and expressed in tobacco plants in our laboratory and was found functionally active (Aziz *et al.*, 2002). Now it has been cloned and expressed in tomato plants and its characterization is underway (Aziz, 2003). Once it succeeds it will give good boost to anthrax vaccination programs and increase prospects of edible vaccine.

7. Dominant negative mutants of PA, such as double mutant of K397D and D425K, D2L2 loop deletion mutant, F427A and D425K double mutant etc., which are non functional themselves but could block action of normal PA and associated toxicity in case of anthrax infection, can be used for therapy (Sellman *et al.*, 2001a, b; Ahuja *et al.*, 2003).
8. PA32 (Domain III and IV) competes with PA for binding to receptor and inhibits its binding to cell (Cirino *et. al.*, 1999). It has the potential of use both as a vaccine candidate and therapeutic agent.

1.1.10 POTENTIAL THERAPEUTIC APPLICATIONS

PA in combination with PA binding domain of LF/EF can be used as an efficient delivery system for toxins, antigens, drugs or DNA for varied purposes.

1. Fusion of Shiga toxin and Diphtheria toxin with LFn are efficiently delivered to cytosol. The fusion could be used for cancer therapy where destruction of certain cell type is needed (Arora *et al.*, 1994).
2. Direct delivery of antigen/epitope inside the cell such as gp120 portion of HIV enveloped protein (Goletz *et al.*, 1997) or 9 amino acids cytotoxic T-lymphocytes CTL epitope from *Listeria monocytogenes* (Ballard *et al.*, 1998) are able to induce CTL response, which is needed in case of intracellular pathogens.
3. Engineering specific protease site cleavable by certain cell type at furin site can target specific cells such as engineered site specific to urokinase plasminogen activator delivers LF fusion with ADP ribosylating domain of *Pseudomonas* exotoxin A to cancer cells and kills them (Liu *et al.*, 2001; 2003).

4. Even short tracts of polycationic residues such as lysine arginine or histidine at N-terminus of Diphtheria A toxin have been shown to be able to bind to PA and get internalized without the aid of LFn fragment (Blanke *et al.,* 1996).
5. LFn fused with DNA binding protein can efficiently deliver DNA into the cell (Gaur *et al.,* 2002).

1.2 ABOUT PROTEIN STABILITY

Proteins are one of the most important building blocks of the cell that mediate functioning of the cells. Depending on the need to survive under diverse environments that the cells face during evolution such as extreme growth temperature ranging from -40 to +115°C, pressure up to 120 MPa, water activity as low as 0.6 corresponding to salinities up to 6M and pH range of 1-11 (Jaenicke, 2000), they have devised different strategies to survive by increasing the stability of proteins, nucleic acids and maintaining membrane fluidity. Here, we will be discussing about the stability of proteins in particular, methods employed to increase their stability and application of this knowledge to increase the stability of proteins *in vitro*.

Stability of proteins is generally classified into two classes:

1. Conformational or Thermodynamic or Physical
2. Kinetic

Physical or conformational stability is generally defined as the free energy change (ΔG_{stab} or $\Delta G_{f \Rightarrow U}$ or simply ΔG) for an unfolding or denaturation reaction

$$F \text{ (folded/Native)} \Leftrightarrow U \text{ (Unfolded/Denatured)} \qquad \text{eq. 1.1}$$

Native/Folded state is the biologically active conformation which is most of the time compact, while denatured unfolded state is biologically inactive conformation and is most of the times less compact as compared to the native state. The fine details of these states vary with conditions. Native or folded state of a protein is a result of delicate balance of large number of interactions such as H-bonding, electrostatic interactions, hydrophobic interactions and van der Waals interactions. The final stability of the native structure over that of the unfolded state is very marginal and generally in the range of 5-20 kcal/mol (Volkin & Klibanov, 1989; Jaenicke 1990; Pace *et al.*, 1996), and is several orders lesser in magnitude as compared to that of individual interactions. It could be assigned to a few H-bonds, ion pairs or van der Waals interactions (Privalov, 1979; Dill, 1989; Vieille and Zeikus, 2001). Pace et al., 1996 calculated that average free energy gain

per H-bond is 1.1-1.6 kcal/mol while it is 1.18 kcal/mol for burial of every -CH2- group (hydrophobic effect).

Kinetic stability or the long-term stability of a protein is dependent on both conformational stability as well as chemical stability of the building blocks of the protein, *i.e.* amino acids, depending upon the environmental conditions and the nature of the protein. Thermodynamic stability provides measure of the stability of the folded conformation against denaturation by denaturants such as temperature, pH, ionic strength, pressure, excipients etc., while kinetic stability measures the resistance to irreversible inactivation or persistence of biological activity as a function of time (Fagain, 1995). Depending upon the forces involved (conformational or kinetic) the denaturation step could be either reversible or irreversible. The simplest realistic model, which includes both possibilities, was first proposed by Lumry and Erying in 1954. As per this model, denaturation occurs in two steps: 1) Reversible unfolding of the native protein (N) with an equilibrium constant K_{eq} = k1/k2 to yield an unfolded or partially unfolded state 'U'; and 2) Irreversible change in unfolded state with rate constant k3 to produce the final state 'F' which is unable to fold back to the native protein. This model can be depicted as under:

$$N \Leftrightarrow U \Rightarrow F \qquad \text{eq 1.2}$$

(k1 and k2: rate constant for forward and backward reactions of equilibrium N $\Leftrightarrow$ U; k3: rate constant for irreversible forward change in the unfolded protein that makes it unable to refold back in U $\Rightarrow$ F)

The processes responsible for the irreversible step are aggregation, deamidation (mostly aspargine and glutamine residues), autolysis and other chemical changes occurring to the residues (Ahern and Klibanov, 1987; Wang, 1999; Vieille and Zeikus, 2001). Depending on the constants k1, k2 and k3, two extreme cases can be envisaged:

1. k3<<k2. The protein unfolding behaves as a two state fully reversible process, *e.g.*, RNase and lysozyme. In this case information about the energetics and the mechanism of unfolding can be obtained in detail (Privalov 1979, 1982).
2. k3>>k1. The unfolding step is a two state irreversible process (Sanchez-Ruiz, 1992). In this case equilibrium thermodynamic analysis is not possible and only the denaturation enthalpy can be measured.

Stabilization methods generally aim at slowing down either of the steps shown in eq 1.2 above. Both reversible and irreversible unfolding processes are prevented or slowed down by additives such as sugars, surfactants, salts etc. and could also be achieved through protein engineering by removing the thermally labile residues as well as by increasing the conformational stability.

1.2.1 MECHANISMS OF INACTIVATION:

1.2.1.1 UNFOLDING, SCRAMBLED STRUCTURE FORMATION AND AGGREGATION: The native conformation of proteins is maintained by a delicate balance of different forces like H-bonding, ion pair formation, hydrophobic and van der Waals interactions. These forces get weakened in protein under various denaturing stresses such as high temperatures, pressure, presence of denaturants, etc. The protein molecule looses its native structure and unfolds, under the influence of denaturing stress. There are a number of analytical methods available for monitoring the unfolding process under the influence of denaturing conditions; these are DSC (Differential Scanning Calorimetry), CD (Circular Dichroism) spectroscopy, UV- spectroscopy, fluorescence spectroscopy, viscosity measurements, etc. Though the ability to unfold a protein in term of loss of the secondary and tertiary structure is lower for temperature in comparison to that achieved by chemical denaturants such as guanidium thiocynate, guanidium hydrochloride and urea, this discussion will be confined to unfolding process initiated by temperature for the sake of simplicity.

Melting temperature or T_m is a useful quantity used to describe and compare the ability of a protein to resist thermal unfolding. It is defined as the temperature for the given protein at which 50% of the protein molecules are unfolded ($\Delta G^\circ = 0$, at this point). For most of the proteins, T_m ranges in between 40 - 80°C. This value has been

shown to vary with the hydration state of a protein and found to increase sharply with lowering of protein hydration due to destabilization of the unfolded state (Rupley and Careri, 1991; Wang, 1999). With an increase in T_m the resistance to thermal unfolding increases, for example highly thermostable glyceraldehyde -3- phosphate dehydrogenase (GAPDH) from *Thermotoga maritima* has a melting temperature of 109°C (Jaenicke, 1996). Protein inactivation is tightly linked to the unfolding of the protein (*i.e.* loss of secondary and tertiary structure) and becomes significant only a few degrees below the T_m of protein. However there is no direct relationship between the T_m and protein stability as measured by $\Delta G_{f \Rightarrow U}$ (Dill *et al.*, 1989). It could be better described by a theoretical diagram shown below suggesting ways adopted by thermophiles to stabilize proteins (taken from Vieille and Zeikus, 2001; originally proposed by Nojima *et al.*, 1977: Fig. 1.9). Kosa *et al.*, (1998) demonstrated that although serum albumin from different sources like human, rabbit, rat, bovine, dog have different T_m values in the range of 59.7-56.8°C, yet their maximum stability (ΔG_{stab}) are all similar near 20°C. Likewise, ΔG of three SH3 domains of *tec* family of tyrosine kinases are below 12-16 kJ/mol but their melting temperatures are relatively high ranging between 69-80°C (Knapp *et al.*, 1998). The T_m of proteins also depends upon the experimental conditions and the analytical methods employed for its determination. Hence, the interpretation of protein stability in terms of T_m should be done with great caution.

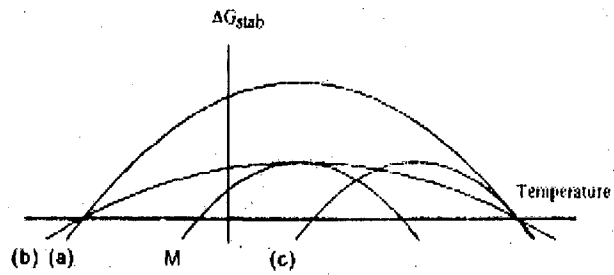


Fig 1.9. Comparison of theoretical ΔG_{stab}-versus-T curves for mesophili and hyperthermophilic proteins. M, theoretical ΔG_{stab}-versus-T curve for mesophilic protein. (a), (b), and (c), theoretical ΔG_{stab}-versus-T curves for hyperthermophilic protein. In curve (a), the hyperthermophilic protein ha the same temperature of maximal stability (Ts) as the mesophilic protein and the ΔG_{stab}-versus-T curve of the hyperthermophilic protein is shifte upward to higher ΔG_{stab} values. In curve (b), hyperthermophilic an mesophilic protein have same T_s values and the same ΔG_{stab} values at T_s The ΔGstab-versus-T curve of the hyperthermophilic protein is flatter. I curve (c), hyperthermophilic and mesophilic proteins have different T values but have the same ΔG_{stab} at their respective T_s. The ΔG_{stab}-versus- curve of the hyperthermophilic protein is shifted toward highe temperatures. the same ΔG_{stab} at their respective T_s. The ΔG_{stab}-versus- curve of the hyperthermophilic protein is shifted toward highe temperatures (taken from Vieille and Zeikus, 2001; originally proposed b Nojima *et al.*, 1977).

Most proteins unfold irreversibly (except a few small proteins) and often result in kinetically stable scrambled structures. Most of the times these scrambled structures aggregate. Aggregate formation is enhanced once the protein is unfolded as now besides exposing large hydrophobic surface area to the solvent, which was otherwise buried in the protein core, it also enhances the possibility of chemical modification/degradation that makes the folding further irreversible for the protein. The kinetic instability of the unfolded protein molecules pushes them toward aggregation in order to stabilize the system. Recent evidences suggest that aggregate formation may involve specific interactions of some conformations of the protein intermediates rather than simply by non-specific aggregation (Speed *et al.*, 1996). Both protein aggregation and folding is a result of the delicate balance of exposed and buried hydrophobic surfaces (Patro and Przybycien, 1996). The balance of exposed and buried hydrophobic surfaces holding the native structure of a protein is so delicate that even a single amino acid change can substantially alter the aggregation behaviour of the protein (Fields *et al.*, 1992).

Irreversible unfolding of proteins is generally modeled as under (Tomazic and Klibanov, 1988)

$$P_{fold} \Leftrightarrow P_{unfold} \Rightarrow P_{scrambled} \qquad \text{eq. 1.3}$$

(Active) (Inactive) (Aggregate or precipitate)

The model is consistent with an intramolecular rate determining step in thermal inactivation which pushes the earlier equilibrium of folded and unfolded state toward scrambled structure formation or aggregation. The natural logarithm of the residual activity (A_r) has been observed to be a linear function of the inactivation time as shown below.

$$\ln(A_r) = -kt \qquad \text{eq. 1.4}$$

where k is the inactivation rate constant and t is the inactivation time .

In this model the inactivation rate constant is dependent on the initial protein concentration.

Based on the theory of Patro and Przybycein (1996), Kurganov (1998) presented an elaborate model of protein aggregation to describe the kinetics of aggregation. As per this model aggregation follows the following steps:

1. Initiation of Denaturation

$N \leftrightarrow D$ (rate constant k_{den}) eq. 1.5.1

2. Nucleation

$nD \leftrightarrow Dn$ (nucleus) eq. 1.5.2

3. Aggregation

$Dn+D \rightarrow D(n+1)$ (rate constant k_{agg}) eq. 1.5.3

For the sake of simplicity initial denaturation is regarded as an irreversible reaction. Kurganov (1998) proposed that initial stages of irreversible protein aggregation involve the reaction of nucleation and the growth of aggregates. The stage of nucleation is thermodynamically unfavourable (ΔG is positive) because of the loss of configuration entropy. When the aggregation phase contains a sufficient number of stable nuclei, the system reaches a critical stage beyond which monomer D (denatured monomer)

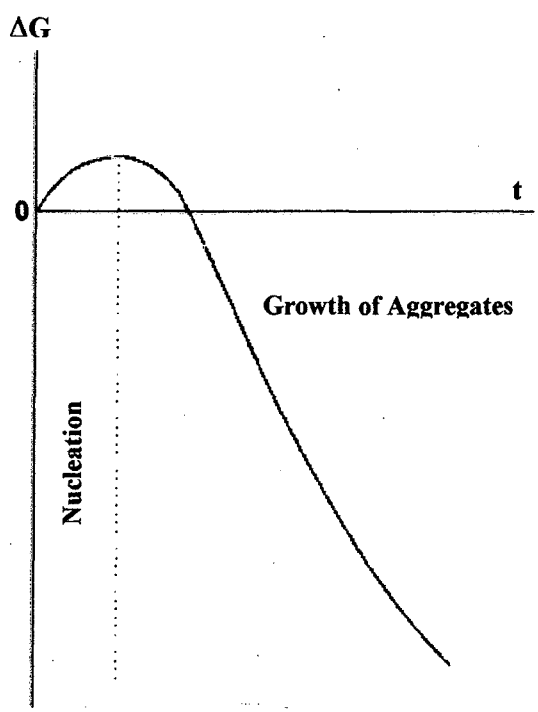


Fig. 1.10 Free energy change (ΔG) as a function of time (*t*) for the initial stages of reversible protein aggregation (taken from Kurganov, 1998; originally proposed by Patro and Przybicein in 1996).

addition is favourable, leading to a decrease in the system free energy (see Fig 1.10). Initially ($t_{lim} \Rightarrow 0$) accumulation of denatured form D proceeds linearly in time, *i.e.*

$$[D] = k_{den}[N_o].t \quad \text{eq. 1.6}$$

where N_o is the initial concentration of the protein. In the initial parts of the kinetic curve, change in absorbance (A) or scattering (I) is proportional to the product $K_{agg}.[D]$ assuming that nuclei concentration is constant or

$$\Delta A/t \propto k_{den}.k_{agg}.t \qquad \text{eq. 1.7}$$

At $t_{lim} \Rightarrow 0$, $A \propto t^2$

$$\text{or } A \propto k_{den}.k_{agg}.[N_o].t^2 \qquad \text{eq. 1.8}$$

E.q. 1.8 describes the relationship of increase in absorbance (A) to aggregation with time.

1.2.1.2 COVALENT MECHANISMS: As amino acids are the building blocks of proteins, their chemical properties do affect the stability of the molecule and sometimes they are the major contributor to the inactivation mechanisms. Some well-known covalent mechanisms involved in inactivation are deamidation, peptide bond cleavage, oxidation, disulfide bridge rearrangement, crosslinking etc. Sequences Asn/Gln-X and Asp/Glu-X are the main hotspots involved in protein inactivation along with sulfide exchange among cysteine residues.

1. **Deamidation:** It is the most common chemical degradation mechanism known to occur in proteins at higher and even at room temperature (Wang, 1999; Vieille and Zeikus, 1996, 2001). Many a times it is the major degradation pathway of proteins, *e.g.*, recombinant human DNase (Shire, 1996; Chen *et al.*, 1998), recombinant human vascular endothelial growth factor (Goolcharran *et al.*, 2000), tissue plasminogen activator (Paranandi *et al.*, 1994). Aspargine and glutamine in the sequence Asn/Gln-X are the most susceptible residues for deamidation in proteins. Asn is > 10 times prone to deamidation as compared to Gln (Vieille and Zeikus, 2001). The rate and the mechanism of deamidation is dependent on a number of factors like local structure, neighboring residues in primary sequence, flexibility/mobility of the backbone and side chains, pH, ionic strength, buffer, ions, and other solution properties (Robinson and Robinson, 2001 a, b and c and the references therein). Several deamidation mechanisms have been reported in the literature and discussed (Wright, 1991; Cleland *et al.*, 1993; Xie and Schowen, 1999; see figure 1.2.3 below). Most of the times it is not clear which mechanism is operating in a particular case.

Deamidation at Asn-X appears to be favoured at neutral or alkaline pH between 5-12 (Son and Kwon, 1995; Daniel *et al.*, 1996). Asn in peptides have been found to be most stable between pH 2-5 while above pH 5 it proceeds rapidly via cyclic imide (succinamide) intermediate formation. Between pH 1-2 it occurs slowly and appears to bypass the succinamide intermediate (Daniel *et al.*, 1996). A large number of proteins undergo deamidation (Wang, 1999; Lai and Topp, 1999) and deamidation has been shown to proceed much faster than hydrolysis in aqueous solutions (Daniel *et al.*, 1996). Some ions or compounds such as HPO_4^{2-}, imidazole, etc. have been found to enhance the deamidation process (Tyler-Cross and Schirch, 1991).

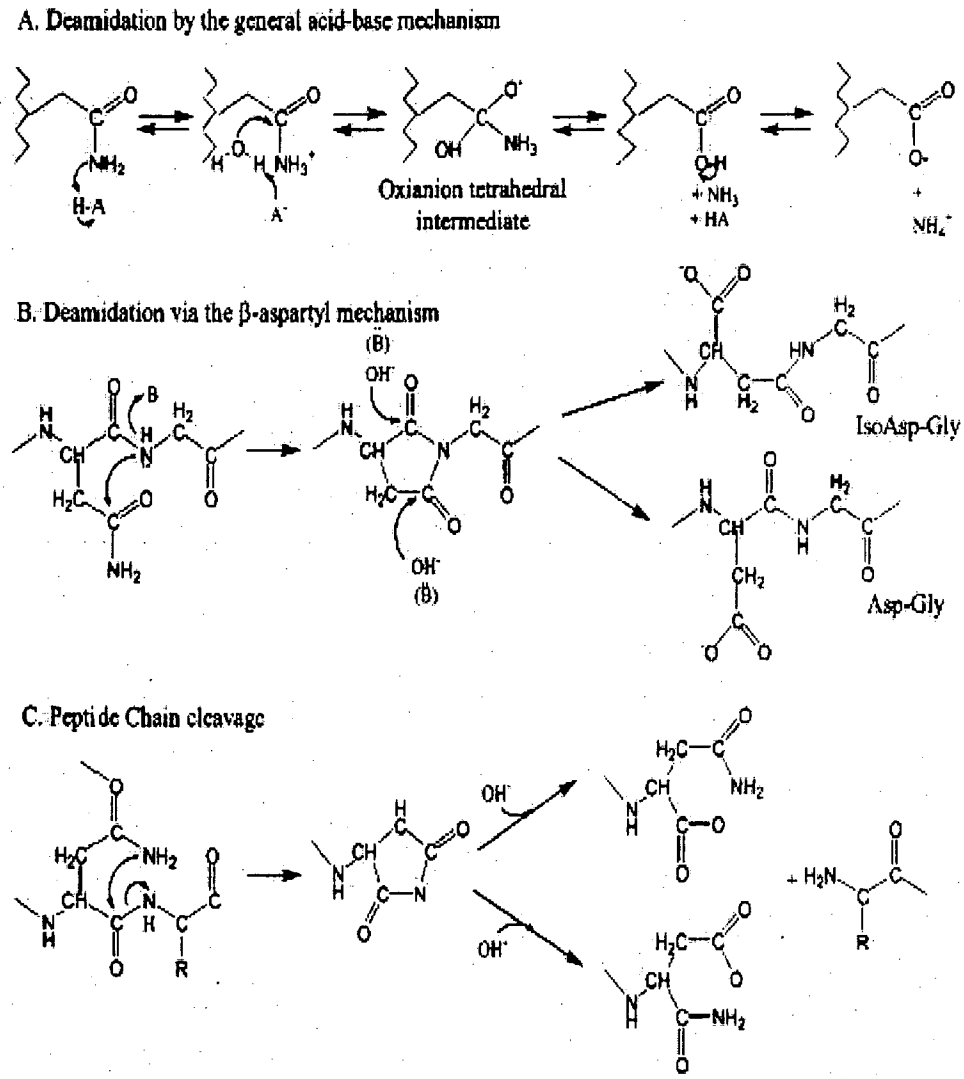


Fig. 1.11 Mechanisms of protein degradation involving Asn residues (taken from Vieille and Zeikus, 2001).

2. **Autolysis/Hydrolysis:** Most of the time hydrolysis of peptide bonds occurs at C-terminal of the Asp residue in the sequence X-Asp-Y (Manning *et al.*, 1989; Li *et al.*, 1995a; Vieille and Zeikus, 2001). The bond between Asp-Pro has been found to be the most labile (Volkin and Middaugh, 1992) possibly because of higher basicity of the N-atom of proline as compared to any other amino acid/residue and increased propensity of Asp to isomerize when linked to N-terminal of proline. Many a times it is a continuation of the deamidation reaction at Asn-Xaa sequence (Fig 1.2.3 c). Some examples are: glyceraldehyde 3 phosphate dehydrogenase of *Methanothermus fervidus* (Vieille and Zeikus, 2001), recombinant human macrophage colony stimulating factor (Schrier *et. al.*, 1993), basic fibroblast growth factor (Shahrokh *et. al.*, 1994a), etc.

3. **Disulfide bond formation, breakage and β-elimination:** All these reactions result in the formation of scrambled structures. It drastically affects the protein stability and activity. Free cysteine residues easily undergo disulfide bond formation under oxidizing conditions. Besides this, they can also mediate/take part in thiol disulfide exchange and result in protein aggregation or polymerization such as in the case of basic fibroblast growth factor (Shahrokh *et al.*, 1994a; Wang *et al.*, 1996). Thiol-disulfide exchange can also occur by a reaction between an ionized thiol group (thiolate anion) and disulfide bond. This reaction increases with increasing pH until pK of nucleophilic thiol group is exceeded (Darby and Creigton, 1997). β-elimination is another mechanism that operates in proteins such as insulin (Constantino *et al.*, 1994) and interleukin receptor antagonist in aqueous solution (Chang *et al.*, 1996) and can lead to scrambled structure formation. In this reaction, disulfide-bridge is broken down into dehydroalanine and thiocysteine as a result of β-elimination. Dehydroalnine thus formed, reacts with nucleophilic groups especially ε-amino group of lysine to form lysinoalanine and consequently produces a scrambled structure. The remaining thiocysteines can react with other cysteines producing another non-native disulfide bond.

4. **Oxidation:** The most oxidizable residues in protein are Met and Cys. Other potential oxidizable residues are His, Trp and Tyr (Manning *et al.*, 1989; Li *et al.*, 1995b, c;

Daniel *et al.*, 1996). Oxidation of these residues is mostly catalyzed by trace amounts of transition metal ions (site specific process) or upon exposure to sunlight (nonspecific). Met residues can be easily oxidized even by atmospheric oxygen. For example human growth hormone in vials containing only 0.4% O_2 gets oxidized on storage. The mechanisms involved and the products formed have been reviewed in detail (Cleland *et al.*, 1993; Stadtman, 1993a, b; Li *et al.*, 1995c). Although oxidation of residues is more common, yet their effect on the activity is not that much significant as compared to deamidation or hydrolysis (Powell, 1996).

5. **Maillard reaction:** It is one of the most widely reported browning reactions in the food industry (Chuyen, 1998). It causes the formation of carbohydrate-protein adduct as a result of reaction between a reducing sugar with amino acids at high temperatures (Paulsen and Pflughaupt, 1980). This adduct formation could change the immunological, biological or physical property of the protein of interest. Hence, storage of proteins in the presence of reducing sugars is not recommended. Tarelli *et al.*, 1994 demonstrated that lysine in vassopressin undergoes rapid glycation in the presence of reducing sugars in both aqueous and solid formulations and the N-terminal adduct can form even at -20°C.

6. **Other reactions:** Isomerization and succinimidation can occur in any residue except glycine. The most prone residues are Asn and Gln and these reactions are most of the times intermediates in the deamidation reaction. Isomerization is also frequent at Asp residue. Both isomerization and succinimidation are dependent on conformation and neighbouring residues just like deamidation. Iso-Asp formation is most likely to occur in relatively unstructured domains of intact proteins or in the domains susceptible to transient unfolding, *e.g.*, methionyl recombinant human growth hormone (Johnson *et al.*, 1989).

Non-disulfide cross-linking has been shown to occur by transamidation in the case of insulin involving Asn^{A-21} and Phe^{B-1}/Gly^{A-1} (Darrington and Anderson 1995; Strickley and Anderson, 1997). Another cross-linking pathway that probably involves formaldehyde-mediated crosslinking has been suggested by Schwendeman *et al.*, (1995) for diptheria and tetanus toxoids during storage.

1.2.2 STABILIZATION OF PROTEINS

There are number of approaches that have derived inspiration/guidance from nature. The strategies that are used to stabilize protein are broadly classified into two categories:

1. Protein Engineering: involves increasing intrinsic stability of protein by changing amino acid residues such that the resulting protein structure is more stable.
2. Co-solvent Engineering: involves changing the solvent milieu around the protein in such a way that it becomes more stable in the given condition.

1.2.2.1 Protein Engineering

1. **Stabilizing structural elements:** Stabilizing structural elements like α–helices and β-strands have been shown to be a good choice when contemplating stabilization of a protein. β-strands can be stabilized by replacing residues with high β-strand propensity (Munoz and Serrano, 1996; Otzen and Fersht, 1995). Various studies have established that the aromatic and β-branched amino acids rank highest in their ability to stabilize β-structure whereas alanine, proline, and glycine are very poor (Kim *et al.*, 1993; Smith *et al.*, 1994; Minor and Kim, 1994; Street and Mayo, 1999; Merkel *et al.*, 1999 and the references therein). Mark and van Gunsteren (1992) have shown by molecular dynamics (MD) simulations that N and C-terminals of helix are particularly prone to open up under denaturing stresses such as heat. It starts with the loss of i+4 → i interactions at helix ends. Harper and Rose (1993) first proposed introduction of "helix capping box" at ends to increase the protein stability. Based on statistical analysis of known sequences at helix terminals, the sequence S/T-X-X-E at N-terminal end of α-helix was proposed to be stabilizing, other residues at N-cap were Asn, Asp, Gln, and Cys and at N3 position they were Gln and Asp. Later more capping motifs were identified. Aurora and Rose (1998) reviewed seven distinct capping motifs in proteins. Glycine has been found to be the most favourable residue at the C-terminus due to its ability to adopt an α_L-conformation without strain. The

most preferred residues at the N-terminus are proline, aspartate, threonine and serine (Fersht and Serrano, 1993; Aurora and Rose, 1998).

