

FT-IR and CD Studies on Fragmentation of Proteins by Proteases

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I. INTRODUCTION

1.1 PROTEINS

Proteins are large biomolecules involved within every living organism. They perform many crucial roles in all biological process, for instance, transport and storage of molecules and ions, generation and transmission of nerve impulses, enzymatic catalyses, immune protection as antibodies, regulation of body processes as hormones, mechanical support, and communication between cells.

1.1.1 Building Blocks of Proteins: Amino Acids

Proteins are built from 20 amino acids which can be combined in a variety of ways determined by gene sequence. An amino acid consists of an amino group (NH_2), a carboxyl group (COOH), a hydrogen atom (H) and a variable side chain group (R) bonded to the same carbon atom which is called α -carbon (Fig. 1.1).

Proteins are formed by a linear chain of these amino acids that can link together into peptides via amide bonds called peptide bonds (Fig. 1.2). Peptides are composed of short chains of amino acids, while longer chains of those build up the polypeptides and then proteins. The free amino group ($-\text{NH}_2$) on one end of the amino acid chain is called N-terminal and the free carboxyl group ($-\text{COOH}$) on the other end of the chain is called C-terminal. As usual, the beginning of a polypeptide is its N-terminal, and amino acid numbering counts from the N-terminal to the C-terminal end.

Amino acids differ as neutral, polar-nonpolar, hydrophilic-hydrophobic, and charged-uncharged, depending on the chemical property of the side chain in which they are attached to the α -carbon. The physical and chemical characteristics of amino acids are determined by side chains. Twenty sorts of amino acids (Fig. 1.3) can vary in size, charge, shape, hydrogen bonding capacity and chemical activity, depending to the nature of the side chains. For instance, alanine (Ala) has a methyl group while the simplest amino acid is *glycine* (Gly) which has only a hydrogen atom as its side chain. *Valine* (Val), *leucine* (Leu) and *isoleucine* (Ile) have larger hydrocarbon side chains. *Proline* (Pro) whose side chain is bonded to both nitrogen and α -carbon atoms, is often found in the bends of the folded protein chains. These aliphatic side chains are hydrophobic (nonpolar), that is, they prefer to be buried in the lipid bilayer avoiding contact with water.

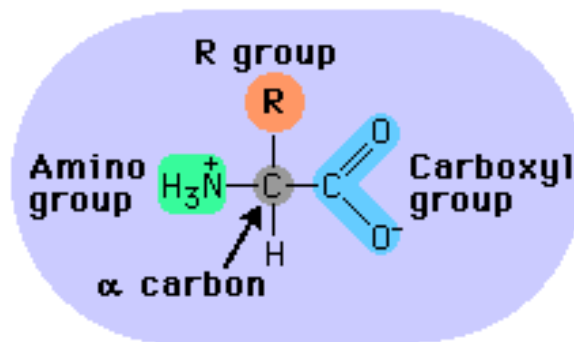


Figure 1.1: General form of an amino acid with α -carbon, R group, amino and carboxyl group.
(from www.phschool.com/science/biology)

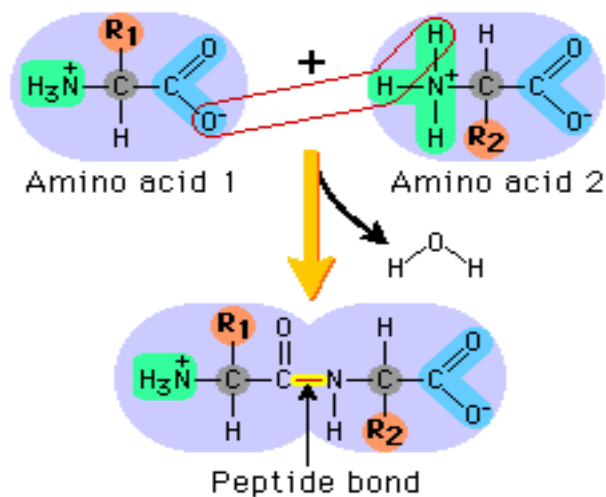


Figure 1.2: A dipeptide is formed when the -NH_2 of one amino acid is linked to the -COOH of a second amino acid. Amino acids are held together by peptide bonds to form polypeptide chains.
(from www.phschool.com/science/biology)

NONPOLAR, HYDROPHOBIC		POLAR, UNCHARGED	
		R GROUPS	
Alanine Ala A MW = 89	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}_3 \end{array}$		Glycine Gly G MW = 75
Valine Val V MW = 117	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}(\text{CH}_3)_2 \end{array}$		Serine Ser S MW = 105
Leucine Leu L MW = 131	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}_2 - \text{CH}(\text{CH}_3)_2 \end{array}$		Threonine Thr T MW = 119
Isoleucine Ile I MW = 131	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}(\text{CH}_3) - \text{CH}_2 - \text{CH}_3 \end{array}$		Cysteine Cys C MW = 121
Phenylalanine Phe F MW = 131	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}_2 - \text{C}_6\text{H}_5 \end{array}$		Tyrosine Tyr Y MW = 181
Tryptophan Trp W MW = 204	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}_2 - \text{C}_8\text{H}_6\text{N}_2 \end{array}$		Asparagine Asn N MW = 132
Methionine Met M MW = 149	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}_2 - \text{CH}_2 - \text{S} - \text{CH}_3 \end{array}$		Glutamine Gln Q MW = 146
Proline Pro P MW = 115	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{CH} - \text{CH}_2 \\ \quad \quad \\ \text{HN} - \text{CH}_2 - \text{CH}_2 \end{array}$		Lysine Lys K MW = 146
POLAR ACIDIC		POLAR BASIC	
Aspartic acid Asp D MW = 133	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}_2 - \text{C}(=\text{O})\text{O}^- \end{array}$		Arginine Arg R MW = 174
Glutamic acid Glu E MW = 147	$\begin{array}{c} ^- \text{OOC} \\ \\ \text{H}_3\text{N}^+ - \text{CH} - \text{CH}_2 - \text{CH}_2 - \text{C}(=\text{O})\text{O}^- \end{array}$		Histidine His H MW = 155

Figure 1.3: Twenty amino acids with their backbone structures and properties with three- and one-letter abbreviations (from <http://matcmadison.edu>).

Aromatic side chains involve *phenylalanine* (Phe) and *tryptophan* (Trp) that are highly hydrophobic and *tyrosine* (Tyr) that is less hydrophobic. The benzene rings of those contain π -electron cloud which enables them to transfer electrons to the other π -systems. *Cysteine* (Cys) and *methionine* (Met) contain a sulphur atom in their side chains and they are also hydrophobic. Cysteine forms covalent disulphide bonds (S-bridge) with another cysteine, shaping some proteins with those disulphide links.

Serine (Ser) and *threonine* (Thr) involve aliphatic hydroxyl groups which make them hydrophilic (polar), that is, they like being exposed to water or solvent depending on the polarity of the solvent. Furthermore, hydrophilic amino acids are *lysine* (Lys) and *arginine* (Arg) which are positively charged at neutral pH and also *histidine* (His) which can be neutral or positively charged depending on its environment. Additionally, *aspartic acid* (Asp) and *glutamic acid* (Glu) are negatively charged acidic side chains while *asparagine* (Asn) and *glutamine* (Gln) have an amide group. The rest are also hydrophilic amino acids. Hydrophilic and hydrophobic capabilities of amino acids are very crucial in protein structure, stability and protein-protein interactions.

