Discussion
4.5 Discussion

Alpha macroglobulins are ubiquitous proteins that occur in diverse animal species, including the vertebrate and invertebrates (Starkey, 1982; Quigley and Armstrong, 1983a;b). The physiological roles of α-macroglobulins are indeed challenging and engrossing. The unusual mechanism of proteinase inhibition by α2M has attracted remarkable attention and led to its purification and characterization from a variety of sources (Table 1.1). Extensive work is currently being carried out on various aspects of α2M and several isolation procedures are available. Earlier study from this laboratory has shown that caprine α2M is unique in several aspects and some of these characteristics were attributed to the special cleaved subunits (Khan et al., 1999).

The present study involves purification and characterization of α2M from buffalo (Bos indicus) blood plasma. The purification of this proteinase inhibitor was achieved using ammonium sulphate fractionation and gel filtration chromatography. A high recovery of 61% with 35-fold enrichment in specific activity is the advantage of this two step scheme of α2M purification (Table 4.1). Homogenity of the α2M preparation was confirmed by the presence of single band in pore-size electrophoresis (Fig. 4.2). Purification to homogeneity of human plasma α2M normally requires additional ion-exchange step (Mirza and Saleemuddin, 1993). The ease of purification of α2M from buffalo plasma is apparently related to the absence of large molecular weight contaminating proteins. The native inhibitor behave like a homotetramer and on treatment with SDS and β-mercaptoethanol migrated as a single band on 7.5% SDS-PAGE (Fig. 4.4). Like its human and bovine homologue (Dunn and Spiro, 1967; Nagasawa et al. 1970) buffalo α2M is also glycosylated with 7.8% carbohydrate.

Various studies using SDS-gel electrophoresis, gel filtration chromatography and sedimentation equilibrium have estimated the molecular weight of the tetrameric α2M in the range 700-800 KDa (Schultze et al., 1955; Schonenberger et al., 1958; Roberts, 1986;
Nagasawa et al., 1970; Hudson and Koo, 1982). The molecular weight of the buffalo \( \alpha_2M \) as determined using a Sephacyl S-300 HR column was 660 KDa (Fig. 4.6). The value is lower than that of human \( \alpha_2M \) (Halls and Roberts, 1978) but comparable with the caprine \( \alpha_2M \) (Khan et al., 1999). Moreover, the Stokes radius of \( \alpha_2M \) also confirms the buffalo \( \alpha_2M \) to be more compact than that from human plasma. The value was 85°A (Fig.4.7) as compared to the reported value of 88° A of the human \( \alpha_2M \) (Roberts, 1986).

Purified buffalo \( \alpha_2M \) when subjected to gel electrophoresis in the presence of SDS and \( \beta \)-mercaptoethanol yielded, a single band (Fig. 4.5). The molecular weight of the subunit of \( \alpha_2M \) determined in this study gave a value of 165 KDa whereas, human \( \alpha_2M \) migrates as a single 185 KDa band (Sotrup-Jensen, 1989). Inspite of its similarity in molecular dimensions the buffalo \( \alpha_2M \) differed from its ruminant counterpart (i.e. caprine \( \alpha_2M \)) in the nature of subunit. The caprine inhibitor is a tetramer of 146 KDa subunits which inturn are made up of two disulfide linked 110 and 36 KDa peptides (Khan et al., 1999). The occurrence of cleaved subunits have also been reported in other species (Hudson et al., 1987) and has been ascribed to the post translational processing of the peptide. Bovine \( \alpha_2M \) however has an intact subunit of 185 KDa. The difference in the nature of the subunit in very closely related species may indicate lack of clear evolutionary trait but suggests abrupt specific adaptation.

Trypsinization of buffalo \( \alpha_2M \) lead to increased mobility in pore limit and rate electrophoresis in 5% acrylamide gels (Fig. 4.8). This is similar to the characteristics "slow" to "fast" transformation of human \( \alpha_2M \) on trypsin treatment (Barrett et al., 1979; Nelles et al., 1980). Sepharose-linked trypsin was capable of bringing about the transformation of \( \alpha_2M \) to the "fast" form albeit at a slower rate (Fig. 4.9) indicating that immobilization process does not abolish the bait region cleaving ability of the proteinase. It has been shown that treatment of human \( \alpha_2M \) with solid support attached trypsin
results in the formation of empty traps that resemble the trypsin treated forms in several respects (Mirza and Saleemuddin, 1993). Similarity in the nature of $\alpha_2$M transformation induced by soluble and immobilized chymotrypsin has been demonstrated by image processing of the electron microscopy (Biosset et al., 1991a;b).

