Chapter 5
Crystallization and structure solution of the non-covalent complex of lysozyme

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5.3 Discussion
5.1 Introduction

Peptide bond formation is a biological event of tremendous significance given the fact that all amino acids are linked through these covalent linkages en route the primary structure of proteins. However, it is the myriad non-bonded interactions amongst the constituent atoms that result in the genesis of the final, three-dimensional conformation of protein molecules. The immense importance of tertiary interactions is highlighted in non-covalent complexes wherein, despite the loss of peptide bonds, a native-like conformation is still maintained. Various biophysical and biochemical tools have been employed in isolation as well as in tandem to gain vital information pertaining to the structural basis of the stability of nicked structures consequent upon cleavage of peptide bonds. Such data gains considerable importance given the plethora of studies directed towards understanding the kinetic and thermodynamic nature of interactions that result in a folded three-dimensional, functional molecule. Proteolysis has been used in a number of studies to sever scissile peptide bonds in protein substrates, thereby leading to the generation of non-covalent complexes with a compromised stability. Although biophysical and biochemical studies of fragment complementary systems provide important insights into the nature of the non-covalent structure, finer, atomic-scale details of the changes in structure owing to fragment complementation or proteolysis remain elusive. It is therefore suggested that high-resolution structural data of the non-covalent complex be obtained prior to a detailed understanding of the fragment complementation process (Ojennus et al., 2001).

Of the several bottlenecks encountered towards crystal structure determination, the formation of good quality, diffractable crystals appear to be a major one. In addition to genetic engineering methods, proteases have been widely used to “do away with” the flexible loop-like structures that may hinder the close packing of macromolecules, thereby facilitating the formation of better crystals (Brown et al., 2000). However, studies performed solely towards understanding the consequences of proteolysis on protein substrates using X-ray diffraction have been few. The nature of interactions prevalent in a non-covalent structure obtained as a result of limited proteolysis would be interesting to study, given the role of non-bonded interactions in stabilizing molecules.

Kim et al. (1992), performed structural investigations on the fully active non-covalent complex RNase S, obtained as a result of subtilitic digestion of RNase A, using
X-ray crystallography. Comparative studies performed with the intact RNase A showed that the extensive hydrogen bond interactions at the core of the molecule remain similar in either case, however, the loop structures show significant variability in structure, therefore demonstrating high B-factors. Furthermore, factors determining the lower stability of nicked RNase A were not obvious from the comparative crystal structures (Kim et al., 1992). Crystal structure of plakalbumin, a proteolytically nicked form of ovalbumin has been solved using isomorphous replacement method and comparisons have been done with the nicked form of α-1-proteinase inhibitor (A1PI) (Wright et al., 1990). The structural similarity demonstrated by the nicked and intact forms of the molecules, in addition to the limited extent of structural change has been shown to be consistent with the spatial proximity of the sequence-conserved residues in plakalbumin and A1PI (Wright et al., 1990).

Lysozyme, a ubiquitous protein molecule has been the subject of abundant studies conducted to provide insights into the role of various factors governing determination of the three-dimensional structure of proteins. Although, the crystal structure of various forms of lysozyme, mutants as well as chemically modified ones is known (www.rcsb.org); changes in the molecule as a consequence of proteolysis has not been illustrated using X-ray crystallography. We therefore took up studies to understand the atomic-scale changes in the structure of lysozyme as a consequence of proteolysis. Studies performed with this objective would add to the ever-growing understanding of the interactions responsible for holding together non-covalent complexes, thereby providing valuable insights into the mechanistics of protein folding.

5.2 Results

5.2.1 Generation of proteolyzed lysozyme

Preliminary experiments were conducted to obtain the non-covalent complex of lysozyme as a consequence of non-specific proteolysis using Subtilisin Carlsberg (SC). 100 μM hen egg white lysozyme (HEWL) was subjected to proteolysis using a molar ratio of 100:1 of substrate: protease in 50 mM Tris pH 8.0 at 37 °C in the presence of
DTT. Figure 5.1 depicts the formation of fragments as a consequence of limited proteolysis of lysozyme. Fragment generation can be shown to have occurred as early as 30 min. from the start of the proteolysis reaction with the genesis of two bands of lower molecular weight, corresponding to approximately 7 kDa and 6 kDa as seen on a 17% SDS-PAGE. Additionally, a smear representing a set of unresolved, set of fragments can also be visualized. Similar fragmentation of HEWL, in the presence of subtilisin by Vogel et al. (1996) resulted in the generation of three fragments, with the cleavage sites occurring at Gln41-Ala42 and Leu56-Gln57. Higher concentrations of lysozyme were used for the proteolysis reaction performed prior to setting up crystallizations of the reaction mixture. Furthermore, removal of protease from the reaction mixture was facilitated by addition of the reaction mixture to 100 μl of pre-equilibrated p-aminobenzamidine beads that are known scavengers of serine proteases (Yang et al., 2006). Mixed components were then spun and the supernatant was used to set up crystallization trials.