1.1.2 Protein Structure and Protein Folding

As described previously, proteins are built from peptide bonds that are formed via covalent binding of the carboxyl group carbon atom of one amino acid to the amino group nitrogen atom of another amino acid by splitting of a water molecule. Additionally, the dihedral angles phi ϕ (between the N and C_α) and psi ψ (between C_α and C) are the degrees of freedom of a protein and they attend the proteins' three dimensional structure. Therefore, the rotations in the chain take place about the bonds in the backbone of the long amino acid residues (Fig. 1.4).

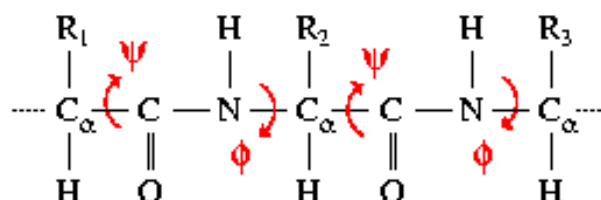


Figure 1.4: The dihedral angles phi ϕ and psi ψ of the backbone of the rigid and planar peptide control the 3-D structure of a protein. (from www.lectures.molgen.mpg.de/).

Conventionally, there are four different levels of protein structure which are called primary, secondary, tertiary and quaternary structures. The simplest but the most fundamental one is the *primary structure* that is a linear sequence of amino acids residues. *Secondary structure* refers to the folded protein structures and occurs when the sequence of amino acids are linked via intermolecular hydrogen bonds between C=O and N-H groups. The α -helices and parallel/antiparallel β -sheets are the stable elements of the secondary structure; whereas turns, random coils and loops don't have a stable secondary structure. The α -helix and β -sheet of those take place at the core of the protein while loops prefer to stand in the outer region. The α -helix is a rod-like, tightly coiled polypeptide chain and utilizes the hydrogen bonds to stabilize itself. Side chains are directed outward away from the helix. The β -sheet is almost completely extended and it is stabilized by hydrogen bonds, too. However, β -sheet differs from the α -helix in that hydrogen bonds stand between the different polypeptide chains instead of within the same chain like in the helices. β sheets can form either parallel or antiparallel sheets with respect to the direction of the polypeptide chain and side chains can take place above or below of its main chain (Fig. 1.5).

Tertiary protein structure happens when certain attractions are present between helices and β -sheets, providing polypeptide folding in three dimensional patterns (Fig. 1.6). In other words, its structure describes the compact packing of the secondary structure elements with respect to each other using a variety of loops and turns. Tertiary structure of a protein is stabilized by hydrogen bonds, ionic interactions and disulfide bonds, and is also driven by proper packing of side chains which results in the burial of hydrophobic residues. Tertiary protein structure comprehends all the noncovalent interactions which are not considered for the secondary protein structure. It defines also the overall fold of the protein. A *quaternary structure* corresponds to spatial organisation of the chains, that is, it is the combination of two or more polypeptides. Subunits refer to the individual chains, and they are not only covalently linked but also disulfide bonds connect them. Complexes of two or more polypeptides are called multimers, namely dimer, trimer, and tetramer if they have two, three or four subunits, respectively. Multimers can be built from identical (i.e. homotetramer from four subunits) or different subunits (i.e. heterotetramer). However, the functional monomers do not have a quaternary structure.

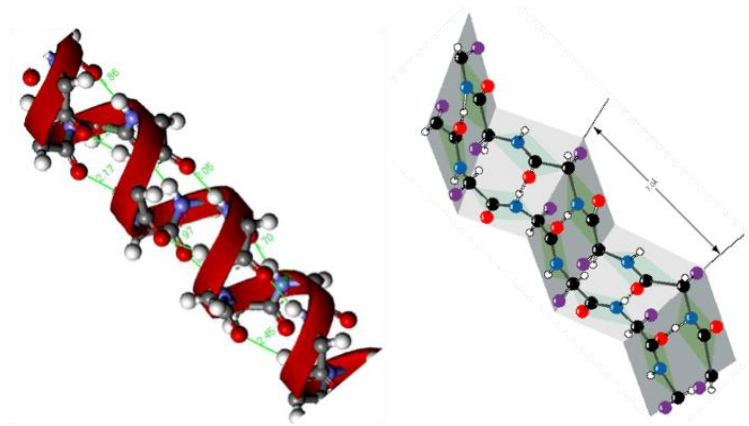


Figure 1.5: Secondary structure is formed via intermolecular H-bonding between C = O and N-H groups. The left image is α -helix and the right image is β -sheet. (from <http://sciencelay.com/biology/proteins/>)

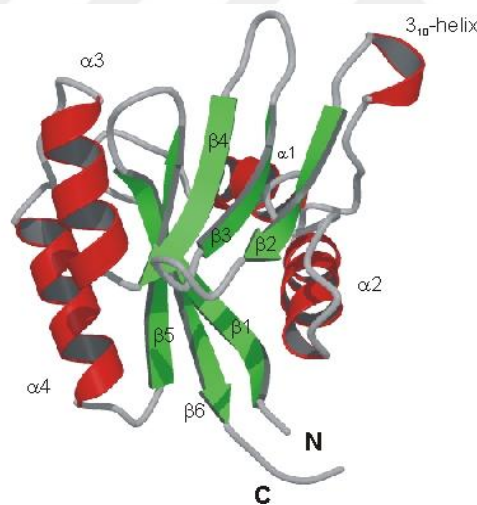


Figure 1.6: Tertiary structure of a protein with beta sheet (green) and alpha helical segments (red ribbons) (copied from www.org.chemie.tu-muenchen.de).

As mentioned above, polypeptide chains can fold into regular structures (α -helix and β -sheets) to construct a stable three dimensional structure. This physical process is called *protein folding*. For most of the proteins, the correct 3-D structure is very important for purposes of functionality. Any failure in folding process can generate some

neurodegenerative and other diseases due to the misfolded proteins. On the other hand, proteins can not fold into their biochemically functional forms under some conditions such as temperature, extreme pH values and high concentration of solutes, mechanical forces, and presence of chemical denaturants. These environmental conditions lead to an unfolded protein, or namely denaturated protein.

1.1.3 Enzymes

Nearly all chemical reactions in biological systems are catalysed by specific macromolecules which are called as enzymes. An enzyme is a large globular protein that increases the rate of a reaction without chemical changes, lowering the activation energy for that reaction. Enzymes are usually specific in their actions and each of them has different specificities. That is, an enzyme can catalyse only a single reaction of a single compound, named enzyme's substrate. Enzymes are classified into six main classes according to the type of the catalysed reaction: hydrolases, isomerases, ligases, lyases, oxidoreductases and transferases. Each of them also has some subclasses, for instance, a protease enzyme from the hydrolase class is responsible for the hydrolysis of an amide group.

The enzyme reactions occur in two stages. First, the substrate binds reversibly to the enzyme, forming the enzyme-substrate complex. Second, the enzyme catalyzes the chemical step in the reaction, producing a product (Fig. 1.7). Enzyme activity can be affected by other molecules such as inhibitors or activators. Enzyme rates also depend on the concentration of the substrate and solution conditions such as temperature, chemical environment (pH) and salt concentrations.

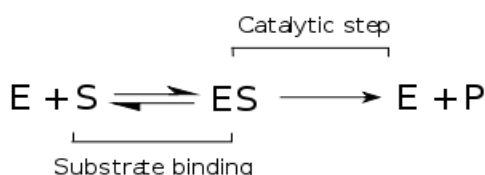


Figure 1.7: A simple representation for substrate binding and catalytic step of an enzyme reaction. The active site of the enzyme (E) binds to the specific substrate (S) to be catalyzed releasing a product (P).

