Introduction
1. INTRODUCTION

1.1. Ribonuclease A

The hydrolysis of RNA \textit{in vivo} is catalyzed by RNA depolymerases, which are most often described as "ribonucleases". The high level of ribonucleolytic activity in the pancrease of ruminants has led to the discovery (Jones, 1920) and detailed characterization of bovine pancreatic ribonuclease A (RNase A) (EC 3.1.27.5). RNase A has been used extensively as a model protein in view of its small size, well understood three dimensional structure and readily reversible denaturation behaviour.

1.1.1. Structure

The crystallization of RNase A dates back to more than 60 years (Kunitz, 1939, Kunitz 1940) and these crystals were shown to diffract to a resolution of 2 Å (Hankuchen 1941). RNase A is the first enzyme and third protein to be sequenced (Hirs \textit{et al}, 1960, Smyth \textit{et al}, 1963). It is also the first protein shown to contain an isoaspartyl residue, derived from the deamidation of an asparagine residue (Asn67) (Capasso, 1996).

RNase A is a small protein, consisting of 124 amino acid residues and with a molecular mass of 13,690 daltons (Pace \textit{et al}, 1995). It contains all the natural amino acids except tryptophan. The number of tyrosines in the enzyme is six and the protein is cross-linked by four disulphides (26-84, 40-95, 58-110, 65-72), there are no free cysteines in the enzyme. RNase A has four proline residues located at positions 42, 93, 114, and 117. The peptide bonds preceding two of the four proline residues are in the cis conformation and are in type VI reverse turns at opposite ends of the native enzyme (Chou & Fasman 1977). The secondary structure consists of long four-stranded antiparallel β-sheets and three short α-helices. The overall shape of the enzyme resembles that of a kidney, with the active site residues lying in the cleft (fig 1).

1.1.2. Catalysis

RNase A acts on the phosphodiester bond located between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent pyrimidine nucleotide.
Fig. 1. **Ribbon diagram of the three-dimensional structure of ribonuclease A.** The model was taken from the Brookhaven protein data bank and drawn with RASMOL.
forming a 2', 3'-cyclic phosphate, which is then hydrolyzed to the corresponding 3'-nucleoside phosphate (fig. 2). The action of the enzyme on ribonucleic acid or pyrimidine nucleotide 3'-esters therefore involves two successive reactions; the formation of cyclic phosphates and subsequent hydrolysis of the cyclic esters to give the 3'-nucleotides. Poly (C) is cleaved by pancreatic RNase A approximately 20-fold faster than is poly (U) (delCardayre & Raines, 1994). Early work on the study of kinetics of catalysis by RNase A used RNA (Kunitz, 1946) or nucleoside 2', 3'-cyclic phosphodiesters (Crook, 1960) as substrates. Homopolymorphic substrates such as poly (U) and poly (C) are now readily available. Uridylyl (3'→5') adenosines, which have well-defined extinction coefficients (Warshaw & Tinoco, 1966) have become the most often used oligonucleotide substrates. A new fluorogenic substrate provides the basis for an extremely sensitive assay for RNase A. 5'[-O-[4-[(2,4-Dinitrophenyl)amino]butyl]phosphoryl]uridylyl(3'→5')2'-deoxyadenosine3'-[N-[(2-aminobenzoyl)-amino] prop-3-yl] phosphate enables the detection of a 50 fM concentration of RNase A (Zelenko et al., 1994).

The most potent proteinic RNase A inhibitor among these is the mammalian ribonuclease inhibitor (RI) (Blackburn & Moore, 1982), while 5'-diphosphoadenosine 3'-phosphate and 5'-diphosphoadenosine 2'-phosphate are the most potent small molecular weight inhibitors (Russo et al., 1997).

1.1.3. Folding and stability

RNase A has remarkable stability against inactivation compared to enzymes from mesophilic sources. It maintains its molecular integrity even under drastic conditions i.e at 95-100°C at pH 3.0 and in 0.25 N sulphuric acid at 5°C (Kunitz & McDonald, 1953). Its amino acid sequence fully encodes its three dimensional structure (Anfinsen et al., 1961), hence it has been used extensively as a model protein for elucidating the principles of protein folding.

The four disulphide bonds in RNase A are critical to the stability of the native enzyme. The enzyme unfolds upon reduction of its disulphide bonds, even in the absence of denaturants, which indicates that folding is coupled to disulphide bond formation. Extensive studies on the thermodynamic stability, structure, and folding/unfolding kinetics of wild-type RNase A suggest that RNase A folds through multiple pathways (Rothwarf &
Fig. 2. Putative mechanism for the transphosphorylation reaction (a) and for the hydrolysis reaction (b) catalyzed by RNase A. In both mechanisms, B is His12 and A is His119. (Raines, 1998).
Scheraga, 1993; Li et al., 1995). Replacing any cystine with another pair of amino acids reduces the thermal stability of the enzyme (Shimotakahara et al., 1997). Specifically, two three-disulphide intermediates, des-[40-95] and des-[65-72] RNase A lacking the 40-95 and 65-72 disulphide bonds, respectively, were observed during the oxidative regeneration or reductive unfolding of RNase A (Li et al., 1995). The two disulphide bonds (Cys26-Cys84 and Cys58-Cys110) contribute more to the thermal stability than do the other two (Cys40-Cys95 and Cys65-Cys72).

RNase A exhibits a slow kinetic phase in its refolding (Tsong & Baldwin, 1972) which can be represented by the following equation:

\[
\text{slow} \quad \text{fast} \\
U_s \xrightarrow{} U_f \xrightarrow{} N
\]

where N is the native enzyme, \( U_f \) are fast refolding species and \( U_s \) are slow refolding species. In RNase A, Pro42 and Pro117 are trans and Pro93 and Pro114 are cis. The isomerization of one or both of the cis peptide bonds may be responsible for the slow kinetic phase observed during the refolding of RNase A. Pro93 peptide bond is trans in the slowest refolding species (Dodge & Scheraga, 1996) and hence trans-to-cis isomerization of that bond is the slowest step in the refolding of the fully denatured enzyme.

RNase A loses almost complete activity in 2-3 M guanidine, without undergoing major conformational changes (Liu & Tsou, 1987). At concentrations above 4.5 M GdnHCl, the \( \alpha \)-helical secondary structure breakdown occurs before the entire tertiary structure of the protein dissolves (Juneja & Udgaonkar, 2002). The rate-limiting step for the unfolding of RNase A is the dissolution of the entire native tertiary structure and penetration of water into the hydrophobic core. In the presence of 8 M urea, RNase A is completely inactive (Resnick et al., 1959). The thermal stability of RNase A decreases with increasing concentration of urea and the size of hydrophobic group substituted on the urea molecule (Poklar et al., 1999). In trifluoroethanol (TFE) concentration greater than 30 %, there is a cooperative collapse of the tertiary structure of RNase A coinciding with the loss of its activity (Köditz et al., 2002). The breakdown of the tertiary structure in TFE is accompanied by the induction of secondary structure.
The solution stability of RNase A in phosphate buffer (pH 4.0, 6.4, and 10.0) at 45°C decreases with increasing pH due to the formation of soluble aggregates (Townsend & Deluca 1990). In a later study, it was shown by concurrent static light scattering that RNase A aggregation was observed at 65°C and above when much of the protein was denatured (Tsai et al., 1998a). In contrast, heat denaturation did not lead to RNase A aggregation in a very acidic medium due to the effect of charge-charge repulsion between the highly protonated RNase A molecules. At 75°C, SDS and dextran sulphate were successful in preventing RNase A aggregation, whereas their cationic, nonionic, zwitterionic analogs were not (Tsai et al., 1998b). When concentrated in mildly acidic solutions, RNase A forms long-lived oligomers (Liu et al., 2002). The major dimeric component forms by a swapping of the C-terminal β-strands between the monomers, and the minor dimeric component forms by swapping the N-terminal α-helices of the monomers. RNase A can form both a linear and a cyclic domain-swapped oligomer.