Introduction of residues with high helix propensity like alanine stabilizes α helices while introduction of helix breakers, like proline and glycine destabilize the helix (Facchiano, *et al.*, 1998) Surface salt bridges spaced at i, i+3, and i, i+4 along the chain of α-helices are stabilizing (Huyghues-Despointes *et al.*, 1993), if they are parallel to the helix dipole field.

The orientation of >C=O and >N-H groups along the axis of α-helix or a β-strand results in net dipole moments at terminals with - pole on the C-terminus and + pole on the N-terminus. This arrangement of charges is destabilizing and hence introduction of negatively charged residues at the N-terminus and positively charged residues at the C-terminus stabilizes them (Vieilli and Zeikus, 1996, 2001; Querol, 1996). Introduction of negative charge at the N-terminus in two helices of T4 lysozyme, *i.e.* Ser38Asp and Asn144Asp increased the T_m by 2°C for the single mutant and 4°C for the double mutant (Manning *et al.*, 1989). This charge neutralization mechanism can be more efficient in stabilizing proteins than the introduction of alanine in the α-helix (Vieilli and Zeikus, 1996).

2. **Reducing flexibility:** Although flexibility of proteins is needed for its function at active site, yet it can be problematic at other places, as it could lead to the opening up of the structure and hence increased tendency to aggregate in the presence of a denaturant. Simulations of protein unfolding have also shown that unfolding begins in exposed loop regions (Daggett and Levitt, 1992; Lazaridis *et al.*, 1997). Thompson and Eisenberg (1999) proposed that reducing the loop size can potentially stabilize a protein as the statistical evaluation of a number of protein families with both thermophilic and mesophilic members showed that thermophilic proteins have shorter loops as compared to those in the mesophilic ones.

Structural domains, which are in close proximity, can also be stabilized by introduction of disulfide bridges and by incorporating ligand/metal binding sites as they decrease the entropy of unfolding and increase the tendency towards reversible denaturation (Lee *et al.*, 1997; Mozhaev *et al.*, 1984). Gokhale, *et al.*, (1994) have

increased the thermal stability of thymidylate synthase by introduction of two disulfide bridges across the dimer interface. Similarly introduction of metal ion binding have been shown to increase the stability of proteins tremendously, *e.g., Bam* H1 (Saxena, 2001) and Iso-1-cytochrome c of Saccharomyces cerevisiae (Muheim *et al.*, 1993). Several others example can be found in a review by Hellinga (1998 and the references therein).

Stability of the unfolded conformation has been decreased by reducing entropy of the unfolded state by glycine to X or X to proline (Gly is the most flexible residue while Pro is the most rigid residue) substitutions. Stability of I-repressor increases by 0.7-0.9 kcal/mol on Gly46Ala and Gly48Ala mutations (Hecht *et al.*, 1986) and in the case of T4 lysozyme it increases by ~0.8kcal/mol for the Ala82Pro mutant (Matthews *et al.*, 1987).

3. **Increased Hydrophobic packing in protein core:** Hydrophobic interaction is the major stabilizing force in proteins and hence an increase in the hydrophobic interactions in the protein core or increased packing of the hydrophobic side chains in the protein core can stabilize proteins tremendously. Pace (1992) has suggested that proteins gain 1.3±0.5 kcal/mol in stability upon the burial of extra methyl group by studying mutants of four different proteins. Mostly indirect experimental evidences are available that show hydrophobic interactions do stabilize the structure, for example the Ile96Val and Ile96Ala mutants of barnase are destabilized by 1.2 and 4.0 kcal/mol respectively (Kellis *et al.*, 1988). Yutani *et al.*, (1987) demonstrated that the stability of α subunit of tryptophan synthase increased with the increase in the hydrophobic character of the substitutions at position 49. Improved core packing has been shown to increase the thermal stability of chicken egg-white lysozyme (Shih *et al.*, (1995 a, b). Filling of hydrophobic cavities, such as in the folded structure of RNase H1 from *E. coli*, has also been shown to increase the stability of proteins (Ishikawa *et al.*, 1993).

4. **Replacing potential site of chemical degradation:** It is the best strategy to prolong the kinetic stability of proteins. Once the proteins capable of reversible unfolding are

unfolded, chemical degradation makes the folding irreversible and leads to decrease in the stability. Many proteins have been stabilized by replacing the residues prone to chemical degradation by chemically stable residues, as they would be making the otherwise reversible unfolding irreversible, e.g. HPr (Sharma *et al.*, 1989), Hen egg white lysozyme (Tomizawa *et al.*, 1995) and others (Mozhaev, 1993 and references therein)

5. **Other strategies:** Besides protein stabilizing strategies described above many other approaches have also been suggested and explored such as docking of the N and C termini and anchoring the loose ends, conformational strain release (Kimura *et al.*, 1992; Kawamura *et al.*, 1996), increased inter-subunit interactions and oligomerization, introduction of ion pairs and hydrogen bonds, decreasing the exposed hydrophobic surface area, aromatic-aromatic interactions between phenyl rings, post translational modifications, *etc.* (Vieille and Zeikus 2001, and references therein).

   Proteins have also been stabilized by substituting residues with those present in other homologous proteins, especially the consensus residues in multiple sequence alignment, with fair degree of certainty (Wirtz and Steipe, 1999; Rath and Davidson, 2000; Wang *et al.*, 1999; Serrano *et al.*, 1993; Larson and Davidson, 2000; Steipe *et al.*, 1994). The screening of the larger conformational space in proteins has been made possible by random mutagenesis. It has been used for the production of many new stable variants (Arnold *et al.*, 1999a,b and references therein).

1.2.2.2 Cosolvent Engineering:

Various classes of compounds like sugars, polyols, surfactants, aminoacids, polymers, salts, *etc.*, have been used to stabilize a variety of proteins (Wang, 1999; Fagain, 2003). Generally the stabilizing effect is protein specific and depends upon the various physiochemical properties of the proteins.

1. **Surfactants:** Surfactants prevent surface induced inactivation of the proteins by preventing aggregation and precipitation. All classes of surfactants, *i.e.* anionic,

cationic and neutral have been successfully used to stabilize proteins. Non-ionic surfactants are generally the preferred ones, as they do not electro-statically interact with proteins. Surface-induced aggregation and adsorption has been decreased by Tween-20 and 80 in a number of proteins, *e.g.*, transforming growth factor β-1 (Gombotz *et al.* 1996), recombinant human granulocyte colony stimulating factor (Johnston, 1996), recombinant human growth factor (Maa and Hsu, 1997; Bam *et al.*, 1998), haemoglobin (Kerwin *et al.*, 1998) *etc.* (Wang *et al.*, 1999). Many other surfactants have also been used to decrease the surface induced inactivation of proteins, *e.g.*, Poloxamers 407 and 188 for methionyl porcine growth hormone (Charman *et al.*, 1995) and recombinant human growth hormone (Katakam and Banga, 1997) respectively, SDS for BSA (Giancola *et al.*, 1997), etc.

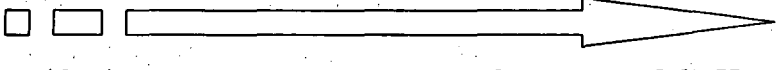
Non-ionic surfactants have been shown to even inhibit the chemical degradation in the case of human epidermal growth factor (Son and Kwon, 1995), but the presence of alkyl peroxides in the surfactants could also increase the oxidation of proteins as reported in the case of recombinant human ciliary neurotrophic factor (Kneep *et al.*, 1996) and recombinant human granulocyte colony stimulating factor (Herman *et al.*, 1996). Inclusion of antioxidants such as methionine, cysteine, or glutathione can retard these reactions significantly. This peroxide mediated oxidation is more severe than that by atmospheric oxygen.

2. **Polyols and Sugars:** These are often used as nonspecific protein stabilizers. Non-reducing sugars are the best choice, as they do not undergo Maillard reaction with proteins. Their protein stabilizing ability lies in their ability to get preferentially excluded from the protein surface, most of the time which results in an increase in the surface tension of water (Xie and Timasheff 1997a, b; Kaushik and Bhat, 1998, 2003; Timasheff, 1998). A large number of proteins have been stabilized by sugars (Wang, 1999). Arakawa *et al.*, 1993 proposed that minimum of 0.3 M or 5% sugar is needed to achieve significant protein stabilization. Different sugars stabilize proteins to different levels depending on the type of protein (Cahn *et al.*, 1996; Mcintosh *et al.*, 1998; Wang, 1999)

Sugars and polyols can also protect proteins against chemical degradation. Li *et al.*, (1996a, b) have shown that sugar can effectively inhibit the metal catalyzed oxidation of human relaxin. The inhibitory effect was shown to be the result of complex formation with metal ions instead of commonly accepted radical scavenger mechanism. Sugar and polyols are not universal stabilizers. For example sorbitol does not have any effect on the stability of recombinant human keratinocyte growth factor (Chen *et al.*, 1994a) and porcine growth hormone (Charman *et al.*, 1993), while mannitol even destabilizes interleukin-1 receptor by decreasing its T_m from 48.1 to 46.7°C (Remmele *et al.*, 1998).

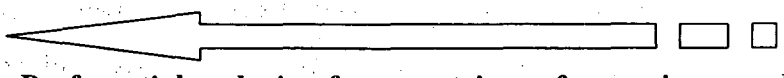
3. **Salts:** Salts affect the stability of protein by non-specific screening of the electrostatic interactions (Deby-Hückel) among the charged groups in proteins, specific ion binding to proteins and its effect on solvent properties at high concentrations hence the net effect on protein stability can be stabilizing, destabilizing or neutral depending upon the type of interaction among the charged groups of proteins and the interaction of ions with them as well as their effect on the solvent properties. At lower concentration, the effect of salts are specific to proteins and determined by specific ion binding and charge screening, while at higher concentration their effect correlates with the Hofmeister series both for anions and cations (Volkin and Klibanov, 1989; Timasheff, 1998) as shown below. Ions on the left enhance the hydrophobic interactions in proteins by reducing the solubility of hydrophobic groups of proteins. They also cause preferential hydration of the protein. Combination of these two effects makes proteins more compact and stable. Ions on the right side destabilize the protein by binding extensively to the charged groups or the dipoles (peptide bonds) of protein.

Preferential binding to protein, so decreased stability



$(CH_3)_4N^+$>NH_4^+> K^+, Na^+> Mg_2^+>Ca_2^+> Ba_2^+> $GdmH^+$

SO_4^{2-}>CH_3COO^->Cl^->Br^->NO_3^->ClO_4^-> SCN^-



Preferential exclusion from protein surface, so increased stability

Many proteins have been stabilized to different degrees by salts, *e.g.*, BSA (Giancola *et al.*, 1992), recombinant factor VIII SQ (Fatoures *et al.*, 1997a), RNase T1 (Mayr and Schmid, 1993), bakers yeast alcohol dehydrogenase and bovine liver glutamate dehydrogenase (Ramos *et al.*, 1997), *etc.*

4. **Polymers:** The ability to stabilize proteins is generally attributed to their surface activity, preferential exclusion from the protein surface, steric hindrance to protein - protein interactions and increased viscosity, limiting movement of protein structural domains. The stabilization ability is also very protein specific in the case of polymers.

   Hydroxy propyl β-cyclodextrin (HP-β-CD) is one of the best protein stabilizer and solublising agent among polymers. It has been used to prevent thermal inactivation and interfacial denaturation and precipitation of porcine growth hormone (Charman et. al., 1993), aggregation of recombinant human keratinocyte growth factor (Zhang *et al.*, 1995), stabilize interleukin-2 and bovine insulin (Brewster, 1991). Other polymers that have been used for protein stabilization include dextran (51 and 18 kD) for increasing T_m of thrombin (Boctor and Mehta, 1992), dextran (38 and 82 kD) for increasing T_m of porcine pancreatic elastase (Chang *et al.*, 1993), PVP (10k) for increasing the T_m of interleukin-1 receptor (Remmele *et al.*, 1998), fibronectin 0.05% for increasing mitogenic activity of human epidermal growth factor (Son and Kwon, 1995) *etc.* In addition to polymers described above sulfated polymers, sulfated polysaccharides, polyamino acids, heparin, etc. have also been used (Wang, 1999). Polymers may inhibit chemical degradation in proteins as well. For example dextran can inhibit metal catalyzed oxidation of human relaxin (Li *et al.*, 1996a, b)

5. **Metal Ions:** Metals directly bind to charged sites and make the structure more rigid and compact and hence, stabilize the protein (Kristjannson and Kinsella, 1991). Mozhaev and Martinek (1984) suggested their involvement in the stabilization of thermophilic proteins. They can even enhance the activity of proteins as observed in

the case of RNase H (Goedken and Marquesee, 1998). Among metal ions, calcium is the well known stabilizer for many proteins both at high and low temperatures.

Examples of stabilization achievd by metal ions include, 19 kDa catalytic fragment of human fibroblast collagenase by Ca^{2+} (Lowry *et al.*, 1992), RNase H by Mn^{2+} (Goedken and Marqusee, 1998), porcine pancreatic DNase by Ca^{2+} (Chan *et al.*, 1996), inhibition of insulin fibrillation by Ca^{2+} or Zn^{2+} (Brange, 1997)

6. **Amino acids:** They stabilize proteins mostly by the preferential exclusion mechanism (Jensen *et al.*, 1996). Aggregation of keratinocyte growth factor is inhibited by his, gly, sodium aspartate, sodium glutamate and lysine hydrochloride (Zhang *et al.*, 1995). Similarly stability of pig heart mitochondrial malate dehydrogenase (Jensen *et al.*, 1996), recombinant human keratinocyte growth factor (Chen *et al.*, 1994a), RNase A (Gopal and Ahluwalia, 1993; Liu and Sturtevant, 1996), cytochrome c (Taneja and Ahmad, 1994; Foord and Leatherbarrow, 1998), interleukin-1 receptor (Remmele *et al.*, 1998) *etc.* have been shown to increase in the presence of different amino acids and upto different degrees. In the case of cyt-c, amino acids Gly, Ala, Lys, Ser, Thr, Pro, Met and Val increased the T_m while amino acids Arg and His destabilized the molecule (Taneja and Ahmad, 1994). Destabilization of RNase A, holo-alpha-lactalbumin, apo-alpha-lactalbumin, lysozyme and metmyoglobin by Arg and His amino acids have been also observed (Rishi et al., 1998). Certain amino acids may prevent chemical degradation as well, especially Met and His have been shown to decrease ascorbate/Cu(II)/O2 dependent oxidation of papain (Kanazawa, *et al.*, 1994a, b).

Several other approaches have been also tried on individual basis. α-crystallin has been used as a chaperone to prevent the aggregation and facilitate refolding of reduced lysozyme (Raman *et al.*, 1997). Recombinant human protein disulfide isomerase has been found to enhance refolding of recombinant human interleukin -2 (C125A mutation: C125A rh IL2) by accelerating thiol disulfide exchange (Du *et al.*, 1998). Cyclic diphosphoglycerate can stabilize glyceraldehyde 3 phosphate dehydrogenase at 90°C (Hensel and König, 1988). 2-O-β-mannosylglycerate has been shown to stabilize rabbit

muscle lactate dehydrogenase, bakers yeast alcohol dehydrogenase and bovine liver glutamate dehydrogenase (Ramos *et al.*, 1997). Several other references could be found in a review by Wang (1999).

1.3 BACKGROUND AND OUTLINE OF THESIS:

PA is known to be a highly thermolabile molecule that loses activity within 48 hrs of incubation at 37°C, while at 4°C it takes a few weeks to lose the activity completely. Loss of activity results in its inability to bind to the cell surface (Radha *et al.*, 1996). As PA is the main immunogen of all vaccines against anthrax, its thermolability is cause of great concern as even transient exposure to higher temperatures could compromise the efficacy of the vaccine. Efforts were made in our laboratory (Radha *et al.*, 1996), therefore, to stabilize the molecule against thermal inactivation. This included testing/evaluation of the ability of certain selected co-solvents as excipients to stabilize PA. It was found that 3M $MgSO_4$ was able to retain ~80% activity even after incubation at 37°C for 48 hrs. In order to apply the strategy of using cosolvents in an effective manner, there is an urgent need to study the mechanism of thermal inactivation of PA, so that rational design strategies to stabilize PA molecule can be applied. With this in view, the work outlined "Studies on Thermostabilization of Protective Antigen of *B. anthracis*", was envisaged. Protein engineering and co-solvent engineering approaches were used to increase the thermostability of PA, so that its shelf life could be increased to such an extent that storing it at room temperature may not cause any loss of activity over a considerable period of time. The stabilized PA could, thus, be stored at room temperature at least for brief periods or could withstand the transient high temperature exposures, which may happen during transportation of the vaccine.

Chapter 2 of the thesis deals with increasing the stability of PA by protein engineering. This was facilitated by the knowledge of the crystal structure of the protein molecule (Petosa *et al.*, 1997). There was, however, a need to model the missing part of the PA structure followed by identification of the buried hydrophilic residues or exposed hydrophobic surfaces/residues. Sequence alignment of the PA sequence with homologous proteins like ADP ribosyl transferase and Iota Ib toxin revealed that most of the buried hydrophilic groups were conserved, except a few. Non-conserved hydrophilic residues among the aligned sequence were changed to alanine to decrease the hydrophilic character in the protein interior assuming that the non-conserved buried residues would not be strongly involved in the stabilizing interactions like H-bond network and could be dispensable. Besides this, an exposed hydrophobic residue

Phe554, which formed a dispensable part of the hydrophobic patch that is involved in the oligomerization of the protein (Ahuja *et al.*, 2001), was also replaced with alanine to decrease the hydrophobic character of the exposed surface, as exposed hydrophobic patches have been known to cause inactivation of many proteins by aggregation (Wang, 1999). The mutants Gln277Ala and Phe554Ala were found to have enhanced stabilities over the native protein, with Phe554Ala mutant being able to retain ~90% activity after incubation at 37°C for 48 hr.

Chapter 3 deals with the characterization of the various processes that lead to the thermal inactivation of PA. It became clear that the main cause of thermal inactivation was aggregation. Keeping this in mind the effect of various co-solvents like salts, glycine and its methyl derivatives and polyols were investigated. To have a quick assay for the ability of the co-solvents to thermostabilize PA, incubation of the protein was carried out in the higher temperature range of 40°C to 75°C for 20 minutes in the presence and absence of co-solvents. Results showed that in case of glycine and its methyl derivatives, ability to thermostabilize PA followed the order glycine > sarcosine > dimethyl glycine > betaine, consistent with their increasing hydrophobic character while in the case of polyols there was no clear trend observed based on the number of –OH groups. For example, adonitol was not having any effect on PA stability at lower concentrations while its epimer xylitol was stabilizing the PA molecule at the same concentration and erythritol was even destabilizing the PA molecule at lower concentration. It was found that 2.7 M glycerol along with 500 mM NaCl in 40 mM Hepes, pH 7.9 (at 25°C) was the best condition able to prevent the thermal inactivation of the PA even at 75°C. Under this condition PA was able to retain 100% activity even after 20 min incubation while in the control it became 0% even at 55°C within 10 minutes. Prolonged incubation in the same condition showed very rapid loss of activity after ~30 minutes, which probably indicates the possibility of chemical modifications of some residues, such as deamidation of labile Asn and Gln residues at critical positions or some other reactions, making the reactivation irreversible on cooling. Further work would be required to test for the long-term stability of the PA molecule at moderate temperatures (37°C - 45°C) under the best-selected conditions, so that PA can be effectively used in vaccines.

CHAPTER 2

THERMOSTABILIZATION OF PA: MOLECULAR MODELING AND PROTEIN ENGINEERING

2.1 INTRODUCTION:

Protective Antigen (PA) is the central molecule in anthrax intoxication/pathogenesis and as the name suggests is able to provide protective immunity against anthrax. It is the cell-binding moiety among the tripartite anthrax toxin complex molecule and helps in the translocation of the enzymatic moieties, *i.e.* Edema Factor (EF) and Lethal factor (LF), to the cytoplasm, where they exert their toxic effects. Inside cytosol, EF and LF cause massive production of nitrogen oxide and oxygen radicals along with cytokines and interleukins by macrophages. This results in anaphylactic shock and death of the sensitive animals.

Petosa *et al.*, in 1997 have elucidated the crystal structure of PA. According to the crystal structure details, PA consists of four domains, which are primarily organized into antiparallel beta sheets with a few short helices of less than 4 full turns in length. Domain 1, a beta sandwich with a jelly roll topology, is responsible for binding to LF/EF during the anthrax intoxication process, while domain 2 is organized as a beta barrel and plays a role in the oligomerization and membrane insertion of PA. Domain 3 is the smallest domain and is also involved in oligomerization (Mogridge *et al.*, 2001; Ahuja *et al.*, 2001) and possibly binding to LF/EF (Little *et al.*, 1996). Domain 4 is the receptor-binding domain (Brossier *et al.*, 1999, Varughese *et al.*, 1999)

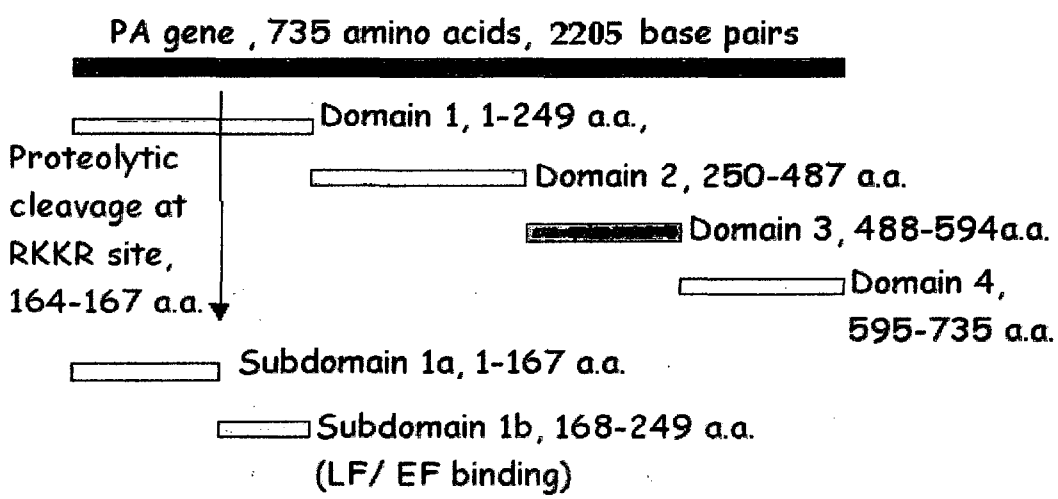


Fig 2.1 GRAPHICAL DEPICTION OF THE STRUCTURE OF PA

PA has been shown to elicit protective immunity in experimental animals (Ivins *et al.*, 1998; McBride *et al.*, 1998; Pitt *et al.*, 2001; Reuveny *et al.*, 2001). It has recently been shown that domain 4 of PA alone can provide immunity (Flick-Smith *et al.*, 2002). EF and LF individually are not immunogenic, yet their administration along with PA increases the antibody titer against LF and EF, which could be more protective (Singh *et al.*, 1998). Though the protective efficacy provided by anthrax toxin components *i.e.* PA, EF, and LF either individually or in combination is lesser than that provided by attenuated strains (Little and Knudson, 1986; Brossier *et al.*, 2002), but the toxigenicity and side effects associated with the attenuated strains have prompted the use of purified PA as a vaccine candidate. Different strategies are underway to increase its potency, which involves producing non-toxic mutants of anthrax toxin components (PA, EF, LF) and then using them in combination (Singh *et al.*, 1998). Different adjuvants are also being evaluated to increase its efficacy and provide long lasting immunity against anthrax (Ivins *et al.*, 1992, 1995; McBride *et al.*, 1998).

PA is a very thermolabile molecule. Its thermolability has been a cause of concern as accidental exposure to higher temperature during transportation or storage of the vaccines could compromise its efficacy. Hence it is imperative to increase the thermostability of PA. There have been two approaches to increase the stability of proteins: a) modulating the milieu by co solvents like salts, amino acids, polyols and sugars (See Chapter 1: section 1.2.2.2) etc. ; b) changing the primary sequence by genetic engineering and hence its intrinsic stability. Our laboratory endeavours (Radha *et al.*, 1996) have shown that use of co-solvents can be a good proposition to prevent loss of activity of PA. Magnesium sulfate at 3M concentration was found to be the best stabilizer among the various stabilizers studied, retaining more than 80% activity after 48 hrs of incubation at 37°C. The control PA without the stabilizer completely loses the activity in the same period. Increasing intrinsic stability of PA by genetic engineering could be a promising alternative but so far, there have been no efforts made in this direction. A number of approaches have been explored to increase the intrinsic stability of different proteins (Vieille and Zeikus 2001, and references therein; van den Burg and Eijsink, 2002 and references therein). Gokhale *et al.*, (1994) engineered two disulfide

bridges across the dimer interface of Thymidylate synthase and improved its stability. Strategies employing proline substitutions and glycine replacements to X (any aminoacid) have been successfully tried with T4 lysozyme by Nicholson *et al.*, (1992). Removal of buried hydrophilic residues with unsatisfied H-bonds has been shown to increase the stability of MYL, a variant of Arc repressor by Hendsch *et al.*, (1996). Empirical approaches such as substitution of an amino acid with a conserved residue present in homologs, preferably thermostable ones, have been successfully used to increase the stability of GroEL minichaperons (Wang, *et al.*, 1999), Abp1p SH3 domain (Rath and Davidson, 2000) and Immunoglobulin V_H domain (Wirtz and Steipe, 1999).

The aim of the present study was to thermostabilize the PA molecule without affecting its functional stability. Since no thermostable homologue of PA is known and its functional stability should not be compromised while attempting its thermostabilization, following strategy was followed:

1. *In silico* model building of the missing parts of PA structure for further analysis.
2. Identification of the totally buried hydrophilic amino acid residues.
3. Sequence alignment with homologous proteins and identification of the non-conserved residues.
4. The non-conserved buried hydrophilic residues Gln277, Ser330, Thr674, which may not be strongly involved in stabilizing interactions like H-bond network were changed to alanine.
5. Hydrophobic character of a hydrophobic patch, which is known to be involved in oligomerization (Ahuja *et al.*, 2001) was reduced by changing one of the residues Phe554 to alanine, as exposed hydrophobic surfaces are known to cause aggregation in proteins.

The present work, thus, focuses on generating thermostable variants of PA by genetic engineering, which could be used as a better vaccine candidate against anthrax.