1.1.4 Digestion of Proteins by Proteases

In a proteolysis process, a large polypeptide can be hydrolyzed into smaller fragments by proteases. These enzymes cleave the polypeptides at the specific peptide bonds breaking them (Fig. 1.8b). A member of this protease family is the serine protease whose active site includes the serine, histidine and aspartate residues (Fig. 1.8a). The negatively charged serine residue can make an attack on the carbonyl group of the peptide bond that is to be cleaved. There are three digestive serine proteases: chymotrypsin, elastase and trypsin that play an essential role in digestion of proteins, and they differ with respect to the target cleavage sites. It will be explained in detail in the material and methods section of the present study.

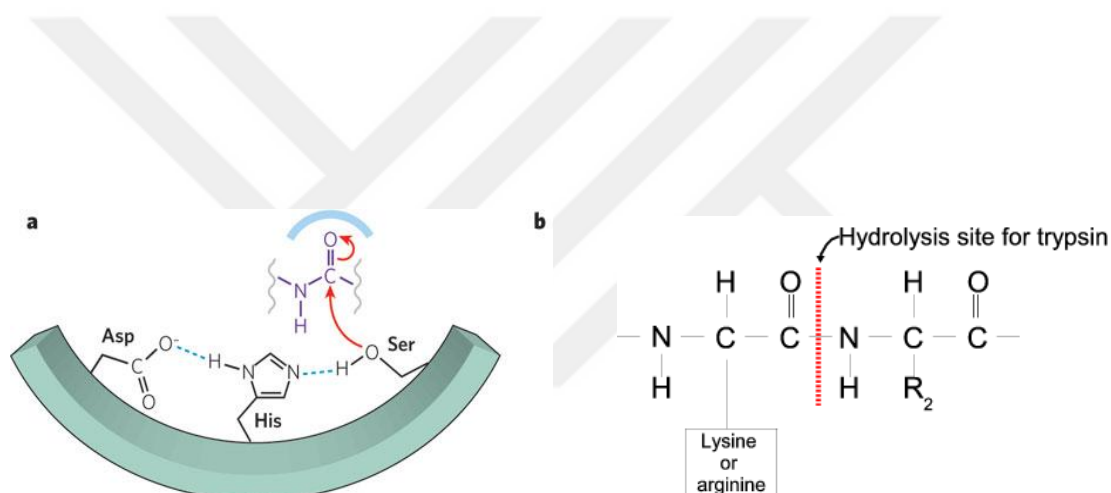


Figure 1.8: (a) A catalytic triad located in the active site of the soluble serine proteases, which has an important role in the protease mechanism. The red arrows shows movement of the electron pairs, the blue curve indicates the oxyanion holes, the larger curve stages the enzyme schematically, blue dotted lines represent the hydrogen bonds or other electrostatic interactions and grey lines represent the continuation of the substrate polypeptide. (b) Cleavage site of the target substrate is the peptide bond (red dot line) between two amino acids. Trypsin only cleaves on the C-terminal site of lysine and arginine residues. (Reproduced from www.nature.com/nature/journal and www.clcbio.com/scienceimages/hydrolysis)

1.2 LIGHT

1.2.1 Electromagnetic Radiation

Let's simply consider a pair of particles carrying equal but opposite charges. When observed from a large distance compared to their separation, pair of charges is called an electric dipole. An oscillating dipole with a changing pattern of electric and magnetic forces generates an electromagnetic (em) wave (Fig. 1.9Left). Electric and magnetic fields oscillate together but perpendicular to each other and em waves propagate in a direction perpendicular to the both fields with a speed of light.

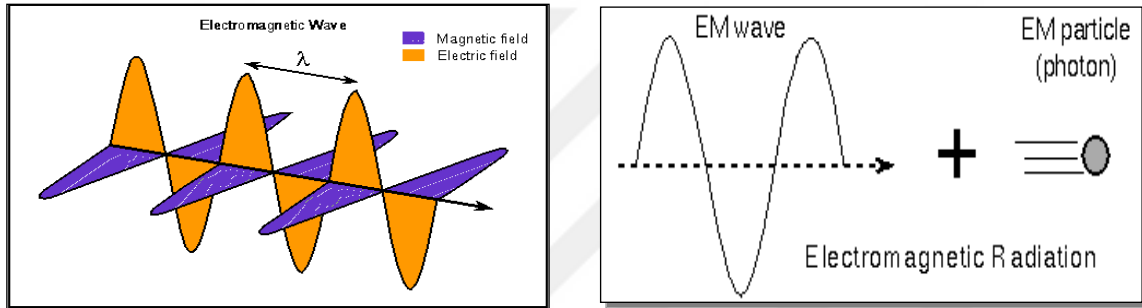


Figure 1.9: Left: The waves propagate in the direction of $\vec{E} \times \vec{B}$; **Right:** Wave-particle duality; light behaves both a particle and wave.

Light is the electromagnetic radiation of any wavelength, whether visible or not. Primary properties of light are the intensity, frequency or wavelength, and polarization. Light exhibits both wave and particle (photons) properties, which is known as *wave-particle duality* (Fig. 1.9Right). According to the description of light as an electromagnetic wave, the electric and magnetic field vectors are, respectively:

$$\vec{E}(x, t) = \vec{E}_0 \cdot \cos \left[2\pi \left(\nu t - \frac{x}{\lambda} \right) + \phi \right] \quad (\text{Eq. 1.1})$$

$$\vec{H}(x, t) = \vec{H}_0 \cdot \cos \left[2\pi \left(\nu t - \frac{x}{\lambda} \right) + \phi \right] \quad (\text{Eq. 1.2})$$

where, ν is the frequency in Hz, λ is the wavelength in nm and ϕ is the phase angle for $x=0$, $t=0$. Light can be considered as energy packets called photon. According to the description of light as a particle, the energy of a photon, E is

$$E = h \cdot \nu \quad (\text{Eq. 1.3}) \quad \text{and with} \quad \lambda = \frac{c}{\nu} = \frac{1}{\bar{\nu}} \quad (\text{Eq. 1.4}), \quad E = h \cdot \frac{c}{\lambda} \quad (\text{Eq. 1.5})$$

where $\bar{\nu}$ is the wavenumber in cm^{-1} which is proportional to the transition energy, h is the Planck's constant ($6,62 \cdot 10^{-34} \text{ J} \cdot \text{s}$) and c is the speed of light in vacuum ($2,99 \cdot 10^8 \text{ m/s}$). Figure 1.10 shows the electromagnetic spectrum with corresponding wavelength and frequency. The energy can be detected in many forms from the highest to lowest energy, such as gamma rays, x-rays, ultraviolet (UV), visible, infrared (IR), microwave and radio waves. Except light in the visible (optical) region (400-700nm), other forms of electromagnetic radiation are not detectable with our eyes.