5.2.2 Crystallization of proteolyzed lysozyme

Proteolyzed lysozyme was set up for crystallization using the hanging drop vapour diffusion method by employing the 96 conditions of the Magic 96 matrix (Hol and Sarfaty, personal communication). Initially, 20 mg/ml of the proteolysis mixture was set up for crystallization at 25 °C (room temperature). Tetragonal crystals, typical of lysozyme were obtained after three days in the presence of 0.2M NaCl, 0.1M Imidazole pH 8.0 and 1.0 M Ammonium Phosphate (Figure 5.2 (A)). Additionally, crystals were also found to form in the presence of 0.2M NaCl, 0.1M Na Phosphate Citrate, pH 4.2 and 20% PEG 8000 (Figure 5.2 (B)). Data collection was performed using X-rays at room temperature. Furthermore, crystals obtained under the same condition were collected and several washes were performed using the mother liquor. Thereafter, the crystals were subjected to analysis employing SDS-PAGE followed by detection with silver staining. Figure 5.2 (C) shows that the crystals used for data collection did not show fragmentation similar to that obtained for the reaction mixture. Thus suggesting that religation of the nicked species had occurred during the process of crystallization of the molecule. Owing to the extensive studies showing protease-catalyzed religation of nicked molecules, the
Figure 5.1: **Proteolytic profile of lysozyme.** Lysozyme subjected to proteolysis with Subtilisin Carlsberg at a molar ratio of 100:1 shows the generation of two fragments as observed on a 17% SDS-PAGE (Lane 1: control lysozyme, lane 2: reaction after 2 min. from initiation, lane 3: 10 min., lane 4: 30 min., lane 5: 45 min., lane 6: 60 min).
Figure 5.2: **Studies of proteolyzed lysozyme crystals.** (A) Crystals of proteolyzed lysozyme obtained in the presence of 0.2M NaCl, 0.1M Imidazole pH 8.0 and 1.0 M Ammonium Phosphate. (B) Crystals of proteolyzed lysozyme obtained in the presence of 0.2M NaCl, 0.1M NaPhosphate Citrate pH 4.2 and 20% PEG 8000. (C) SDS-PAGE profile of crystals depicting religated lysozyme (lane 3) and control lysozyme (lane 2).
above result gains significance. Literature suggests that religation has been shown to occur in the presence of organic co-solvents as well as crowding agents such as dextran and PEG (Somalinga and Roy, 2002), therefore indicating that the nicked lysozyme is subjected to religation in the presence of the crystallization conditions employed.

In order to obtain lysozyme crystals bearing the nicked molecule, an alternative method was devised wherein, 5.0 µl of the proteolyzed molecule (at concentrations of 20 mg/ml and 100 mg/ml) was applied on to the siliconized coverslip followed by the inversion of the coverslip on reservoirs containing different concentrations of sulphuric acid (0.05% to 2.2%), at 4 °C and 25 °C (room temperature). It was supposed that, owing to the hygroscopic nature of sulphuric acid, solvent would be extracted from the drop; therefore resulting in saturation and subsequent crystallization of the nicked molecule in the absence of any religation-facilitating agent. Interestingly, crystals of lysozyme were observed at a sulphuric acid concentration of 1.4 % and 1.6 % after approximately 4 days (Figure 5.3 (A)). Morphologically, the protein crystals were similar to those obtained earlier, however an increased fragility was indicated by the easy disintegration of the crystals upon mounting onto the glass capillary for data collection. Nevertheless, the crystals were subjected to X-rays and data collection was performed. Low intensity reflections were obtained as a consequence of diffraction, furthermore, exposing the mounted crystal to increased exposure times did not yield better diffraction spots. Crystals were subjected to SDS-PAGE analysis inorder to determine the status of the nicked molecule post-crystallization. Figure 5.3 (B) indicates that crystals obtained in the presence of sulphuric acid (in the reservoir), were indeed nicked and showed no religated material as a consequence of crystallization. It could therefore be inferred that poor quality diffraction could have been the result of conformational heterogeneity of the nicked species.