2.2 MATERIALS

2.2.1 Chemicals

Agarose (Sea Kem GTG) was from FMC Corp, USA. Acrylamide, Ampicillin, Bovine Serum Albumin (BSA), Coomassie brilliant blue R-250, Calcium chloride, Glycine, Glutamine, Glycerol, Glucose, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Potassium acetate, HEPES, PMSF, SDS, Sodium acetate, Sodium chloride, Sodium hydroxide, Sodium bicarbonate, Penicillin, Streptomycin, Imidazole, Diaminobenzidine, Tris, Tween-20, Protein determination reagent and other chemicals were purchased from USB Chemicals, USA. Nitrocellulose membranes were purchased from Amersham Pharmacia Biotech, UK. Ni-NTA agarose was purchased from Qiagen, Germany. Cell culture plasticwares were obtained from Corning, USA. Fetal calf serum (FCS) was from Biological Industries, Israel. RPMI 1640, 3-(4,5-dimethylthiazol-2-yl), -5-diphenyltetrazolium bromide (MTT) and other chemicals were purchased from Sigma Chemical Co., USA. Media components for bacterial growth were purchased from Hi-Media Laboratories, India. PA purified from *E. coli* transformed with pMW1 (Gupta *et al.*, 1999) was used as standard.

2.2.2 DNA modifying enzymes and reagents

The enzymes and chemicals used for DNA manipulation were purchased from Life Technologies, USA; Boehringer Mannheim, Germany; MBI fermentas and New England Biolabs, USA and Stratagene, USA. The oligonucleotides were obtained from Critical Technologies for Molecular Medicine, Yale University Medical School, USA and Microsynth Inc., Sweden. The PCR was performed on Perkin Elmer thermal cycler using DNA amplification kit from Perkin Elmer, USA. The expression vector pQE30 was obtained from Qiagen, Germany.

2.2.3 Bacterial strains and cell lines

E.coli strain DH5α and macrophage like cell lines RAW264.7 and J774A.1 were obtained from ATCC (American type culture collection), USA.

2.3 SECTION A. MOLECULAR MODELING, ANALYSIS OF PA STRUCTURE AND IDENTIFICATION OF BURIED HYDROPHILIC GROUPS

2.3.1 RESEARCH METHODOLOGY

2.3.1.1 Modeling of PA and identification of buried hydrophilic groups

The crystal structure of PA (1ACC; pdb; www.rcsb.org) was retrieved and the missing residues were reconstructed using homology module of insight II program from MSI (Accelrys). The modeled PA structure thus obtained was further subjected to energy minimization using AMBER force field (Weiner *et al.*, 1984, 1986). In the first stage, only hydrogen atoms were minimized (as most of the time they are the main cause of nuisance in energy minimization) with all heavy atoms fixed. In the second stage the structure thus obtained was subjected to minimization with all atoms free to minimize. Steepest descent (SD) method and conjugate gradient (CG) method of minimization was used. The energy-minimized structure was used for further analysis of PA for accessible surface area (ASA) of residues using ACCESS program of Lee and Richards (1971). A probe of 1.4 Å and a slice thickness of 0.1 Å was used. Percent (%) accessibility of each residue was calculated and residues with 0 % accessibility were selected.

2.3.1.2 Sequence alignment

Multiple sequence alignment (Table 2.2) was done using CLUSTAL-W program (Thompson *et al.*, 1994; available at www.ebi.ac.uk) to find out the conserved and non-conserved residues in homologous proteins and to select the variable residues that could be mutated to increase the hydrophobic character in the protein interior.

2.3.2 RESULTS

The table given below shows the totally buried hydrophilic residues with 0 % solvent accessibility.

Table 2.1: PA residues with 0 % Solvent Accessible Surface Area (SASA)

RESIDUE NO.	RESIDUE	TOTAL SURFACE AREA* (Å²)	SOLVENT ACCESSIBLE SURFACE AREA (SASA) (Å²)
1	GLU	407.776	0.00
6	ASN	340.623	0.00
7	ARG	545.428	0.00
62	SER	260.644	0.00
67	GLY	175.632	0.00
89	MET	445.340	0.00
181	ASP	327.708	0.00
182	GLY	175.632	0.00
188	GLU	407.776	0.00
192	TYR	568.484	0.00
221	SER	260.644	0.00
222	SER	260.644	0.00
232	PRO	387.849	0.00
233	TYR	568.484	0.00
234	SER	260.644	0.00
237	GLU	407.776	0.00
244	ASP	327.708	0.00
277	GLN	420.691	0.00
330	SER	260.644	0.00
368	ASN	340.623	0.00
373	PRO	387.849	0.00
434	MET	445.340	0.00
488	THR	343.284	0.00
494	ASN	340.623	0.00
674	THR	343.284	0.00

Most often hydrophilic groups, when present in the protein interior, are part of a H-bond network and are very critical for the stability of the molecule. In order to avoid changing the residues that may be critical for the stability of the protein, buried hydrophilic residues were sequence aligned with Iota Ib (*Clostridium perfringens)* and ADP ribosyltransferase (*Clostridium difficle)* by CLUSTAL-W program presuming that the residues critical for stability will be conserved in homologous proteins. Sequence alignment (Table 2.2) shows that majority of the buried hydrophilic residues are more or less conserved except Gln277, Ser330 and Thr674. Presuming that residues Gln277, Ser330 and Thr674 are not important for structure and change at these positions will be tolerated, these residues were selectively changed to alanine. It was assumed that such a change would stabilize the molecule, as the cost of burying a hydrophilic group would be minimized. Besides these residues Phe554, which is a dispensable part of a solvent exposed hydrophobic patch (Ahuja *et al.,* 2001) involved in oligomerization of PA, was also chosen for change to alanine to decrease the hydrophobic character of the hydrophobic patch, as in many instances exposed hydrophobic patches are involved in inactivation of proteins by inducing aggregation.

Table 2.2 Sequence alignment of PA (*Bacillus anthracis*) with Iota Ib (*Clostridium perfringens*) and ADP ribosyltransferase (*Clostridium difficle*)

Position > Protein v	1	6	7	62	89	181	188	192	221	222	232	233	234	237	244	277	330	368	373	434	488	494	674
PA	E	N	R	S	M	D	E	Y	S	S	P	Y	S	E	D	Q	S	N	P	M	T	N	T
Iota Ib	D	E	E	S	M	D	E	Y	S	S	P	Y	T	Q	D	A	H	N	P	I	S	D	I
ADP rib.	H	K	E	S	M	D	E	Y	S	N	P	Y	T	E	D	A	H	N	P	I	S	D	I

Legend:

	Identical
	Highly Conserved
	Conserved
	Variable

2.4 SECTION B: CONSTRUCTION OF THE MUTANTS Q277A, S330A, F554A AND T674A USING SITE-DIRECTED MUTAGENESIS

2.4.1 RESEARCH METHODOLOGY

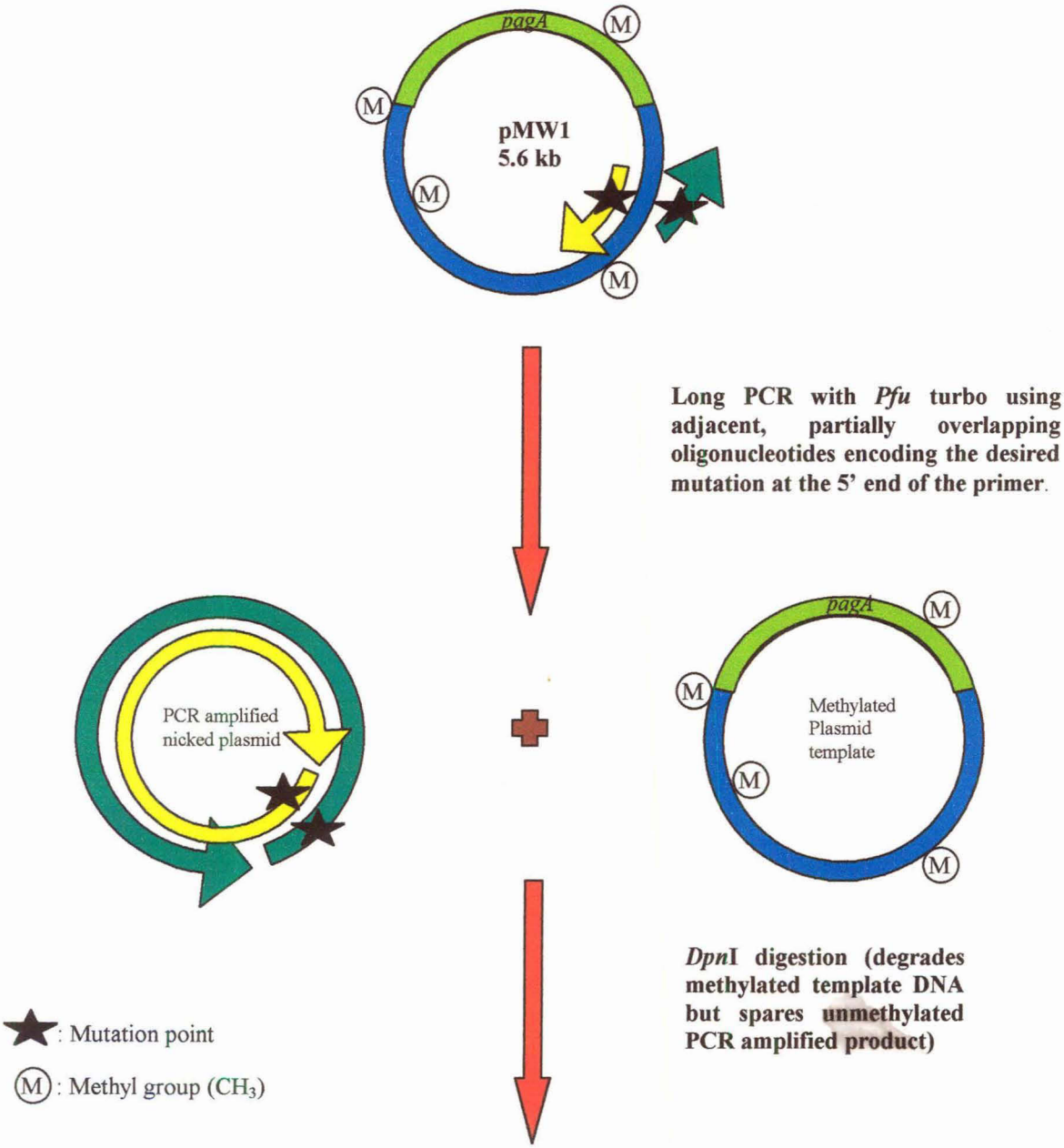


FIG 2.2 SCHEMATIC PRESENTATION OF THE CONSTRUCTION OF MUTANTS OF PROTECTIVE ANTIGEN

2.4.1.1 Generation of mutant PA genes: Long PCR

For generating each mutant, two complimentary mutagenic oligonucleotides, having mutation point near their 5′ end were used to prime DNA synthesis by *Pfu* Turbo (mix of *Pfu* and *Taq* DNA polymerase). Entire lengths of both the strands of the plasmid pMW1 (Gupta *et al.*, 1999) were amplified in a linear fashion during several rounds of thermal cycling, generating a mutant plasmid with staggered complimentary ends on the opposite strands which could anneal and produce a circular plasmid with nick (Hemsley *et al.*, 1989). These nicked mutagenized plasmids get ligated inside the cell producing the fully functional and circular mutant plasmid.

Mutants of PA were generated using 4 sets of two mutagenic primers, each spanning the desired mutation point but on opposite strands, for PCR. The sequence of the above mentioned primers is as shown below. The mutation point has been underlined.

TABLE 2.3: Sequence of primers used for constructing mutations in pMW1.

Mutation	Primer	Sequence
Gln277Ala	F	5′ CA AAA AAT GAG GAT **GCA** TCC ACA CAG AAT ACT GAT AGT GAA ACG AGA AC 3′
	R	5′ G TGT GGA **TGC** ATC CTC ATT TTT TGA GAG AAT AAT ATT CTC CAT ATC TAC 3′
Ser330Ala	F	5′ CG AAT TCA **GCT** ACG GTC GCA ATT GAT CAT TCA CTA TCT CTA GCA GGG 3'
	R	5′ C AAT TGC GAC CGT **AGC** TGA ATT CGA ATT ACT AAA TCC TGC AGA TAC ACT CCC ACC 3′
Thr674Ala	F	5′ CAA GAT GGA AAA **GCA** TTT ATA GAT TTT AAA AAA TAT AAT GAT AAA TTA CCG TTA TAT ATA AG 3′
	R	5′ C TAT AAA **TGC** TTT TCC ATC TTG CCG TAA ACT AGA AAT ATT CAA C 3′
Phe554Ala	F	5′ GAA TTT GAT TTT AAT **GCA** GAT CAA CAA ACA TCT CAA AAT ATC AAG AAT CAG 3′
	R	5′ GAG ATG TGT TTG TTG ATC **TGC** ATT AAA ATC AAA TTC GGT TAT GTC TTT CCC TTG 3′

Note: F and R refer to forward and reverse primers. Mutant codons are in bold and underlined.

PCR was performed in a 100 µl reaction mix using DNA thermal cycler (Perkin Elmer) in 0.2 ml thin walled tubes. The reaction mix consisted of 25 ng of template DNA (pMW1), 0.2 mM of each dNTP (Amersham Inc.), 25 pmol of each oligonucleotide, 2 µl of *Pfu* polymerase PCR buffer (10X) and 2 units of *Pfu* Turbo DNA polymerase (Stratagene). The PCR conditions were:

i) Initial denaturation at 94°C for 5 minutes.

ii) 94°C for 0.5 minute.

iii) Annealing at 58°C for 0.75 minute.

iv) Extension at 68°C for 4.5 minutes.

v) Final extension at 68°C for 7 minutes.

Steps (ii), (iii) and (iv) were repeated for 20 cycles.

The amplification was checked by agarose gel electrophoresis of the PCR product.

2.4.1.2 *Dpn*I digestion of the PCR product

The PCR product was treated with *Dpn*I that specifically cleaves fully methylated $G^{m}ATC$ sequences (Vovis and Lacks, 1977). The digestion reaction was carried out in 20µl reaction volume with 100ng of the amplified product, 2 µl of 10X *Dpn* I reaction buffer and 0.5 U of *Dpn* I. *Dpn* I digests the methylated pMW1 which was used as template and not the unmethylated DNA synthesized during the course of PCR. *Dpn* I treated PCR product was transformed into competent *E. coli* DH5α cells and the transformed cells were selected on LB Agar plates supplemented with 100 µg/ml ampicillin.

2.4.1.3 Preparation of competent *E. coli* DH5α cells:

The procedure adopted for the preparation of competent *E. coli* DH5α cells was that of Cohen *et al.*, (1972) with slight modifications.

1. For making competent cells, *E. coli* DH5α cells were freshly streaked from the frozen glycerol stock kept at -70°C, on an LB Agar plate. Plate was incubated overnight at 37°C. A single colony was inoculated into 3 ml LB broth and incubated overnight at 37°C with shaking. For preparing competent cells one ml of the overnight grown culture was inoculated into 100 ml LB broth. It was allowed to grow at 37°C with shaking for 2-3 hr. until A_{600} reached 0.4-0.8. The culture was chilled on ice-cooled water to arrest the growth then transferred to ice cold 50 ml polypropylene tubes and centrifuged at 4000 rpm for 10 min in a Sorvall SS34 rotor.

2. The supernatant was decanted and the pellet was resuspended gently in 10 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 10 min.

3. The cells were then centrifuged at 4000 rpm in Sorvall SS34 rotor. The pellet was resuspended in 3 ml of ice cold 0.1 M $CaCl_2$. Chilled glycerol was added to the cells to a final concentration of 20%.

4. About 200 µl aliquots were taken for checking viability, contamination and efficiency of transformation. The rest of the suspension was kept at 4°C for 12-24 hours to enhance the competency of the cells and then stored in aliquots of 200 µl at - 70°C.

2.4.1.4 Transformation of competent cells

Transformation of competent *E.coli* DH5α cells with pMW1 and the mutagenized plasmids was performed according to procedures described by Sambrook *et al.*, 1989 as described below

1. A 200 µl aliquot of competent cells in 1.5 ml Eppendorf tube was thawed over ice-water mix and 10 ng of the *Dpn* I digested PCR product DNA was added to it, mixed by tapping and kept on ice-water mix for 30 min.

2. The cells were subjected to heat shock at 42°C for 90 seconds in a water bath and were immediately chilled on ice for 1-2 min.

3. About 800 µl of LB broth was added to the cells and incubated at 37°C for 1hr on a shaker.

4. The cells were pelleted and resuspended in 100 µl of LB broth and plated on LB Agar plates containing 100 µg/ml of ampicillin and incubated at 37°C for 16 hours.

2.4.1.5 Screening of the transformants

The presence of the mutant plasmids was confirmed by mini-preparations of plasmid DNA. This method is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

1. A single transformed colony was inoculated in 3 ml of LB broth medium containing appropriate antibiotics and shaken overnight at 37°C. Cells from 1.5 ml of the culture were pelleted in a microfuge tube by centrifugation at 12,000g for 30 seconds.

2. The media was discarded and the pellet was resuspended in 300 µl of ice-cold solution I (50 mM glucose, 25mM Tris-Cl, pH 8.0 and 10 mM EDTA, pH 8.0).

3. 300 µl of freshly prepared solution II (0.2N NaOH, 1% SDS) was added to the tube. The contents of the tube were gently mixed by inverting the tube four to five times and incubated at room temperature for five minutes.

4. After 5 minutes, 300 µl of ice-cold solution III (5M potassium acetate, glacial acetic acid and water) was added and vortexed to mix the contents of the tube and incubated on ice water mix for 15 minutes.

5. The mixture was centrifuged at 12,000g for 15 minutes. The supernatant was transferred to a fresh tube and 0.7 volume of isopropanol was added.

6. The DNA was precipitated by centrifugation at 13,000g for 30 minutes at room temperature. The pellet was washed with 70% ethanol and air-dried. The air-dried pellet was resuspended in 20 µl of autoclaved Milli-Q water and a 2 µl aliquot of the preparation was checked on 0.8% agarose gel.

The plasmids were screened further by restriction analysis with *Kpn*I and *BamH*I restriction endonucleases. Since these enzymes have been used in cloning of PA gene into pQE30 (pMW1), digestion with the same should give fallout of the right size corresponding to size of the gene (PA gene-2.2kb) and another band corresponding to the vector (pQE30-3.4kb).

2.4.1.6 DNA Sequencing

The mutations were confirmed by sequencing of the above constructs by automated sequencing at a commercial DNA sequencing facility available at South Campus, University of Delhi, New Delhi-67.

2.4.2 RESULTS

2.4.2.1 Construction of alanine substitution mutants of PA

To elucidate the role of buried hydrophilic residues Q277, S330, T674 and one exposed hydrophobic residue F554 in stability of PA, with the aim of increasing thermostability, were changed to alanine by site-directed mutagenesis using long PCR. The set of mutagenic primers used for generating each mutation contained the desired mutation point near their 5′ end and primed the synthesis of DNA on opposite strands. The primers used for long PCR amplification, had relatively high melting temperatures and wherever possible ended in a G/C (singlet) nucleotide to avoid false priming and primer-dimer formation. Plasmid pMW1, that contains the gene for mature PA cloned into the expression vector pQE30, was used as template for long PCRs. The PCR amplified DNA contained the PA gene with the desired mutation.

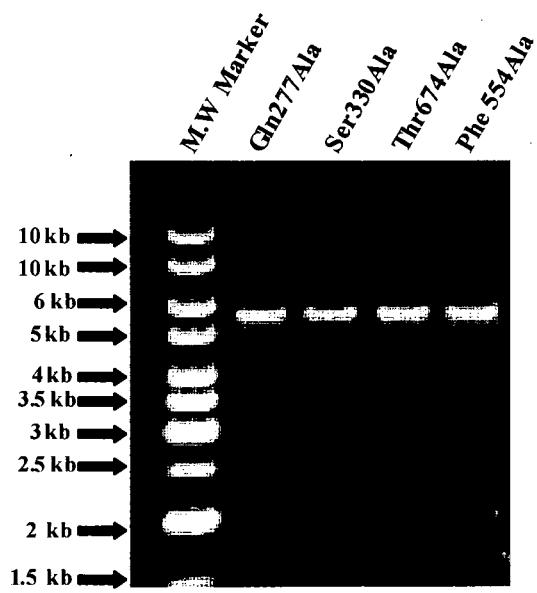


Fig. 2.4 Agarose gel showing long PCR amplification products of 5.6 kb

The PCR products obtained were resolved on a 0.8% agarose gel and were 5.6 kb as expected, which corresponds to the size of the template DNA pMW1. After *Dpn*I digestion, the PCR products were transformed directly into competent *E.coli* DH5α cells. *Dpn*I digested the methylated template pMW1 DNA, sparing the PCR product as it was unmethylated. The PCR product didn't need ligation as they were like mutant plasmids with staggered nicks on the opposite strands. The nicks are repaired inside the bacterial cells after transformation resulting in formation of functional plasmid with mutant PA gene. The transformants were screened for plasmid DNA by minipreparations and restriction analysis.

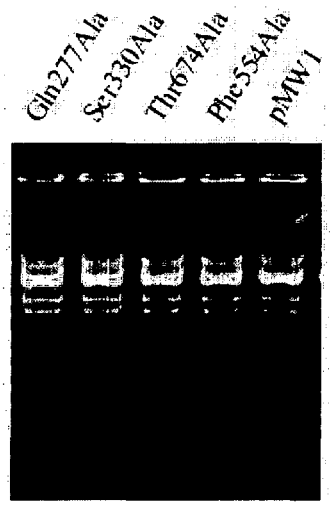


Fig 2. 4 Agarose gel showing mutated recombinant pMW1 plasmids

The recombinant plasmids were digested with *Kpn*I and *BamH*I restriction endonucleases. Since these enzymes have been used in cloning of PA gene into pQE30 (pMW1), digestion with the same gives fallout of the right size corresponding to size of the gene (PA gene-2.2kb) and another band corresponding to the vector (pQE30-3.4kb).

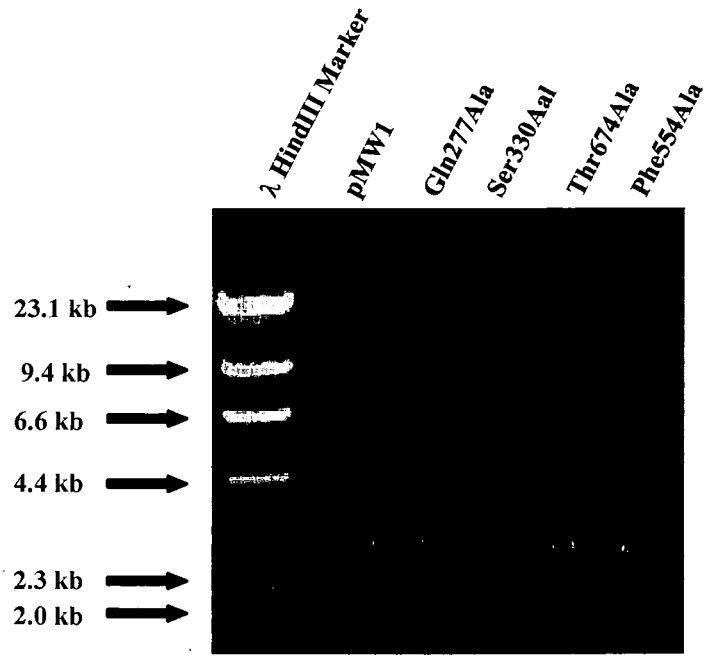


Fig 2. 6 Agarose gel showing the restriction digestion pattern of the mutated recombinant plasmids with *Bam* HI and *Kpn* I.

2.4.2.2 DNA sequencing of the mutant plasmids

The different constructs were confirmed by sequencing of the gene on the automated sequencer. Sequencing results are shown below (Fig 2.7) showing the region of interest.

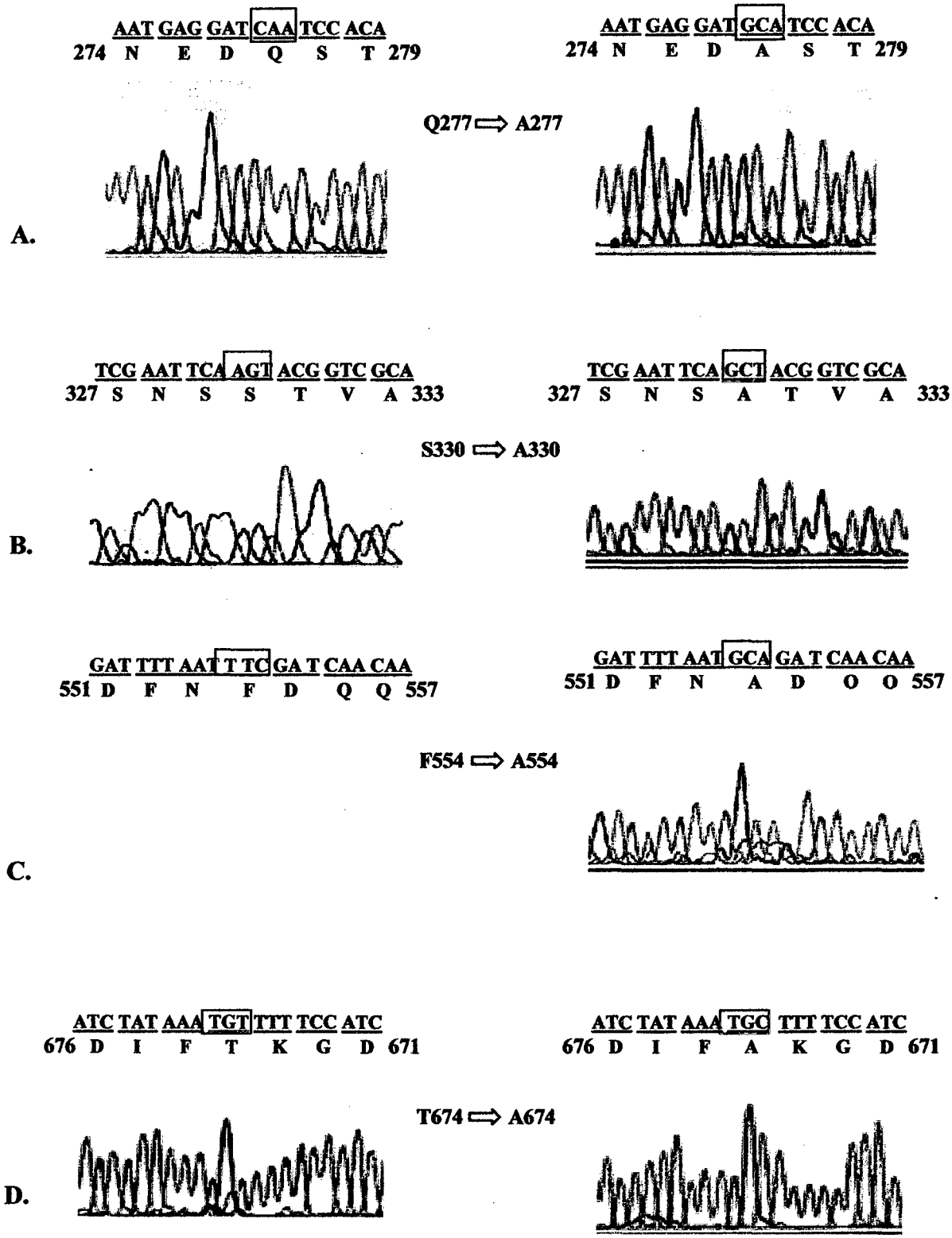


Fig 2.7 Sequencing results of the mutated PA genes. Right side of the panel shows the DNA sequences at mutation points while those on left side are the corresponding wild type sequences. A) Q277A. B) S330A. C) F554A. D) T674A.