$\alpha$Ms from a variety of sources function as "molecular traps" for broad spectrum proteinases (Barrett and Starkey, 1973) rather than as inhibitors (Sottrup-Jensen, 1987; Sottrup-Jensen, 1989; Roberts, 1986). Buffalo $\alpha_2$M appears to be no exception and exhibits inhibitory activity and protection of amidolytic activity to trypsin (pH 8.0), chymotrypsin (pH 8.0), pronase E (pH 8.0), proteinase K (pH 8.0) and bromelain (pH 6.8) as shown in Table 4.3. This is apparently related to the presence of susceptible bonds in the bait region of the molecule.

It is evident from several studies that the reaction of $\alpha_2$M with methylamine is a complex phenomenon involving random attack on the thiolesters (Feinman, 1983; Sottrup-Jensen, 1987; Strickland and Bhattacharya, 1984; Larson et al., 1985). Incubation of buffalo $\alpha_2$M with methylamine, resulted only in a very small increase in electrophoretic mobility (Fig. 4.10). Nearly half the original activity of buffalo $\alpha_2$M is retained by the $\alpha_2$M after treatment with methylamine (Table 4.2). This suggests that the thiol esters of buffalo $\alpha_2$M are more stable to nucleophilic attack by methylamine than those of human $\alpha_2$M (Gonias et al., 1982). Somewhat similar behaviour is shown by bovine and caprine inhibitor (Nagasawa et al., 1970; Khan et al., 1999). It is interesting to note that the methylamine treated human $\alpha_2$M is completely devoid of the ability to inhibit or entrap proteinase (Gonias et al., 1982), while the inhibitory activity of equine $\alpha_2$M remains completely unaffected by methylamine (Motoshima et al., 1988).

The number of methylamine or trypsin sensitive thiol esters present in buffalo $\alpha_2$M was determined by the quantitation of the number of excess free thiols generated as a result of these treatments. Methylamine liberated only 2 moles of thiols per mole of
α₂M while those liberated in response to trypsin treatment were 4 (Table 4.4). Although, buffalo α₂M like its human and bovine counter part has 4 moles of thiol ester per mole but the accessibility of two among these seems to be restricted even to low molecular weight molecule i.e. methylamine. Although the physiological significance of the presence of thiol esters of unequal reactivity is difficult to predict, their occurrence has also been reported in caprine α₂M (Khan et al., 1999). Trypsinization of methylamine pretreated α₂M caused further release of two thiol esters and converted the intermediate mobility species to "fast" from on 5% gel (Fig. 4.10).

In human α₂M the structural constraints released by the thiol ester cleavage are transmitted to the other parts of the molecule, ultimately causing perturbation in the thiol ester domain of the adjacent subunit and the increase in the accessibility of thiol esters to methylamine (Gonias et al., 1982 Dangott et al., 1983). Apparently these exert an opposite effect as in buffalo α₂M and conformational changes occurring as a result of cleavage of two thiol esters restrict the accessibility of the remaining two esters. Alternatively, the recalcitrance of two thiolesters of buffalo α₂M may result from their location in deep hydrophobic cleft in manner that makes them inaccessible even to small molecular weight amine.

The stoichiometry of inhibition of trypsin showed that 1.0 mole of intact buffalo α₂M entrapped a maximum of 1.0 mole of enzyme shown in Fig. 4.11 which is lower than that of human inhibitor. This behaviour may be related to the structural features of buffalo α₂M that promote a higher extent of non-productive activation by cleavage of the bait region before binding of proteinase (Ganrot, 1966) or due to smaller dimensions of the buffalo inhibitor (Fig. 4.7).

The amino acid composition of buffalo α₂M bore remarkable similarities with those of human, goat and mouse α₂Ms (Dunn and Spiro, 1967; Hudson et al., 1987; Khan et al., 1999). The concentrations of Asp, Thr and Pro were lower significantly while those
of Gly and Ala were higher (Table 4.5). The similarities are also reflected in the cross reactivity of the anti-buffalo α₂M with goat and human α₂M (Fig 4.13).