Since, the unconventional method of mixing no precipitant with the reaction mixture yielded crystals, albeit of low quality, polyethylene glycol (PEG) of different molecular weights at variable concentrations was employed for generation of more robust, diffractable crystals. Proteolyzed lysozyme at concentrations of 25 mg/ml and 100 mg/ml were set up for crystallization against 10% to 35% of PEG 20000, 8000, 6000 and 3350. Crystallization set up was subjected to two temperatures as indicated in earlier trials. Within two days, several proteins crystals were observed in trials conducted at 4°C,
Figure 5.3: Crystals of proteolyzed lysozyme. (A) Tetragonal crystals of nicked lysozyme obtained in the presence of 1.6 % sulphuric acid as reservoir solution. (B) SDS-PAGE profile showing the proteolytic status of the above crystals (Lane 1: marker, lane 2: control lysozyme, lane 3: crystal grown in the presence of 1.6 % sulphuric acid).
in the presence of 15% PEG 3350 as the reservoir solution (Figure 5.4 (A)). SDS-PAGE analysis of the crystals showed that the molecules constituting the crystal were completely proteolyzed, with no band corresponding to the mobility of the native counterpart detected (Figure 5.4 (B)).

5.2.3 Data collection, integration and scaling

Data collection was performed at room temperature with 2° oscillations of the crystal. Data collection statistics (Table 5.1) show 93.9 % completeness of data in the resolution range 2.07-2.0 Å. Further integration and scaling of the data was performed using Denzo and Scalepack (Otwinowski and Minor, 1997). The unit cell parameters were similar to that of the native lysozyme, therefore the nicked molecule was assigned the space group, P4_3212. The Matthews coefficient (V_M) calculated to be 2.07 Å^3/Da was found to be within the usual limits for protein molecules (1.62 < V_M < 3.53 Å^3/Da) (Matthews, 1968). Additionally, the solvent content was computed to be 41.35 % (www.rigaku.co.jp). Comparisons of native HEWL structure from the Protein Data Bank (193L) showed that both, Matthews coefficient (V_M) as well as the solvent content of the crystals were similar, thereby further attesting the similar morphology of crystals obtained for the native and nicked species.

5.2.4 Structure solution and refinement

Scaled data was used for structure solution using a 1.33 Å structure of HEWL as the initial model (PDB code: 193L) for obtaining the phases. Since the model and the target protein molecules are similar from the sequence as well as unit cell parameter perspectives, 193L was itself used for fitting the experimental data. Coordinates of the model were refined to achieve a good fit with the experimental data using CNS (Brunger et al., 1998) suite of programs. Initial 2Fo-Fc map calculations showed no density for the residue 71, therefore the residue, Gly 71 was mutated to alanine at that site followed by complete deletion of that residue from the chain for further refinement cycles. Furthermore, map contoured at a sigma level of 1.0 did not show any density for the residue (Figure 5.5 (A)). Additionally, visualization of the map contoured at 0.7σ did not show any density in that region (Figure 5.5 (B)). Finally, several cycles of maximum
Figure 5.4: Crystal of proteolyzed lysozyme. (A) Crystal of proteolyzed lysozyme obtained in the presence of 15% PEG 3350 as the reservoir solution. (B) Silver stained SDS-PAGE profile of crystals obtained in the presence of PEG (Lane 1: marker, lane 2: control lysozyme, lane 3: crystals obtained in the presence of 15% PEG as the reservoir solution).
<table>
<thead>
<tr>
<th>Parameter</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
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<tr>
<td>Cell dimensions</td>
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</tr>
<tr>
<td>(b(\text{Å}))</td>
<td>78.558</td>
</tr>
<tr>
<td>(c(\text{Å}))</td>
<td>38.216</td>
</tr>
<tr>
<td>Resolution limit</td>
<td>2.0 Å</td>
</tr>
<tr>
<td>Molecules/ asymmetric unit</td>
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<tr>
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<td>Completeness of data in last shell</td>
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<tr>
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</tr>
<tr>
<td>Redundancy of data for last shell</td>
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</tr>
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<td>Overall Rmerge (%)</td>
<td>9.5 %</td>
</tr>
<tr>
<td>Rmerge in last shell (%)</td>
<td>37.7 %</td>
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Table 5.1: Crystallization and data collection statistics for proteolyzed lysozyme
Figure 5.5: Electron density (2Fo-Fc) maps of proteolyzed lysozyme. 2Fo-Fc maps of proteolyzed lysozyme depicting the site of proteolysis (A) 2Fo-Fc map contoured at 1.0σ level, (B) 2Fo-Fc map contoured at 0.7σ level.
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likelihood function refinement of coordinates and simulated annealing using data in the resolution range 50-2.0 Å interspersed with individual temperature factor refinement and model building resulted in an R-value of 0.21 and an R-free of 0.23 (Table 5.2). Additionally, optimal geometric restraints were computed using the optimize_wa script of CNS towards the later stages of refinement. Water molecules from the Fo-Fc map that had a corresponding density in the 2Fo-Fc map were selected with the additional requirement that these moieties remain in contact with a protein atom or another water molecule. Further refinement was terminated when no additional water peaks were observed. PROCHECK used for validation of the final model indicated that the molecule had an acceptable geometry with 89.3% residues falling in the most favoured regions and 10.7% residues present in the additionally allowed regions, as suggested by the Ramachandran plot (Laskowski et al., 1993) (Figure 5.6).