2.5 SECTION C. EXPRESSION AND PURIFICATION OF THE MUTANT PROTEINS

2.5.1 RESEARCH METHODOLOGY

2.5.1.1 Protein expression

Transformed *E.coli* DH5α cells harboring the mutant plasmids were inoculated in 3 ml LB media containing 100 µg/ml ampicillin and incubated overnight (O/N) at 37°C with shaking (250 rpm). These O/N grown cultures were pelleted and subjected to an SDS-PAGE (Sodium Dodecyl Polyacrylamide Gel Electrophoresis) to see the level of expression and also electroblotted and probed with polyclonal anti-PA antibodies (western blotting).

2.5.1.2 Polyacrylamide Gel Electrophoresis

Proteins were analysed by SDS-PAGE according to the method of Laemmli (1970). Electrophoretic analysis of proteins was carried out in 12% separating with 5% stacking gel. Protein samples for SDS-PAGE were prepared by the addition of 20 µl of 6X loading buffer to 100µl of cell suspension (made from 1.5 ml of O/N grown culture), boiled for 5 minutes followed by centrifugation at 12,000g for 5 minutes at room temperature. 8µl of the supernatant was loaded to each well. Gel was run at a constant voltage of 120 V in a Bio-Rad mini gel apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The resolved proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250 for 5-10 minutes followed by destaining the gels with destaining solution, to remove excess stain.

2.5.1.3 Western Blot Analysis

For probing the proteins in cell lysate with anti-PA antibody, the samples resolved on SDS-polyacrylamide gels were transferred to nitrocellulose membrane at a constant

voltage of 25-30V overnight or 100V for an hour with cooling jacket, in the Bio-Rad mini trans-blot cell. The membrane blocking was carried out by wetting it in TTBS containing 10% non-fat milk for 1 hour. After washing the membrane with TTBS thrice for 5 min each to remove the excess of non-fat milk, the blot was incubated with the anti-PA antibody (1:1000 dilution), for 45 minutes, diluted in Tris buffer (pH 7.6) containing 0.5% BSA and 0.1% Tween-20. Blot was washed with three changes of TTBS to remove residual free antibody and then probed with HRP conjugated secondary antibody for 45 minutes, diluted in Tris buffer (pH 7.6) containing 0.5% BSA and 0.1% Tween-20. The membrane was washed three more times with TTBS and was developed by addition of diamino benzidine (DAB) and H_2O_2 made in TTBS. As soon as the bands appeared the reaction was stopped by washing the membrane in running water, air dried and preserved.

2.5.1.4 Purification of mutant proteins

The mutant PA proteins were purified using Ni-NTA metal-chelate affinity chromatography under denaturing conditions as follows:

1. The culture pellets from 100 ml cultures were resuspended in 10 ml of denaturing buffer containing 100 mM sodium phosphate buffer, 350 mM sodium chloride and 8M urea (pH 8.0).

2. The resuspended pellets were incubated at 37°C for 60 minutes on a rotary shaker (250 rpm).

3. The lysates were centrifuged at 12000g for 30 min. at room temperature and the clarified supernatants were mixed with Ni-NTA slurry.

4. Ni-NTA slurry with bound protein was packed into separate columns and allowed to settle.

5. The flow through from each column was reloaded on the respective column to allow maximal protein binding.

6. Ni-NTA matrix was washed with 50 ml denaturing buffer containing 8 M urea, followed by on-column renaturation of the proteins by decreasing the urea concentration gradually from 8 M to 0 M.

7. The mutant proteins were eluted with elution buffer containing 250 mM Imidazole chloride, 100 mM sodium phosphate of pH 7.5 and 300 mM sodium chloride. Fractions of 1.0 - 1.3 ml were collected.

8. 10 µl of each fraction was analyzed on 12 % SDS-PAGE to check the protein content and purity of the preparation. Fractions containing the protein were collected, pooled and dialyzed against 20 mM HEPES buffer pH 7.5 containing 250 mM NaCl and stored in aliquots at 4°C.

The concentration of purified mutant proteins were estimated using Bradford's method, subjected to an SDS-PAGE and also electroblotted to probe with anti PA antibody.

2.5.2 RESULTS

2.5.2.1 Expression of PA gene mutants in *E.coli* DH5α cells

To check the expression of the mutant proteins from the recombinant mutant plasmids in *E. coli* DH5α cells, the cultures were grown overnight and the protein profile was studied. Expression was established by SDS-PAGE and Western Blot analysis.

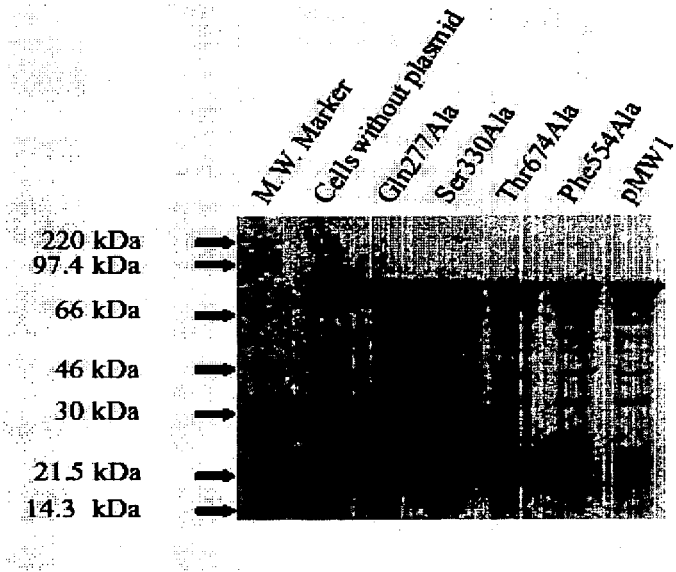


Fig 2.8 A 12% SDS-PAGE of the cell lysate of transformed *E.coli* cells expressing mutant PA proteins

The mutant proteins were expressed simultaneously with the growth of the culture and could be seen on the SDS-PAGE gel as 83 kDa bands corresponding in mobility to the standard PA. All mutants were expressing the protein in amounts comparable to the native PA.

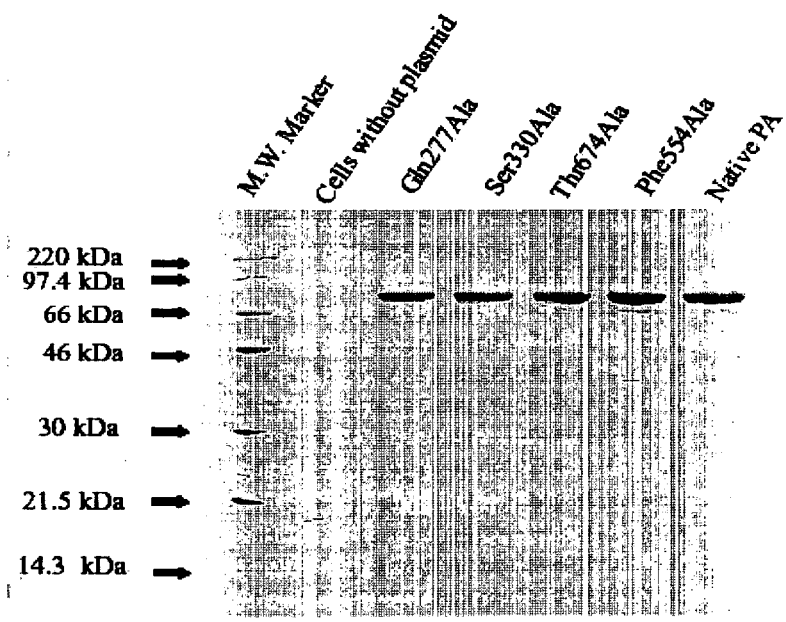


Fig 2.9 Western blot of the cell lysate of transformed *E.coli* cells expressing mutant PA proteins developed with polyclonal anti-PA antibodies

Electroblotting followed by developing the Western Blot with anti-PA antibodies confirmed the presence of the specific (mutant PA) protein.

2.5.2.2 Purification of the mutant proteins

The mutant proteins were expressed constitutively as inclusion bodies inside the cells. Inclusion bodies are the insoluble aggregates of proteins, which are formed by some proteins when levels of protein expression are high. Strong denaturants such as 6M GdHCl or 8M urea completely solublize inclusion bodies. Under denaturing conditions, the 6X histidine tag on the protein is fully exposed that improves binding of the protein to the Ni-NTA matrix and hence maximizes the efficiency of the purification procedure by reducing the potential non-specific binding. The recombinant PA and mutant PA proteins were purified from inclusion bodies under denaturing conditions using Ni-NTA affinity chromatography. Since the mutant proteins were expressed at high levels, the level of co-purifying contaminant proteins was relatively low. Protein renaturation and refolding was carried out on the Ni-NTA column itself prior to elution. Immobilizing one end of the protein during renaturation prevents intermolecular interactions that lead to aggregate formation. The proteins obtained after affinity

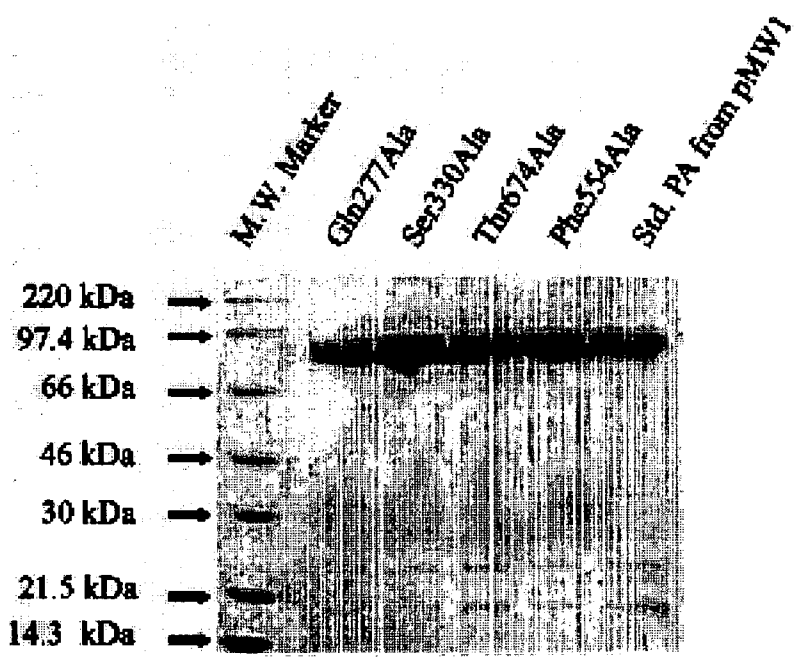


Fig 2.10 A 12% SDS-PAGE of the Ni-NTA purified mutant PA proteins.

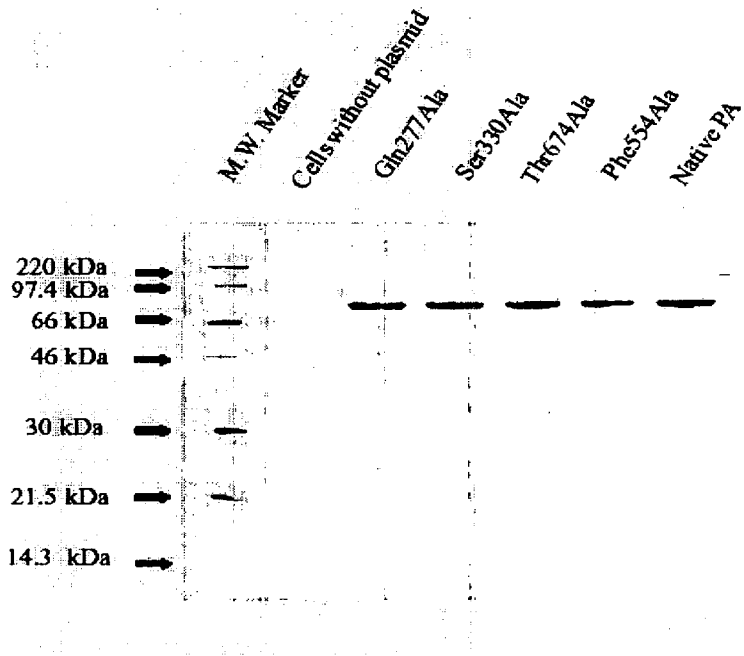


Fig 2.11 Western blot of the Ni-NTA purified mutant PA proteins developed with polyclonal anti-PA antibodies

chromatography were more than 90% pure. Mutant protein yields of ~ 40-60 mg/L were obtained. These proteins were used for further experiments. The purified proteins were electroblotted and the Western blot was developed with anti-PA antibodies and anti-His antibodies. The purified protein gave a single band of ~83kDa on the developed blot corresponding to purified PA.

2.6 SECTION D. CHARACTERIZATION OF THE MUTANTS

2.6.1 RESEARCH METHODOLOGY

2.6.1.1 Cell culture and cytotoxicity assay

Macrophage like cell line RAW264.7 was maintained in RPMI 1640 medium containing 10% heat inactivated FCS, 25 mM HEPES, 100U/ml penicillin and 200 μg/ml streptomycin in a 95% humidified 5% CO_2 environment at 37°C. For biological assay, a 96 well culture plate was prepared with ~90% cell density. PA mutants were added in varying concentrations with LF (1μg/ml) and incubated for 3 hrs. at 37°C. PA from *Bacillus anthracis* along with LF was used as a positive control. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye. MTT dissolved in RPMI was added to each well at a final concentration of 0.5mg/ml and incubated for another 45 min at 37°C to allow uptake and reduction of the dye by viable cells. The medium was replaced by 0.5% (w/v) sodium dodecyl sulphate (SDS), 25 mM HCl in 90% isopropyl alcohol and the plate vortexed. The absorption was read at 540 nm using microplate reader (BIORAD).

2.6.1.2 Thermostability studies on PA and mutant PA proteins

Native PA and the mutated PA molecules were used at 10 μg/ml (20 mM HEPES and 25mM NaCl, pH 7.4 at 25°C) concentration for incubation at different temperatures (37-49°C) for 5-160 min in order to evaluate the thermostability of the protein. They were also incubated at 37°C for 48 hrs in 0.6 ml Eppendorff tubes and sample aliquots were withdrawn at different time intervals ranging from 3-48 hrs and kept at 4°C before doing cytotoxicity assay.

2.6.2 RESULTS

2.6.2.1 Cell culture and cytotoxicity assay

Macrophages and macrophage like cell lines are sensitive to lethal toxin and hence provide a convenient assay for anthrax toxin. RAW264.7 cells were maintained in RPMI 1640. For biological assay, a 96 well culture plate was prepared with ~90% cell density. PA mutants were added in varying concentrations along with LF (1 μg/ml).

The mutant T674A was completely non-toxic in combination with LF, even at concentrations of 10 μg/ml. The mutants Q277A, S330A, and F554A were found to be comparable in toxicity to that of wild type PA (Table 2.4).

Table 2.4 EC_{50} of mutant and native PA (pMW1)

PA and mutant proteins	EC_{50} * (μg/ml)
Q277A	0.06
S330A	0.07
F554A	0.07
T674A	>10
Standard PA/pMW1	0.06

*EC_{50} is defined as the concentration of PA required to kill 50% of RAW264.7 cells in combination with LF (1μg/ml).

Thermostability of native PA and mutant PA proteins: The mutant T674A was nontoxic. Hence only mutants Q277A, S330A, and F554A were incubated at different temperatures (37-49°C) for different time intervals 95-160 min. It was found that S330A behaved like the wild type while Q277A and F554A showed increased stability (Fig 2.12). When the promising candidates, *i.e.* Q277A and F554A were further incubated at

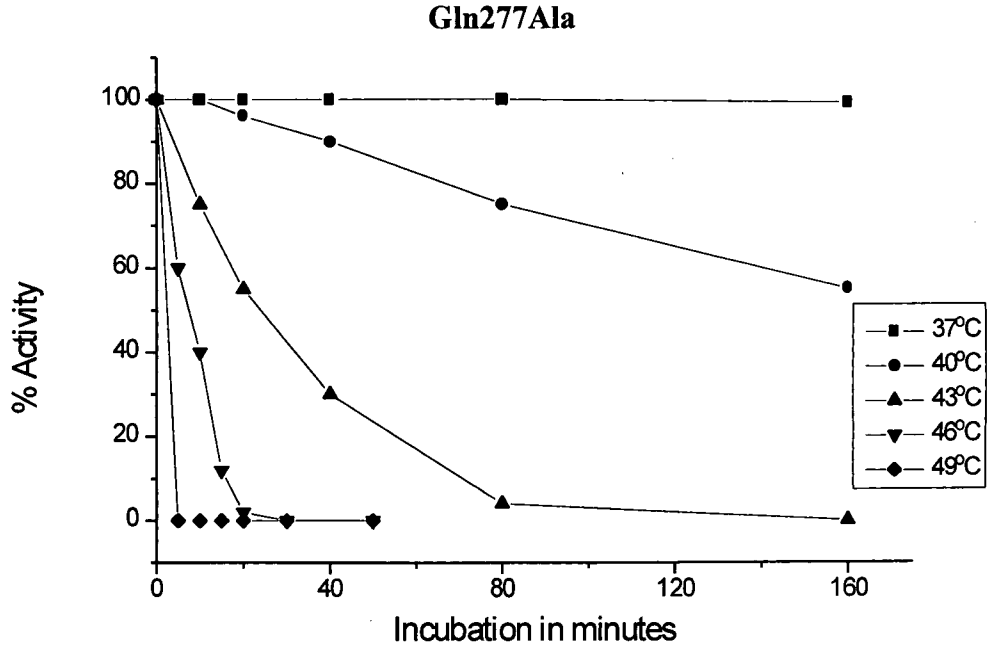


Fig. 2.12a

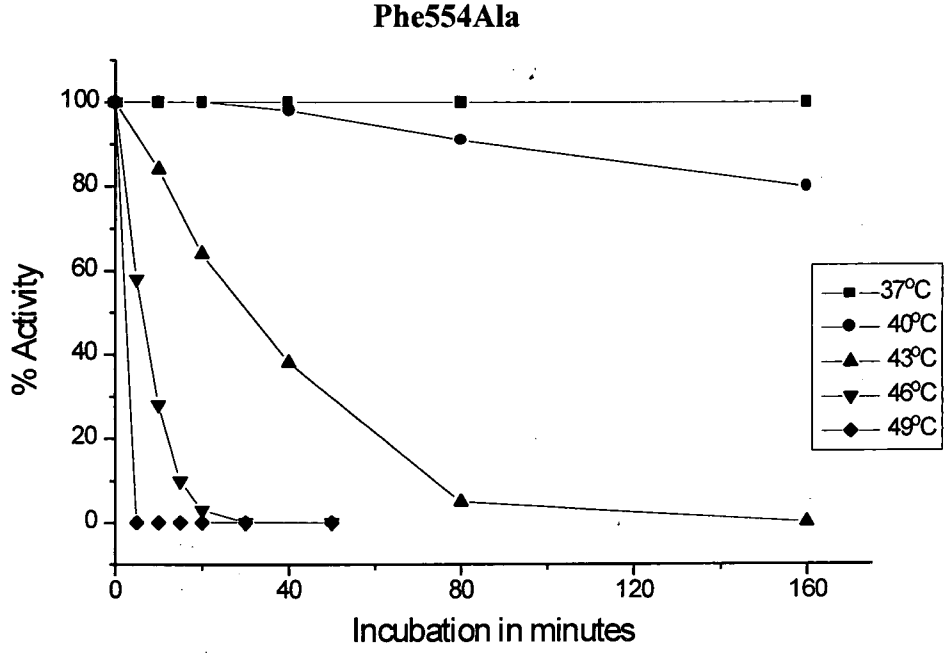


Fig. 2.12b

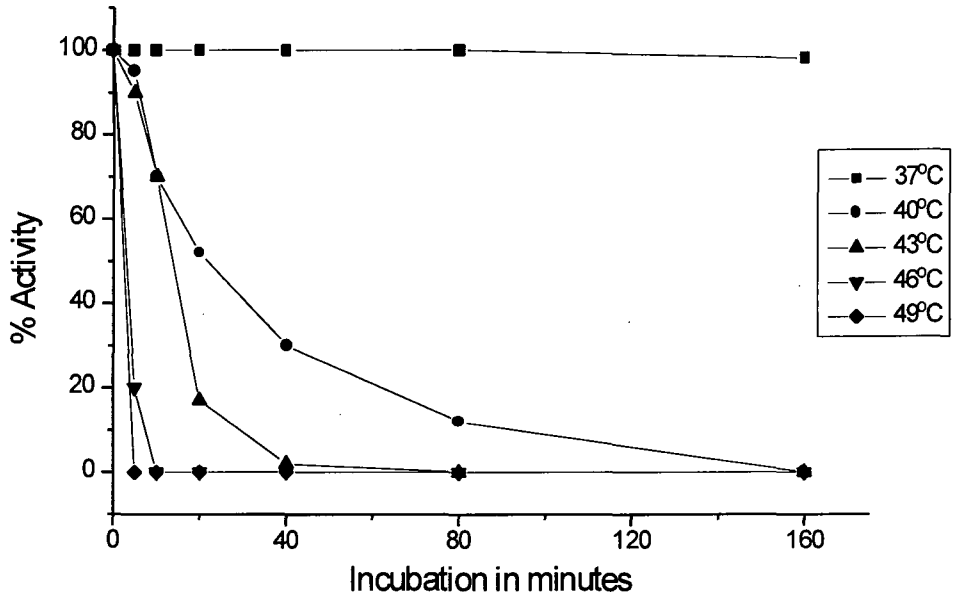


Fig. 2.12c

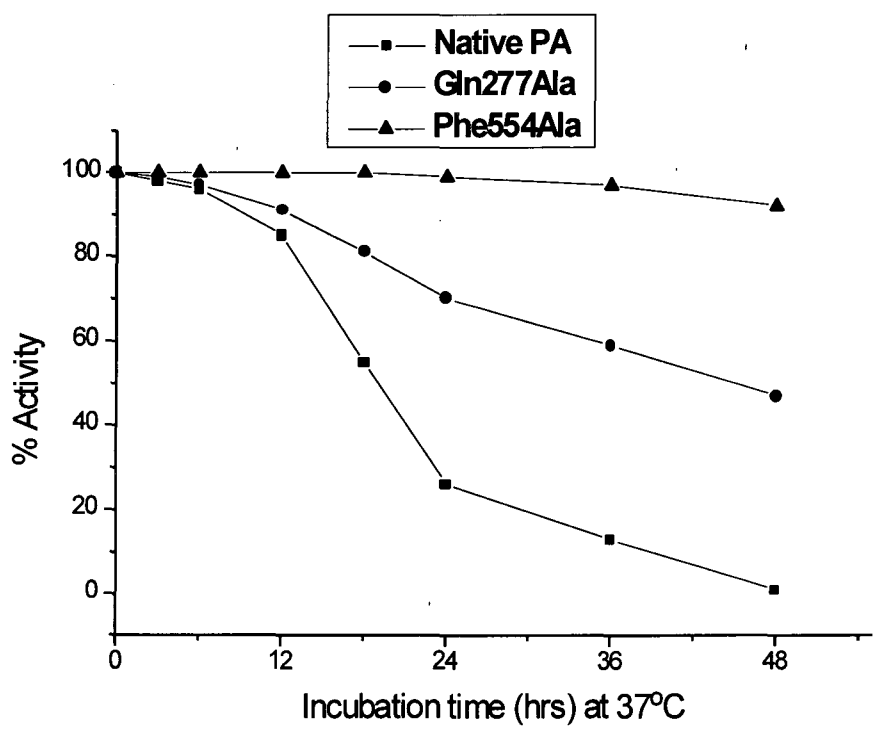


Figure 2.12 d

Figure 2.12 a) Inactivation profiles of mutant Gln277Ala at different temperatures. b) Inactivatio profiles of mutant Phe554Ala at different temperatures. c) Inactivation profiles of native PA a different temperatures. d) Inactivation of native PA and mutants at 37°C. Concentration o protein was 10 μg/ml (20 mM HEPES, 25 mM NaCl, pH 7.4 at 25°C) during incubation. % Activit was calculated as % retention of ability of PA and mutant proteins (along with 1 μg/ml of LF) t kill RAW264.7 cells. % Error in the measurements of activity was ±5%.

37°C for extended time it was found that F554A retained more than 90% activity after 48hrs while Q277A retained ~45% activity (Table 2.5). It is clear from the table that F554A mutation is the most stabilizing.

TABLE 2.5: Activity of PA mutants retained after 48 hrs of incubation at 37°C in comparison with native PA.

PA Mutants	Residual Activity after 48 hrs of Incubation at 37°C
Gln277Ala	~45%
Phe554Ala	~90%
Native PA	0%

Note: Native PA and mutant PA proteins (10μg/ml in 20 mM HEPES with 25 mM NaCl, pH 7.4 at 25°C) were incubated at 37°C for 48 hrs. For calculating loss of activity the protein kept at 4°C was taken as a reference.

2.6 DISCUSSION

Protective Antigen (PA) of *Bacillus anthracis* is the central moiety in anthrax intoxication process. PA itself is non-toxic and acts as a carrier for other exotoxin moieties namely LF and EF, to deliver them into cytosol. LF and EF themselves are enzymatic moieties but unable to effect their entry into the cytosol and hence are nontoxic in the absence of the carrier molecule PA. Since LF and EF cannot act alone their binding to PA is an important step in the anthrax intoxication of mammalian cells.

As expected for the central moiety in intoxication, immunization with PA has been found effective in providing protection against anthrax (Bhatnagar and Batra, 2001 and references therein). However, its thermolability has been a cause of concern as it loses activity in 48 hrs at 37°C. Accidental exposure to higher temperature could hence,

compromise its efficacy. In the present study efforts were made to increase the thermostability of PA by genetic engineering.

The PDB structure available for PA (1acc: pdb) was found to be incomplete as there were some missing residues. The structure was therefore model built using Homology module of Insight II (Accelrys) program. The analysis of structure for locating buried hydrophilic groups using accessible surface area calculations, showed that some hydrophilic residues are buried in the core (Table 2.1). Buried hydrophilic groups are most of the time usually involved in stabilizing the structure through hydrogen bond network and ion pairs. Any buried hydrophilic group not involved in stabilizing interactions could be a source of destabilization (Hendsch *et al.*, 1996). The buried hydrophilic residues of PA were sequence aligned with Iota Ib (*Clostridium perfringens)* and ADP ribosyltransferase (*Clostridium difficle)* to find out the residues, which are variable, as usually the conserved residues are known to be involved in stabilizing interactions. Conserved residues are supposed to be critical for structural stability as shown earlier in the case of GroEL minichaperons (Wang, *et al.*, 1999), Abp1p SH3 domain (Rath and Davidson, 2000) and Immunoglobulin V_H domain (Wirtz and Steipe, 1999). Residues Gln277, Ser330 and Thr674 turned out to be variable in homologous proteins while other polar and charged residues were either identical or conserved in the homologous proteins.

Among the hydrophobic residues of the hydrophobic patch in domain 3 of PA, Phe554 has been found not to be crucial for activity compared to the other residues (Ahuja *et al.*, 2001). Since Phe554 could be the site of aggregation, leading to the destabilization of the protein, it was mutated to alanine to decrease its hydrophobic character.

The mutant Gln277Ala appears to be stabilized over the native PA molecule (see Table 2.5, Fig 2.12) as it retains ~45% activity on incubation at 37°C for 48 hrs, while during the same time the native PA loses activity completely. The change to Ala is conservative one as in homologous protein this position is occupied by alanine (see Table 2.2). The mutant Ser330Ala however behaved more like the native PA (data not shown). The mutant Thr674Ala was inactive in the cytotoxicity assays (upto 10 μg/ml) and was, therefore, not used for further studies. In homologous proteins this position is

occupied by Isoleucine. Probably the residue at this position is critical for the proper folding of the molecule.