1.1.4. Thermal Unfolding

The reversible thermal denaturation of RNase A proceeds in a manner consistent with a stepwise unfolding mechanism rather than as a transition between the two states. The unfolding process has been studied by static temperature methods (Chen & Lord, 1976, Lustig & Fink, 1992, Fink & Painter, 1987), fluorescence (Houry et al., 1994), X-ray scattering and fourier transform IR (Sosnick & Trewhella, 1992, Seshadri et al., 1994), NMR (Udgaonkar & Baldwin, 1988, Mayo & Baldwin, 1993, Akasaka et al., 1991, Udgaonkar & Baldwin, 1990) and CD (Scholtz & Baldwin, 1993) Spectroscopy. During thermal denaturation, the native protein first relaxes towards an unfolded intermediate state which then slowly equilibrates with more fully denatured structures. The fast unfolding step(s) involves the dismantling of the protein secondary structure, whereas the slower ones have been attributed to specific isomerization involving the proline residues (Juminaga et al., 1997).

The unfolding of RNase A at pH 5.0 in 0.1 M NaCl over the temperature range 32–70°C as studied by Raman Spectroscopy shows that the unfolding begins at 55°C, the melting temperature is at or near 62°C and substantial helical and pleated-sheet conformations remain even at 70°C (Chen & Lord, 1976). Thermal denaturation as
followed by ultraviolet absorbance at pH 8.0 at 287 nm reveals that the unfolding becomes significant above 47.5°C and the transition temperature was 62.5°C (Arnold et al., 1996). The transition temperature (T_α) increases from pH 0.5 to pH 6.0, but appears to be independent of pH below pH 0.5 and above pH 6.0 (Hermans & Scheraga, 1961). The thermally denatured form of RNase A has been shown to be not completely unfolded with retention of nonrandom structure (Seshadri et al., 1994; Privalov et al., 1989; Tamura & Gekko, 1995). RNase A denatured by heat can therefore be further unfolded by treatment with guanidine chloride (Robertson & Baldwin, 1991). During the thermal unfolding of RNase A at neutral or acid pH, evidence of the appearance of intermediates were found with the help of calorimetry (Tsong et al., 1970), spectroscopy (Chen & Lord, 1976), NMR and CD measurements (Adler & Scheraga, 1988).

The irreversible thermoinactivation of RNase A at 90°C and pH 4.0 is caused by hydrolysis of peptide bonds at aspartic acid residues (the main process) and deamidation of asparagine and/or glutamine residues (Zale & Klibanov, 1986). However, at 90°C and neutral pH (pH 6.0 and 8.0), the enzyme inactivation is caused by a combination of disulphide interchange (the main process), β-elimination of cystine residues, and deamidation of asparagine and/or glutamine residues.

1.1.5. Proteolytic susceptibility

Subtilisin cleaves a single bond in native RNase A (Richards, 1955; Ruple et al., 1962) resulting in the formation of RNase S comprising of two tightly associated fragments. These fragments are designated as S-peptide derived from residues 1–20 of RNase A and S-protein from residues 21–124. Although neither fragment alone has any ribonuclease activity, RNase S has enzymatic activity similar to that of intact RNase A. The proteolytic susceptibility of RNase A towards the proteases subtilisin, elastase and proteinase K was comparable (Arnold et al., 1996) and the enzyme was fragmented even at 20°C. Therefore, these proteases were not suitable for investigating thermal unfolding by proteolysis. However, when Ala20 was substituted for Pro by site directed mutagenesis, the resulting mutant enzyme was nearly identical to the wildtype enzyme in the near-UV and far-UV CD spectral properties, in its activity towards 2', 3'-cCMP and in its thermodynamic stability, but the proteolytic resistance to proteinase K and subtilisin
carlsberg was remarkably increased (Markert et al., 2001). Subtilisin treated RNase A has been useful in conformational studies. Complete reduction, followed by reoxidation of S-protein resulted in significant regain of enzyme activity when the material was assayed in the presence of the S-peptide. This finding suggested that “information” determining the formation of secondary and tertiary structure of RNase A is present in the amino acid sequence of S-protein (Holmes & Yarwood, 1989). Although limited covalent changes may not markedly disrupt the apparent conformation of RNase A in aqueous solution at room temperature, they may be responsible for a marked decline in the conformational stability of the derivatives, readily apparent on denaturation by heat and urea. Studies on the combined effects of heat and urea on the relative conformational stability of RNase A, RNase S and S-protein, showed that RNase A was the most resistant to unfolding, while S-protein was the most labile (Hubbard et al., 1994). The difference in thermal stability between RNase S and RNase A were attributed to entropic effects, i.e. a greater conformational flexibility of both backbone and side chains in RNase S (Lang et al., 1986).

Ficin, an endopeptidase of plant origin, cannot cleave native RNase A but can act on the thermally denatured enzyme (Winchester et al., 1970). There is no loss of ribonucleic activity when RNase A is incubated with ficin at 25°C and pH 4.2. However, on performing the digestion at 60°C and pH 4.2, conditions under which RNase A is known to undergo a reversible thermal transition, virtually all enzyme activity was lost within 10 min. Digestion by ficin results in the formation of transient intermediates possessing enzyme activity. The existence of such intermediates confirms that some of the initial points of proteolysis are not associated with the active site (Winchester et al., 1970).

Chymotrypsin also does not hydrolyze native RNase A at an appreciable rate (Spackman, et al., 1960) but rapidly cleaves the thermally unfolded enzyme (Rupley et al., 1962). Amino acid analyses of the intermediates found in chymotrypsin digests of thermally unfolded RNase A shows that the sites of chymotryptic attack are Tyr25-Cys26, Leu35-Thr36, Phe46-Val47, Tyr76-Ser77, Met79-Ser80, Tyr97-Lys98 and an unidentified bond whose N-terminal member is Glu or Gln (Rupley & Scheraga, 1963). Therefore, three regions of the molecule were identified that unfold and these are: around residues 73 to 80, around half-cystine 26, and around tyrosine 97. The C-terminal region of RNase A
including tyrosine 115 seem to be unaffected by the thermal unfolding. In order to identify the region of RNase A that unfolds first during thermal unfolding, Arnold et al. (1996) tried to use chymotrypsin but all attempts to isolate large fragments reflecting the first cleavage site were unsuccessful. Therefore, chymotrypsin proved to be unsuitable for the localization of the region becoming first accessible to proteolysis during thermal unfolding.

Trypsin cleaves the peptide bonds in which carbonyl group is contributed by Arg or Lys (Holmes & Yarwood, 1989). There are 14 potential trypsin cleavage sites in RNase A molecule (Lys1, Lys7, Arg10, Lys31, Arg33, Lys37, Arg39, Lys41, Lys61, Lys66, Arg85, Lys91, Lys98, Lys104). However, it is essential that the local unfolding of at least 12 residues around the cleavage site of a protein should have occurred before getting cleaved by trypsin (Burns & Schachman, 1982). Native RNase A is also resistant against cleavage by trypsin at room temperature (Arnold et al., 1996; Ooi et al., 1963; Lang et al., 1986). Cleavage of RNase A by trypsin at 60°C occurs at Lys31-Ser32 and at Arg33-Asp34. Therefore, one of the region of RNase A which unfolds in the thermal transition includes the region of the chain between Lys31 and Asp34, which is not an essential part of the active centre since activity remains in the cleaved molecule (Ooi et al., 1963). As has been already mentioned that in chymotryptic attack one of the cleavage site was Tyr25-Cys26 and in tryptic attack there are two cleavage sites in the region from Lys31 to Asp34. This suggests that the region between residues 25-35 unfold during the thermal transition. The N-terminal tail from Lys1 to Tyr25 has two positions which are cleaved by trypsin in RNase A (Hirs, 1956), viz., Lys7-Phe8 and Arg10-Glu11. But these bonds are not cleaved in the early stages of digestion of RNase A at 60°C (Ooi et al., 1963), therefore, the N-terminal region seems not to unfold during the thermal transition. RNase A unfolding has also been studied by denaturation with guanidinium chloride (Yang & Tsou, 1995). After 5 hrs of digestion in 1 M guanidinium chloride, trypsin cleaves at Arg33-Arg34 as observed in thermal denaturation experiments, and additionally at Arg10-Glu11.