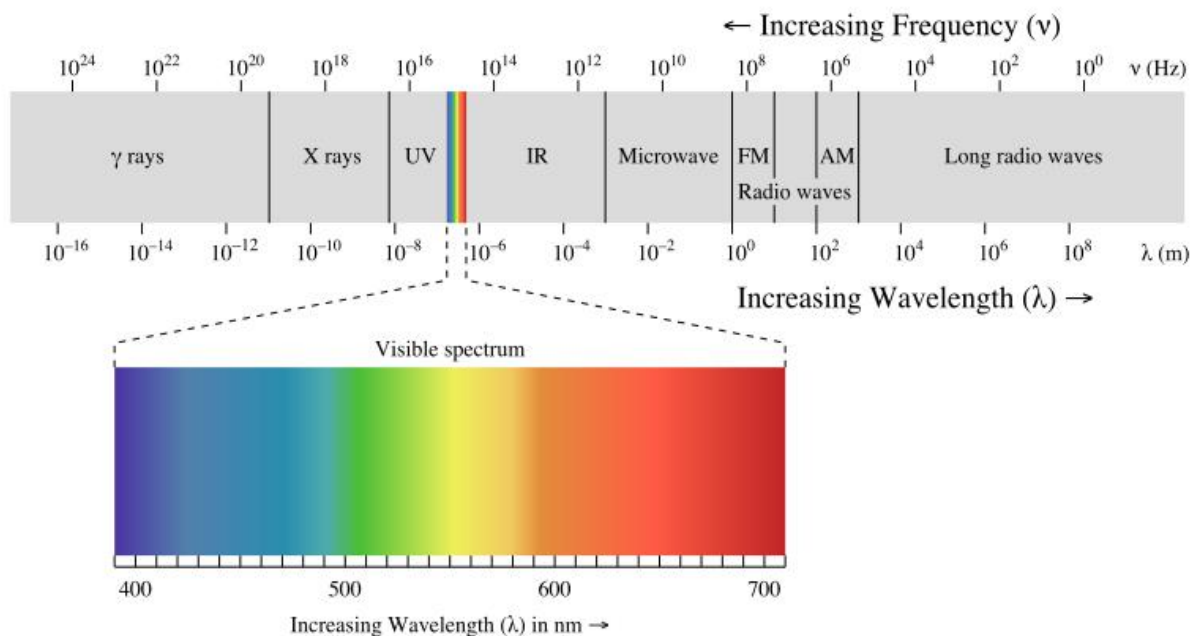


Figure 1.10: Spectrum of the electromagnetic radiation.

(adopted from <http://www.answers.com/topic/electromagnetic-radiation>)

1.2.2 Interaction of Light with Matter

The interaction of em radiation with matter leads to changes in the energy states of the electrons with an absorption or emission process. That is, if matter is exposed to a beam of photons, there is a probability of light being absorbed by the matter causing excitation of atoms from the ground state (E_0) to the excited state (E_1). The energy difference between these two energy levels is $\Delta E = E_0 - E_1 = h \cdot \nu$ and ΔE corresponds to the energy of the absorbed photon. Electromagnetic radiation carries energy and momentum which might be imparted to the matter; in this regard, molecules can interact with the electromagnetic radiation as in table 1.1:

Spectral region	Structure and motion involved in absorption or emission
Ultraviolet-Visible	Electrons-orbital motion (UV-Visible Spectroscopy)
Infrared	Nuclei-vibrational-rotational motion (Infrared-, Raman Spectroscopy)
Microwave	Electron spin-processional motion (ESR Spectroscopy)
Radio-wave	Nuclear- spin-processional motion (NMR Spectroscopy)

Table 1.1: Structures and motions involved in absorption or emission; used instruments and corresponding spectral regions.

1.3 IR-SPECTROSCOPY

1.3.1 General Principles of IR-Spectroscopy and Absorption of Infrared Light

Infrared (IR) spectroscopy deals with the infrared region of the electromagnetic spectrum. It is used to get information about the structure of a compound such as peptides or proteins and to investigate the sample composition. The infrared region includes the range between 0.7-500 μm ($14000\text{--}20\text{ cm}^{-1}$). This region is subdivided into three regions as near-IR (0.78-2.5 μm), mid-IR (2.5-50 μm) and far-IR (50-500 μm). IR-spectroscopy works in the mid infrared (MIR) region with wavelengths between 2.5 and 50 μm or wavenumbers between 4000 and 200 cm^{-1} (10^{13} to 10^{14} Hz). An IR spectrum is plotted against the reciprocal of the wavelength. Absorbed IR radiation is converted into energy of molecular vibration. In other words, the absorption of IR radiation excites the vibrational transitions of molecules. Absorption happens if the light frequency coincides with the frequency of vibrational transitions. In this respect, IR-spectroscopy is based on the interaction of infrared radiation with the electric

dipole moment of the molecules. It exploits molecules that have specific frequencies (resonant frequencies) at which they rotate or vibrate corresponding to the discrete energy levels (vibrational modes). These frequencies are depicted by the shape of the molecular potential energy curves.

Energy levels of vibrational molecules are simply approximated by the simple harmonic oscillator model. In this approximation, according to the Hooke's law, two atoms with m_1 and m_2 masses connected to the end of a spring (bond) behave as a harmonic oscillator and oscillate with an angular frequency ω . In terms of the Hooke's law, the extension of a bond is related to the restoring force, F

$$F = -k \cdot x \quad (\text{Eq. 1.6})$$

where k is the force constant of the bond between molecules or atoms and x is the distance away from the equilibrium position. Moreover, the reduced mass μ of the system becomes

$$\mu = \frac{m_1 \cdot m_2}{m_1 + m_2} \quad (\text{Eq. 1.7})$$

In this regard, potential energy of the oscillating system is

$$U = \frac{k \cdot x^2}{2} \quad (\text{Eq. 1.8})$$

and the angular frequency is

$$\omega = \sqrt{\frac{k}{\mu}} \quad (\text{Eq. 1.9})$$

Together with equation 1.7 and $\omega = 2\pi\nu$, atoms oscillate with a frequency of

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \quad (\text{Eq. 1.10})$$

This is the vibration of such a diatomic molecule given by classical mechanics. Therefore, the frequency of the oscillating system is only dependent on the force constant and on the masses of the oscillating atoms. The larger the mass of an atom, the smaller the frequency (or wavenumber) of the vibration.

Since the vibrational energies of molecules are quantized in contrast to the classical mechanics, the transition between energy levels allowed by quantum mechanics, E_n is

$$E_n = \left(n + \frac{1}{2} \right) h \cdot \nu \quad (\text{Eq. 1.11})$$

where n is the quantum number (0,1,2,3...) with selection rules of $\Delta n = \pm 1$. Then, the lowest energy level, ground state (E_0) is $E_0 = \frac{1}{2} h \cdot \nu$. (Eq. 1.12)

E_0 is known as the zero point energy. This implies that a molecule can never have zero vibrational energy and the atoms can never be without a vibrational motion.

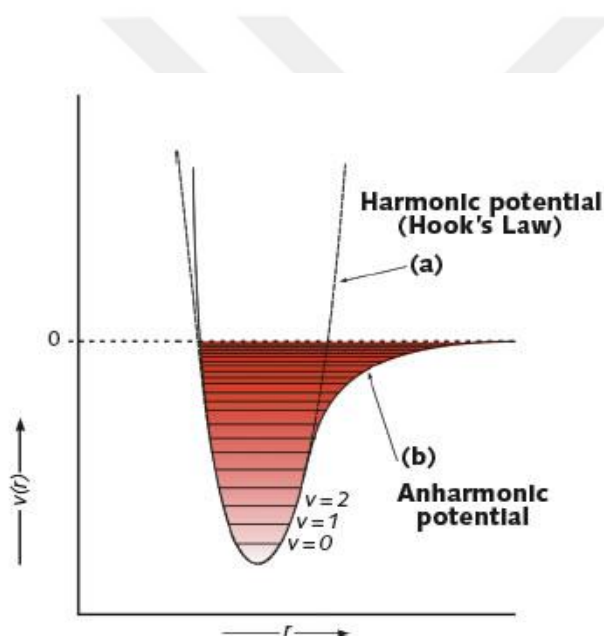


Figure 1.11: The potential energy curve of the quantum mechanical (a) harmonic and (b) anharmonic oscillator. An anharmonic oscillator does not obey to the Hook's law and can be described by Morse potential.