Mutation Phe554Ala is the most stabilizing one (Table 2.5, Fig 2.12b). This mutant protein retains ~90% activity even after 48 hrs incubation at 37°C. In proteins homologous to PA, Phenylalanine is present at this position yet this residue is not critical for activity as observed earlier (Ahuja *et al.*, 2001). This mutation could be stabilizing the PA molecule by decreasing the exposed hydrophobic surface and hence leading to a decrease in the total free energy of the native state resulting in the stabilization of the PA molecule. An alternative explanation could be that by decreasing the exposed hydrophobic surface area the propensity of aggregation of the protein is lowered at higher temperatures.

This study shows that decreasing the hydrophilic character in the protein interior and increasing that in the protein exterior could be a good preposition for thermostabilization when coupled with multiple sequence alignment. Further improvement in thermostability could be achieved by incorporating salt bridges or disulfide bridges. This study has resulted in producing two thermostable PA mutants Gln277Ala and Phe554Ala, which could prove to be better candidates for recombinant anthrax vaccine.

CHAPTER 3

THERMOSTABILIZATION OF PA: EFFECT OF SALTS, GLYCINE AND ITS METHYL DERIVATIVES AND POLYOLS

3.1 INTRODUCTION

Anthrax infection proves fatal once the symptoms appear and hence prophylactic measures are an effective way to counter the disease. Three methods have been employed for the prevention of the anthrax infections: the first one is the administration of antibiotics to kill the vegetative bacteria and prolong the incubation period of disease so that in the meantime the body could either mount immune response on its own or the person gets vaccinated. The second one is passive immunization with immune sera or monoclonal antibodies against the bacterial pathogen, and the third and the most effective one is the active immunization using vaccines, since vaccination can effectively provide immunity against anthrax.

There are currently two types of human vaccines available for countering anthrax. One of the vaccines, that is in use in erstwhile countries of Soviet block and some third world countries is the Sterne strain, the attenuated strain of *Bacillus anthracis* which is also used for cattle. Another available human vaccine that is in use in the US and the European countries is the alum precipitated sterile culture supernatants of toxigenic, non-encapsulated strain of *Bacillus anthracis*, which contains trace amounts of LF, EF and cellular debris besides the main immunogenic molecule PA. The aluminium hydroxide present in the vaccines acts as an adjuvant and enhances the sustained production of antibodies. Although these vaccines are quite effective, requirement of several booster doses and associated side effects like edema and pain at the site of vaccination due to the presence of LF, EF and cellular debris in trace amounts are the main irritants. Efficacy of these vaccines also vary from lot to lot, as the amount of PA which is the main immunogen varies in each lot and there is no control over the amount of PA produced and the level of its purity. Recombinant PA, that has been expressed and purified from *E. coli* in our laboratory, can be a suitable alternative to the available vaccines (Gupta *et al.*, 1999; Chauhan *et al.*, 2001).

PA is however a highly thermolabile protein molecule. Our laboratory (Radha *et al.*, 1996) has earlier shown that it loses activity within 48 hrs at 37°C and hence

accidental exposure of the PA vaccine to higher temperatures during transportation or storage could compromise the quality and efficacy of the vaccine. The deterioration of protein pharmaceuticals on storage has been a well-documented fact (Manning *et al.*, 1989; Clealand *et al.*, 1993; Manning *et al.*, 1995; Wong and Parserampuria, 1997; Wang, 1999; Lai and Topp, 1999) and a cause of great concern. Various investigations have been carried out to characterize the primary cause of deterioration in protein quality (Manning *et al.*, 1989; Wang, 1999) and its prevention (Gianfreda and Scarfi, 1991; Arakawa *et al.*, 1993; Clealand *et al.*, 1993; Timasheff, 1993; Manning *et al.*, 1995; Wong and Parasrampuria, 1997) so that the overall quality of the proteins could be improved.

The instability in a protein stems from its inherent physical and chemical instability, which are also modulated by the extrinsic factors like pH, temperature, ionic strength, presence of other compounds, etc. Careful examination of the factors responsible for protein instability would help in surmounting the stability related problems. The most common form of physical instability encountered is the aggregation of protein molecules, which can be induced and/or affected by a variety of extrinsic factors and chemical transformations. Recombinant human keratinocyte growth factor or rhKGF (Chen *et al.*, 1994a), porcine growth hormone or pGH (Charman *et al.*, 1993), human growth hormone or hGH (Katakam *et al.*, 1995), insulin (Strickley and Anderson, 1997), human relaxin (Li *et al.*, 1995a) are some examples of aggregation prone proteins which readily aggregate, when induced by heat, passage of time, vortexing, covalent modification and oxidation of His and Met residues, respectively. Many chemical reactions are also responsible for the inactivation or the deterioration of proteins. Chemical instability is dependent on the primary sequence of the protein, its local mobility and flexibility (Xie and Schowen, 1999; Robinson and Robinson, 2001; Xie *et al.*, 2003), accessibility to solvent and presence of extrinsic factors. Major source of chemical instability are deamidation, disulfide bond breakage and rearrangement, hydrolysis or autolysis, succinimidation and isomerization (Wang, 1999; Lai and Topp, 1999; See review of literature: Chapter 1). Many a times chemical modifications occur on a single residue in succession, e.g. isomerization, succinimidation, deamidation and hydrolysis or autolysis at Asn/Gln-X or Asp/Glu-X sequences, the so-called hotspots. Some

modifications, which are less frequent, are nondisulfide cross-linking of insulin (Brange *et al.*, 1992, 1997; Darrington and Anderson, 1995), formaldehyde mediated cross-linking of lyophylized diphtheria toxoid and tetanus toxoid (Schwendeman *et al.*, 1995), Maillard reaction (Chuyen, 1998), etc. Maillard reaction is one of the most widely reported ageing reactions in food industry. It results in the formation of protein-carbohydrate adducts as a result of the reaction of reducing sugars with some amino acids (mainly Lysine) in proteins.

Stabilization of a protein mainly depends upon the identification of major inactivation pathway as it helps in devising methods for their stabilization. In some cases the extent of protein degradation or inactivation mechanism depends on the purity, e.g., human insulin (Brange *et al.*, 1992) and purification scheme, *e.g.*, recombinant human thrombopoietin (Senderoff *et al.*, 1996). Genetic engineering has been used extensively to change the intrinsic stability of proteins (Mozhaev and Martinek, 1984; Kristjansson and Kinsella, 1991; Vieille and Zeikus, 1996, 2001; Lehman and Wyss, *2001;* van den Burg and Eijsink, 2002). It has been proposed that decreasing exposed hydrophobic surface and increasing hydrophobic character of internal or buried residues (Kristjansson and Kinsella, 1991) increase stability of proteins, which correlates well with the increased packing efficiency and stability (Vieille and Zeikus, 1996; Takano *et al.*, 1998). Increase in hydrogen bonding (Kristjansson and Kinsella, 1991), stabilization of secondary structure elements (Querol *et al.*, 1996; Prieto *et al.*, 1997; Vieille and Zeikus, 1996, 2001) also leads to an increase in protein stability. Replacement of residues involved in chemical instability as in the case of deamidation of human growth hormone releasing factor analog (Freidman *et al.*, 1991), recombinant human phenylalanine hydroxylase (Carvalho *et al.*, 2003) etc. and hydrolysis or autolysis of subtilisin J (Bae *et al.*, 1995) etc. have been successful in stabilizing the proteins. Stabilization by extrinsic factors (cosolvent, cosolute, excipients, etc.) also has been a good alternative. Cosolvents stabilize proteins by pushing the Native (compact) $\Leftrightarrow$ Denatured/ Unfolded (loose) equilibrium towards the native state by increasing the $\Delta G_{N\rightarrow D}$ in the presence of a cosolvent. This is achieved by: a) strengthening protein structure stabilizing forces by the preferential hydration mechanism (Bhat and Timasheff, 1992; Xie and Timasheff

1997a, b; Timasheff, 1998), electrostatic shielding of unfavourable electrostatic interactions (Schellman, 1987, 1990; Yang and Honig 1992; Timasheff, 1992, 1995; Garcia-Moreno, 1994; Gilson 1995; Sharp 1995; Sharp *et al.*, 1995; Kohn *et al.*, 1997, Remmele *et al.*, 1998) and specific binding of ions to protein (Pace and Grimsley, 1988; Goto *et al.*, 1990a, b; Makhatadze and Privalov, 1992; Hagihara *et al.*, 1993; Mayr and Schmid, 1993; Makhatadze *et al.*, 1998); b) destabilizing the denatured state by the solvophobic effect due to unfavourable peptide backbone interactions with solvents in presence of osmolytes (Liu and Bolen, 1995; Qu *et al.*, 1998; Bolen and Baskakov, 2001) or in other words cosolvents stabilize the protein by enhancing the native interactions and perturbing the nonnative interactions (Wrabl and Shortle, 1996). The stabilizing effects are usually dependent on cosolvent concentration and the type of protein, although high concentrations of excipients are not necessarily more effective and in some cases may lead to opposite effects, e.g., sorbitol reduces heat induced aggregation of acidic fibroblast growth factor (aFGF) at 55°C effectively at concentrations below 0.5 M but is less effective at higher concentrations (Tsai *et al.*, 1993). Various excipients like polyols, sugars, salts, amino acids, polymers and surfactants have been used to stabilize different proteins like human growth hormone or GH (Pikal *et al.*, 1991), recombinant human DNase (Chen *et al.*, 1998), RNase (Lin and Timasheff, 1996; Kaushik and Bhat, 1998, 1999, 2003), thrombin (Boctor and Mehta 1992), recombinant human granulocyte colony stimulating factor or rhG-CSF (Herman *et al.*, 1996), maize leaf phosphoenolpyruvate carboxylase or ml-PEPC (Jensen *et al.*, 1996, 1997), pig heart mitochondrial malate dehydrogenase or phm-MDH (Jensen *et al.*, 1996), acidic fibroblast growth factor or aFGF (Won *et al.*, 1998) etc. (other references could be found in reviews by Wang (1999) and Lai and Topp (1999)).

There have been many efforts to produce PA in pure form and in large quantities to improve the quality of PA used in vaccines, by applying different purification schemes to purify it from the primary source or from other sources (i.e., recombinant technology). Stanley *et al.*, (1960) tried to purify PA from guinea-pigs dying from anthrax. The method was tedious and many a times produced cross-contaminated anthrax toxin components (i.e., PA, LF and EF together with each other). Pezard *et al.*,

(1993) tried to avoid the problem of cross-contamination by engineering *Bacillus anthracis* strains in which one or more of the toxin components were inactivated. These were used to produce subsets of the toxin components. Leppla (1988) described large-scale purification of anthrax toxin components from 50-liter fermenter culture supernatants using hydroxyapatite and DEAE sepharose matrices. The yields of PA obtained were however only 8 mg/L.

Ivins and Welkos (1986) expressed the PA gene in *B. subtilis* but the yields obtained were 20 μg/ml. Singh *et al.,* (1989) cloned the PA gene in shuttle plasmid pYS5 derived from pUB110 and pVC8f(+)T which was able to replicate in both *Bacillus sp.* and *E. coli.* Production of PA from pYS5 plasmid in *Bacillus anthracis* and *B. subtilis* requires a rich medium and vigorous aeration. Yields of PA obtained were not more than 50 μg/L of the culture supernatant. But most of the above-mentioned systems in which *Bacillus anthracis* is the host require expensive containment facilities and also give very low yields of the recoverable protein. The other systems suffer from the requirement of too many chromatographic steps for purification leading to degradation of the recombinant protein. PA expression has been also tried in baculovirus vector and vaccinia virus (Iacono-Connors *et al.,* 1990), *Bacillus subtilis* strain IS53 (Baillie *et al.,* 1994) and heat-inducible *Bacillus subtilis* bacteriophage phi105 expression system (Baillie *et al.,* 1998) but yields were meager.

Gupta *et al.,* (1999) from our laboratory, provided the first-ever recombinant overexpression system for PA in *E. coli,* which could give expression levels of upto 2 mg/L. It was cloned under the transcriptional regulation of the T5 promoter with a hexa-histidine tag and required IPTG (isopropyl-thio-β-D-galactopyranoside) for induction of PA production. As IPTG is an expensive chemical and cannot be used with proteins prepared for therapeutic purposes, it was finally constitutively expressed in *E. coli* (Vibha *et al.,* 2001) and by growth parameter optimization a yield of 125 mg/L of batch culture was achieved. The expressed PA protein has been shown to be as fully biologically and functionally active as the native PA purified from *Bacillus anthracis* and also provides protection against anthrax comparable to native PA from *Bacillus anthracis.*

Efforts from our laboratory demonstrated that PA loses its activity, *i.e.*, ability to bind to cell surface and cause internalisation of LF and EF into the cell, within 48 hrs when incubated at 37°C (Radha *et al.*, 1996). Some selected co-solvents have been shown to be able to effectively stabilize the PA molecule against thermal inactivation, *e.g.*, in the presence of 3M Magnesium sulfate, PA retains > 80% activity after 48 hrs of incubation at 37°C. It has also been shown (Singh *et al.*, 2002) that decreasing the hydrophobic character of the exposed hydrophobic patch and increasing hydrophobic character of the buried residue could effectively increase the thermal stability of PA. Mutant Phe554Ala was found to be the most promising one which could retain >90% activity on 48 hrs of incubation at 37°C.

PA is the most important molecule in anthrax intoxication and immunization. Its thermolability (Radha *et al.*, 1996) could limit its effective utilization for vaccination purposes. Thus it becomes imperative to elucidate the mechanism operational in its thermal inactivation, so that methods could be devised to protect it against high temperature susceptibility. Much remains to be explored and explained about the behaviour of PA towards thermal inactivation. Various cosolvents have been shown to increase the stability of proteins. Salts, polyols, and methyl glycines are the members of naturally occurring cosolvents, popularly known as osmolytes, which are employed by organisms under various stress conditions to prevent denaturation of proteins and to keep the cell machinery functional. These osmolytes have been extensively studied for protein stabilization with their mechanism of action already elucidated in detail (Arakawa and Timasheff, 1983, 1990; Timasheff, 1992; Liu and Bolen, 1995; Qu *et al.*, 1998; Kaushik and Bhat, 1998, 1999, 2003). They are the well-known protein stabilizers even outside their physiological concentration range (Santoro *et al.*, 1992) but the stabilizing effect is highly dependent on the nature of the protein (Santoro *et al.*, 1992; Kaushik and Bhat, 2003). Salts are known for their ability to stabilize proteins by non-specific electrostatic shielding (Kohn *et al.*, 1997, Remmele *et al.*, 1998) and specific ion binding (Makhatadze and Privalov, 1992; Makhatadze *et al.*, 1998). Polyols and methyl glycines are non-specific protein stabilizers, which are able to exert their effect by preferentially hydrating the protein and have been very useful in increasing the

thermostability of proteins. With this background on the effect of cosolvents on the thermal stability of proteins, the present work mainly focused on determining the cause of thermal inactivation of PA followed by studying the effect of protein concentration, salts, polyols and methyl glycines (*e.g.*, glycine and its methyl derivatives) on the thermal stability of PA so that its thermostability could be improved substantially.

3.2 MATERIALS

3.2.1 Chemicals

Acrylamide, Ampicillin, Bovine Serum Albumin (BSA), Coomassie brilliant blue R-250, Glycine, Glutamine, Glycerol, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, HEPES, PMSF, SDS, Sodium chloride, Sodium hydroxide, Sodium bicarbonate, Penicillin, Streptomycin, Imidazole, Protein determination dye and other chemicals were purchased from USB Chemicals, USA. Ni-NTA agarose was purchased from Qiagen, Germany. Cell culture plastic wares were obtained from Corning, USA. Fetal calf serum (FCS) was from Biological Industries, Israel. RPMI 1640, 3-(4,5-dimethylthiazol-2-yl), -5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma Chemical Co., USA. Media components for bacterial growth were purchased from Hi-Media Laboratories, India. PA purified from *E. coli* transformed with pMW1 (Gupta *et al.*, 1999) was used as standard.

3.2.2 Bacterial strains and cell lines

E.coli strain DH5α and macrophage like cell lines RAW264.7 and J774A.1 were obtained from ATCC (American type culture collection), USA.

3.3 RESEARCH METHODOLOGY

3.3.1 Protein expression

Transformed *E. coli* DH5α cells harboring the pMW1 plasmids (Gupta *et al.*, 1999) was inoculated in 3 ml LB media containing 100 μg/ml ampicillin and incubated overnight (O/N) at 37°C with shaking (250 rpm). The O/N grown starter culture was further used for inoculation of 100 ml culture with 1% (v/v) inoculum. Culture was grown at 37°C with shaking (250 rpm) for 6-7 hrs (A_{600}~2-3) then cells were pelleted by centrifugation at 5000g for 10 minutes and stored at -20°C.

3.3.2 Purification of PA

The PA was purified using Ni-NTA metal-chelate affinity chromatography under denaturing conditions as follows:

1. The culture pellet from 100 ml culture was resuspended in 10 ml of denaturing buffer containing 100 mM sodium phosphate buffer, 350 mM sodium chloride and 8 M urea (pH 8.0).

2. The resuspended pellet was incubated at 37°C for 60 minutes on a rotary shaker (250rpm) to complete the lysis of cells.

3. The lysate was centrifuged at 12000g for 30 min. at room temperature and the clarified supernatant was mixed with Ni-NTA slurry.

4. Slurry was packed into separate columns and allowed to settle.

5. The flow-through from each column was reloaded on the respective column to allow maximal protein binding.

6. Ni-NTA matrix was washed with 50 ml denaturing buffer containing 8 M urea, followed by on-column renaturation of the protein by decreasing the urea concentration gradually from 8 M to 0 M.

7. PA was eluted with elution buffer (100 mM sodium phosphate containing 250 mM Imidazole chloride and 350 mM sodium chloride, pH 7.5). Fractions of 1.0 - 1.3 ml were collected.

8. 10 µl of each fraction was analyzed on a 12 % SDS-PAGE to check the protein content and purity of the preparation. Fractions containing more than 90% PA were collected, pooled and dialyzed against 20 mM HEPES buffer, pH 7.5 containing 250 mM NaCl and stored in aliquots at 4°C.

The concentration of the purified PA was estimated using Bradford's method, subjected to an SDS-PAGE and also electroblotted to probe with polyclonal anti-PA antibody.

3.3.3 Protein Concentration determination

The concentration of PA used for assays was determined by Bradford's method using Protein Determination Dye from USB. Bovine Serum Albumin (BSA) was taken as standard. Standard curve was made in the range of 0-10 μg/ml by adding required amount of BSA to the buffer in which the protein of interest was present, and adding 200 μl of Protein Determination Dye and making up the volume to 1 ml. It was vortexed and the absorbance was taken at 595 nm (A_{595}). A_{595} = 0.05 corresponds to 1 μg/ml of protein.

3.3.4 Polyacrylamide Gel Electrophoresis

Proteins samples were analysed by PAGE according to the method of Laemmli (1970). Electrophoretic analysis of proteins in denaturing conditions, *i.e.* presence of SDS, was carried out in 12% separating and 5% stacking gel, while non-denaturing PAGE was carried out in 4-15% pre-cast gradient gel from Biorad, USA. Protein samples for PAGE were prepared by the addition of 6X loading buffer to the protein (i.e. PA) sample that had to be analysed. Loading buffer for SDS-PAGE sample contained SDS and β-ME/DTT while for the non-denaturing PAGE these were omitted. SDS-PAGE gel was run at a constant voltage of 120 V at room temperature while the non-denaturing PAGE was run at a constant voltage of 30 V at 2-8°C in a Biorad mini gel apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The resolved protein bands were visualized by staining the gels with Coomassie Brilliant Blue R-250 for 5-10 minutes followed by destaining the gels with the destaining solution to remove excess stain.

3.3.5 Cell culture and cytotoxicity assay

Macrophage-like cell line RAW264.7 was maintained in RPMI 1640 medium containing 10% heat inactivated FCS, 25 mM HEPES, 100U/ml penicillin and 200 μg/ml

streptomycin in a 95% humidified 5% CO_2 environment at 37°C. For biological assay, a 96 well culture plate was prepared with ~90% cell density. PA samples incubated at different temperatures were added with LF (0.3 μg/ml), unless mentioned otherwise, and incubated for 3-5 hrs at 37°C. PA sample that was kept at 4°C and had not been exposed to any higher temperature treatment along with LF was used as a positive control. Same amount of additive (salt or cosolvent) along with LF but without PA was used as a negative control. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye. MTT dissolved in RPMI was added to each well at a final concentration of 0.5 mg/ml and incubated for another 45 min at 37°C to allow uptake and reduction of the dye by viable cells. The spent medium was discarded and after drying the plate, 100 μl of 25 mM HCl in 90% isopropyl alcohol was added to each well to dissolve the cells and formazone crystals formed as a result of reduction of the MTT dye by viable cells. The plate was vortexed and the absorption of the solutions was read at 540 nm using a microplate reader (Biorad).

3.3.6 Characterization of loss of Activity

A. **AUTOLYSIS:** To find out whether the loss of activity of PA on heating is because of autolysis, PA (100 μg/ml) in 20 mM HEPES buffer with 25 mM NaCl, pH 7.4 was subjected to heating at 55°C for 10 min. This sample along with an untreated control, which was kept at 4°C, was mixed with SDS loading buffer, boiled for one minute and then subjected to SDS-PAGE to see emergence of extra bands as a proof of autolysis.

B. **AGGREGATION:**

B1. **LOSS OF SOLUBLE PA FORM (PAGE AND SDS-PAGE):** To find out whether loss of PA activity on incubation at higher temperature is because of aggregation, PA (50 μg /ml) in 20 mM HEPES buffer with 25 mM NaCl, pH 7.4 and in 40 mM HEPES with 2.7 M Glycerol + 500 mM NaCl, pH 7.9 (the best condition able to prevent thermal inactivation of PA: see section 3.4.6) were subjected to heating at

55°C for 10 min and 20 minutes respectively. These samples along with the untreated control PA sample were then subjected to non-denaturing 4-15% PAGE to see the emergence of high molecular weight forms if any because of aggregation. These heat-treated PA samples were further centrifuged at 12000g for 10 minutes and the supernatant were separated. To see the apparent decrease in intensity of the PA band on heating if any due to loss of the soluble form, supernatant and pellet of the samples were resuspended in SDS lysis buffer then boiled for one minute and subjected to SDS-PAGE along with untreated control.

B2. **AGGREGATION VERSUS DENATURATION: MONITORING ABSORBANCE INCREASE AT DIFFERENT WAVELENGTHS WITH INCREASE IN TEMPERATURE:** The PA sample (50 µg /ml in 20 mM Hepes with 25 mM NaCl, pH 7.4) was heated with scan rate of 1°C /min and increase in absorbance (scattering) at different wavelengths i.e. 278, 340, 440, 600 and 700 nm were monitored. It was done to ascertain whether the loss of activity is because of denaturation or aggregation as change in absorbance will not occur over a wide range of wavelengths in case of denaturation and would rather be confined to a narrow range, while in the case of aggregation it will follow Rayleigh scattering Elbaum *et al.*, 1976; Malinski and Nelsestuen, 1986; Lehner *et al.*, 1999; Militello *et al.*, 2003) and change in absorbance (intensity, I) due to scattering will be visualized over a wide range of wavelengths. In Rayleigh scattering, intensity of scattering, I (measured by change in absorbance) depends on wavelength (λ) given by the relation:

$$I \propto 1/\lambda^4$$

The pattern of absorbance at different wavelengths would tell whether on heating PA gets denatured or aggregated. In each case the pattern of change will be different.

[Note: The scattering at 400 nm is 9.4 times as great as that at 700 nm for equal incident intensity in air (http://hyperphysics.phy-astr.gsu.edu/hbase/atmos/blusky.html)]

B3. CORRELATION OF LOSS OF ACTIVITY WITH AGGREGATION: PA (40 μg/ml) in 20 mM HEPES buffer with 25 mM NaCl, pH 7.4 and 2.7 M Glycerol + 500 mM NaCl in 40 mM HEPES, pH 7.9 was put in a thermal cuvette at 46 and 58°C respectively and the increase in absorbance at 340 nm (A_{340}) with time was measured. At regular interval of one minute/10 minute, samples were withdrawn from the cuvette and stored at 4°C until residual activity was assessed by cytotoxicity assay to ascertain the loss of activity because of aggregation.

3.3.7 Effect of pH, protein concentration, salts, glycine and its methyl derivatives and polyols on thermostability of PA

To study the effect of pH, protein concentration, salt, glycine and its methyl derivatives and polyols, on thermal stability of PA, fractions obtained after Ni-NTA affinity chromatography of >90% purity (densitometry of Coomassie brilliant blue stained 12% SDS-PAGE gel) were taken and the buffer was exchanged to 20 mM HEPES with 250 mM NaCl, pH 7.5 using Centricon concentrators of 30 kDa cut-off limit. PA samples with different pH, salt or co-solvent concentrations were made by diluting the PA stock in suitable buffer so that the desired pH, concentration of PA, salt, or cosolvent was achieved. These samples were then incubated for 20 minute at different temperatures and the residual activity was calculated by checking their cytotoxic ability (cell lysis) on macrophage like cell lines i.e. J774A.1 or RAW 264.7, along with 0.3 μg/ml LF. Temperature at which 50% activity is retained after 20 minutes of incubation (designated $T_{1/2}$) was determined for comparison of the ability of different osmolytes to stabilize PA.

Note: All experiments were done more than three times. The results shown are the representative ones.

3.4 RESULTS AND DISCUSSION

3.4.1 Characterization of loss of Activity

A. AUTOLYSIS

Autolysis of a sensitive protein at autolytic site results in cleavage of protein into two fragments of smaller length/size. To rule out the possibility of autolysis in PA as cause of loss of activity at higher temperature PA (100 μg /ml) was incubated at 55°C for 10 minutes then dissolved in SDS lysis buffer and subjected to SDS-PAGE along with untreated control. The result of SDS-PAGE is shown below in Fig 3.1. The band of PA remains intact without appearance of extra or smaller size bands after heating at 55°C for 10 minutes. It makes clear that under the said conditions loss of activity of PA was either not because of autolysis or the fragment getting cleaved off was very small if at all autolysis was happening.