5.2.5 Comparative structural features of nicked and native lysozyme

Visualization of the model after final refinement suggested that the nicked molecule had an overall molecular structure similar to that of the native molecule. However, no density was visualized for residue Gly 71 either in the 2Fo-Fc or the Fo-Fc map, therefore indicating that proteolysis had taken place at this site. Additionally, comparison of the individual temperature factors of main chain atoms for the nicked lysozyme as well as 1BWH showed that there was no significant change in the b-factor as a consequence of proteolysis (Figure 5.7). Secondary structure rendering of the refined coordinates and subsequent model visualization in ICM (www.molsoft.com) showed that the nicked region mapped to an exposed loop (Figure 5.8). Comparison of the superimposed structures of the native and nicked lysozyme suggested minimal perturbation of the overall three-dimensional structure post-proteolysis. Additionally, superimposition of the loop bearing residues Val 65 to Leu 75 with those derived from two other lysozyme structures (193L and 1BWH) using ICM showed that the deviations were observed primarily at residues Pro 70 and Ser 72 flanking the region of proteolysis (Figure 5.9). The carbonyl group of Pro 70 in addition to its cyclic ring shows a departure in conformation from the other two superimposed structures. Furthermore, the Oγ atom of Ser 72 shows a change in the conformation, however, main chain deviations of the residue were not observed. Interestingly, deviations of either the side chain or the main
Table 5.2: Structure refinement and validation statistics of proteolyzed lysozyme

<table>
<thead>
<tr>
<th>Parameter</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Total number of reflections used</td>
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</tr>
<tr>
<td>Reflections in working set</td>
<td>7295 (85.7 %)</td>
</tr>
<tr>
<td>Reflections in test set</td>
<td>409 (4.8 %)</td>
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<tr>
<td>R-factor (%)</td>
<td>21.3</td>
</tr>
<tr>
<td>R-free (%)</td>
<td>23.8</td>
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<tr>
<td>RMSD bond lengths (Å)</td>
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<tr>
<td>RMSD bond angles (deg.)</td>
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</tr>
<tr>
<td>Number of protein atoms</td>
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</tr>
<tr>
<td>Number of solvent molecules</td>
<td>65</td>
</tr>
<tr>
<td>Ramachandran plot</td>
<td></td>
</tr>
<tr>
<td>Residues in most favoured region</td>
<td>89.3 %</td>
</tr>
<tr>
<td>Residues in additionally allowed region</td>
<td>10.7 %</td>
</tr>
</tbody>
</table>
Figure 5.6: **Ramachandran plot of nicked lysozyme.** Most of the residues are in the allowed region and a few in the additionally allowed region.
Figure 5.7: **B-factor plot of proteolyzed lysozyme.** Graphic depicts comparison of the individual temperature factors of main chain atoms of nicked lysozyme (dotted trace) and 1BWH (solid trace) against residue number.
Figure 5.8: Graphic representation of the structure of proteolyzed lysozyme. (A) Secondary structure rendering of the refined model shows the site of proteolysis to be in the loop structure. (B) Stick model of a part of the loop bearing the nicked region.
Figure 5.9: **Superimposed structures of lysozyme.** Comparison of the nicked region of lysozyme (magenta) with similar regions from 1BWH (cyan) and 193L (orange) displaying structural changes in the vicinity of the proteolyzed region.
chain were not observed in the superimposed structures beyond the residues 70 and 72. To further confirm that proteolysis had indeed led to removal of the Gly 71 residue and that the non-availability of density was not an artifact generated due to the mobile loop structure, the structure factors of 1BWH were used to calculate the 2Fo-Fc map for the molecule. Results showed the presence of electron density for this residue, thereby indicating that the removal of residue 71 had occurred in our molecule. Omit map calculations performed by deleting residues 66 to 74 showed the map to be similar to the 2Fo-Fc map computed earlier. Additionally, water peaks observed in the nicked molecule were quite similar to those observed for 1BWH. Interestingly, however, no water molecules were found near the site of proteolysis. Overall, studies indicate that the nicked molecule had minimal structural perturbation as a consequence of proteolysis. It can be inferred therefore that proteolysis of lysozyme leads to minor local disturbances near the site of proteolysis in the structure.