The dimer-dimer interactions in the tetrameric α₂Ms play an important role in the trapping of proteinase and regulation of conformational changes that accompany proteolytic attack (Jensen, 1993). The tetramerization of α₂M may have functional implications on the biological functions of the inhibitor, which may involve the simultaneously evolved contact zone. The significance of the contact zone has been emphasized for human α₂M (Shanbhag et al., 1997). Curiosity to investigate the role of contact zone as a functional site in non-human tetrameric α₂M's led us to carry out the studies on the dissociation of the buffalo inhibitor.

Dissociation of α₂M along non-covalent axis by urea, guanidine hydrochloride, SDS, metal ions of +2 oxidation State (Zn, Cd, Hg, Cu, Ni) and chaotropic anion has been shown to generate half molecules (Sottrup-Jensen et al., 1980; Pochon et al., 1987; Pratt and Pizzo, 1984; Roche et al., 1988; Sjoberg et al., 1991; Shanbhag et al., 1996). The chaotropic anion thiocyanate has been used to dissociate buffalo α₂M and α₂M-MeNH₂ complex through non-covalent axis in this study. The result in Fig. 4.14 clearly shows that thiocyanate dissociates α₂M into half molecules with an electrophoretic mobility comparable to that obtained by incubating the protein with 1% SDS (Harpel et al., 1979). α₂M-MeNH₂ dissociates at far higher concentration of thiocyanate than that required for native α₂M (Fig. 4.15).

It is known that the chaotropic thiocyanate anion modifies the structure of water thereby disrupts the hydrophobic interactions (Hamaguchi and Geiduschek, 1962). Dissociation of the tetramer in presence of NaSCN suggests the involvement of hydrophobic amino acids in the association between the non-covalent bonded subunits in α₂M. The observed difference in the dissociation profile between native and methylamine reactive α₂M is indicative of the increased interaction between the contact zone which
inturn are also controlled by the conformational changes induced in the protein on the thiol ester cleavage or by cleavage of both thiol ester and bait region. Human $\alpha_2$M-MeNH$_2$ also dissociates to a lower extent than the native $\alpha_2$M in presence of NaSCN (Shanbhag et al., 1996).

The Stokes radius of the buffalo $\alpha_2$M half molecule generated by sodium thiocyanate was similar to that obtained by dissociation with 4M urea (Table 4.6). Neither urea nor NaSCN cleave disulphide bonds (Liu et al., 1987; Shanbhag et al., 1996). Thus, the $\alpha_2$M half molecule generated in this study is the likely result of the disruption of only the non-covalent interactions. These observations taken together indicate that no major conformational change may have occurred during dissociation of tetrameric $\alpha_2$M into half molecules under the conditions used and the dimers may remain in an undenatured state. In support of this the observed stokes radius for the fraction of $\alpha_2$M that remained undissociated on treatment with 1.6M NaSCN was comparable with that of native $\alpha_2$M (Table 4.6).

Fig. 4.16 and 4.17 shows the difference in the intrinsic fluorescence of native $\alpha_2$M and that treated with 1.6M NaSCN. The dissociation of tetramer into half molecule causes a decrease in the intrinsic fluorescence with a slight red shift of the emission maximum, after 80 min. incubation with the anionic salt. This decrease in fluorescence suggest that some conformational changes occur as a consequence of dissociation in which the environment of the hydrophobic amino acid is altered. The dissociation of human $\alpha_2$M into half molecules induced by NaSCN results in conformational changes resembling those that occur to thiol ester cleavage in the inhibitor and treatment of the resulting half molecules with methylamine caused no significant additional conformational alteration (Shanbhag et al., 1997). The results shows the presence of aromatic amino acid residues at the contact zone and that these fluorophores are gradually brought into a polar environment. The fluorescence behaviour of the exposed aromatic amino acid of a protein may also be influenced by the composition of the
solvent even in the absence of any significant conformational changes and such effect have been used to probe the solvent accessibility of aromatic residues of a protein (Creighton, 1991.). Addition of sodium thiocyanate is known to cause an increase in the polarity of the solvent (increased refractive index) and hence may cause a decrease in fluorescence intensity and shift in emission maximum (Fig. 4.16 and 4.17). It is also possible that the tyrosyl hydroxyl groups of proteins are involved in hydrogen bonding with the solvent molecules (Creighton, 1991).