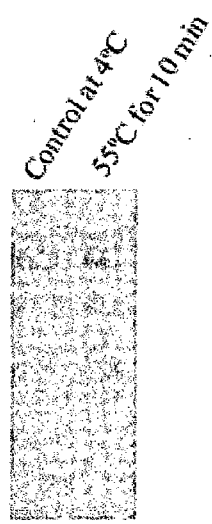
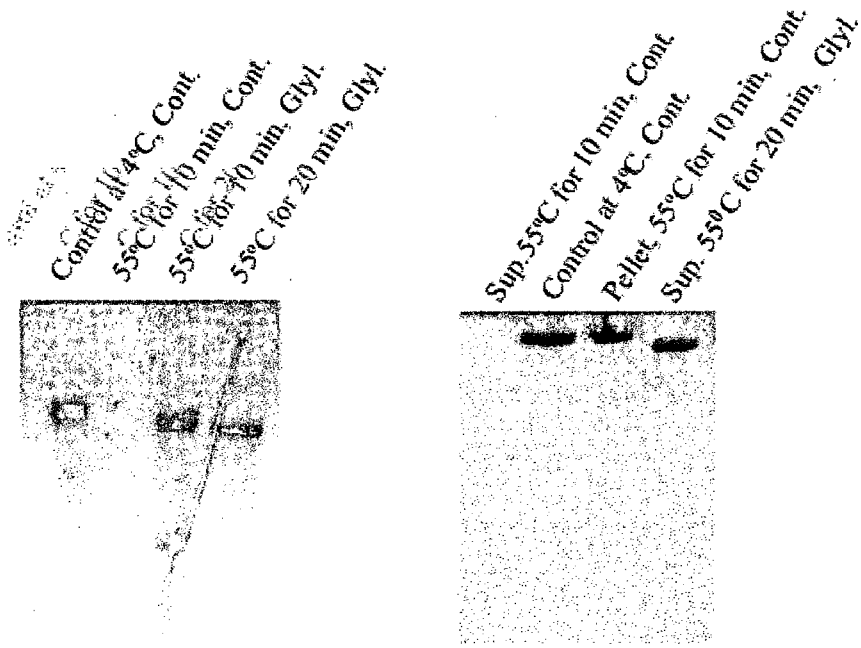


Fig. 3.1 A 12% SDS-PAGE of PA sample (100 μg /ml in 20 mM Hepes with 25 mM NaCl, pH 7.4) incubated at 55°C for 10 minutes.

B. AGGREGATION

B1. LOSS OF SOLUBLE PA FORM (PAGE AND SDS PAGE) ON HEATING:

Aggregation has been shown to be the main cause of inactivation in a number of proteins. Aggregation of the protein results in formation of large visible aggregates or small soluble oligomers. In both the cases the apparent size of the molecules/cluster increases. To find out whether on heating PA aggregates or not, PA (100 μg /ml) was heated at 55°C for 20 minutes in the presence of 25 mM NaCl in 20 mM Hepes, pH 7.4 and 2.7 M Glycerol+500 mM NaCl in 40 mM Hepes, pH 7.9. These heat-treated samples were subjected to non-denaturing 5-15% PAGE and 12% SDS-PAGE. Results of non-denaturing 5-15% PAGE and 12% SDS-PAGE are shown in Fig 3.2 a and b, respectively. In the non-denaturing 5-15% PAGE, there is no band visible in the heated PA sample (55°C for 10 minutes), corresponding to control PA while at the same time in the presence of 2.7 M Glycerol + 500 mM NaCl in 40 mM Hepes, pH 7.9 (the best condition known to prevent thermal inactivation; see section 3.4.6 on the effect of polyols), the band corresponding to PA is present at 10 and 20 min. of incubation



a. Native PAGE **b. 12% SDS PAGE**

Fig 3.2 a) 5-15% non-denaturing PAGE. b) 12% SDS-PAGE showing loss of soluble PA (Cont.) on heating and its prevention by 2.7 M Glycerol + 500 mM NaCl (Glyl.) when incubated at 55°C for 10 and 20 minutes. (Sup.: Supernatant of the sample; Cont.: PA sample in 20 mM HEPES with 25 mM NaCl, pH 7.4; Glyl.: PA sample in 40 mM HEPES with 2.7 M Glycerol + 500 mM, pH 7.9)

at 55°C. This indicates that probably on heating PA is forming such large aggregates that are unable to enter the non-denaturing gel. This inference is further corroborated/substantiated by SDS-PAGE gel where the supernatant from heated PA sample (55°C for 10 minutes in 20 mM Hepes with 25 mM NaCl, pH 7.4) after centrifugation at 12000g shows no PA band while the pellet from the same sample shows band corresponding to PA. Whereas in the presence of 2.7 M Glycerol + 500 mM NaCl (40 mM Hepes, pH 7.9) PA was present in the supernatant even after heating for 20 minutes at 55°C. This unequivocally proves that upon heating the loss of soluble form of PA occurs because of aggregation and it may be the cause of loss of activity at higher temperatures.

B2. AGGREGATION VERSUS DENATURATION: MONITORING ABSORBANCE INCREASE AT DIFFERENT WAVELENGTHS WITH INCREASE IN TEMPERATURE: Denaturation of proteins is accompanied by change in spectral properties of the protein due to change in the environment of residues (e.g. Trp, Tyr), which can be easily monitored by a spectrophotometer. Most of the time these changes are centered around a narrow wavelength band while in the case of aggregation, scattering of the incident light occurs (Rayleigh scattering) which is proportional to the inverse of the wavelength to power four ($I \propto 1/\lambda^4$). In other words when aggregation occurs, the observed change in absorbance because of scattering is more at lower wavelength than at longer wavelengths. In order to characterize the process of thermal inactivation of PA, it was heated at a sweep rate of 1°C/min and the change in absorbance at 278, 340, 440, 600 and 700 nm was monitored with increase in temperature as a function of time. The pattern of the change in absorbance can tell whether the loss of PA activity with increase in temperature is because of denaturation or aggregation since in each case the pattern of change will be different.

Fig 3. 3 below shows the change in absorbance at the wavelengths 278, 340, 440, 600 and 700 nm plotted versus temperature, when PA (50 μg/ml in 20 mM Hepes with 25 mM NaCl, pH 7.9) was heated at sweep rate of 1°C/min. The increase in absorbance starts around 40°C and reaches maximum at ~49°C. The increase in

absorbance is smooth which starts at the same temperature for all the wavelengths and was observed over the entire range of wavelengths used, indicating that PA is getting aggregated. The observed behaviour points to aggregation prone nature of PA and possibly this is the cause of loss of activity at higher temperatures.

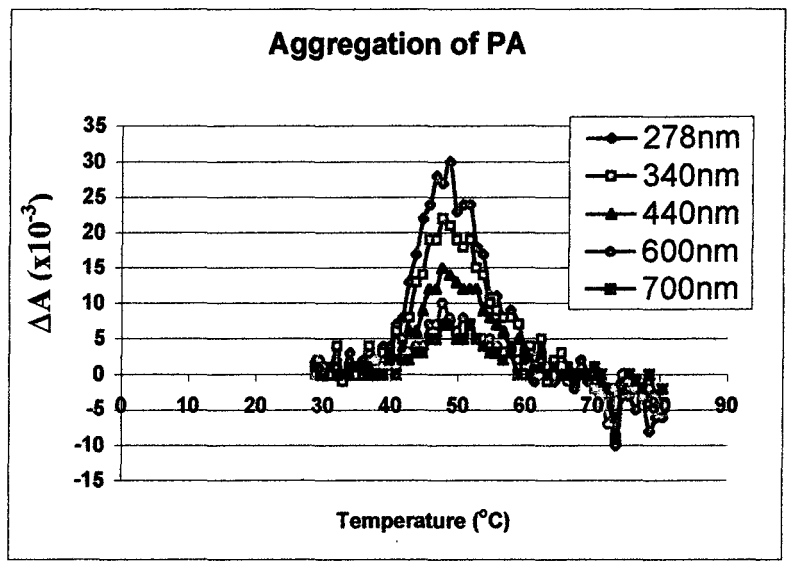


Fig 3.3 Absorbance increase at different wavelengths with increase in temperature: The PA was heated at sweep rate of 1°C/min. The increase in absorbance was monitored (ΔA) at different wavelengths and plotted verses temperature. ΔA: (absorbance at given temperature T °C) - (Absorbance at T-1 °C).

B3. CORRELATION OF LOSS OF ACTIVITY WITH AGGREGATION:

Further proof of aggregation as a cause of thermal inactivation came from the data (Fig 3.4) correlating aggregation with loss of activity. PA (40 μg/ml) in 20 mM HEPES with 25 mM NaCl, pH 7.4, and 40 mM HEPES with 2.7 M Glycerol + 500 mM NaCl, pH 7.9 was incubated in thermal cuvette at 46°C and increase in absorbance at 340 nm (A_{340}) with time was measured. Small aliquots were withdrawn at regular intervals from the samples being incubated and stored at 4°C for assessing residual activity (R.A.) by cytotoxicity assay later. 2.7 M Glycerol + 500 mM NaCl is known (see section 3.4.6 on the effect of polyols) to prevent thermal inactivation of PA even at higher temperature. PA sample in 2.7 M Glycerol + 500 mM NaCl was therefore

incubated at 46°C and 58°C and A_{340} was monitored. Small aliquots were withdrawn at regular intervals from the sample being incubated and assessed for residual activity (R.A.) by cytotoxicity assay to find the correlation of loss of activity with aggregation. PA (40 μg/ml) in 20 mM HEPES with 25 mM NaCl, pH 7.4 lost activity within 6 minutes at 46°C with concomitant increase in A_{340} with time while in the case of solution containing 2.7 M Glycerol + 500 mM NaCl there was no loss of activity upto 30 min at 46°C and no appreciable increase in A_{340} (data not shown) was observed. Even at 58°C there was no loss of activity within the limits of the experimental error. These findings unequivocally prove that the loss of activity of PA at moderately high temperature 46-58°C is mainly because of temperature-induced aggregation and prevention of aggregation also helps in preventing loss in the activity of the protein.

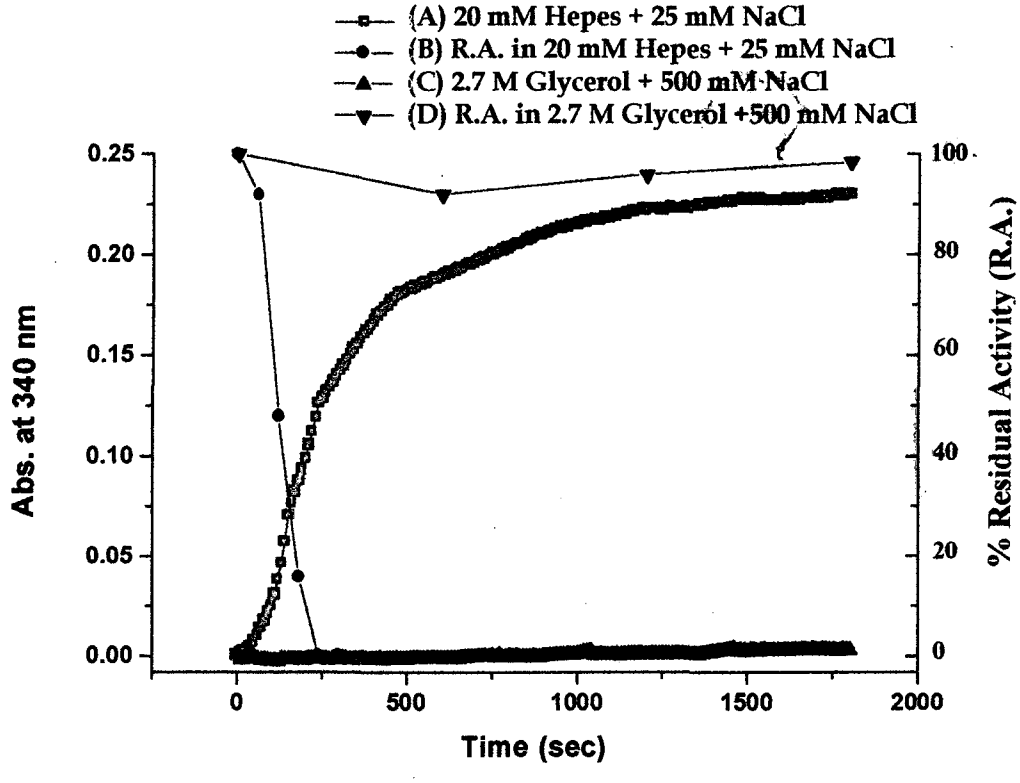


Fig 3.4 Correlation of loss of Activity with Aggregation: PA (40 μg/ml) was incubated at (A) 46°C in 20 mM HEPES with 25 mM NaCl, pH 7.4 (C) 58°C in presence of 2.7 M Glycerol + 500 mM NaCl, pH 7.9 and absorbance monitored at 340 nm; (B) and (D) are residual activity in A and B respectively during incubation. Residual activity (R.A.) was determined by cytotoxicity assay on Macrophage like cells RAW264.7. 0.8 μg /ml of the incubated PA was used along with 0.3 μg/ml of LF for cytotoxicity assay.

3.4.2 Effect of pH:

pH and ionic composition of the protein solution has strong influence on the stability of proteins (Privalov, 1979; Stigter *et al.*, 1991; Yu *et al.*, 1996). To investigate the effect of pH on the stability of PA, it was incubated at 40-52°C for 20 minutes in 20 mM and 40 mM HEPES of pH 6.6, 7.0, 7.4, 7.6, 7.9 and 8.2 containing 25 mM NaCl. The residual activity in the samples incubated was determined by cytotoxicity assay. The residual activity left after incubation at various pH values at different temperatures are presented in the form of a bar diagram below (Figure 3.5). It is clear from the figure that stability of the PA is highly pH dependent and it is stable in a very narrow pH range. The most favourable pH is ~7.9 (at 25°C) for higher temperature incubation. All further experiments were therefore carried out at pH 7.9 in 40 mM HEPES.

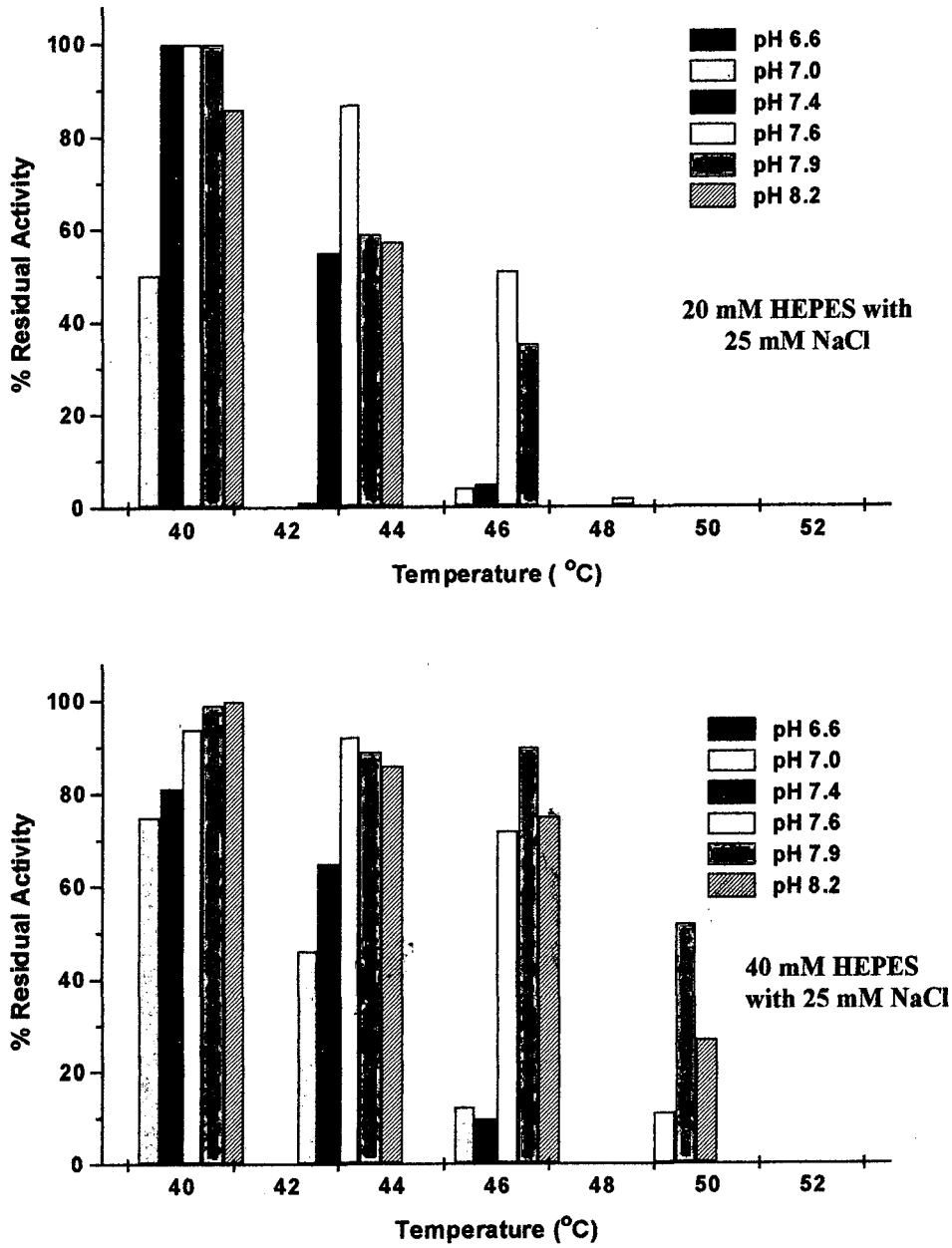


Figure 3.5 Effect of pH and buffer concentration on the thermal stability of PA: PA (8 µg/ml) was incubated at 40-52°C for 20 minutes in the presence of (a) 20 mM HEPES with 25 mM NaCl and (b) 40 mM HEPES with 25 mM NaCl. Residual activity was calculated by cytotoxicity assay on Macrophage like cells RAW264.7. 0.8 µg/ml of incubated PA sample was used along with 0.3 µg/ml of LF for cytotoxicity assay.

3.4.3 Effect of PA concentration:

Concentration of a protein affects its aggregation behaviour, e.g., recombinant human insulin like growth factor -I (Charman *et al.*, 1993; Fransson *et al.*, 1996a, b), TP40 or cysteine (replaced with alanine in 40 kDa segment of Pseudomonas exotoxin) mutant of transforming growth factor - α - Pseudomonas exotoxin fusion protein (Sanyal *et al.*, 1996) and recombinant human growth hormone (Bam *et al.*, 1998). Lattice model of protein aggregation predicts that at sufficiently high concentrations a protein molecule could aggregate or precipitate (Fields *et al.*, 1992). Ruddon and Bedows (1997) suggested that protein concentrations higher than 0.02 mg/ml may facilitate potential aggregation, and hence to avoid potential aggregation even in proteins where aggregation is usually not observed, concentrated protein samples must be avoided. Protein concentration may or may not affect the transition temperature (T_m) of protein. In the case of recombinant human keratinocyte growth factor (rhKGF), initial unfolding temperature is independent of protein concentration (Chen *et al.*, 1994a) while that of interferon-β-1a it decreases from about 77°C to 68°C when the protein concentration is increased from 10 μg to 100 μg /ml (Runkel *et al.*, 1998). In the case of cutinase, a drop of 4°C in T_m was observed over 30 fold increase in protein concentration (Petersen *et al.*, 2000).

Effect of PA concentration on thermal inactivation was examined by incubating PA solutions containing varying concentrations of PA viz., 4, 8, 16 and 32 μg/ml in 40 mM HEPES with 25 mM NaCl, pH 7.9 (25°C) at 40-52°C for 20 minutes. The samples were then tested for their residual activity in cytotoxicity assays. The results of the activity assays are shown in Fig 3.6. It is apparent from the residual activity left in the different samples of varying PA concentrations that the loss of activity is highly dependent on PA concentration. The 4 μg/ml PA sample retains ~95% activity after 20 minute incubation at 49°C while under the same conditions 8 μg/ml retains only ~60% activity, while samples having 16 and 32 μg/ml completely lose activity. It is clear from Fig 3.6 that a variation of PA concentration in the range of 4-32 μg/ml results in a decrease in the $T_{1/2}$ by ~6°C from ~51°C to 45°C, further corroborating the fact that PA is prone to aggregation and loss of activity is mainly due to its aggregation.

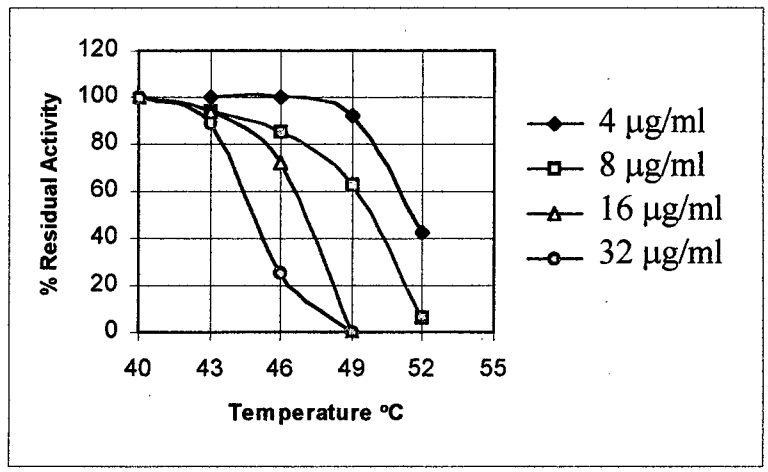


Fig 3.6 The dependence of PA thermostability on protein concentration. Different concentration of PA in 40 mM Hepes with 25 mM NaCl, pH 7.9 was incubated at different temperatures for 20 min and residual activity was determined by cytotoxicity assay on Macrophage like cells RAW264.7. 0.8 µg/ml of the incubated PA was used along with 0.3 µg/ml of LF for cytotoxicity assay.

3.4.4 Effect of NaCl and $MgCl_2$:

Salts or ionic composition of the milieu in protein solutions is known to be important for function and stability of proteins for a long time (von Hippel and Wong 1964; von Hippel and Schleich, 1969a, b) but the exact mechanism of the stabilization or destabilization is not yet clear. A number of possible mechanisms such as specific and non-specific binding of ions to protein molecules, electrostatic shielding, change in solvent properties etc. have been proposed (Schellman, 1987, 1990; Yang and Honig, 1992; Timasheff, 1992, 1995; Garcia-Moreno, 1994; Gilson, 1995; Sharp, 1995; Sharp *et al.*, 1995). All of the possible mechanisms work in tandem rather than in isolation and hence, the net effect of salts on the stability of proteins is a sum of all the factors described above (Kohn *et al.*, 1997). Salts modulate stability of proteins both by directly interacting with the protein and indirectly by changing the solvent properties. Direct interaction of salts involves interaction of ions with charged patches on the protein surface (prominent effect) or nonspecific shielding of electrostatic repulsion among groups of similar charges. Indirect interactions are generally weak in nature and become appreciable only

at higher concentration of the salts and result in a change in the solvent properties such as viscosity, surface tension, volume etc. caused by the addition of the salt to the solvent. These changes in the solvent property in turn can affect the stability of proteins.

The effect of NaCl and $MgCl_2$ on the thermal stability of PA was investigated by incubating PA in the presence of varying concentrations of the salt for 20 minutes at different temperatures (49-58°C) and comparing the residual activity determined by cytotoxicity assay (Fig. 3.7). It was found that adding 600 mM of NaCl or 300 mM $MgCl_2$ increase $T_{1/2}$ of PA by >8°C as compared to that in 25 mM NaCl. This shows strong dependence of PA stability on salt concentration. NaCl is a monovalent salt while $MgCl_2$ is a divalent salt. Both monovalent and divalent salts were used so that effect of the salt on the thermal stability of PA could be easily attributed to its ionic strength or cation and anion concentration. Ionic strength, I, depends on molar concentrations of the cations and anions and is given by the relationship (Atkins, 1990):

$$I = \tfrac{1}{2} \cdot (m_+ \cdot z_+^2 + m_- \cdot z_-^2)$$

where z_+ and z_- are charge numbers and m_+ and m_- corresponding molar concentrations for cation and anion, respectively in the salt solutions. So at same molar concentration ionic strength of $MgCl_2$ will be three times than that of NaCl. Similarly the concentration of anion (Cl^-) at the same molar concentration of $MgCl_2$ will be twice higher than for NaCl, but the concentration of cations will be the same. Residual activity of PA left on incubation at 49-58°C for 20 minutes as a function of salt concentration (Fig. 3.7 A, B and C) as well as anion concentration (Fig. 3.7 D) and cation concentration (Fig. 3.7 E) are shown below. The results show that the stabilizing action of salts are certainly of different nature than the consequence of general ionic screening as the stabilizing effect is highly dependent on anion concentration (Fig. 3.7 D) rather than on cation

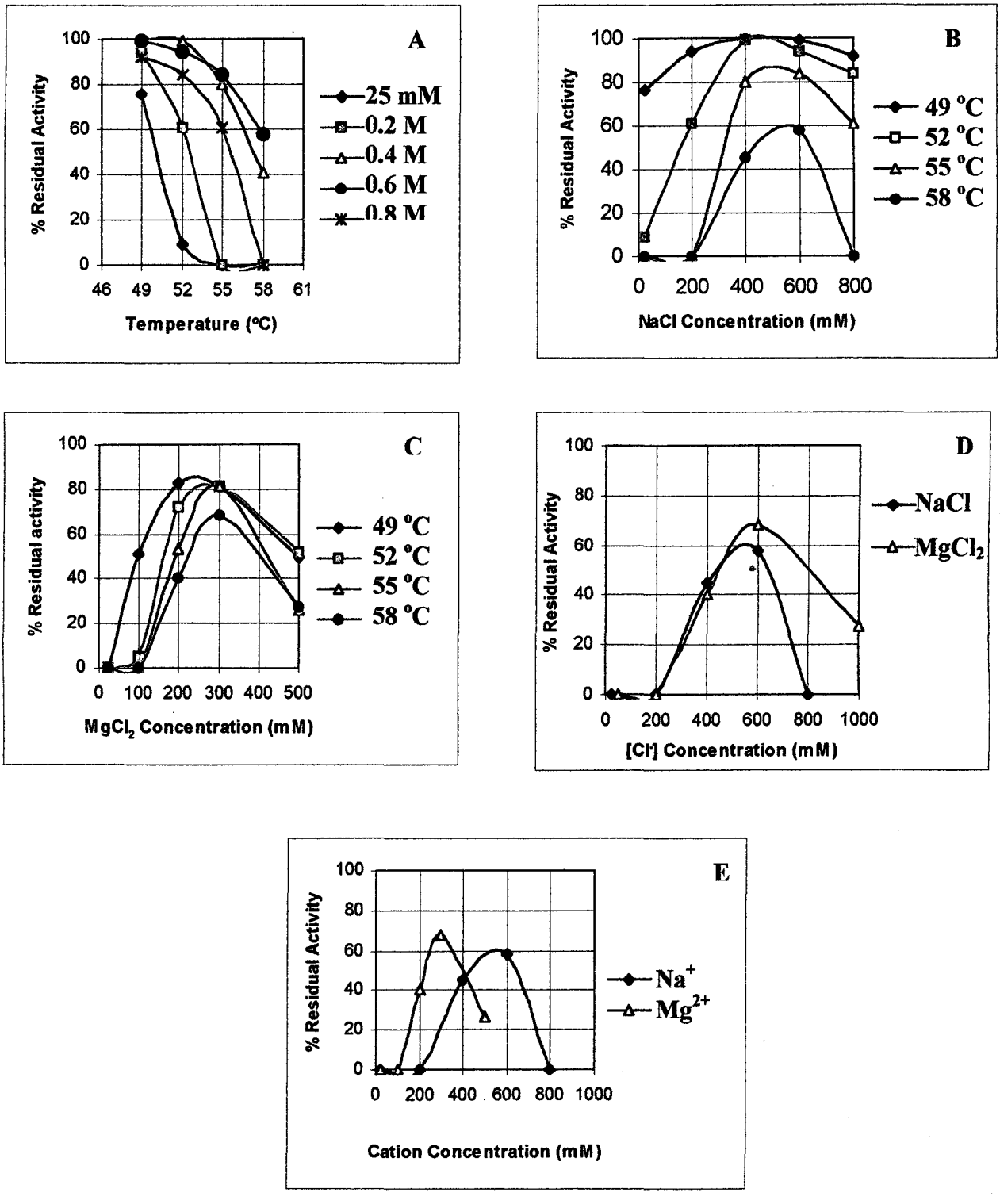


Fig 3.7 The dependence of PA stability on (A) NaCl (B) NaCl (C) $MgCl_2$ (D) Cl^- (at 58°C) (E) Cation (at 58°C) concentrations. PA (8 μg/ml) in 20 mM Tris, pH 8.1 was incubated at different temperatures for 20 min. and residual activity was determined by cytotoxicity assay on Macrophage like cells RAW264.7. 0.8 μg/ml of the incubated PA was used along with 0.3 μg/ml of LF for cytotoxicity assay.

concentration (Fig. 3.7 E) or ionic strength (data not shown). This dramatic stabilization effect could be interpreted in terms of binding of Cl^- to PA. Similar dramatic effects have been observed in case of human and yeast Ubiquitin (Makhatadze *et al.*, 1998) and RNase T1 (Mayr and Schmid, 1993; Pace and Grimsley, 1988). In case of human Ubiquitin, addition of 300 mM NaCl increases the transition temperature by 17°C at pH 2.0 while addition of 1 M NaCl to RNase T1 increase its transition temperature by 10°C at pH 5.0. These effects are attributed to specific binding of ions to the proteins.