5.2.6 Biophysical characterization of proteolyzed lysozyme

Biophysical characterization of proteolyzed lysozyme was carried to further attest the crystallographic data. Size exclusion experiments were performed with native as well as nicked lysozyme to assess changes in hydrodynamic volume as a consequence of proteolysis. 0.2 mg/ml of the samples were applied to Superdex 200 gel filtration column pre-equilibrated with 50mM Tris pH 8.0 and 100mM NaCl. Data shows that the elution volume of nicked lysozyme (22.93 ml) is similar to that of the native molecule (22.96 ml), therefore suggesting that proteolysis of lysozyme does not result in an increase in the hydrodynamic radius of the molecule (Figure 5.10 (A, B)). Furthermore, investigations into the three-dimensional structure of nicked lysozyme were performed using intrinsic fluorescence. Data shows that nearly similar spectra are obtained for the nicked and native lysozyme (Figure 5.10 (C)). Results indicate that the microenvironment of the tryptophan population remains largely undisturbed as a consequence of proteolysis, therefore corroborating the crystallographic data that suggested the absence of perturbances of the three-dimensional structure. Far-UV circular dichroic studies were performed with native and proteolyzed forms. Samples showed two band minima at 208 nm and 222 nm suggesting the presence of a considerable α-helical content. Figure 5.10 (D) illustrates the spectra obtained for nicked and native molecules indicating the lowered
Figure 5.10: *Biophysical characterization of proteolyzed lysozyme.* Gel filtration chromatogram of (A) native lysozyme and (B) nicked lysozyme. (C) Plot depicting the comparative emission spectra of native and nicked lysozyme. (D) Circular dichroic spectra showing change in profile of lysozyme as a consequence of proteolysis.
secondary structural content of the proteolyzed molecule. Overall, biophysical data pertaining to nicked lysozyme indicates that the non-covalent complex formed as a consequence of proteolysis shows a native-like structure with a slightly lowered secondary structural content, therefore suggesting that despite the cleavage of peptide bonds, the non-covalent interactions in the molecule are sufficient enough to maintain the native-like conformation.

5.3 Discussion

Ever since the pioneering studies on proteolyzed RNase A (Richards and Vithayathil, 1959) depicting fragment complementarity, extensive studies have been performed using several models to provide insight into the mechanism of protein folding. Biophysical and biochemical data exists to show that the non-covalent complex generated as a result of limited digestion exhibits properties similar to those of the native molecule. We attempted to study changes in structure of the native molecule as a consequence of proteolysis using X-ray crystallography in order to further understand the implications of a non-covalent complex at the atomic scale.