However, the potential energy curve for the molecules connected by real bonds corresponds to the Morse potential, not to the harmonic oscillator potential well. The potential energy curve for harmonic and anharmonic oscillator is shown above in figure 1.11. If bonds between molecules stretch beyond a limiting distance, they can break and these molecules dissociate. Thereby, the anharmonic oscillator is a convenient model to make a good approximation for vibrational structure of molecules. In this case, the spacing between the vibrational levels decreases and the interatomic distance increases while the energy reaches to maximum limited value approaching to the dissociation energy of molecules. The

selection rules for anharmonic oscillator are $\Delta n = \pm 1, \pm 2, \pm 3...$, and there is an additional possibility of larger jumps to higher energy level in the anharmonic oscillator.

1.3.2 Molecular Vibrations

Molecular motions occur as translational, rotational and vibrational motions of atoms in the molecules. A molecule with N atoms has $3N$ degrees of freedom according to the Cartesian coordinates of each atom in the molecule. A nonlinear molecule has $3N-6$ degrees of freedom because 3 of these degrees are rotational and 3 of those are translational motions, and the remaining corresponds to the normal modes of vibration. In a linear molecule, 3 degrees of these are translational and 2 of those are rotational. Therefore, a linear molecule has a total of $3N-5$ normal modes of vibration. In a normal mode of vibration, the atoms move with same frequency and phase, whereas amplitudes and directions of their motions differ.

There are mainly two types of vibration modes, stretching and bending. Both can be symmetric (ν_s) or asymmetric (ν_{as}). Stretching or valence vibrations (ν) are changes in the bond length between atoms in the molecule while bending vibrations, also called deformation vibrations (δ), are changes in the angle between two bonds. Bending modes have 4 types; in-plane rocking, in-plane scissor bending, out-of-plane wagging and twisting, and they have less energy than stretching vibrations.

The nonlinear water molecule (H_2O) has 3 vibrational degrees of freedom (Fig. 1.12).

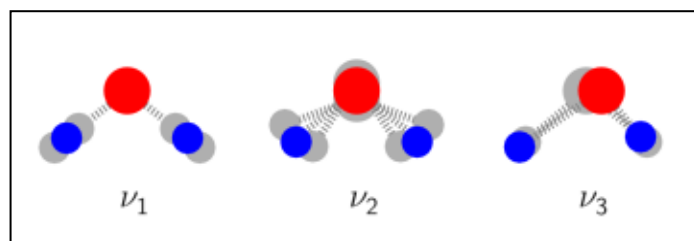


Figure 1.12: Normal vibrational modes of water: (ν_1) symmetric stretching; (ν_2) symmetric bending; (ν_3) asymmetric stretching.

A linear molecule such as a CO_2 has 4 vibrational degrees of freedom (Fig. 1.13).

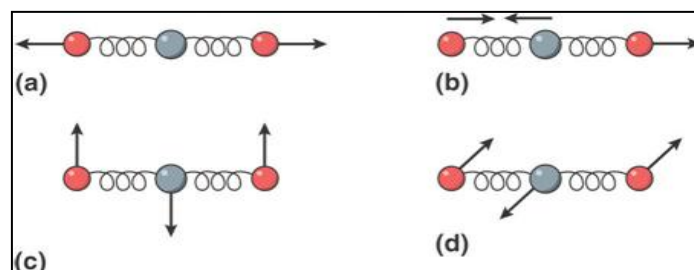


Figure 1.13: Normal vibrational modes of CO_2 : (a) symmetric stretching; (b) asymmetric stretching; (c) in-plane bending; (d) out- of-plane bending.

The symmetrical stretching mode of CO_2 (Fig. 1.13a) is inactive in the IR because this vibration does not cause a change in the dipole moment of the molecule. However, all other vibrational modes of H_2O create a dipole moment change in the molecule; therefore it is called IR-active. In general, the larger the dipole change, the stronger the intensity of the band in an infrared spectrum.

1.3.3 FTIR-Spectroscopy

The original instruments were dispersive IR spectrometers that utilize a grating monochromator. Yet, modern IR spectrometers are commonly Fourier-transform type because of their various advantages; such as increasing sensitivity, rapid data collection, high speed and high light intensity at the detector.

Fourier Transform Infrared Spectroscopy (FTIR) is a measurement technique that uses the entire spectrum of IR light to collect the IR spectrums of the vibrational transitions of molecules, providing structural and functional information about proteins. An FTIR spectrometer includes an interferometer which is the main part of it. The *Michelson interferometer* design is the most common type among interferometers used for FTIR spectrometers. Basically, an FTIR instrument is composed of a Globar source (a silicon carbide rod which is electrically heated to about 1500 K), a beam splitter, two mirrors, one

fixed and one movable, and a detector (Fig. 1.14). In principle, radiation leaves the light source and is split in two halves by the beam splitter. Half of it is reflected towards the stationary mirror from which they are reflected back to the beam splitter. Thereby the radiation travels a fixed distance. However, the other half of the radiation from the light source transmits through the beam splitter and is reflected back by a movable mirror. In the latter case, the path length of a beam varies due to the position of the movable mirror. Reflected beams interfere when they recombine at the beam splitter and that results in *constructive* (at zero path difference and multiples of λ) or *destructive interferences* (at multiples of $1/2\lambda$) depending on the optical path difference between two halves. Finally, light intensity transmits through the sample and accesses the detector.

There are two types of detectors used in spectrometers: thermal and photon detectors. For the purpose of thermal detectors, pyroelectric detectors are widely utilized, which are made from pyroelectric materials such as triglycine sulfate (TGS) and deuterated triglycine sulfate (DTGS). In principle, the absorbed radiation by detector leads to an increase in temperature. Thus, if the temperature is changed, the surface charge density of the substance changes, called pyroelectric effect. When an electric field is applied across the pyroelectric material sandwiched between two electrodes (capacitor), the electric polarization occurs. After the removal of the electric field, the polarization persists in the material. Since the polarization is temperature dependent, the capacitance of the pyroelectric material is altered by the heating effect of the incident IR radiation. Finally, the detector monitors changes in intensity, and therefore the interferogram. Additionally, MCT detectors are used as photon detectors, which are the alloy of mercury, cadmium and tellurium. These are the semiconductor detectors that they operate at liquid N_2 temperature about 77 K. In principle, the absorbed IR radiation generates the charge carriers in the material, and thus, increases its conductivity while decreases its resistivity. The increased conductivity is monitored with respect to the flowing current under the applied constant voltage, and finally, the detector is very sensitive to monitor the changing of the IR radiation intensity.