When nonspecific interactions like charge screening and change in the solvent properties are the major effectors of stability, the effect is usually marginal like in the case of lysozyme and RNase A (Mayr and Schmid, 1993; Makhatadze *et al.*, 1998). Specific interactions like binding of anions or cations to charged surface drastically affect the stability of proteins as had been shown in case of human and yeast Ubiquitin (Makhatadze *et al.*, 1998) and RNase T1 (Pace and Grimsley, 1988; Mayr and Schmid, 1993). The stabilizing effects of ions (especially anions) on acid denatured state of proteins have been studied extensively (Goto *et al.*, 1990a, b; Hagihara *et al.*, 1993). It has been shown that anion binding promotes the formation of molten globule like state from the acid denatured state. Hagihara *et al.*, (1993) interpreted the results in term of non-specific binding of anions to charged groups of the protein. Whatever may be the case it is certain that favourable anion and cation binding could push the equilibrium between native and denatured state towards the native state, thus stabilizing the protein.

In the case of PA, although the results presented here are preliminary yet the observed dramatic increase in $T_{1/2}$ of PA by > 8°C in 600 mM NaCl or 300 mM $MgCl_2$ strongly points to the possibility of specific ion binding which may be pushing the equilibrium between the native and the denatured states towards the native state and thus stabilizing the native PA protein. The stability of PA above 600 mM NaCl and 300 mM $MgCl_2$ starts decreasing and becomes zero at 800 mM in the case of NaCl while in the case of $MgCl_2$ the studied range was only upto 500 mM but the residual activity was decreasing after reaching a maximum ~300 mM. The observed behaviour of PA thermal stability towards the presence of salts is rather surprising as it does not follow the usual trend of protein stability modulation by salts. It decreases with increase in salt

concentration after reaching a maximal value. In addition to this, at lower salt concentration, NaCl and $MgCl_2$ affect the PA thermal stability differently, *i.e.* at 25 mM concentration NaCl was found to be better stabilizer than $MgCl_2$. These aspects would need further investigation.

3.4.5 Effect of glycine and its methyl derivatives:

Glycine and its methyl derivatives are well known osmolytes, which are produced by organisms under various denaturing or stressful conditions like high salt or urea concentration, high temperature, low water activity etc. (Yancey *et al.*, 1982; Somero, 1986; da Costa *et al., 1998;* Trotsenko and Khmelenina, *2002*). Osmolytes provide stability to proteins without substantial alteration in their catalytic action (Borowitzka and Brown 1974; Bowlus and Somero, 1979; Pollard and Jones, 1979; Wang and Bolen 1996) both *in vivo* (Singer and Lindquist, 1998) and *in vitro* (Santoro *et al.*, 1992; Timasheff, 1995, Rishi *et al.*, 1998) and even outside their physiological concentration range as shown by Bolen and co-workers (Santoro *et al.*, 1992; Rishi *et al.*, 1998) without deleterious effects on biological activity. The family of glycine and its methyl derivatives include: glycine, a naturally occurring compatible osmolyte; sarcosine (N-methylglycine) and betaine (N, N, N trimethyl glycine), the naturally occurring counteracting osmolytes (Bowlus and Somero, 1979; Yancey *et al.*, 1982) and N, N dimethylglycine (DMG) a synthetic counteracting osmolyte (Santoro *et al.*, 1992; Gopal and Ahluwalia, 1993). Osmolytes are generally believed to stabilize protein by getting preferentially excluded from the protein surface and favouring the preferential hydration of the protein (Arakawa and Timasheff, 1983, 1985; Timasheff, 1992, 1995, 1998). Osmolytes increase the chemical potential of denatured ensemble (of protein), which increase with increase in the osmolyte concentration, resulting in an increased preferential hydration and hence stabilization. Preferential hydration of proteins and the solvophobic effect of the osmolyte for the peptide backbone raise the Gibbs free energy of the denatured ensemble causing compaction in the structure (Liu and Bolen, 1995; Qu *et al.*, 1998; Bolen, 2001; Bolen and Baskakov, 2001). As unfolding/denaturation generally leads to loosening of the structure resulting in an increased surface area and exposure of the hydrophobic surfaces, osmolytes push the Native (compact) $\Leftrightarrow$ Denatured/ Unfolded

(loose) equilibrium towards the native state and thus stabilize the protein molecule.

The effect of glycine and its methyl derivatives on the thermal stability of PA was investigated by incubating PA with varying concentration of the co-solvents at different temperatures (46-58°C) for 20 minutes and then comparing the residual activity determined by the cytotoxicity assay. The effect of glycine and its methyl derivatives on stabilizing PA against thermal inactivation is shown in fig 3.8. The $T_{1/2}$ values for glycine, sarcosine, dimethyl glycine and betaine at 1.0 M were 52.3, 51.3, 51.3 and 50.6°C, respectively while at 2.4 M concentration they were 56.7, 56.6, 56.6 and 55.7°C, respectively (Table 3.1). The ability of glycine and its methyl derivatives to stabilize the PA followed the order glycine > sarcosine≥ dimethyl glycine > betaine, at concentrations studied, as expected from their increasing hydrophobicity. The same had been observed in the case

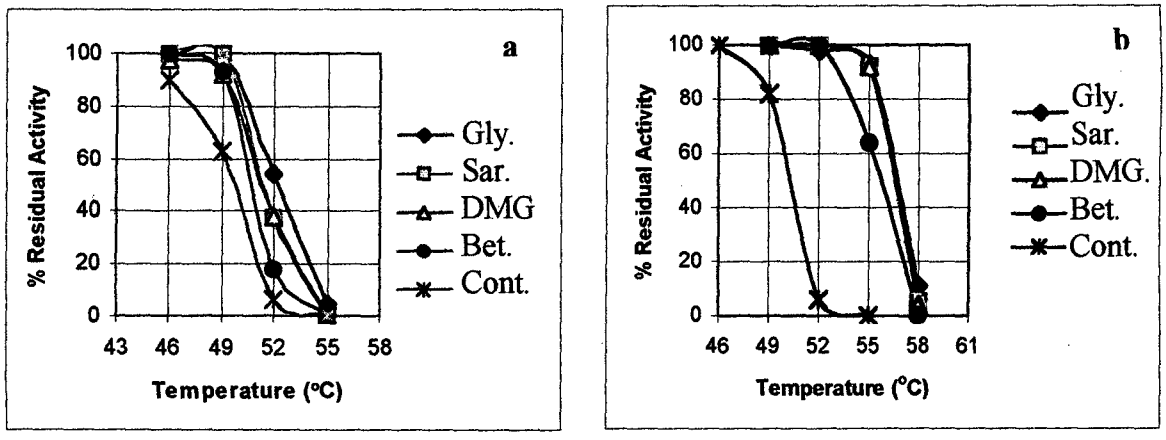


Fig 3.8 Effect of glycine and its methyl derivatives on stability of PA (a) 1.0 M and (b) 2.4 M. PA (8 μg/ml) in 40 mM HEPES with 25 mM NaCl, pH 7.9 was incubated with different concentrations of glycine and its methyl derivative at different temperatures for 20 minutes and residual activity was determined by cytotoxicity assay on macrophage like cells RAW264.7. 0.8 μg/ml of the incubated PA was used along with 0.3 μg/ml of LF for cytotoxicity assay. (Gly: Glycine; Sar: Sarcosine; DMG: Dimethyl Glycine; Bet: Betaine; Cont: PA in 25 mM NaCl only)

of RNase and HEW lysozyme (Santoro *et al.*, 1992) and BSA (Arakawa and Timasheff, 1983). Methyl amine derivatives with increase in the methyl groups are preferentially more excluded from the protein surface, resulting in increased preferential hydration of

the protein but the resulting increase in hydrophobicity decreases their preferential exclusion from the denatured protein or in other words causes an increased preferential binding to the denatured state and hence lesser ability to push the **Native (compact) ⇔ Denatured/ Unfolded (loose) equilibrium** towards the native state. Although the mechanism of stabilization is same for all the proteins studied so far (or at least they fit in the explanation provided) yet the magnitude of opposing stabilization forces, i.e. preferential hydration of the protein and possible weak binding of the **glycine and its methyl derivatives** with the partially or completely denatured states varies greatly and depends upon the nature of the protein (*i.e.* extent and nature of exposed surfaces). For example, 8.2 M sarcosine causes a 22°C increase in the T_m of RNase A while the same increase in the T_m can be achieved with 5 M sarcosine for HEW lysozyme. (Santoro *et al.*, 1992)

3.4.6 Effect of Polyols:

Polyols are another class of osmolytes, which are produced during various denaturing stresses (Yancey *et al.*, 1982; Timasheff, 1992, 1993). Polyols along with other polyhydric compounds such as sugars are known to stabilize the proteins for a long time (Tanford, *et al.*, 1962; Utter *et al.*, 1964; Gerlsma, 1968, 1970, Gerlsma and Sturr, 1972, 1974; Frigon and Lee, 1972). They are often used as non-specific protein stabilizers. The protein stabilizing ability of polyols is believed to due to their preferential exclusion from the protein surface (Xie and Timasheff, 1997a, b; Timasheff, 1998) which leads to preferential hydration and hence stabilization of the protein molecule. There have been efforts to correlate the relative ability of polyols to stabilize proteins to steric exclusion of the polyol molecules from the protein surface (Schachman and Lauffer, 1949), number of OH groups (Gekko and Morikawa, 1981a, b; Gekko, 1982) and their stereochemical properties (Back *et al.*, 1979; Uedaira and Uedaira, 1980; Kaushik and Bhat, 1998, 2003), differential ability to strengthen water lattice structure (Gerlsma, 1970) depending upon the OH group orientation and distance between O-O of polyol with respect to that of O-O bond distance (4.86 Å) in water lattice structure (Tait *et al.*, 1972), etc. The prevalent view is that preferential hydration of proteins by polyols and solvophobic nature of the peptide backbone promotes folding and compaction of the protein molecule, pushing the equilibrium between the native and the unfolded protein in favour of the native state

and thus stabilizing the protein molecule (Liu and Bolen, 1995; Qu *et al.*, 1998; Bolen, 2001; Bolen and Baskakov, 2001).

The effect of various polyols on the thermal stability of PA was investigated by incubating PA with varying concentration of polyols and polyols + 500 mM NaCl at different temperatures (46-75°C) for 20 minutes and then comparing the residual activity determined by the cytotoxicity assay. Fig 3.9 shows the ability of different polyols to stabilize PA against the thermal inactivation. Fig 3.9 a, b and c show the effect of 0.9, 1.8 and 2.7 M polyol, respectively. The $T_{1/2}$ for different polyols independently and in combination with 500 mM NaCl is shown in Table 3.2. In general, the stabilizing effect of polyols increases with the increase in concentration, though they differ greatly in magnitude. At any given molarity, the ability of polyols to stabilize PA followed the order glycerol > sorbitol > xylitol ≥ trehalose > adonitol > erythritol. It was observed that on addition of 500 mM NaCl to polyols the stability of PA invariably increased and the magnitude was always more than that of polyol or NaCl alone. It suggests that the mechanism of stabilization of PA is different in the case of polyols and salts. In the presence of 500 mM of NaCl the above order of power of stabilizing PA changed to glycerol > xylitol > sorbitol > adonitol > erythritol. It indicates that the presence of 500 mM NaCl modulate the primary nature of polyol interaction with the protein or in other words although polyols and NaCl were acting in tandem and stabilizing the protein, yet the presence of NaCl affected the interaction of polyol with the protein in some subtle way which was different for each polyols. Salts stabilize proteins by screening of unfavourable or repulsive charge-charge interactions or by favuorable electrostatic binding of ions, while polyols are known to strengthen hydrophobic interactions. Four more observations that need mention were:

a) i-erythritol was destabilizing PA at lower concentrations as compared to control. At 0.9, 1.8 and 2.7 M concentration of i-erythritol, $T_{1/2}$ for PA were 43.7, 46.3 and 51.5°C, respectively (Table 3.2), while that of control was 50.3°C. It points towards specific destabilizing action of i-erythritol with PA, which appears very prominent at lower concentration but was overwhelmed by weak stabilizing

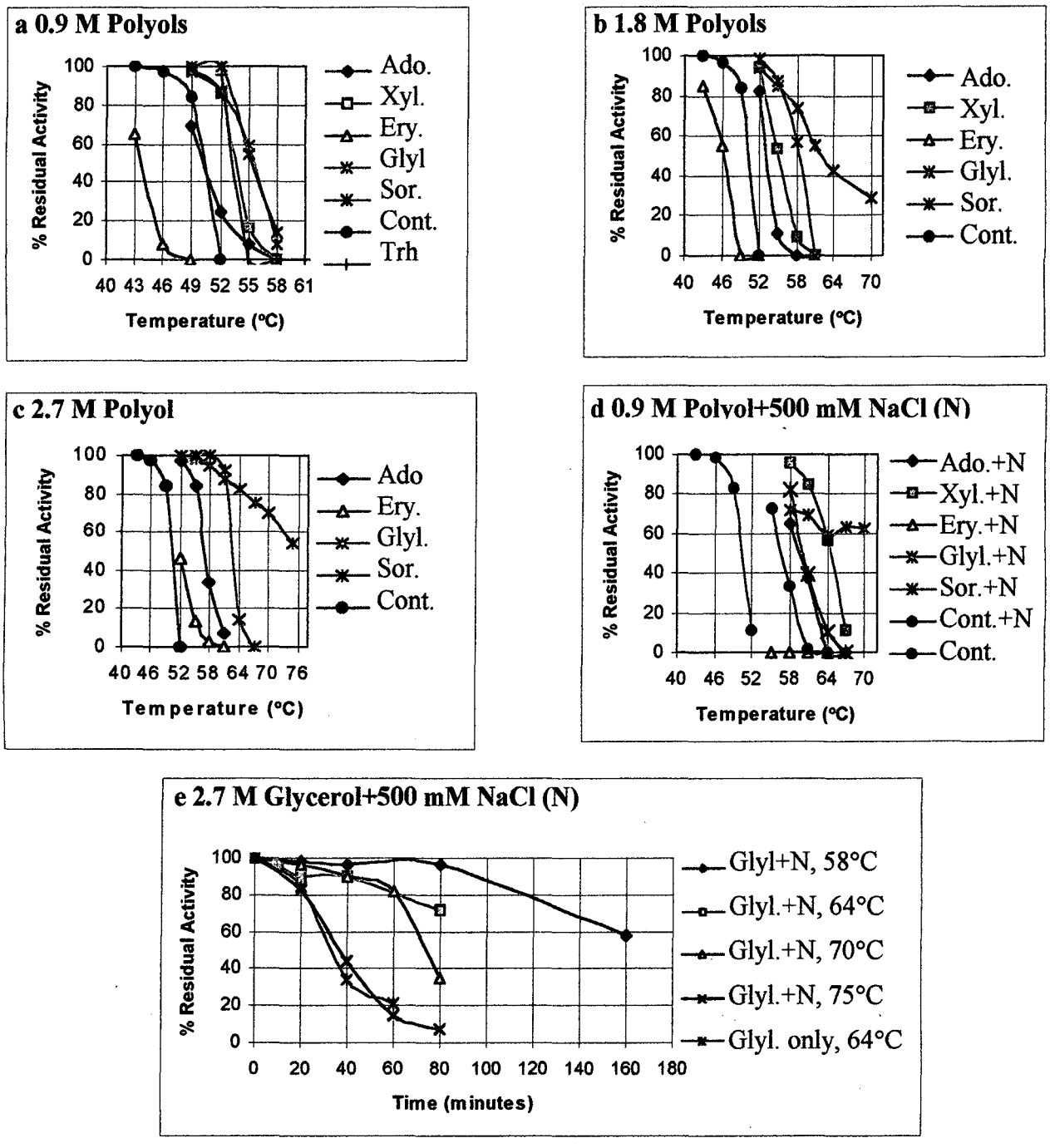


Fig. 3.9 Effect of Polyols on the stability of PA (a) 0.9 M (b) 1.8 M (c) 2.7 M (d) 0.9 M Polyols + 500 mM NaCl (e) 2.7 M Glycerol + 500 mM NaCl and 2.7 M Glycerol only. PA (8 μg/ml) in 40 mM HEPES, pH 7.9 was incubated with different concentrations of polyols (with 25 mM NaCl) and polyols + 500 mM NaCl at different temperatures for 20 min and residual activity was determined by cytotoxicity assay on Macrophage like cells RAW264.7. 0.8 μg /ml of the incubated PA was used along with 0.3 μg/ml of LF for cytotoxicity assay. (Ado: Adonitol; Xyl: Xylitol; Ery: i-Erythritol; Glyl: Glycerol; Sor: Sorbitol; Trh: Trehalose; Cont: PA in 25 mM NaCl; N: 500 mM NaCl unless otherwise mentioned)

Table 3.1 Effect of glycine and its methyl derivatives on $T_{1/2}$ of PA (Fig 3.8)

Co-Solvent	Concentration	$T_{1/2}$ (20minute) in °C
Control	25 mM NaCl without solvents	49.5-50.2[a]
Glycine	1.0 M	52.3
Glycine	2.4 M	56.7
Sarcosine	1.0 M	51.3
Sarcosine	2.4 M	56.6
Dimethyl Glycine (DMG)	1.0 M	51.3
Dimethyl Glycine (DMG)	2.4 M	56.6
Betaine	1.0 M	50.8
Betaine	2.4 M	55.7

Table 3.2 Effect of polyols on $T_{1/2}$ of PA (Fig 3.9)

Co-Solvent	Concentration	$T_{1/2}$ (20 minute) in °C
Control	25 mM NaCl without solvents	50.3[a]
Control	500 mM NaCl without solvents	57.5
Glycerol	0.9 M	55.7
Glycerol	1.8 M	61.9
Glycerol	2.7 M	>75
Glycerol	0.9 M+500 mM NaCl	>75
Erythritol	0.9 M	43.7
Erythritol	1.8 M	46.3
Erythritol	2.7 M	51.5
Erythritol	0.9 M+500 mM NaCl	<55
Adonitol	0.9 M	50.2
Adonitol	1.8 M	53.2
Adonitol	2.7 M	57.0
Adonitol	0.9 M+500 mM NaCl	59.7
Sorbitol	0.9 M	55.3
Sorbitol	1.8 M	58.4
Sorbitol	2.7 M	62.5
Sorbitol	0.9 M+500 mM NaCl	60.2
Trehalose	0.9 M	53.3
Xylitol	0.9 M	53.5
Xylitol	1.8 M	55.1
Xylitol	0.9 M+500 mM NaCl	64.5

a: $T_{1/2}$ for PA in 20 mM HEPES with 25 mM NaCl, pH 7.4 (25 °C) was 43±2 °C, while in 40 mM HEPES wit 25mM NaCl, pH 7.9 (25 °C) was 48.5±2 °C when incubated at final concentration of 8 μg/ml. PA was used at final concentration of 0.8 μg/ml along with 0.3 μg/ml of LF for cytotoxicity assay on RAW264.7 cell line.

interactions at higher concentration.

b) Adonitol and xylitol, which are epimers stabilize PA to different degrees. At 0.9 M concentration adonitol behaved like control but increase in the concentration increased the stabilization of PA, while xylitol behaved like a good stabilizers among the polyols studied. Different behaviour of epimers has been reported earlier in the case of collagen. The differential effect of epimers towards stabilizing the protein has been attributed to differences in the solvent organization property because of variation in the hydrophobicity that is related to hydroxyl group position (Kurznetsova *et. al.*, 1998).

c) Effect of Glycerol towards stabilizating PA is highly concentration dependent. At 0.9 M it was similar to that of sorbitol with a $T_{1/2}$ of 55.7°C, but with increasing concentration its effect deviated from that of sorbitol (Fig. 3.9c). An interesting behaviour was observed when 500 mM NaCl was present along with 0.9 M glycerol. The residual activity obtained on incubation in the range of 58°C to 70°C, was ~60-75 %, which did not follow the usual trend of decrease in activity with increase in temperature. It could be due to glycerol assisted refolding of the heat-denatured PA. Glycerol has been shown to increase the refolding yield of a number of proteins, *e.g.*, tailspike protein and citrate synthase (Mishra, 2002) etc. Earlier, it has been shown that polyols increase the refolding of heat-denatured proteins, *e.g.*, Staphylococcus nuclease in the presence of xylose (Frye and Royer, 1997).

d) Another interesting feature was observed when 500 mM NaCl was present along with polyols. It invariably resulted in enhancement of residual activity obtained at any given temperature as compared to the residual activity obtained when only polyols were used (Fig. 3.9d and Table 3.2). This observation indicates that the mechanisms operational in the stabilization of the PA by polyols and NaCl are different and additive.

When PA was incubated at 58-75°C in the presence of 2.7 M glycerol + 500 mM NaCl for 0-160 minutes, the rate of loss of activity (at all the temperatures studied) was initially slow followed by a fast phase (Figure 3.9e). It was found that loss of PA activity in the presence of 2.7 M glycerol on incubation at 64°C follows approximately the same profile as 2.7 M glycerol + 500 mM NaCl follows on incubation at 75°C, which strongly support the notion that mechanisms operating for the stabilization of PA by polyols and salts are different in nature (Fig 3.9 a, d: sorbitol and xylitol). The fast phase of loss of activity appeared early with an increase in the temperature of incubation while on shorter incubations the activity regained was comparable in all the cases. It points to irreversible changes occurring in the protein at elevated temperature, which make the protein unable to regain the activity on cooling. It could probably be due to some chemical modifications to which the proteins are prone to at higher temperatures like deamidation, isomerization, oxidation, autolysis etc., which are known to occur in proteins at elevated temperatures (Wang, 1999; Lai and Topp, 1999).

In conclusion, the investigation into the cause of thermal inactivation of PA led to the finding that PA is a highly aggregation prone protein, which is also the main cause of its thermal inactivation at elevated temperatures (46-58°C). Salts have been shown to affect its stability in a positive way depending however on their concentration used. The stabilization by NaCl and $MgCl_2$ on the stability of PA points to specific stabilizing interaction of salt ions with charged residues on PA. In the case of glycine and its methyl derivatives PA stabilizing ability decreases with an increase in their hydrophobicity (*i.e.* increase in the number of methyl groups). Glycine was found to be the best stabilizer followed by sarcosine, dimethyl glycine and betaine. Polyols were found to be also good stabilizers of PA activity. Their ability to stabilize PA differed greatly, on one hand there was erythritol that decreased thermal stability of PA while on the other glycerol in combination with NaCl was able to retain ~100% activity of PA even at 75°C when incubated for a shorter duration. On extended incubation at high temperature the activity of PA was lost at a very high rate perhaps due to some chemical modifications in the protein molecule. Despite these observations and findings much more remains to be done in order to understand the mechanisms of thermal

inactivation of PA and its stabilization by osmolytes so that they can be effectively used to stabilize and prolong the shelf life of PA in vaccines.

SUMMARY

The present work entitled "Studies on Thermostabilization of Protective Antigen of *B. anthracis*" involves characterization of the thermolabile nature of PA and enhancement of its thermal stability by protein and co-solvent engineering. PA being the central moiety in anthrax intoxication and main immunogen that can provide protective immunity against anthrax has an important place in pathogenesis and its control. Its thermolability has remained a cause of concern as transient exposure of vaccines to higher temperatures during transportation could compromise their efficacy. The present work was, thus envisaged to increase the themostability of PA and to characterize its thermolabile nature.

Initial phase of the work started with the model building of the missing parts of PA crystal structure (1ACC:www.rcsb.org) with the help of Homology module of Insight II program from MSI (Accelrys Inc). The model built, energy minimized structure was analysed for totally buried residues using the ACCESS program of Lee and Richards (1971). The buried hydrophilic residues of the protein could be the source of destabilization if not involved in stabilizing interactions such as H-bond networks or ion-pairs. Assuming that the critical residues important for the stability of the protein and involved in stabilizing interactions would be conserved in homologous proteins as well, PA sequence was aligned with that of Iota –Ib (*Clostridium perfringens*) and ADP-ribosyltransferase (*Clostridium difficle*). Sequence alignment showed that except residue Gln277, Ser330, and Thr674 other hydrophilic residues were fairly conserved in all the homologous proteins (Table 2.2). Therefore these residues were changed to alanine to decrease the hydrophilic character in the protein interior. Besides these the residue Phe554, a dispensable surface exposed residue of the hydrophobic patch of PA involved in oligomerization and biological activity, was also changed to alanine to decrease the hydrophobic character of the exposed patch, as in many cases exposed hydrophobic patches have been shown to be involved in aggregation of the protein. These mutants were evaluated for their thermal stability by incubating them at 37°C for 48 hrs. Phe554Ala and Gln277Ala retained ~90% and 45% activity while the native PA and Ser330Ala mutant completely lost the activity (Fig 2.12 and Table 2.5). Hence, by

decreasing the hydrophilic character in the protein interior and hydrophobic character on the protein exterior (surface) two thermostable variants of PA were made. These could be better candidates for PA based anthrax vaccine.