Lang et al. (1986) applied trypsin pulses after varying durations of refolding of the polypeptide chain of RNase A. The sites of cleavage which become inaccessible in the course of refolding are located in the 31–39 chain segment of the RNase A chain. Protection of this region against attack by trypsin is achieved on the major slow refolding pathway in parallel with the formation of a native-like folded, active intermediate, when refolding is carried out under conditions which strongly stabilize the folded state. The 31–39 region of the RNase A chain is accessible at early stages of refolding. It is
structured and hence does not become inaccessible until the formation of the overall folded native or native-like structure. This suggests that the 31–39 region of the RNase A chain is not important for early steps which direct the pathway of refolding. The major part of the 31–39 region in the native RNase A forms a solvent-exposed loop structure with a high degree of flexibility, as indicated by increased temperature factors of this segment of the chain (Wlodawer et al., 1982; Wlodawer & Sjölin, 1983). This region also shows a strong variability in the amino acid sequence in homologous RNases (Blackburn & Moore, 1982) and the trypsin-cleavage sites located there are among the first proteolytic sites which become accessible upon entering the thermal transition of RNase A (Arnold et al., 1996; Ooi et al., 1963; Burns & Schachman, 1982; Ooi & Scheraga, 1964). In similarly folded S-protein (des-(1-20)-RNase), Lys31-Ser32 and Arg33-Asn34 are probably the first bonds to be split by trypsin (Allende & Richards, 1962). Taken together this evidence suggests that the 31–39 region of RNase A is not of central importance for the stability of the folded state.

Thermolysin appears to be the most appropriate protease for studying the thermal unfolding of RNase A as it degrades only the unfolded enzyme molecules (Arnold et al., 1996). Furthermore, thermolysin is adequately thermoresistent enough to withstand temperatures at which RNase A is denatured. Thermolysin attacks native RNase A at 50°C or higher temperatures (Arnold et al., 1996). The first two cleavage sites were found to be Asn34-Leu35 and Thr45-Phe46.

A number of recent studies have focussed on the limited proteolysis of proteins in the presence of trifluoroethanol (TFE) which is known to favour the formation of α-helix and facilitates the adoption of a partially folded state. Thermolysin has usually been used due to its high stability in TFE. When RNase A is incubated in 50% TFE, thermolysin primarily cuts at Asn34-Leu35 although the native protein is completely resistant to proteolysis (Polverino De Laureto et al., 1997). Since the first cleavage sites are Lys31-Ser32 and Arg33-Asn34 for trypsin as stated earlier and for thermolysin these are Asn34-Leu35 and Thr45-Phe46, the region from Lys31 to Leu35, together with the adjacent β-structure containing Thr45-Phe46 is suggested to represent a labile region of the RNase A molecule (Arnold et al., 1996). Proteolysis is a useful method for the determination of unfolding constants. Thermolysin which possesses at least 32 potential cleavage sites in RNase A molecule is unspecific and stable enough to be suitable for the determination of unfolding constant of RNase A. The unfolding constant determined by Imoto et al. (1986)
for RNase A at 50°C and pH 8.0 is \((10.1 \pm 0.4) \times 10^{-4} \text{ S}^{-1}\) by thermolysin. This is comparable with the value \((9.19 \pm 0.54) \times 10^{-4} \text{ S}^{-1}\) determined by Arnold et al. (1996).

The analysis of the proteolytic studies suggests that the different regions of RNase A have varying levels of stability (Hubbard, 1998). The mobile loop around residue 20 is readily cleaved by several proteinases, even at 20°C. At slightly higher temperature this region is extended out towards residue 25 susceptible to chymotrypsin cleavage. At still higher temperature, the end of the helix from Asn24-Asn34 may partly unfold to allow cleavage by trypsin at this region, and also destabilize the following β-strand to permit hydrolysis by thermolysin at residue 45. The region from Lys31 to Leu35, along with the adjacent β-structure containing Thr45-Phe46 is suggested to represent a thermolabile region of RNase A which is not of central importance to the stability of the molecule.

1.1.6. Roles of constituent amino acid residues

Bovine pancreatic RNase A has 124 constituent amino acid residues. The structural and functional roles of various amino acid residue of RNase A have been analyzed by several strategies, which include chemical modification in combination with chemical synthesis using the S-peptide/S-protein system (Chatani & Hayashi, 2001). However, these methodologies have mainly addressed the polar amino acid residues. More recently, an efficient expression system for RNase A using \(E. coli\) has been developed (delCardayre et al., 1995), that facilitated the generation of mutant enzymes with altered polar/nonpolar amino acid residues on a large scale.

The amino acids (His12, Lys41, His119, Gln11, Phe120 and Asp121) have been shown to participate directly in RNase A action (Raines, 1998). His12, His119 and Lys41 have been shown to constitute the active site of RNase A. Chemical modification of His12, His119 (Crestfield et al., 1963) or Lys41 (Heinrikson, 1966) results in the loss of most of RNase A activity. Recombinant DNA techniques further revealed that His12 and His119 act as an acid and a base and that Lys41 stabilizes the transition state (Richards & Wyckoff, 1971; Thompson & Raines, 1994). His119 also participates directly in transition state stabilization via hydrogen bonding (Panov et al., 1996). Crystallographic investigation of the complex of RNase A with substrate analogues (Richards & Wyckoff, 1971; Fontecilla-Camps et al., 1994; McPherson et al., 1986) and a series of site-directed mutagenesis studies, revealed six substrate binding subsites, \(P_1, P_0, P_1, P_2, B_1, B_2, \) and \(B_3\). The amino acid residues associated with the sites include Arg85 at \(P_1\) (Fisher et al., 1998a), Lys66 at \(P_0\), Gln11, ⋯ and Asp121 at \(P_1\), Lys7 and Arg10 at \(P_2\), Thr45.
Asp83 and Phe120 at B1, Asn71 and Glu111 at B2 with their respective ionic clusters (Raines, 1998).

The indirect role of Lys7, Arg10, and Lys66 in the catalytic reaction of RNase A has been elucidated by the mutation of these residues, which not only significantly affect the \( K_m \) but also the \( K_{cat} \) of the enzyme (Boix et al., 1994; Fisher et al., 1998b). The replacement of Asp121 with Asn in a semisynthetic RNase A reduces the catalytic efficiency (Stern & Doscher, 1984), while the mutagenic replacement of Phe120 by other amino acid residues alters both catalytic efficiency and thermal stability (Tanimura et al., 1998; Chatani et al., 2001). Replacement of Tyr97 with Phe, Ala or Gly also leads to a decrease in activity since Tyr97 is involved in maintaining the correct position of Lys41 (Eberhardt et al., 1996). The replacement of the cysteine residues of the disulphide Cys26-Cys84 or Cys58-Cys110 with alanine decreases the thermal stability by 40°C without affecting \( K_{cat}/K_m \) for Poly (C), but the removal of Cys40-Cys95 or Cys65-Cys72 drastically decreases the \( K_{cat}/K_m \) rather than conformational stability (Klink et al., 2000).

Some synthetic [Orn10]-S-peptide analogues, containing Phe, Tyr, Ile, Ala, cpGly, and Gly at position 8 showed that Phe8 plays an important role in \( \alpha \)-helix stability, and that the capability of these peptides to bind to the S-protein is in the order of Phe>Tyr>cpGly>Ile>(Gly, Ala) at position 8. This result reveals the significance of hydrophobic interactions of S-peptide with the S-protein (Filippi et al., 1976). A study of P42A, P93A, P114A, and P117A showed that the isomerization of the Tyr92-Pro93, Asn113-Pro114, and Val116-Pro117 peptide bonds effects the folding process (Dodge & Scheraga, 1996). The replacement Pro93 with glycine has an effect not only on folding rate, but also on the thermal stability (Schultz et al., 1998). From a study of Y25F and Y97F mutant RNase A, it has been shown that Tyr25 and Tyr97 contribute to accelerating conformational refolding and the conformational stability as well as the cis-trans isomerization of the slow-refolding phases (Juminaga et al., 1997).