The 90% change in the fluorescence (ΔF max) resulting from incubation with NaSCN for native α₂M both at (λ ex) 280 and 295 nm was reached far more rapidly than that of α₂M-MeNH₂ (Fig. 4.18 and 4.19). That attainment of ΔF max necessitates approximately twice the time for α₂M-MeNH₂ complex than for native α₂M. This is a further indication of the enhanced interactions between the non-covalently associated subunits in the methylamine treated α₂M. The methylamine induced enhancement in interaction between the dimers may be far more stronger in case of human α₂M that requires a six-fold increased concentration of thiocyanate to induce a similar rates of changes in fluorescence comparable to the native α₂M (Shanbhag et al., 1997). The difference is evidently related to the fact that only two thiol esters are cleaved on treatment of the buffalo α₂M with methylamine while in case of human α₂M all the four thiol esters are equally reactive to the methylamine treatment. It is also likely that the areas of contact between the disulphide bonded dimers are smaller due to smaller dimensions of the buffalo α₂M. It is however not clear if the two thiol esters cleaved in buffalo α₂M are those in disulphide linked or non-covalently associated subunits.

α₂M is also a major zinc binding protein of human plasma (Parisi and Vallee, 1970; Adham et al., 1977). Equilibrium dialysis of the binding of ⁶⁵Zn by α₂M at pH 7.9 showed heterogenous binding which could be attributed to two classes of binding sites (Pratt and Pizzo, 1984). According to the Delain model (1988) the contact zone between two dimers may contain two strong Zn²⁺ binding sites (Gettins and Cunningham, 1986).
The physiological zinc concentration in buffalo plasma as estimated by the method of Song et al. (1976) varies in the range of 18-20 μM. About 400-625 μg Zn/g of purified protein was quantitated in buffalo while about 320-770μg of zinc has been reported per g of human α2M (Parisi and Vallee, 1970).

The effect of zinc on the amidolytic activity of trypsin shown in Fig. 4.20 indicates that trypsin binding activity does not depend on the presence of zinc in this protein, since removal of nearly all the zinc by EDTA results in no loss of trypsin binding activity. Increase in zinc concentration beyond 30 μM results in marked loss of enzyme binding activity. Removal of unbound zinc from α2M against buffer containing EDTA did not restore its trypsin binding activity (Fig 4.20).

The buffalo α2M exposed to either low or high zinc concentration responded to methylamine and Sepharose linked trypsin treatment as the native α2M (Fig 4.21) by undergoing the characteristic "slow" to "fast" transformation.

Surprisingly, the α2M treated with 200 μM zinc lacked the ability to protect trypsin against inhibition by SBTI (Fig. 4.20). Considering that bait region cleavage and accompanying gross conformational changes occur in the preparation treated with trypsin (Fig 4.20). The behaviour may be related to very rapid closure of the “Trap” after bait region cleavage without entrapment. Alternatively, small alteration in the interactions between the covalently associated dimers may restrict the steric protection to the inhibitor associated trypsin from reaction with SBTI.

The intrinsic fluorescence spectra were clearly different for methylamine and proteinase treated buffalo α2M (Fig. 4.23). The emission maximum for native α2M is 333 nm (at λ ex 280 nm). Treatment of native α2M with Sepharose-linked trypsin caused a drastic decrease in fluorescence intensity with a small red shift. Exposure of aromatic amino acids to a polar environment results in a decrease in fluorescense intensity.
Methylamine causes a 3nm blue shift with slight enhancement in fluorescence suggesting burial of aromatic residues inside the protein. These findings clearly suggest that the conformational alteration accompanying trypsin and methylamine treatments are remarkably different in buffalo α2M. The emission maximum for native α2M at (λ ex) 295 nm was observed at 339 nm (Fig. 4.24) which is characteristic for tryptophanyl fluorescence. Both methylamine and immobilized trypsin treated α2M showed a 3 nm blue shift in the emission maximum (Fig. 4.24). Interestingly, results from Fig. 4.23 and 4.24 indicate that while tryptophans may get buried in the hydrophobic environment whereas tyrosine are exposed to the solvent after trypsin or methylamine treatment. Trypsin treatment induced gross conformational changes in buffalo α2M as has been observed in case of human, bovine and equine α2M using fluorescence measurements (Bjork and Fish, 1982; Bjork et al., 1985; Motoshima et al., 1988). The buffalo inhibitor however appears unique in that it exhibits decrease in fluorescence on trypsinization but other αMs showed enhancement. Use of Sepharose-linked trypsin in the experiments eliminates the interference by the proteinase as this does not result in the entrapment of the former and leads to the formation of "empty closed trap" (Mirza and Saleemuddin, 1993). The principal difference between this study and those of Bjork et al. (1982; 1985) and Motoshima et al. (1988) is the use of soluble trypsin by the latter for inducing the conformational alterations. It is now well known that the entrapment of the challenging soluble proteinase in α2M results the observed alterations in fluorescence that may actually represent a combined contribution of the accompanying conformational alteration and those of the entrapped proteinase. The effect of methylamine on buffalo α2M was however marginal suggesting minimal conformational alterations as observed in case of the bovine and equine α2Ms (Bjork et al., 1985; Motoshima et al., 1988).