Interestingly, crystallization trials conducted of the nicked species with Magic 96 matrix yielded crystals containing intact molecules of lysozyme. Religation of proteolyzed molecules in the presence of organic co-solvents has been extensively investigated (Vogel et al., 1994; Ray et al., 1999). Additionally, the usage of crowding agents such as PEG and dextran in facilitating religation has been illustrated using proteolyzed rabbit muscle TIM. Given the principle of microscopic reversibility of reactions, proteases have been implicated in synthesis of peptide bonds in the presence of conditions wherein the enthalpic barrier is lowered by protonation of the carboxylic group and the entropic barrier is lowered owing to the stereo-chemical proximity of the reacting termini. In our studies, crystallization trials were set up with the nicked form obtained after removal of the protease, SC using immobilized p-aminobenzamidine, a well-known inhibitor of serine proteases. Similar techniques using p-aminobenzamidine moieties immobilized onto magnetic carriers have been successfully employed to adsorb SC from solutions (Yang et al., 2006), thereby underlining the efficacy of this method in the removal of Subtilisin Carlsberg from proteolysis reaction mixtures. Our results showing
religation of proteolyzed lysozyme in a protease-deficient milieu, therefore suggest that
the presence of proteases may be not be a pre-requisite for reverse-synthesis of the
peptide bond, thus presenting a stark deviation from the hitherto accepted concept of
peptide bond reformation being a protease-catalyzed phenomena. Furthermore, results
clearly show that in the presence of religation-facilitating agents such as PEG and salts,
crystals bearing religated molecules are obtained. However, under the alternate conditions
of crystallization employed, wherein the nicked lysozyme has no precipitating agent like
PEG or salt in the milieu, crystals bearing nicked species are formed. Literature exists to
suggest that both salts as well as PEG brings about crystallization by abstracting water
available to the macromolecules in solution, thereby fostering interaction amongst the
protein molecules that eventually leads to the formation of aggregates or ordered
crystalline entities. Furthermore, owing to its property of altering the dielectric constant
of solutions, PEG mimics the effect of organic solvents (McPherson, 1999). We therefore
argue that religation of nicked molecules in the presence of PEG occurs as a consequence
of the lowering of dielectric constant of the milieu and subsequent alteration of the pKa of
the carboxylic group, thereby aiding religation.

Proteolysis is generally avoided in crystallographic investigations owing to the
inherent microheterogeneity in the proteolyzed population, therefore studies pertaining to
crystallographic examinations of nicked molecules have been few. On the other hand, it is
believed that the atomic-scale details of non-covalent complexes would further enhance
the understanding of the contribution of long-range interactions towards genesis of the
folded conformation of proteins. Our results show that the non-covalent complex of
lysozyme generated as a result of non-specific proteolysis exhibits a structure similar to
that of the native molecule. Superimposition of the nicked molecule with the native form
clearly shows that there are no large-scale deviations of either the main-chain or the side
chain atoms in the nicked molecule from that of the native form. Detailed analysis of the
site of proteolysis exhibits the absence of density for the residue Gly 71, therefore
suggesting that the residue might have been removed as a consequence of proteolysis.
Additionally, possibility of the non-existent density being primarily a consequence of the
inherent flexibility of the glycine residue cannot be precluded. In either case, the cleavage
of peptide bond is clearly indicated. Comparative studies performed using the structure
factors of lysozyme (PDB code: 1BWH) indicated that the native molecule had density for
Glycine 71 residue, thereby further attesting the perturbation of this residue in the nicked
structure. Furthermore, secondary structure representations indicated that the site of proteolysis occurred in the loop region. Additionally, superimposition of residues in the vicinity of the site of proteolysis showed that no major changes in the structure of the loop region occurred. Since deviations in the structure were mapped only to residues flanking the nicked region, it can therefore be inferred that the long-range interactions remain largely unperturbed. Consequently, it is expected that deviations in the overall tertiary structure of the molecule as a function of changes in atomic interactions would not be observed, since the site of proteolysis lies in the loop region of the molecule that contributes minimally to the energetics of the folded conformation. The Oγ atom of Ser 72 along with the Pro 70 residue shows changes in conformation from the other structures of lysozyme taken into consideration, however the effect of proteolysis cannot be extended to other structural features, therefore emphasizing that proteolysis brings about local changes in the structure of the molecule. It is generally expected that proteolysis at loop regions would lead to structures wherein the loops would show an enhanced conformational flexibility, therefore leading to non-existent density in such regions. However, the crystallographic data pertaining to nicked lysozyme shows considerable density in the loop region encompassing the site of proteolysis, therefore suggesting that no such enhancement of flexibility occurs as a consequence of proteolysis in lysozyme. It can therefore be concluded that the termini generated as a consequence of proteolysis are in close proximity and would exhibit entropic parameters similar to that of the native conformation. Changes in the tertiary structure observed using intrinsic fluorescence and gel filtration further indicated that the nicked molecule bears a native-like conformation. Finally, it can also be inferred that lowering of stability of the nicked structures as a consequence of depletion of peptide bonds is adequately compensated for by the non-bonded interactions in the molecule.