The importance of the C-terminal region in constituting the active site has been suggested by the fact that des [121-124] RNase A, a mutant enzyme lacking the 121-124 C-terminal residues, possesses only about 0.5 % of the activity of the native enzyme towards cytidine-2,3-cyclic monophosphate (Lin, 1970). The replacement of the C-terminal Val124 residue with other amino acids, such as Gly, Ala, Lys or Trp decreases the recovery of activity in refolding from S-S reduced form, implying that the C-terminal amino acid residue significantly affects the formation of the correct disulphide bonds during the refolding process, and that the hydrophobic interaction of Val124 is important.
for efficient packing of RNase A molecule (Fujii et al., 2000). An NMR study of the (C65S, C72S) mutant RNase A, lacking the Cys65-Cys72 disulphide bond, has indicated that some interactions exist between the 65-72 disulphide loop and the C-terminus. Moreover, the hydrogen-bonding network within the hydrophobic core is destroyed, suggesting that this disulphide bond also contributes significantly to stabilizing the hydrophobic core of the native protein (Shimotakahara et al., 1997).

Immunological reactivity analysis suggests that some ordered structures exist for the S-S reduced form of RNase A (Chavez & Scheraga, 1977; Chavez & Scheraga, 1979; Chavez & Scheraga, 1980). These structures are referred to "chain folding initiation sites (CFIS)". The peptide fragments of 1-13 (Rico et al., 1986), 21-42 (Jimenez et al., 1988) and 50-61 (Jimenez et al., 1987) can fold to native-like α-helices, while the 80-124 (Chavez & Scheraga, 1977) and 105 (or 109)-124 (Beals et al., 1991) fragments also have partially ordered structures, suggesting that the CFIS can form the ordered structures with relatively short-range interactions. The residues from 106 to 118 have been shown to stabilize the conformation of RNase A (Torrent, 2001). The refolding of Y92W by double-jump refolding experiments revealed a hydrophobically-collapsed intermediate, which supports the hypothesis that the region around position 92 is also a CFIS in the folding pathway (Sendak et al., 1996).

The mutagenic replacement of Phe46 with Glu or Lys changes a part of the β-sheet to random coils. Moreover, the replacement of Phe46 with Val folds to the native structure with full activity, but the replacement with Ala loses the ability to fold to the native structure. These results and a comparison between temperature and pressure denaturation (Chatani et al., 2002) suggests that the hydrophobicity of the side chain of Phe46 is crucial to the formation of the correct secondary and tertiary structures in protein folding.

Onconase is a highly cytotoxic homolog of RNase A (Mikulski et al., 1995). RNase A can also be made toxic to cancer cells by replacing Gly88 with an arginine residue (Leland et al., 1998). K7A/G88R RNase A is nearly 10-fold more cytotoxic than G88R RNase A and nearly equal to Onconase (Haigis et al., 2002).

1.1.7. Immobilization of RNase A

RNase A immobilized on a variety of solid supports has been used primarily to elucidate mechanisms of enzyme stabilization by immobilization. RNase A immobilized on cyanogen bromide activated Sepharose CL-4B exhibited a decrease in specific activity relative to the soluble enzyme upon increasing the points of attachment between the
enzyme and the matrix (Koch-Schmidt & Mosbach, 1977). As the number of attachments increased from 1 to 8, the specific activity decreased from 60 to 15%. However, there was a proportionate increase in the thermal stability of the immobilized RNase A coupled by multiple linkages. The preparation exhibited a broader endotherm and a higher transition temperature (by about 5°C).

RNase A has been coupled to silica beads with the help of glutaldehyde and mechanism of enzyme stabilization studied (Rialdi & Battistel, 1994). A partial decoupling of the protein domains as well as a thermal stabilization of the protein molecule resulted upon immobilization. One of the two domains of the protein was thermally stabilized with respect to the other. The effect is apparently due to favourable protein-support interactions rather than to the single-point covalent attachment.

During the refolding and oxidation of reductively denatured RNase A in solution, there is a marked lag in appearance of enzymatic activity as compared to the oxidation of sulphydryl groups, whether such oxidation is spontaneous or is catalyzed by sulphydryl oxidase (Janolino et al., 1985). However, if RNase A is covalently attached to a derivatized glass surface, a lag period is not observed during reformation of native structure from the completely reduced, denatured state. Therefore, in solution, intermolecular interactions alter the pathway of polypeptide chain folding and disulphide bond formation, leading to nonnative disulphides which do not rapidly interchange to form native pairings. The segregation of refolding polypeptide chains by covalent immobilization apparently prevents such interactions.

RNase A immobilized covalently has been shown to exhibit severe loss of activity (Messing, 1970; Axén et al., 1971; Martinez et al., 1990). Since the amino acids most amenable to covalent modification (cysteine, lysine, and histidine, and three [Lys7, Lys41, and Lys66] of its ten lysines and two [His12 and His119] of its four histidines) are also critical for ribonucleolytic activity (Raines, 1998). The immobilization of a T45G mutant of RNase A via the cysteine residue installed at position 19 facilitated the retention of comparatively high ribonucleolytic activity (10%) and rapid and thorough removal from solution (Sweeney et al., 2000).

1.1.8. Immunogenicity

RNase A is a good antigen despite its low molecular weight (Smolens & Sevag, 1942). Fully methylated RNase A retains nearly 30% of its immunological activity suggesting
that the modified protein contains antibody recognizable residual native structure, which presumably accommodates some antigenic determinants (Acharya et al., 1977).

The two hexapeptides NH₂-Lys-Asp-Cys-Lys-Pro-COOH and NH₂-Asp-Cys-Arg-Glu-Thr-Gly-COOH corresponding to the strong hydrophilic regions 37-42 and 83-88, respectively, on RNase A were shown to be antigenic, and hence supported the view that highly hydrophilic regions on the protein have a good correlation with their potentially antigenicity (Sagar et al., 1989). However, antisera raised against the peptide 37-42-RSA conjugate did not cross-react with native RNase A but readily cross-reacted with the denatured enzyme. This suggested that the site 37-42 is not exposed completely to the surface of the molecule and hence unavailable for cross-reaction. X-ray crystallographic studies on RNase A have also shown that the region 37-42 is exposed to the surface only partially (Kartha et al., 1967).

1.2. Phospholipase D

Phospholipases constitute a diverse series of enzymes that can be classified into phospholipase D, C, A₂, A₁ and B according to their sites of hydrolysis on phospholipids (fig. 3) (Wang, 2001). Phospholipids provide the backbone for biomembranes, serve as rich sources of signaling messengers and occupy important junctions in lipid metabolism. The activities of phospholipases not only affect the structure and stability of cellular membranes, but they also regulate many cellular functions. Phospholipase D has been chosen for the study in view of its actual and potential applications in the synthesis of natural phospholipids, such as phosphatidylglycerol, phosphatidylserine and novel artificial phospholipids (Juneja et al., 1989; Takami et al., 1994).

1.2.1. Functions

Phospholipase D (PLD) (EC 3.1.4.4), is distributed widely both in the plant and animal kingdom, and catalyzes the hydrolysis of the terminal phosphodiester bond of glycerophospholipids. Because of its additional capability of transferring the phoshatidic acid moiety of the substrate to various primary acceptor alcohols, the enzyme is predestined for preparation of phospholipids with modified head groups (Eibl & Kovatchev, 1981; Shuto et al., 1987). PLD hydrolyzes phosphatidylcholine to produce phosphatidic acid and choline. Phosphatidic acid can be further hydrolyzed to lysophosphatidic acid by phospholipase A₂ or to diacylglycerol by phosphatidic acid phosphohydrolase. Lysophosphatidic acid is a known mitogen that interacts with G-protein-coupled receptors in many cell types (Moolenaar, 1995), and diacylglycerol activates protein kinase C (Berridge, 1993). In addition, the stimulation of PLD by small
Fig. 3. Hydrolysis of phosphatidylcholine by PLD, PLC, PLA₂, PLA₁, PLB, and lysoPLA and the respective reaction products. The arrow lines for PLD, PLC, and PLA₂ indicate the site of hydrolysis, but those for PLB, lysoPLA, and PLA₁ do not. PLA₁ hydrolyzes the sn-1 acylester bond, whereas lysoPLA removes the last fatty acid from lysophospholipids that can be produced by PLA₂ and PLA₁, as marked by the curved arrows. PLB sequentially removes two fatty acids from phospholipids, and its final reaction products are the same as those of lysoPLA. Cho, Choline; P-Cho, Phosphocholine; FA, fatty acid. (Wang, 2001).
G proteins such as ARF and Rho suggests that at least some forms of PLD may be involved in intermembrane protein trafficking and cytoskeletal rearrangement (Exton, 1997; Singer et al., 1997).