As it was described above, FTIR generates an infrared spectrum of samples that absorb infrared light. After the radiation transmittance through the sample, light reaches the detector that measures the intensity of striking light. The output from the interferometer is called an *interferogram*. The intensity of a beam at the detector is represented by $I(x)$

(interferogram) as a function of the mirror position x which defines the optical path

$$\text{difference: } I(x) = \int_{-\infty}^{+\infty} I(\nu) \cos(2\pi x \nu) d\nu. \quad (\text{Eq. 1.13})$$

In order to obtain the IR spectrum, the interferogram is transformed by a computer with the help of an algorithm called Fourier transformation from space domain to the frequency domain. The spectral intensity is illustrated by $I(\nu)$ (single beam spectrum) as a function of

$$\text{the wavenumber: } I(\nu) = \int_{-\infty}^{+\infty} I(x) \cos(2\pi x \nu) dx. \quad (\text{Eq. 1.14})$$

Since the experimental data is measured at discrete points rather than for infinite amount of points, in practice, *discrete Fourier transform* is used. FTIR instruments have a high signal to noise ratio (SNR or S/N), and thus obtain high quality FTIR spectra. In this respect, SNR is related ($\text{SNR} \propto \Delta\nu \cdot t^{1/2}$) to the analysis time (t), resolution ($\Delta\nu$ in cm^{-1}) and the number of scans (N). For instance, it takes longer to acquire many scans than a few scans, and thus, the measured time of one spectrum is related to the number of scans added together ($t \propto N$). Besides, a longer time is required in order to obtain a high resolution spectrum than a low resolution spectrum ($t \propto 1/\Delta\nu$) because the movement of the mirror position along the longer distance takes more time. In the FTIR spectroscopy, a high resolution spectrum involves noise; therefore, the noise also depends on the resolution. When the length of the interferogram is increased, more noise is recorded, leading to the increasing noise in the spectrum. Hence, one should choose the appropriate parameters in order to avoid the noise contributions in the spectra and to maintain the data quality.

Michelson interferometer

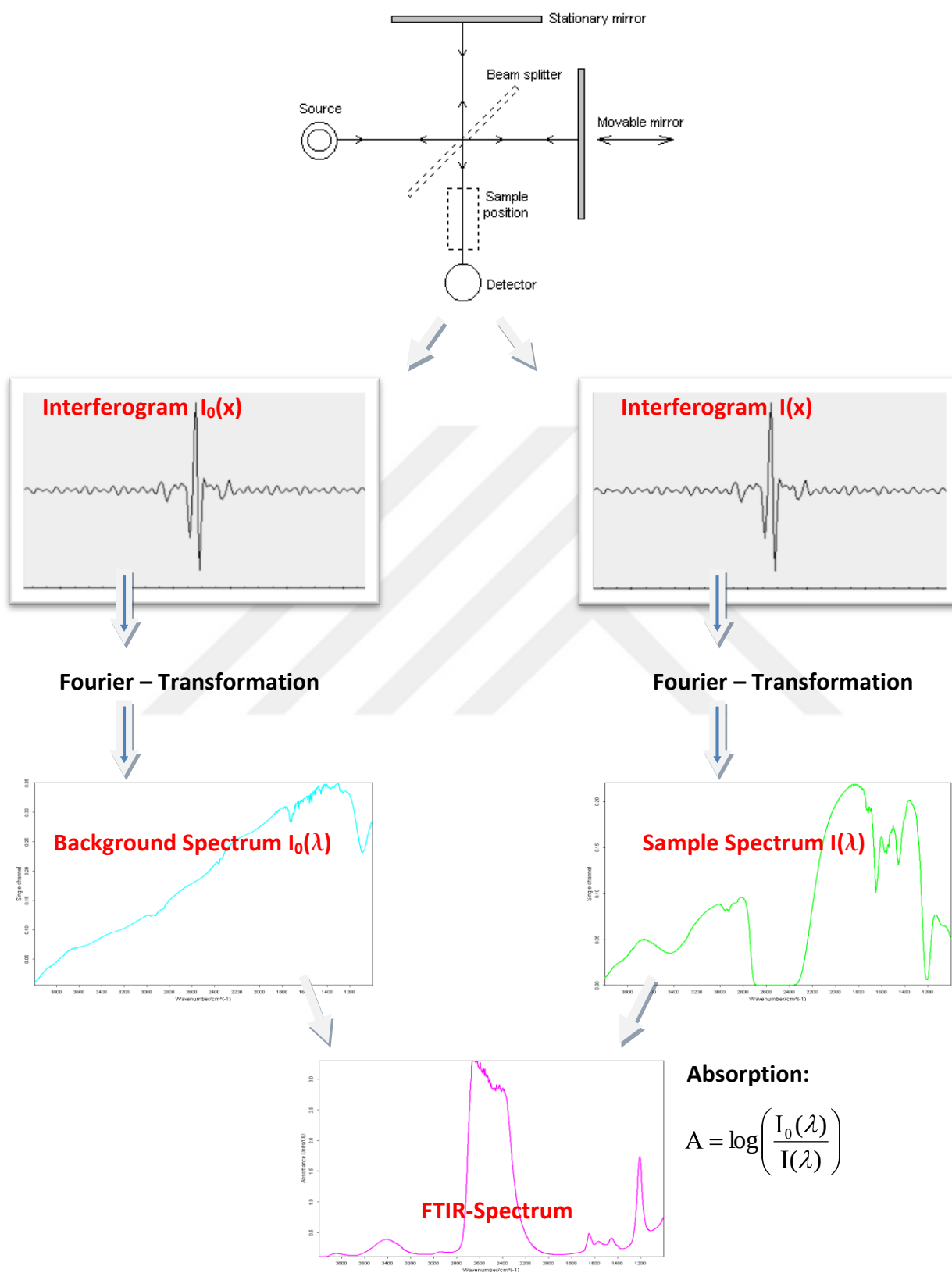


Figure 1.14: Formation of a FTIR spectrum and a schematic diagram of the Michelson interferometer. (Michelson interferometer was copied from <http://teaching.shu.ac.uk/hwb/chemistry/tutorials/>)

1.3.4 Absorption Spectrum

For the IR spectroscopy, after infrared radiation interaction, the dipole moment changes of molecules are utilized to obtain absorbance spectrum. Hence, it can be thought as a fingerprint in order to determine molecules and proteins according to their band position and absorbed intensity. The IR absorbance of a sample is calculated by the *Beer-Lambert*

$$\text{equation: } A = -\log\left(\frac{I}{I_0}\right) = \epsilon \cdot c \cdot d \quad (\text{Eq. 1.15})$$

where A is the absorbance, I_0 and I are the light intensities of the single beam spectra of the reference and of the sample, respectively; ϵ is the molar extinction coefficient [$\text{mol}^{-1}\text{cm}^{-1}$], c is the concentration of the sample [mol/l] and d is the path length of the cuvettes [cm]. In transmission measurement techniques, a sample is placed between two IR windows typically made of ZnSe , BaF_2 or CaF_2 using a spacer which determines the path length. The sample is placed between detector and output of the interferometer and it absorbs the IR light according to the equation 1.15. Since atmospheric water vapour and CO_2 have a strong absorbance in the MIR spectral region, the sample chamber of the interferometer is continuously purged with dry air or N_2 , and then a single beam spectrum of the reference (background) is recorded before the sample measurement to get an IR spectrum of a sample. In that way, environmental contributions are eliminated, providing normalization of the sample single beam spectrum with respect to the reference spectrum. Figure 1.15 represents a FTIR absorption spectrum of an α -helical-rich protein and of atmospheric water vapour.