Looking at the causes of themolability of PA, its proneness to autolysis and aggregation was evaluated. Incubation of PA for 10 minutes at moderately high temperature (55°C) did not show any evidence of autolysis while the activity of PA was completely lost (Fig 3.1). This indicated involvement of some other mechanism in the thermal inactivation of PA. Aggregation as a possible cause of thermal inactivation was also probed. It was found that PA is highly prone to aggregation (Fig 3.2 and 3.3). PA (40 μg/ml) lost activity within 6 minutes when incubated at 46°C with concomitant increase in aggregation while the addition of 2.7 M glycerol along with 500 mM NaCl prevented the aggregation as well as preserved the PA activity even after 30 minutes of incubation at 58°C (Fig 3.4). Effects of pH, protein concentration, salt concentration, glycine and its methyl derivatives and polyols on the thermal stability of PA were also studied. PA appeared to be stable only in a narrow pH range (~7.9: see Fig. 3.5) at higher temperature (49°C). As expected from the aggregation prone nature of PA, ~6°C decrease in $T_{1/2}$ (~51 to 45°C) was observed over eight fold increase in concentration of PA (4-32 μg/ml: see Fig. 3.6). Both the salts studied, *i.e.* NaCl and $MgCl_2$, stabilized the PA and the increase in stability with concentration pointed towards binding of Cl^- to PA. At 600 mM concentration of NaCl and 300 mM concentration of $MgCl_2$ the observed increase in $T_{1/2}$ was >8°C (Fig 3.7). Ability of glycine and its methyl derivatives to stabilize PA followed the order glycine > sarcosine > dimethyl glycine > betaine, as expected from their increasing hydrophobicity because of increasing number of methyl groups. Glycine at 2.4 M concentration stabilized PA the most with ~6.5°C increase in $T_{1/2}$ as compared to control (Fig.3.8, Table 3.1). The polyols were also found to be good stabilizers of PA. In general the stability increased with the increase in concentration, but the magnitude of stabilization differed greatly for different polyols. The ability of polyols to stabilize PA followed the order glycerol > sorbitol >xylitol ≥ trehalose >adonitol >erythritol. Addition of NaCl to polyols at 500 mM final concentration always resulted in increased thermostabilization of PA. In the presence of 500 mM NaCl, the ability of polyols to

stabilize PA changed to glycerol > xylitol > sorbitol >adonitol > erythritol. This observation suggests that the mechanism of stabilization of PA in the two cases is different and complementary in nature. In the presence of 2.7 M glycerol + 500 mM NaCl the $T_{1/2}$ for PA was >75°C. On extended incubations (0-160 minutes) of PA in the presence of 2.7 M glycerol and 2.7 M glycerol + 500mM NaCl at different temperatures (58-75 °C), the rate of loss of activity was initially slow followed by a fast phase (Fig 3.9). The fast phase of activity loss appeared early with an increase in temperature of incubation while on shorter incubations the residual activity obtained was comparable in all the cases. It points to some irreversible changes occurring in PA at elevated temperatures, which are making the protein unable to regain the activity on decreasing the temperature. Proteins are known to be prone to various chemical modifications such as deamidation, autolysis, isomerization etc. at higher temperature. It may be happening in the case of PA as well. This study provides insights into the basic mechanisms responsible for the thermolabile nature of PA and has helped in generating strategies for producing two thermostable mutants of PA as well as formulations using cosolvents to stabilize the protein.

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APPENDIX
(Adapted from Chauhan, V., 2001)

PREPARATION OF BACTERIAL CULTURE MEDIA

LB medium (Luria Broth)

Dissolve 20 gms of LB powder (Hi-Media) in double distilled water. Sterilize the media by autoclaving for 20 minutes at 15 lb/sq.in.

LB Agar

Dissolve 35 gms of LB agar powder (Hi-Media) in double distilled water. Sterilize the media by autoclaving for 20 minutes at 15 lb/sq.in. Allow LB agar to cool and pour in 90 mm disposable petri plates (Tarsons) along with appropriate antibiotics and allow to solidify.

ANTIBIOTICS SOLUTION

Ampicillin

Prepare 100 mg/ml stock in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store by freezing at -20°C.

Kanamycin

Prepare 50 mg/ml stock solution in double distilled water and sterilize by filtration through 0.22 μm filter (sterile). Store at - 20°C.

SOLUTIONS FOR PLASMID ISOLATION AND PURIFICATION

Solution I

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

Prepare Solution I in batches of 100ml, autoclave for 20 minutes at 10 lb/sq.in. and store at 4°C.

Solution II

0.2 N	NaOH (freshly diluted from 10 N stock).
1%	SDS

Chauhan, V., (2001). Studies on Domain 1b of Protective Antigen of Anthrax Toxin. Ph.D thesis submitted to Centre for Biotechnology, Jawaharlal Nehru University, New Delhi.

Solution III

5M Potassium acetate	60 ml
Glacial Acetic acid	11.5 ml.
ddH_2O	28.5 ml

The resulting solution is 3M with respect to potassium and 5 M with respect to acetate. Autoclave at 15 lb/sq.in. for 20 minutes. Store at 4°C.

STOCK SOLUTION OF COMMONLY USED REAGENTS

1M Tris.

Dissolve 121.1 gms of Tris base in 800 ml of double distilled water and adjust the desired pH (6.8, 7.4, 8.0) with concentrated HCl. Make up the volume to 1 liter and autoclave.

0.5 M EDTA.

Add 186.1 gms of disodium EDTA.2 H_2O in 800 ml of double distilled water. Stir vigorously on a stirrer, adjust the pH to 8.0 with NaOH (about 20 gms of NaOH pellets), make up the volume to1 liter and autoclave.

3M sodium acetate

Dissolve 204.5 gms of $C_2H_3O_2Na$. $3H_2O$ in 400 ml of ddH_2O, Adjust the pH to 5.3 with glacial acetic acid. Make up the volume to 500 ml and autoclave.

10% SDS

Dissolve 10 gms of electrophoresis grade SDS in 70 ml of ddH_2O heat at 60°C to dissolve and make up the volume to 100 ml.

Ethidium Bromide (10 mg/ml)

Dissolve 10 mg of ethidium bromide in 1 ml ddH_2O. Store in a dark bottle.

30% Acrylamide Stock

Dissolve 29.2 gms of acrylamide and 0.8 gms of bis acrylamide in 50 ml of ddH_2O. Make up the Volume to 100 ml, filter the solution through Whatman no. 1 paper, degas and store in a dark bottle.

Calcium Chloride (0.1 M)

Dissolve 147.0 gms of $CaCl_2.2H_2O$ in 100 ml of ddH_2O and sterilize by autoclaving.

Sodium Phosphate (1M)
Monobasic

Dissolve 138 gms of $NaH_2HPO_4.H_2O$ in 800 ml of ddH_2O and make up the volume to 1liter.

Dibasic

Dissolve 268 gms of $Na_2HPO_4.7H_2O$ in 700 ml of ddH_2O and make up the volume to 1 liter.

Ammonium persulfate (10%)

To 1 gm of ammonium persulfate add 10 ml of ddH_2O the solution may be stored for several weeks at 4°C.

100mM Phenyl methyl-sulfonyl fluoride (PMSF)

Dissolve 17.4 mg of PMSF in 1 ml of isopropanol. Divide the solution in aliquots and store at -20°C.

BUFFERS

50 X TAE buffer (Tris acetate, EDTA)

Dissolve 242 gms of Tris base in 700 ml of ddH_2O and add 57.1 ml of glacial acetic acid and 100 ml of 0.5 EDTA pH 8.0. Make up the final volume to 1liter.

Phosphate Buffer Saline (PBS)

Dissolve 8 gms of NaCl, 2 gms of KCl, 1.44 gms of Na_2HPO_4 and 0.2 gms of KH_2PO_4 in 800 ml of ddH_2O. Adjust the pH to 7.4 with HCl. Make up the final volume to 1 liter and sterilize by autoclaving at 15 lb/ sq.in for 20 minutes and store at room temperature.

SDS-PAGE electrophoresis buffer

Dissolve 3 gms of Tris base, 14.4 gms of glycine and 1 gm of SDS in 1 liter of ddH_2O.

Protein transfer buffer (Western Transfer Buffer or WTB)

Dissolve 5.8 gms of Tris base, 2.9 gm of glycine and 0.33 gms of SDS in 0.5 liter of ddH_2O. Add 200 ml of ethanol and make up the final volume to 1 liter.

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

100mM	Tris-Cl (pH6.8)
200mM	DTT
4%	SDS

0.2%	Bromophenol blue
20%	Glycerol
10%	β-mercaptoethanol

10 X Restriction enzyme *Kpn* I buffers (L)

100mM	Tris.Cl (pH 7.5)
100mM	$MgCl_2$
10mM	DTT

10X Restriction enzyme *Bam* H1 buffer (K)

200mM	Tris.Cl pH 8.5
100mM	$MgCl_2$
10mM	DTT
1000mM	KCl

10 X Amplification/PCR buffer

100 mM	Tris.Cl pH 8.3
15 mM	$MgCl_2$
500 mM	KCl
0.1 %	gelatin

DNA loading dye (6X)

Dissolve 0.2 gms bromophenol blue, 0.2 gms xylene cynol and 30 ml of glycerol and make up the volume to 100 by autoclave ddH2O.

SDS-PAGE reagents

Composition of resolving gel (12%) 10 ml

4.0 ml	30% acrlyamide solution
2.5 ml	1.5 M Tris-Cl pH 8.8
3.3 ml	ddH_2O
100µl	10% SDS
100µl	10% APS
10µl	TEMED

Composition of stacking gel (5%) (5.0 ml)

0.83 ml	30% acrlyamide solution
0.68 ml	10M Tris.Cl pH 6.8
3.4 ml	ddH_2O
50 µl	10% SDS
50 µl	10% APS
5µl	TEMED

Staining solution

Dissolve 1 gm of coomassie blue in 450 ml of methanol. Add 100 ml of glacial acetic acid and make up the volume to 1 liter by double distilled water. Filter through Whatman no. 1 and store at room temperature.

Destaining solution

Add methanol: Water: Acetic Acid in the ratio of 45:45:10. Store at room temperature.

ANIMAL TISSUE CULTURE MEDIUM RPMI 1640

A) Maintaining the Macrophage like cell line J774A.1 and RAW264.7.

Dissolve the following components in 800 ml of double distilled water

RPMI salt	10.4 gms
$NaHCO_3$	2.0 gms
HEPES	12.0 gms
*L-Glutamine	0.3 gms
*L-Leucine	0.050 gms
*L-Lysine	0.040 gms
*L-Methionine	0.015 gms
Penicillin	0.062 gms
Streptomycin	0.100 gms

* components added only when the medium provided is deficient for them.
Adjust the pH 7.2- 7.4 with HCl and make up the volume to 1000 ml. Filter sterilize the media and store at 4°C.

B) Cytotoxicity assay

Medium is prepared as (A) above except Glutamine is omitted, as deamidation of it (in old media say 2-3 months) would produce ammonia (NH_4^+) which will hinder the activity assay.

REAGENTS FOR PROTEIN ESTIMATION BY BRADFORD METHOD

A) Protein Determination Reagent (USB, Cat #30098)

B) 1 mg/ml BSA standard stock.

Standard graph for protein estimation was made in the range of 1-10 □g/ml using BSA. To every 1 ml sample (final volume) 200 □l of Protein Determination reagent was added. After thorough mixing of the reagent with protein solution absorbance was taken at 595 nm. Same way protein concentrations in samples were determined by comparing absorbance at 595 nm with that of BSA standard.

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Gln277 and Phe554 residues are involved in thermal inactivation of protective antigen of *Bacillus anthracis*

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Abstract

Protective antigen (PA) is the main component of all the vaccines against anthrax. The currently available vaccines have traces of other proteins that contribute to its reactogenicity. Thus, purified PA is recommended for human vaccination. PA loses its biological activity within 48 h at 37 °C and its thermolability has been a cause of concern as accidental exposure to higher temperatures during transportation or storage could decrease its efficacy. In the present study, we have used protein engineering approach to increase the thermostability of PA by mutating amino acid residues on the surface as well as the interior of the protein. After screening several mutants, the mutants Gln277Ala and Phe554Ala have been found to be more thermostable than the wild-type PA. Gln277Ala retains ~ 45% and Phe554Ala retains ~ 90% activity, even after incubation at 37 °C for 48 h while in the same period wild-type PA loses its biological activity completely. It is the first report of increasing thermostability of PA using site-directed mutagenesis. Generation of such mutants could pave the way for better anthrax vaccines with longer shelf life.

Keywords: Thermostabilization; Protective antigen (PA); *Bacillus anthracis*

Anthrax is primarily a disease of animals caused by *Bacillus anthracis*, a Gram positive spore forming bacteria [1–3]. Humans are accidental hosts, as they mainly acquire infection either by handling contaminated animal products or by ingesting contaminated meat products [1,4]. Recently, a new dimension has been added to this by its use as a bioterrorism agent. Pathogenesis of anthrax is a result of interplay of three protein molecules, namely, protective antigen (PA), lethal factor (LF), and edema factor (EF), produced by *B. anthracis* [3–7]. All three molecules are individually non-toxic. LF (90 kDa) is a Zn^{2+} metalloprotease [6] that cleaves the MAPKKs [8,9] and is lethal to sensitive macrophages when given in combination with PA (lethal toxin, LeTx) [1–9]. EF (89 kDa) is a calmodulin-dependent adenylate cyclase, which causes an increase in the cellular cAMP levels, and hence, edema when given in combination with PA (edema toxin, EdTx) [10]. PA (83 kDa) is the cell binding molecule that facilitates the entry of LF/EF into the cell where they exert their action. PA as the name suggests is the most immunogenic protein among the anthrax toxin complex components (PA, LF, and EF) and provides the immunity against anthrax when administered to host. It is the main component of all the vaccines against anthrax [1]. Purified PA protein is recommended for use in humans as currently used vaccines have many side effects due to the presence of LF and EF [11]. We have addressed this problem by cloning and expressing recombinant PA in *Escherichia coli*. Recombinant PA, purified using affinity chromatography, was found to be biologically and functionally active [12,13]. PA has been shown to be thermolabile and it loses activity, even at 37 °C within 48 h [14]. Its thermolability has been a cause of concern as accidental exposure to higher temperature during transportation or storage could decrease its efficacy.

Thermostability of proteins depends on a number of chemical and physical factors [15]. Chemical instability

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is known to arise due to several factors like deamidation of Asn and Gln, isomerization, hydrolysis of peptide bonds, oxidation of side chains, disulfide bond formation and breakage, etc. These reactions have been known to play a major role in the instability of a number of therapeutically important proteins like tissue plasminogen activator, insulin [16,17], etc. Issues of chemical instability have been comprehensively reviewed by Wei Wang [15]. Just like chemical stability, conformational stability also depends upon the unique sequence of amino acids in a protein. It is generally defined as the free energy difference between the folded and unfolded conformations of the protein molecule. Interactions like hydrophobicity, hydrogen bond, electrostatic interaction, and Van der Waals interaction play an important role in the conformational stability of a protein. The final conformational stability of most of the naturally occurring globular proteins could be assigned to a few hydrogen bonds or salt bridges usually of the order of 5–20 kcal/mol [15,18]. With the increase in the understanding of interactions involved in the conformational stability of proteins, a number of approaches have been explored to increase their thermostability. The stability of the folded conformation has been increased by stabilizing different structural elements like α-helix [19,20] and β-sheet [21], introduction of disulfide bridge [22], and by incorporating ligand/metal binding sites [23]. Stability of the unfolded conformation has been decreased by reducing entropy of the unfolded state by glycine to X or X to proline mutations [24]. Empirical approaches such as changing amino acid residues to those that are present in other homologous thermostable proteins [25,26] and screening larger conformational space by random mutagenesis [27] have been used. These approaches have been successfully employed to thermostabilize various proteins [15–27]. In addition to these approaches, protein can also be made thermostable by using different cosolvent additives [15,28,29]. Cosolvents affect stability by interacting preferentially with the protein; i.e., preferential binding or preferential exclusion [28,29].

We have earlier stabilized PA at higher temperatures by the use of cosolvent additives [14]. However, making thermostable mutants by protein engineering would reduce the cost of production, as it would eliminate the use of excipients. In this study, we have introduced mutations at four different places in the PA molecule and evaluated their effect. Among the mutations made, Gln277Ala (buried polar residue) and Phe554Ala (in the exposed hydrophobic patch [30]) were observed to be thermostable. We report for the first time the increase in thermostability of PA achieved by genetic engineering means. These mutants could prove to be promising candidates for improved recombinant anthrax vaccine.

Materials and methods

Reagents and supplies. The enzymes and chemicals used for DNA manipulation were purchased from Life Technologies (Invitrogen, USA), Roche Chemicals (Germany), Stratagene (USA), Perkin–Elmer (Applied Biosystems, USA). The oligonucleotides were obtained from Microsynth (Switzerland). The PCR was performed in Perkin–Elmer thermal cycler. DNA purification kit, expression vector pQE30, and Ni–NTA agar were obtained from Qiagen (Germany). Cell Culture plasticware was obtained from Corning (USA). Fetal calf serum (FCS) was from Biological Industries (Israel). RPMI 1640, 3-(4,5-dimethylthiazol-3-yl)-5-diphenyltetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), Hepes, NaCl, and other chemicals were purchased from Sigma Chemical (USA). *E. coli* strain DH5α and RAW264.7, a macrophage like cell line, were obtained from ATCC (American Type Culture Collection; USA). Growth media components were from Hi Media Laboratories (India). InsightII software was from MSI (Accelrys, USA).

Modeling and analysis of PA structure. The crystal structure of PA (1ACC; pdb; www.rcsb.org) was retrieved and the missing residues were reconstructed using homology module of insightII program from MSI (Accelrys). The modeled PA structure thus obtained was subjected further to energy minimization using amber force field [31]. At first stage, only hydrogen atoms were minimized (as most of the time they are the cause of nuisance in energy minimization) with all heavy atoms fixed. At second stage, the structure thus obtained was subjected to minimization with all atoms being free to minimize. Steepest descent (SD) method and conjugate gradient (CG) method of minimization were used. This was used for further analysis of PA for accessible surface area (ASA) using ACCESS program of Lee and Richards [32]. Probe radius of 1.4 Å and slice thickness of 0.1 Å was used. Percent (%) accessibility of each residue was calculated and residues with 0% accessibility were picked.

Multiple sequence alignment (Table 1) was done using CLUSTAL W program [33] (www.ebi.ac.uk). Sequence alignment shows that, except Gln277, Ser330, and Thr674 residues other 100% buried polar and charged residues are more or less conserved. Changing these non-conserved residues to alanine could stabilize the molecule, as the cost of burying a hydrophilic group would be minimized. Besides these residues, Phe554 was also changed to Ala to reduce the hydrophobic character of the hydrophobic patch [30].

Table 1
Multiple sequence alignment of related proteins (only100% buried but not identical residues shown)

Protein	Position									
	1	6	7	222	277	330	434	488	494	674
PA	E	N	R	S	Q	S	M	T	N	T
Iota-Ib	D	E	E	S	A	H	I	S	D	I
ADP-rib.	H	K	E	N	A	H	I	S	D	I

Note. Position refers to residue number of PA for convenience. PA is protective antigen from *B. anthracis*. Iota-Ib is from *Clostridium perfringens*. ADP-ribosyltransferase from *Clostridium difficile*.

Table 2
Sequence of primers used for constructing mutations in pMW1

Mutation	Primer	Sequence
Gln277Ala	F	5′ CA AAA AAT GAG GAT GCA TCC ACA CAG AAT ACT GAT AGT GAA ACG AGA AC 3′
	R	5′ G TGT GGA TGC ATC CTC ATT TTT TGA GAG AAT AAT ATT CTC CAT ATC TAC 3′
Ser330Ala	F	5′ CG AAT TCA GCT ACG GTC GCA ATT GAT CAT TCA CTA TCT CTA GCA GGG 3′
	R	5′ C AAT TGC GAC CGT AGC TGA ATT CGA ATT ACT AAA TCC TGC AGA TAC ACT CCC ACC 3′
Thr674Ala	F	5′ CAA GAT GGA AAA GCA TTT ATA GAT TTT AAA AAA TAT AAT GAT AAA TTA CCG TTA TAT ATA AG 3′
	R	5′ C TAT AAA TGC TTT TCC ATC TTG CCG TAA ACT AGA AAT ATT CAA C 3′
Phe554Ala	F	5′ GAA TTT GAT TTT AAT GCA GAT CAA CAA ACA TCT CAA AAT ATC AAG AAT CAG 3′
	R	5′ GAG ATG TGT TTG TTG ATC TGC ATT AAA ATC AAA TTC GGT TAT GTC TTT CCC TTG 3′

Note. F and R refer to forward and backward primers. Mutant codons are underlined.

Generation of mutants. Gln277Ala, Ser330Ala, Thr674Ala, and Phe554Ala mutants of PA were constructed by site-directed mutagenesis of plasmid pMW1 [12,13] using quick change kit of Stratagene (La Jolla, USA) with little change in strategy and designated pPA1, pPA2, pPA3, and pPA4, respectively. The length of mutagenic primers was between 44 and 62 bases (Table 2). All mutants were confirmed by manual sequencing.

Purification of PA and mutant PA proteins. Escherichia coli DHα transformed with native and mutant construct of PA, i.e., pMW1, pPA1, pPA2, pPA3, and pPA4 were grown at 37 °C (250 rpm) in 100 ml LB medium containing 100 μg/ml ampicillin. The culture was allowed to grow upto $A_{600} \sim 1.5$–2.0. Cells were harvested by centrifugation at 4000*g* for 10 min. The pellet was resuspended in 10 ml lysis buffer (8 M urea, 0.1 M Na phosphate, 300 mM NaCl, and pH 7.8). Cells were stirred at 37 °C for 1 h. The lysate was centrifuged at 12,000*g* for 30 min. The supernatant was loaded on to an Ni–NTA column pre-equilibrated with lysis buffer. The PA bound to the Ni–NTA column was refolded by gradual removal of urea by passing a gradient of 8.0–0.0 M urea in buffer containing 0.1 M Na phosphate, pH 7.8. The recombinant protein bound to column was eluted with 250 mM imidazole in 0.1 M Na phosphate, 300 mM NaCl, pH 7.0. The eluted fractions were analyzed on SDS–PAGE. Fractions containing >95% pure PA were pooled and the buffer was changed to 20 mM Hepes, pH 7.4, with 500 mM NaCl using Centricons (30 KDa cutoff; Millipore). These were stored at 4 °C for further use.

Thermostability studies on PA and mutant PA proteins. Native PA and mutated PA were used at 10 μg/ml (20 mM Hepes, 25 mM NaCl, pH 7.4 at 25 °C) concentration for incubation at different temperatures (37–49 °C) for 5–160 min in order to evaluate the thermostability of the protein. They were also incubated at 37 °C for 48 h in 0.6 ml Eppendorff tubes and sample aliquots were withdrawn at different time intervals ranging from 3 to 48 h and kept at 4 °C before cytotoxicity assay.

Cell culture and cytotoxicity assay. Macrophage like cell line RAW264.7 (ATCC) was used for cytotoxicity assay. It was maintained in RPMI 1640 media containing 10% heat-inactivated FCS, 50 mM Hepes, 100 U/ml penicillin-G, and 200 μg/ml streptomycin [12,13].

Recombinant native PA [12] and its mutants were assayed for their functional activity, as described earlier [5]. Proteins (1 μg/ml) were used along with 1 μg/ml of LF for cytotoxicity assay. The recombinant PA from pMW1 [12] was used as control. All assays were done in triplicates. After 3 h of incubation, the cell viability was determined by using MTT dye and the resulting precipitate was solubilized in 0.5% (w/v) SDS, 25% mM HCl in 90% isopropyl alcohol. Absorbance was read at 540 nm and percent activity was determined.

Results and discussion

Analysis of the residues of modeled PA structure for their accessible surface area (ASA), coupled with multiple sequence alignment (Table 1), revealed that except residues Gln277, Ser330, and Thr674, other fully buried polar and charged residues were either identical or conserved in homologous proteins. Conserved residues are supposed to be crucial for structural stability, as shown earlier in the case of GroEL and SH3 domains [25,26]. Hence, Gln277, Ser330, and Thr674, which are not conserved at all in multiple sequence alignment, were mutated to alanine.

Among the hydrophobic residues of the hydrophobic patch in domain 3 of PA, Phe554 has been found not to be crucial for activity compared to the other residues [30]. Since Phe554 could be the site of aggregation, leading to the destabilization of protein, it was mutated to alanine to decrease its hydrophobic character.

Mutant Gln277Ala appears to be stabilized over the native PA molecule (see Table 3 and Fig. 1A), as it retains ~45% activity on incubation at 37 °C for 48 h, while during the same time the native PA loses activity completely. The change to Ala is conservative one as in homologous protein this position is occupied by alanine (see Table 1). Mutant Ser330Ala, however, behaved more like the native PA (data not shown). Mutant Thr674Ala was inactive in the cytotoxicity assays (upto 10 μg/ml) and therefore was not used for further studies. In homologous proteins, this position is occupied by isoleucine. Probably, the residue at this position is crucial for the proper folding of the molecule.

Mutation Phe554Ala is the most stabilizing one (Table 3 and Fig 1B). This mutant protein retains ~90%

Table 3
Activity of PA mutants retained after 48 h of incubation at 37 °C in comparison with native PA

S. No.	Mutant	Residual activity after 48 h of incubation at 37 °C
1.	Gln277Ala	~45%
2.	Phe554Ala	~90%
3.	Native PA	0%

Native PA and mutant PA proteins (10 μg/ml in 20 mM Hepes, 25 mM NaCl, pH 7.4 at 25 °C) were incubated at 37 °C for 48 h. For calculating the loss of activity, the protein kept at 4 °C was taken as a reference.

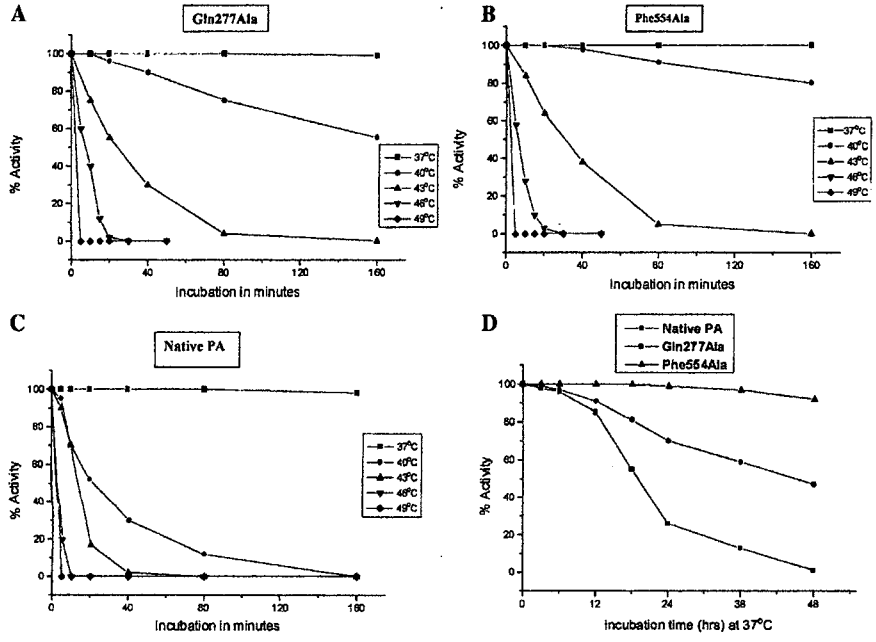

Fig. 1. (A) Inactivation profile of mutant Gln277Ala at different temperatures. (B) Inactivation profile of mutant Phe554Ala at different temperatures. (C) Inactivation profile of native PA at different temperatures. (D) Inactivation of native PA and mutants at 37 °C. Concentration of protein was 10 μg/ml (20 mM Hepes, 25 mM NaCl, pH 7.4 at 25 °C) during incubation. Percent activity was calculated as percent retention of ability of PA and mutant proteins (along with 1 μg/ml of LF) to kill RAW264.7 cells.

activity, even after 48 h of incubation. In proteins homologous to PA phenylalanine is present at this position, yet this residue is not crucial for activity, as observed earlier [30]. This mutation could be stabilizing the PA molecule by decreasing the exposed hydrophobic surface, and hence, leading to a decrease in the total free energy of the native state, resulting in the stabilization of the PA molecule. An alternative explanation could be that by decreasing the exposed hydrophobic surface area, the propensity of aggregation of the protein is lowered at higher temperatures.

This study shows that decreasing the hydrophilic character in the protein interior and increasing that in the protein exterior could be a good preposition for thermostabilization when coupled with multiple sequence alignment. This study resulted in producing two thermostable PA mutants Gln277Ala and Phe554Ala, which could prove to be better candidates for recombinant anthrax vaccine.

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