PLD catalyzed hydrolysis of phospholipids has been observed during seed germination, aging, and senescence and under a broad spectrum of stress conditions, including freezing, drought, wounding, pathogen infection, nutrient deficiency, and air pollution (Chapman, 1998; Frank et al., 2000; Wang, 2000). Specifically, PLDs play pivotal roles in plant response to stresses, and one way in which they achieve this is by mediating the action and production of stress-related hormones, abscisic acid (ABA) (Fan et al., 1997; Jacob et al., 1999), jasmonic acid (Wang et al., 2000), and ethylene (Lee et al., 1998).

In yeast, PLD is required for the late phases of meiosis and sporulation (Rose et al., 1995); its function in these processes may result from a role in membrane trafficking (Xie et al., 1998). In mammalian cells, PLD function is important for various processes, including vesicular trafficking, secretion, mitogenesis, oxidative burst, and cytoskeletal rearrangement (Liscovitch et al., 2000). Activation of PLD produces messengers that in turn activate various enzymes such as protein kinases, lipid kinases, phosphatases, and phospholipases. A recent study also suggests that PLD activation may promote degradation of the translation factor eEFIA (Ransom-Hodgkins et al., 2000).

1.2.2. Subfamilies

Based on the requirements for Ca$^{2+}$ and lipids of in vitro assays, PLDs can be grouped into three classes: (a) the conventional PLD that is most active at millimolar levels of Ca$^{2+}$ (20 to 100 mM), (b) the polyphosphoinositide (PI)-dependent PLD that is most active at micromolar levels of Ca$^{2+}$, and (c) the phosphatidylinositol (PtdIn)-specific PLD that is Ca$^{2+}$-independent (Wang, 2000). The conventional PLD is the most prevalent and best studied class in plants and has been purified to apparent homogeneity from several plant sources (Wang, 2000). The PI-dependent PLD was characterized recently in Arabidopsis (Pappan et al., 1997) while the PtdIn-specific PLD was identified in suspension cells of Catharanthus roseus (Wissing, 1996).

According to similarities of deduced amino acid sequence, gene architecture, and biochemical properties, PLDs in Arabidopsis are divided in five groups, PLD α, β, γ, δ and ε. Most of the PLDs cloned from other plant species belong to the PLDα group, and multiple PLDαs have been cloned from cabbage (Kim et al., 1999; Pannenberg et al., 1998; Schäffner et al., 2002), Craterostigma plantagineum (Frank et al., 2000), and rice
Two isoenzymes of PLD from cabbage, PLD1 and PLD2 have recently been identified, on the basis of their cDNAs (Schäffner et al., 2002). Both have been assigned to the α-type of plant PLDs.

1.2.3. Catalysis and substrate specificity

The hallmark of the PLD superfamily is the consensus motif, H(X)K(X)D, embedded within a more loosely conserved region that is usually present in two separate copies in PLD, PSS, and cardiolipin synthases but found as a single copy in the nucleases (Koonin, 1996; Ponting & Kerr, 1996). The PLDs cloned from eukaryotes all contain two H(X)K(X)D motifs, which constitute two active-site regions necessary for PLD activity (Xie et al., 2000). Recently, crystal structures have been determined for a 16-kd endonuclease member of the PLD superfamily (Stuckey & Dixon, 1999) and a 54-kd bacterial PLD (Leiros et al., 2000) (fig. 4). Such structural information provides valuable insights into the mode of action of PLD catalysis. The enzymes of the PLD superfamily use a conserved histidine for nucleophilic attack on the substrate phosphorus. PLD hydrolyzes phospholipids at the P-O rather than the C-O bond via a two-step ping-pong reaction mechanism involving a phosphatidylated enzyme intermediate (Stuckey & Dixon, 1999).

Most plant PLDs have broad substrate specificity (Abousalham et al., 1997; Pappan et al., 1998), but different groups of PLDs exhibit varied abilities to hydrolyze different phospholipids (Pappan et al., 1998). PLDα, β, and γ all utilize phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol as substrates, but the substrate presentation and Ca\(^{2+}\) levels required for PLDβ and γ are strikingly different from those of PLDα (Pappan et al., 1998). In addition, PLDβ and γ use phosphatidylserine and N-acyl phosphatidylethanolamine as substrates. Although PLDβ and γ hydrolyze the same substrates, PLDγ, but not PLDβ, prefers ethanolamine-containing lipids-phosphatidylethanolamine and N-acyl phosphatidylethanolamine to other lipids. None of these cloned PLDs uses phosphatidylinositol 4,5-bisphosphate, or cardiolipin as a substrate. In contrast, the Ca\(^{2+}\)-independent PLD from *C. roseus* hydrolyzes phosphatidylinositol, but not phosphatidylcholine, phosphatidylethanolamine or phosphatidylglycerol (Wissing et al., 1996). The PLD also was reported to lack the transphosphatidylation activity characteristic for all other PLDs (Pappan et al., 1998; Wang, 2000). Varied substrates specificities and preferences suggest that activation of different PLDs may result in selective hydrolysis of membrane phospholipid:
Fig. 4. Stereographic illustration of PLD from *Streptomyces* sp. strain PMF coloured from dark blue to red according to the amino acid sequence. Two flexible loops (dashed and labeled L1 and L2, accordingly) are seen at the right side, which is the part of the enzyme that docks onto the membrane when hydrolysis takes place. Two phosphate ions (red) are found on the surface of the protein, one located at the interface between two independent crystallographically related molecules, and the other in the active site of the protein, which is located in the center of the figure. (Leiros et al., 2000).
1.2.4. Regulation and Activation

A number of factors influence the activities of PLD. Notable among these are Ca$^{2+}$ (Wang, 2000; Zheng et al., 2000), pls (Pappan et al., 1997; Qin et al., 1997), substrate lipid composition (Pappan et al., 1998), pH changes (Pappan & Wang, 1999), and mastoparan, a tetradecapeptide G-protein activator (Van Himbergen et al., 1999). PLDs bind Ca$^{2+}$ and phosphatidylinositol 4,5-bisphosphate (Pappan et al., 1997; Zheng et al., 2000). Sequence analysis indicates that plant PLDs contain a Ca$^{2+}$/phospholipid-binding fold, called the C2 domain, at the N-terminus (Pappan et al., 1997). The C2 domains of PLDα and β have been demonstrated to bind Ca$^{2+}$ and this binding causes conformational changes of the proteins (Zheng et al., 2000). PLDβ C2 have a higher affinity for Ca$^{2+}$. The Ca$^{2+}$ requirement of PLDα is influenced strongly by pH and substrate lipid composition (Pappan & Wang, 1999). PLDα is active at near-physiological, micromolar Ca$^{2+}$ concentrations at pH of 4.5-5.0 in the presence of mixed lipid vesicles. In contrast, PLDβ and α are most active around neutral pH, and their Ca$^{2+}$ requirements are independent of pH.

Intracellular translocation between cytosol and membranes have been proposed as one important mechanism of PLD activation (Wang, 2000). The relative distribution of PLD between the soluble and membrane fraction changes during development and in response to stress (Ryu & Wang, 1996; Wang et al., 2000). Study of the Ca$^{2+}$ and C2 domain interaction showed that Ca$^{2+}$ binding increases the affinity of the C2 domains for membrane phospholipids (Zheng et al., 2000). This indicates that C2 domains in PLDs is responsible for mediating a Ca$^{2+}$-dependent intracellular translocation between cytosol and membranes. The increased association with membranes of preexisting PLD in the cell may represent a rapid and early step in PLD activation in stress responses (Wang et al., 2000). In addition, gene expression also plays a role in regulating the cellular levels of some PLD isoforms (Wang et al., 2000).