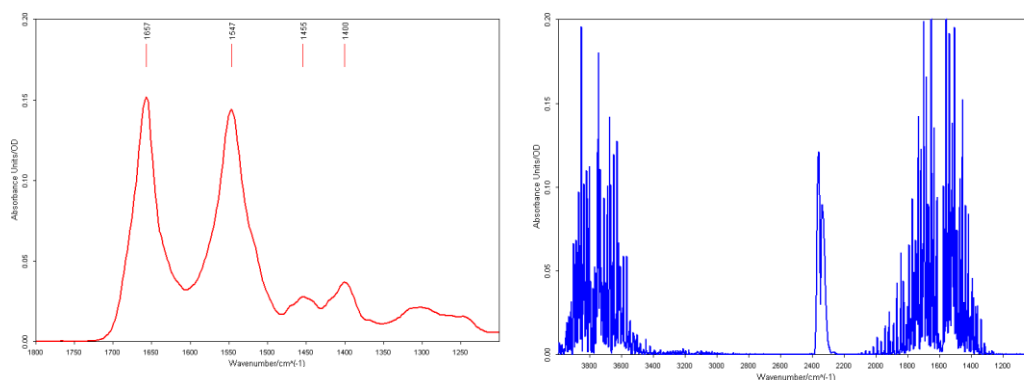


Figure 1.15: FTIR absorption spectrum of an α -helical-rich protein with its amide I and amide II bands (left) and atmospheric water vapour (right).

1.3.5 FTIR Signatures of Proteins and Amide Bands

The IR spectrum of polypeptides exhibits amide bands which represents different vibrational modes of the peptide group, yielding information about the structure of proteins. The spectrum also contains relatively weak contributions from amino acid side chains. The peptide group gives rise to 9 characteristic bands, called amide A, B, I, II, through VII in the order of decreasing frequency (Fig. 1.15Left).

Among the amide bands, the most useful spectral region for protein secondary structure analysis is the *amide I* band ($1600\text{--}1700\text{ cm}^{-1}$) which arises mainly from the C=O stretching vibrations of the protein backbone (70-85 %). It also consists of minor contributions from the out-of-phase C-N stretching modes ($\sim 10\text{--}20\%$ of the amide I range) and of N-H in-plane bending (Krimm and Bandekar, 1986). It shifts towards lower wavenumbers by $2\text{--}10\text{ cm}^{-1}$ upon hydrogen-deuterium exchange. *Amide II* ($1480\text{--}1580\text{ cm}^{-1}$) results from in-plane N-H bending ($40\text{--}60\%$), C-N stretching ($18\text{--}40\%$) and C-C stretching vibrations ($\sim 10\%$). It is shifted $\sim 100\text{ cm}^{-1}$ towards lower wavenumbers upon H/D exchange, named amide II' mode ($1490\text{--}1450\text{ cm}^{-1}$). Amide I and amide II vibrations of proteins are hardly affected by the nature of the side chains. *Amide A* ($\sim 3225\text{--}3280\text{ cm}^{-1}$) arises mainly from N-H stretching vibration ($\sim 95\%$) and is very sensitive to the strength of the hydrogen bonds. Therefore, it is insensitive to the conformation of the peptide backbone. Amide III and amide V are not useful for protein secondary structure prediction because they are complex modes and are characterized by a strong dependency on the nature of side chains. The other amide bands also superimpose with water ($^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$) and have weak intensities in the IR-spectrum. Figure 1.16 indicates the IR-vibrations of a peptide group resulting in amide I and amide II bands of the IR-spectrum.

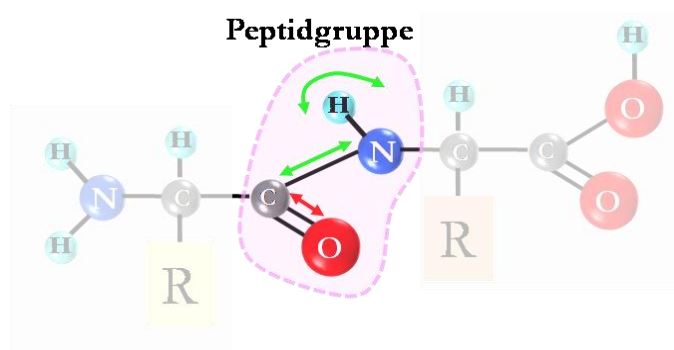


Figure 1.16: Molecular motions of the peptide group: Amide I ($\leftrightarrow$) und Amide II ($\leftrightarrow$)

Conformational changes of the proteins and secondary structure elements, such as α -helices, β -sheets, turns and random coils (disordered) show frequency changes of amide bands in the IR-spectrum, shown in table 1.2. In the $^1\text{H}_2\text{O}$ buffer, α -helix and random coil structures overlap in the amide I region because of broad bands located at close wavenumbers, while β -sheets have distinct characteristics in the amide I region.

Secondary structure	Band position in $^1\text{H}_2\text{O}$ (cm^{-1})	Band position in $^2\text{H}_2\text{O}$ (cm^{-1})
α -helix	1654	1652
β -sheet (strong)	1633	1630
β -sheet (weak)	1684	1679
Turns	1672	1671
Disordered	1654	1645

Table 1.2: Average positions of amide I band assignments to secondary structure $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ buffer (Barth, 2007).

Among 20 amino acids, only nine (Asp, Glu, Asn, Gln, Lys, Arg, Tyr, Phe, His) have significant absorbance between $1750\text{-}1480\text{ cm}^{-1}$ (Table 1.3). Deuteration has little effect on the frequency of the carboxyl group [COO^- , COOH(D)], while protonation shifts only a few cm^{-1} the carboxyl contribution. A crucial difficulty is that side chain absorption overlaps with amide I and amide II bands making it difficult to analyze.

Side chain/assignment	Band position in $^1\text{H}_2\text{O}$ (cm^{-1})	Band position in $^2\text{H}_2\text{O}$ (cm^{-1})
Asp (COOH/COOD) (ν)	1716	1710
Asp (COO^-) (ν_{as})	1574	1585
Arg (CN_3H_5^+) (ν_{as})	1673	1605
Arg (CN_3H_5^+) (ν_s)	1633	1586
Glu (COOH/COOD) (ν)	1712	1706
Glu (COO^-) (ν_{as})	1560	1570
Lys (NH_3) (δ_{as})	1629	-
Lys (NH_3) (δ_s)	1526	-
Tyr (Ring)	1614	1614
Tyr (Ring)	1518	1515

Table 1.3: Assignments of some side chain absorptions with approximated frequencies (Fabian and Mäntele); ν_s and ν_{as} are symmetric and asymmetric stretching frequencies, respectively; δ_s and δ_{as} are symmetric and asymmetric bending frequencies, respectively.

1.4 CIRCULAR DICHROISM SPECTROSCOPY

1.4.1 Physical Principles of CD Spectroscopy

Circular dichroism (CD) Spectroscopy measures the difference in absorption of left- and right-handed circularly polarized light as a function of wavelength in the far-UV region (175-250 nm) of the electromagnetic spectrum. The energy of this radiation leads to electronic transitions in molecules. It is a widely used technique to analyse the secondary structure or conformation changes of the biological macromolecules in order to derive structural and kinetic information about proteins. This phenomenon is exhibited in absorption bands of the optically active molecule.

Natural light is unpolarized and contains oscillations of electric field components in all directions perpendicular to the direction of propagation. There are three kinds of polarized light. Light in the form of a plane wave in space is explained as linearly polarized. Another form of polarization is the circularly polarized light that consists of two perpendicular plane waves of equal amplitude and 90° difference in phase. Elliptically polarized light differs from circularly polarized light with unequal amplitude of perpendicular waves in phase by 90° (Fig. 1.17).