Exposure to zinc yielded interesting alteration in buffalo α2M in response to the methylamine and trypsin. Pretreatment of the inhibitor with 30 μM zinc resulted in no major alteration in the fluorescence behaviour (Fig. 4.25), while exposure to 200 μM zinc
caused a 30% decrease in fluorescence intensity (Fig. 4.26). It may be recalled that buffalo α₂M exposed to 200 μM zinc loses the ability to protect trypsin from SBTI inhibition although it undergoes the characteristic proteolysis and methylamine induced alteration (Fig. 4.20).

The observed decrease in fluorescence intensity in reaction of α₂M with Sepharose-linked trypsin may be related to movement of tryptophan and tyrosine in the proximity of ionized amino acids thereby resulting in the quenching of the fluorescence. However, it is also possible that tryptophans shift in a region of a protein where movement of the indole ring is hindered (Friedfelder, 1984).

Significant change in shape and magnitude in circular dichroic spectra of α₂M treated with methylamine and trypsin has been reported for human α₂M (Bjork and Fish, 1982). For buffalo α₂M, minimal alteration was observed with methylamine similar to bovine and equine α₂M (Bjork et al., 1985; Motoshima et al., 1988) whereas Sepharose-linked trypsin caused a sharp decrease in ellipticity with a change in the shape of the spectra (Fig. 4.27).

The major difference between human and buffalo α₂Ms lies in the behaviour after treatment with methylamine. Following the cleavage of thiol ester bond by methylamine, human α₂M undergoes extensive conformational change that alters both the secondary and tertiary structure as well as the shape of the protein (Bjork and Fish, 1982, Gonias et al., 1982; Dangott et al., 1983).

The observed changes in the buffalo α₂M circular dichroic spectra resulting from reaction with methylamine and Sepharose-linked trypsin was analyzed by the method of Chen et al. (1972). Reaction with trypsin decreased α₂M α-helix content from approximately 9 to 4% whereas methylamine caused no change. Gonias et al. (1982) reported a decrease in α-helix of about 9-10% from 14% for human α₂M. For all three
forms of $\alpha_2$M (native, methylamine and trypsin treated) the majority of the remaining may be $\beta$-ordered and unordered structure (Bjork and Fish, 1982). The circular dichoric spectrum of the 30$\mu$M zinc treated buffalo $\alpha_2$M on reaction with both methylamine and Sepharose-linked trypsin showed similar pattern as native $\alpha_2$M (Fig. 4.29).

There was a loss of about 2% secondary structure of 200$\mu$M zinc pretreated $\alpha_2$M. Methylamine treatment of zinc treated form resulted only marginal change in the spectra as compared to trypsin treatment that caused a very significant decrease in mean residue ellipticity as shown in Fig. 4.30.

The near-U.V. circular dichroism spectral changes as displayed in Fig. 4.31 may not be the true representation of major conformational changes in the protein. The spectra indicate alterations in the immediate structural and electronic environment of the aromatic amino acid residue resulting from interaction with methylamine or proteinase with the inhibitor (Creighton, 1991). The mean residue rotation of $\alpha_2$M treated with Sepharose-linked trypsin is decreased indicating the presence of less ordered structure as compared to native and methylamine treated $\alpha_2$M (Fig. 4.31).

In conclusion it has been shown that buffalo $\alpha_2$M inspite of its smaller dimensions exhibits majority of the characteristics features. Investigations of the zinc treated buffalo $\alpha_2$M suggests that in presence of high concentration of the cation the inhibitor retains several characteristics features but irreversibly looses the ability to entrap and protect the protease from large molecular weight inhibitors.