1.2.5. Immobilization

The first extensive study of transphosphatidylation reactions catalyzed by immobilized PLD from white cabbage was published by Tobback et al. (1988) who investigated many different carrier materials for their suitability for the immobilization of the enzyme. These included celite, alumina oxide, active carbon (for adsorption), octyl-Sepharose, porous glass (for covalent coupling), DEAE-Sephadex (ionic binding), Ca$^{2+}$-alginate.
polyvinylalcohol (entrapment). The quality of biocatalysts obtained was tested by their ability to convert dioleoyl-PC into dioleoyl-PE and best results were obtained with PLD bound to porous glass or to DEAE-Sephadex.

Reuter (1997) immobilized PLD from white cabbage on SiO₂ (Cab-osil M5). For this purpose, the SiO₂ carrier was first derivatized with 3-aminopropyltriethoxysilane or with 3-glycidoxypropyltrimethoxysilane. The highest activities were retained when PLD was attached to SiO₂ functionalized with 3-aminopropyltriethoxysilane. Although the type of binding had to be an ionic or adsorptive, significant release of PLD was not observed. Lambrecht & Ulbrich-Hofmann (1992) used the property of PLD to bind preferentially by adsorption to carriers with hydrophobic surface, and enhancement in binding in presence of Ca²⁺ ions for the purification of PLD from white cabbage leaves. Other findings of this investigation were that the storage stability increased markedly, and a considerable broadening of activity profile resulted between pH 5 and 7 (Lambrecht & Ulbrich-Hofmann, 1993).

1.2.6. Characteristics of PLD2

Two isoenzymes (PLD1 and PLD2) have been identified on the basis of their cDNAs (Pannenberg et al., 1998) from cabbage (Brassica oleracea var. capitata), the traditional source for plant PLD (Heller, 1978). The cloning and expression of PLD1 and PLD2 has recently been successful (Schäffner et al., 2002). The sequence similarity between PLD1 and PLD2 within the coding regions was determined to be 87.5 % at the nucleotide level and 95.8 % at the amino acid level (Pannenberg et al., 1998). The identities of the protein sequence of PLD2 to other cloned PLD sequences are shown in table 1.

The genomic structure of PLD2 consists of 3614 bp coding for 812 amino acid residues and has a molecular weight of 92.1 kd (Schäffner et al., 2002). The 3614 bp genomic DNA contains three intron regions (bp33-bp456, bp560-bp647, bp2554-bp3154). The length of mRNA is 2689 bp and the cDNA is 2439 bp. PLD2 is assigned to the α-type of plant PLDs. It possesses 8 cysteines, a high proportion of hydrophobic branched chain amino acids (21.4 %), and a high proportion of negatively charged amino acids (15 %), which is in accordance with its low calculated pl value of 5.37. PLD2 has neither N-terminal, nor other sequences to target it to nucleus, endoplasmic reticulum, mitochondria, peroxisomes, chloroplasts or vacuoles and hence, it is assumed to be a cytosolic enzyme (Schäffner et al., 2002).
Table 1. Comparison of the amino acid sequence of the recombinant PLD2 from cabbage with other plant, animal and microbial PLDs.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sequence identity with cabbage PLD2 (%)</th>
<th>Acc. Nr. (NCBI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arabidopsis thaliana</em> (PLDα)</td>
<td>92</td>
<td>AB017071</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> (PLDβ)</td>
<td>39</td>
<td>U90439</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> (PLDγ1)</td>
<td>41</td>
<td>AL161532</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> (PLDγ2)</td>
<td>44</td>
<td>AL161532</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em> (PLDα)</td>
<td>80</td>
<td>Z84822</td>
</tr>
<tr>
<td><em>Oryza sativa</em> (PLDα)</td>
<td>77</td>
<td>AB001920</td>
</tr>
<tr>
<td><em>Oryza sativa</em> (PLDβ or γ)</td>
<td>68</td>
<td>AB001919</td>
</tr>
<tr>
<td><em>Pimpinella brachycarpa</em> (PLDα)</td>
<td>74</td>
<td>U96438</td>
</tr>
<tr>
<td><em>Ricinus communis</em> (PLDα)</td>
<td>80</td>
<td>L33686</td>
</tr>
<tr>
<td><em>Vigna unguiculata</em> (PLDα)</td>
<td>81</td>
<td>U92656</td>
</tr>
<tr>
<td><em>Zea mays</em> (PLDα)</td>
<td>77</td>
<td>D73410</td>
</tr>
<tr>
<td><em>Homo sapiens</em> (GPI-PLD)</td>
<td>18</td>
<td>L11701</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em> (PLD2)</td>
<td>20</td>
<td>P70498</td>
</tr>
<tr>
<td><em>Arcanobacterium haemolyticum</em></td>
<td>12</td>
<td>L16583</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>15</td>
<td>AB010810</td>
</tr>
<tr>
<td><em>Corynebacterium pseudotuberculosis</em></td>
<td>12</td>
<td>L16586</td>
</tr>
<tr>
<td><em>Corynebacterium ulcerans</em></td>
<td>13</td>
<td>L16586</td>
</tr>
<tr>
<td><em>Streptomyces acidmyceticus</em></td>
<td>18</td>
<td>E03429</td>
</tr>
<tr>
<td><em>Streptomyces antibioticus</em></td>
<td>17</td>
<td>BAA03913</td>
</tr>
<tr>
<td><em>Streptomyces species</em></td>
<td>17</td>
<td>E05514</td>
</tr>
<tr>
<td><em>Streptoverticillium cinnamoneum</em></td>
<td>16</td>
<td>AB007132</td>
</tr>
</tbody>
</table>
In accordance with all members of the PLD superfamily, PLD2 is also characterized by a duplicated HKD motif (333-348 and 663-680) (fig. 5) (Schaffner et al., 2002). Site-directed mutagenesis studies on human PLD proved His and Lys of this motif to be essential for hydrolytic and transphosphatidylation activities (Sung et al., 1997). PLD2 also contains a C2 domain (8-153) which binds Ca\(^{2+}\) and the binding promotes the associating of the protein with membranes. In addition, a phosphatidylinositol 4,5-bisphosphate binding site characterized by the motif R/KxxxxR\(\times\)RK is located in the C2 domain (Schaffner et al., 2002). PLD2 shows pH optimum of 5.5 to 5.6 that was not affected by up to 100 mM Ca\(^{2+}\). Ca\(^{2+}\) are however mandatory for activity and stimulation of catalytic activity approached a maximum at 45 mM Ca\(^{2+}\).

Watanabe et al. (1999) have characterized the PLD expressed in cabbage leaves to be PLD2. In presence of 50 mM Ca\(^{2+}\), PLD from cabbage is bound from crude enzyme solution to octyl-Sepharose and subsequently selectively eluted by removing the calcium ions (Lambrecht & Ulbrich-Hofmann, 1992). PLD from cabbage is active in reverse micelles formed from its substrate phosphatidylcholine and triton X-100 in diethyl ether (Subramani et al., 1996). The transformation of phospholipids is preferably performed in aqueous-organic emulsion systems (Hirche et al., 1997). 1,3-diacylglycerol-2-phosphocholines with acyl chain lengths of C\(8\)–C\(18\) were found to be inhibitors of PLD from cabbage (Haftendorn et al., 2000). PLD from cabbage was also shown to catalyze the transesterification of alkylphosphate esters, which are potent antitumor agents (Aurich et al., 1997).

1.3. Immunoaffinity Immobilization of Enzymes

The most serious limitation in the long term and large scale applications of enzymes is their lability to various forms of inactivation. A number of strategies have been employed to improve the stability of enzymes (Wiseman, 1994). These include immobilization on appropriate support (Klibanov, 1979), chemical modification (Tyagi & Gupta, 1998), protein engineering (Arnold, 1993), use of organic solvents (Gladilin & Levashov, 1998) or additives (Carpenter et al., 1992).

Use of additives has the advantage that they have the potential to enhance the stability of proteins/enzymes without the need for chemical or genetic manipulation (Monsan & combes, 1998). The additives used include various salts, polyols and sugars-inert polymers such as
polyethylene glycol, and amino acids. Solute or cosolvent stabilization arises from preferential hydration of the protein (Fágin, 1995). The additive is usually excluded from the neighbourhood of the protein molecule which is surrounded by water only. Since an unfolded protein will be less compact than in the native form, this results in further cosolvent exclusion which will be thermodynamically unfavourable. Formation of the more compact native protein is therefore favoured. Glucose oxidase was thermally stabilized by using additives like NaCl, K\(_2\)SO\(_4\) and even lysozyme (Gouda et al., 2003). The stabilization of the enzyme by NaCl and lysozyme was ascribed to charge neutralization. K\(_2\)SO\(_4\) enhanced the thermal stability of the enzyme by primarily strengthening the hydrophobic interactions and made the holoenzyme a more compact dimeric structure. Addition of 0.2 % N,N-dimethylformamide to lipase from Candida rugosa immobilized on Celite allowed a 93 % higher ester yield compared with that obtained when no extra addition was made (Triantafyllou et al., 1993). Poly (ethyleneimine) (PEI) increases the shelf life of a number of proteins at temperatures above ambient. Porcine muscle lactate dehydrogenase (LDH) retained its activity completely for one month in the presence of PEI at pH 7.2 while in its absence, more than 50 % of the activity was lost already after two weeks (Andersson, 1999). Sorbitol, on the other hand improved the thermodynamic stability of LDH but not the storage stability.

One of the most effective among these is immobilization on solid surfaces (Gemeiner et al., 1994). Inspite of the remarkable achievements in improving the stability of several enzymes by immobilization, the mechanisms involved are poorly understood, necessitating individual evaluation of the procedure for each enzyme by trial and error. Among the various strategies available for favourable orientation of enzymes (Saleemuddin, 1999), the use of monoclonal antienzyme antibodies is particularly promising (Solomon et al., 1987; Ruoff et al., 1989).
C2 domain

MAQHLLLHGLTHATIYEVDALHTGGLRSAGFLGKIISNVEETIGFKGETQLYATIDLQKA 60

RVGRTK1TDEPKNKPYESFHIYCAHMASDI1FTVDDNPIGATLGRAYPVDEVING 120

EEVEKWVEILDNPIHGESKIHVKLQYFAVEADRNWNMGVKSAPFPGVYPESQSERQG 180

CKVSLYQGHVDPDNFPKVPIPLAGGKNYPEHRCWEIDIFDAITNAKHLIYTGWSYTEITL 240

VRDSRRPKPGDMTLGELLKKATEGVRVLLLVWDDRTSVDVLKKGMLATHDDETDNYF 300

NGSEVHCLCPRNPDDGGSSIVQLQVSAMFTHHQKIVVDSESVPQGGGSEMRRMFSVG 360

GIDLCDQRYDTPFHSFLRTLDVHDDDFHQPNNFTGATKGGPQEPWQDIHSSRLEGPIAW 420

DVLNFEQRWSKQGGKDILVKLRELSDIIITPSVPMFQEDHVDVWNVFRLRSIDGGAAAGF 480

PDSPEVAAEAGLVSGKDNVIDRSI0QDAYIHAIRRAKDFIYIENQYFGLGSSFAAAGITP 540

EDINALHIPLKELSLKIVDKIEKGEKFRVYVVPMWPEGIPESASVQAILDWQRRRLEMM 600

YKDVQALRAQGLEDPNYLTFFCLGNREVKKEGEYPAERPDPDTDMRQAQERRFMI 660

Second HKD motif

YVHSKMMIVDDEY1IVGSANINQSMRDGARDSEIAMGGYQPHLISHRQPARGQVHGFRMS 720

LWYEHGLMDETFLEDPSLECIKVNRIADKYWDFYSSSESLEHDLPGHHLLRYPISVDNEG 780

NITELPGFEFPDSDKARILGNDVYLYLPPILLT 812

Fig. 5. Amino acid sequence of PLD2 from cabbage (Brassica oleracea var. capitata) showing the C2 domain, first HKD motif and the second HKD motif. (SWISS-PROT: Accession no. 082549, www.expasy.ch).
Both polyclonal and monoclonal antibodies have been employed in the immobilization of enzymes and their relative merits and limitations examined (table 2).

Heterogeneity of the polyclonal antibody population is however more of a rule than exception. Formation of active site recognizing and hence inhibitory antibodies is quite likely if an animal is immunized with a native enzyme (Solomon et al., 1984, Arnon, 1973, Cinader, 1967), although reports describing the non-inhibitory nature of the antisera raised against several enzymes are available (Shami et al., 1991, Jafri et al., 1993, Feinstein et al., 1971, Ben-Yosef et al., 1975). Some plausible explanations offered for the absence of inhibitory antibodies in the antisera include the active site acting as blind spot for the immune system, steric hindrance by high affinity antibodies recognizing adjacent locations of active site directed antibodies and continued accessibility of the active site in the complex formed between active site recognizing antibodies and the enzyme (Shami et al., 1989). Some ingenious techniques for the prevention of the formation of active site directed polyclonal antibodies have been described in the recent years. Fusek et al. (1988) immunized pigs with active site blocked chymotrypsin prepared by treating the enzyme with diisopropylphosphofluoridate. Stovickova et al. (1991) raised non-inhibitory antitrypsin antisera with trypsin complexed with its specific inhibitor antitrypsin. Glycosyl recognizing polyclonal anti-invertase antibodies were found to be non-inhibitory towards the enzyme and more effective in the immobilization of the enzyme than those recognizing the polypeptide domains (Jafri et al., 1995, Jafri & Saleemuddin, 1997). Methods also exist for the fractionation of polyclonal antibodies recognizing different epitopes of a single antigen or those of differing in affinity for a single epitope (Suzuki et al., 1996, Sada et al., 1988).

Expression of nearly full activity by the enzyme complexed directly with antibody (Shami, 1989, Shami, 1991) or when bound to support matrix-coupled antibody have been observed (Stovickova et al., 1991). This is due to the fact that, the antibody molecule acts as a large spacer holding the enzyme at a distance from the support matrix thereby minimizing steric hindrance and facilitating remarkable freedom to act even on high molecular weight substrates (Solomon et al., 1986). Enzymes immobilized on antibody supports usually exhibit high stability. The stability enhancement may arise out of crosslinking like effect caused by antibody binding on enzyme. Shami et al. (1989) argued that reduction in the free energy of the antigen resulting from the binding of even a moderate affinity antibody may be sufficient to confer stability as free energy changes between the folded and the unfolded states of protein lie in the same range (Tantold
Table 2. Enzyme immobilized favourably with the help of antibodies (Saleemuddin, 1999).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Antibody</th>
<th>Support matrix</th>
<th>Stabilization against</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase</td>
<td>PC</td>
<td>-</td>
<td>Temperature</td>
<td>Melchers &amp; Messers, 1970</td>
</tr>
<tr>
<td>Gulonolactone oxidase</td>
<td>PC</td>
<td>-</td>
<td>In vivo Proteolysis</td>
<td>Sato &amp; Waltton, 1983</td>
</tr>
<tr>
<td>Transglutaminase</td>
<td>PC/MC</td>
<td>Agarose beads</td>
<td>Temperature</td>
<td>Ikura et al., 1984</td>
</tr>
<tr>
<td>Carboxy peptidase A</td>
<td>MC</td>
<td>Eupergit C</td>
<td>Temperature, pH, Storage in cold</td>
<td>Solomon et al., 1984;</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>MC</td>
<td>Eupergit C</td>
<td>Storage</td>
<td>Solomon et al., 1987</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>MC</td>
<td>Sepharose</td>
<td>Recycling</td>
<td>deAlwis et al., 1987;</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>PC</td>
<td>Sepharose</td>
<td>-</td>
<td>Fusak et al., 1988</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>PC</td>
<td>-</td>
<td>Sodium hypochloride, Temperature</td>
<td>Shami et al., 1989</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>MC</td>
<td>Sepharose</td>
<td>Temperature</td>
<td>Ruoff et al., 1989</td>
</tr>
<tr>
<td>Alpha amylase</td>
<td>PC</td>
<td>-</td>
<td>Temperature, lyophilization, Freezing &amp; Thawing</td>
<td>Shami et al., 1989; Shami et al., 1991</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>PC</td>
<td>-</td>
<td>Temperature, Ethanol</td>
<td>Shami et al., 1989; Shami et al., 1991</td>
</tr>
<tr>
<td>Horse radish peroxidase</td>
<td>MC</td>
<td>-</td>
<td>-</td>
<td>Solomon et al., 1990</td>
</tr>
<tr>
<td>Trypsin</td>
<td>PC</td>
<td>Sepharose</td>
<td>-</td>
<td>Stovickova et al., 1991</td>
</tr>
<tr>
<td>Urease</td>
<td>PC</td>
<td>Nylon</td>
<td>pH</td>
<td>Agnellini et al., 1992</td>
</tr>
<tr>
<td>NAD glycohydrolase</td>
<td>PC</td>
<td>Nylon</td>
<td>Storage</td>
<td>Agnellini et al., 1992</td>
</tr>
<tr>
<td>Invertase</td>
<td>PC</td>
<td>Sepharose</td>
<td>Temperature</td>
<td>Jafri et al., 1995; Jafri &amp; Saleemuddin, 1997</td>
</tr>
<tr>
<td>L-Hydantoinase</td>
<td>PC</td>
<td>Sepharose</td>
<td>Incubation</td>
<td>Stemann et al., 1994</td>
</tr>
</tbody>
</table>

PC - Polyclonal; MC - Monoclonal.
1970) Furthermore, although due to large size of the antibody, a single matrix bound antibody may not bind more than one molecule of enzyme, lateral interactions with more than one antibody molecule may contribute significantly to the stability of the enzyme protein (Sadana & Madgula, 1993) It is interesting to note that some monoclonal antibodies exhibit chaperon like activity and assist in antigen refolding (Solomon & Schwartz, 1995, Carlson & Yarmush, 1992) and inhibit enzyme aggregation (Katzav-Gozansky et al, 1996) In instances where soluble antibody is used for the enzyme immobilization, a single enzyme molecule may interact with more than one antibody molecules resulting in a high degree of stabilization (Shami, 1991) like an enzyme attached via multiple covalent (Guisan et al, 1991) or non-covalent linkages (Iqbal & Saleemuddin, 1983) While a correlation may exist between the thermal stability of proteins and its susceptibility to proteolysis (Daniel et al, 1982), the exact mechanism by which antibodies enhance stability against other forms of inactivation is still not clear.

1.4. The concept of the unfolding region

The covalent or the adsorptive binding of enzymes to polymers is often, but not always, connected with an increase of stability. Ulbrich-Hofmann et al (1986, 1993) studied the thermal inactivation of monomeric enzymes e.g. α-amylase, trypsin, or chymotrypsin before and after immobilization on various polystyrene, silica, or polyacrylamide matrices. In all cases, the soluble enzymes were irreversibly inactivated according to first order kinetics, whereas the immobilized enzymes, if stabilized, showed a distinct biphasic inactivation progress. The inactivation kinetics of the biphasic inactivation curves could be excellently fitted by the sum of two exponential terms, where one rate constant was in the range of the inactivation constant of the soluble enzyme whereas the second one was markedly smaller. The biphasic inactivation kinetics is caused by two enzyme species arising as a consequence of immobilization, which differ in stability but not in catalytic properties.

These results led to a model of the unfolding region (fig 6) It is based on the concept that unfolding of an enzyme molecule under denaturing stress starts at a define region of the molecule, denoted as the unfolding region or the labile region. After immobilization, the unfolding region of the molecule may be either free or fixed by covalent or noncovalent bonds. All molecules, in which the unfolding region is free, are unfolded in the same way and with the same rate constant as the soluble enzyme. These molecules...
Fig. 6. **Models of the unfolding region.** Unfolding of the soluble protein molecule is initiated at its most labile site. Immobilization results in stabilization if the unfolding region is fixed, whereas no stabilization is observed if the unfolding region is free (Ulbrich-Hofmann et al., 1999).
represent the labile fraction. In the other enzyme molecule, forming the stable fraction, the unfolding region is strengthened. Hence according to this model, the unfolding of several enzymes may begin at specific labile regions in the molecules and modifications/protection of such regions may confer marked stability to them (Schellenberger & Ulbrich, 1989; Ulbrich-Hofmann et al., 1993).

The existence of such labile regions has been demonstrated in a number of enzymes including ribonuclease A, ribonuclease B, thermolysin like protease from Bacillus stearothermophilus, and hematopoietic cell kinase (Arnold et al., 1996; Arnold et al., 1998; Eijsink et al., 1995; Engen et al., 1997). It has been shown that a marked improvement in the stability of the thermolysin like protease from Bacillus stearothermophilus can be achieved by immobilizing the enzyme via the cysteine introduced by site directed mutagenesis in the labile region of the enzyme (Mansfeld et al., 1999).

As mentioned earlier, the primary cleavage sites of RNase A were determined to be Lys31-Ser32 and Arg33-Leu34 for trypsin and Asn34-Leu35 and Thr45-Phe46 for thermolysin (Arnold et al., 1996). The structural region from Lys31 to Leu35, together with the adjacent β-structure containing Thr45-Phe46, therefore presumably represents a labile region. Interestingly, naturally occurring RNase B, which has the identical amino acid sequence and tertiary structure but is more stable than RNase A, differs by an N-linked oligosaccharide chain in this unfolding region, at Asn34 (Arnold & Ulbrich-Hofmann, 1997). In RNase B, the structural region in the vicinity of Lys31 and Leu35 unfolds first (Arnold et al., 1998). Therefore, the unfolding pathway of RNase A is not changed by the carbohydrate attachment and the higher stability of RNase B results from the first N-acetylglucosamine residue (Arnold et al., 1999).

1.5. The present study

Binding to soluble or matrix associated antibodies has been shown to improve the resistance of a number of enzymes to various forms of inactivation. Both monoclonal and polyclonal antibodies (table 2) as well as those raised against the glycosyl residues of glycoenzymes have been shown to be quite effective in this regard (Jafri & Saleemuddin, 1997). More recently F(ab)2 (Jan et al., 2001) and Fab (Gupta et al, 2003) fragments have also been used to successfully improve the stability of enzymes. Unlike polyclonals, monoclonal antibodies immobilized on appropriate support offer the additional advantage
of uniformly orienting enzymes on solid supports (Solomon et al., 1987, Ruoff et al., 1989).

The proposal of Ulbrich-Hofmann that enzymes contain “labile” region where the process of unfolding begins (Ulbrich-Hofmann et al., 1993) has been substantiated in a number of subsequent studies (Arnold et al., 1996, Mansfeld et al., 1999). It was therefore envisaged that antibodies recognizing the labile region may remarkably stabilize the enzyme against thermal and other forms of inactivation. In addition, such antibodies are expected to offer the advantage of favourably orienting the enzyme molecules.

The labile region of RNase A has also been elucidated to comprise of the structural region from Lys31 to Leu35, together with the adjacent β-structure containing Thr45-Phe46 (Ulbrich-Hofmann et al., 1999). Synthetic dodecapeptide corresponding to the labile region 32-43 was therefore synthesized chemically to be used as an antigen. The peptide corresponding to the N-terminal 12 residues was also synthesized. The peptides as well as native RNase A were used for the generation of antibodies in rabbits. The IgG isolated for the immune sera were investigated for their stabilizing potential on RNase A and the mutant L35S-RNase A.

With the aim of locating labile region(s) in a recombinant phospholipase D2 (PLD2) from cabbage, limited proteolysis of the enzyme was performed. Thermolysin cleavage sites on PLD2 were investigated for the purpose of identifying some loop (weak) regions. PLD2 mutants of the cleavage sites were produced to protect the enzyme against proteolysis by thermolysin. PLD2 was immobilized onto polyclonal antiPLD2 antibodies precoupled to Sepharose to investigate the effect of the immobilization on the stability of the enzyme.

Among the various strategies of protein stabilization currently available, all except site-directed mutagenesis are empirical necessitating specific standardization usually by trial and error for each protein. Site-directed mutagenesis on the other hand requires knowledge of the complete structure of the protein and necessitates highly sophisticated technology. In view of the possibility of enzymes having specific labile regions whose modification results in improvement in their stability, it was envisaged that antibodies directed against such regions may be used to advantage for improving the stability as well as orienting the enzyme favourably on support matrices.