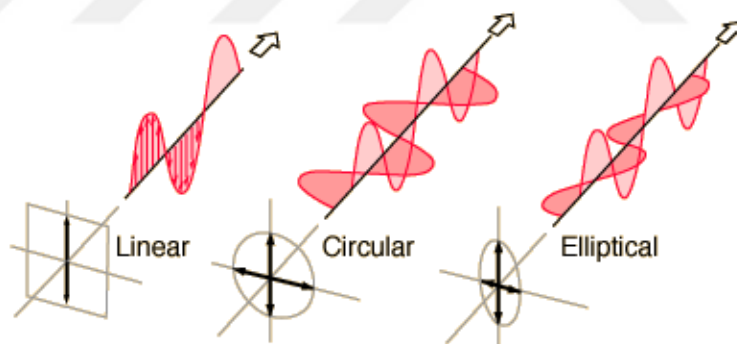


Figure 1.17: Linearly, circularly and elliptically polarized light. If the thumb of your right hand is pointing in the direction of propagation of light, the electric vector rotates in the direction of your fingers, counterclockwise or clockwise (from www.hyperphysics.phy-astr.gsu.edu).

After a plane polarized light passed through an optically active sample, the plane of polarization is rotated with respect to the plane before entering the sample which is named as *optical rotary dispersion*. After emerging from the sample, light is no longer linearly

polarized but elliptically polarized which is termed as *ellipticity*. This result from the difference in absorption of left and right circularly polarized light, known as *circular dichroism*. Two types of circularly polarized light (left and right) are absorbed to different extents at some wavelengths because of differences in their extinction coefficients. One of two polarizations is absorbed more than the other one, and this wavelength-dependent difference of absorption ($\Delta A = A_{\text{Left}} - A_{\text{Right}}$) yields the CD spectrum of the sample.

1.4.2 Circular Dichroism Spectrometer

A CD spectrometer is a variation on a normal absorption spectrometer. It has a source of light, a monochromator to disperse the light into its various wavelengths, a detector, and a sample compartment. Additionally, it has a linear polarizer and a quarter wave retarder to convert the linearly polarized light into the circularly polarized light. The retarder oscillates, alternately making left and right circularly polarized light in order to measure accurately the difference in intensity of two types of light. For practical causes, an acousto-optical or electro-optical modulator is used to modulate the polarization of light at a frequency of 50-100 kHz. The spectrometer has to be purged with nitrogen due to oxygen absorption and strong water absorption below 200 nm; therefore the lower limit for aqueous solutions is 185 nm.

The absorbance difference ($\Delta A = A_L - A_R$) of an asymmetric molecule is typically very small and about 10^{-4} of the absorption. The CD spectrometer measures actually as the differential absorbance ΔA of left and right circularly polarized light. Besides, one can arrive to the molar circular dichroism ($\Delta \epsilon = \epsilon_L - \epsilon_R$) taking into account the cell path length and sample concentration (see equation 1.15). ϵ_L and ϵ_R are the molar extinction coefficients for left and right circularly polarized light, respectively. In addition to $\Delta \epsilon$ and ΔA , circular dichroism can be expressed as degrees of ellipticity (θ). Using Lambert's Beer Law, the relation between them can be simply explained as

$$\Delta A = \theta / 32.982 \quad (\text{Eq. 1.16})$$

$$\Delta \epsilon = [\theta] / 3298.2 \quad (\text{Eq. 1.17})$$

$$[\theta] = 100 * \theta / (c * d) \quad (\text{Eq. 1.18})$$

where $[\theta]$ is the molar ellipticity in $\text{M}^{-1}\text{m}^{-1}$ or $\text{cm}^2 \text{ dmol}^{-1}$ and θ in degrees (deg) or millidegrees (mdeg). A CD spectrum can exhibit either negative or positive bands, depending on which type of light is absorbed more strongly.

1.4.3 Circular Dichroism Signatures of Proteins

A molecule has to be chiral or asymmetric to be optically active because they absorb left and right handed polarized light to a different extent. Biological macromolecules such as proteins and DNA are composed of optically active elements. The chromophore of the peptide group is the carbonyl group which absorbs between 190 and 240 nm. Amide bond electrons absorb energy in the range of far UV, resulting in transitions from ground state to the higher excited states. The intensity and energy of these transitions depends on ϕ and ψ angles of the peptide backbone (Fig. 1.18).

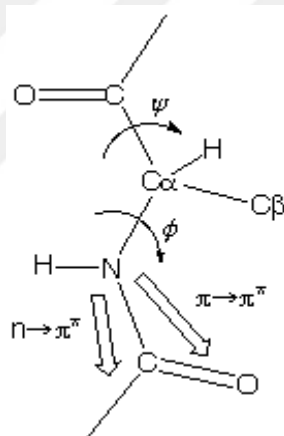


Figure 1.18: $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the amide bond electrons.

The CD spectrum is sensitive to protein secondary structure, such as α -helix, β -sheet and random coil (Fig. 1.19). For instance, the spectrum of an α -helix is composed of the signal of the $\pi \rightarrow \pi^*$ transition which is split into two due to the exciton coupling, observed around 190 nm (positive band) and 210 nm (negative band), and $n \rightarrow \pi^*$ transition centred around 220 nm which is insensitive to chain length. On the contrary, the $\pi \rightarrow \pi^*$ transition of α -helix is quite strongly chain length dependent. The amplitude of the CD signal decreases with smaller helix length. Particularly, the CD of β -sheets is more variable than others, both in

amplitude and in position of the bands. It has a negative peak around 215 nm ($n \rightarrow \pi^*$) and a positive peak around 196 nm ($\pi \rightarrow \pi^*$). Although the CD spectra for random coil structures shows a negative CD band between 190-200 nm and a positive band around 212 nm, they vary considerably in magnitude.

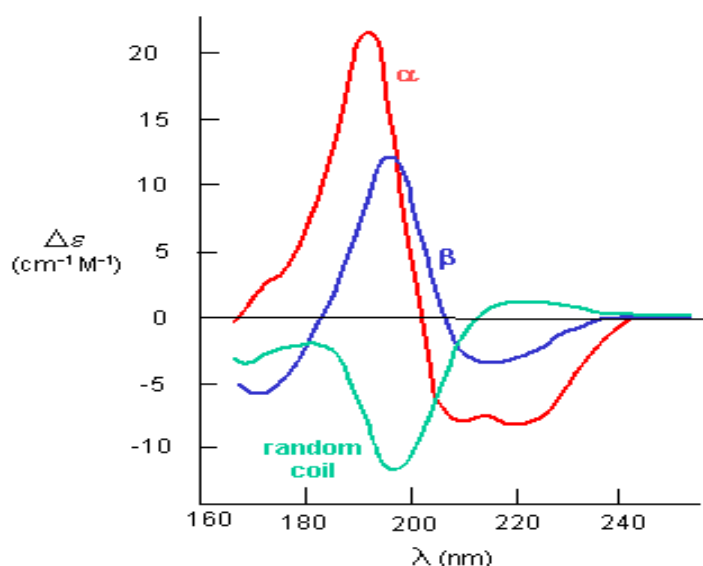


Figure 1.19: CD spectra of secondary structures: α -helix, β -sheet and random coils. (copied from www.employees.csbsju.edu).

Additionally, the CD signal of the peptide group is superimposed by the signals of the aromatic groups and disulfide bridges. If aromatic residues are unusually abundant, they can have significant effects on the CD spectra below 230 nm. The effects of the backbone conformational changes can be observed in the region of 178-240 nm. Therefore, this region is used to analyse the secondary structures of proteins.

1.5 Goal of This Study

The complexity of the enzyme specificity upon peptide bond, named proteolysis modeling, is still far from being solved. Enzymatic hydrolysis, proteolysis, induces degradation of food proteins in digestion and nutrition processes. However, it is not yet clear to figure out the liberated patterns of fragments and the secondary structure (α -helix, β -sheet, random coil)

changes of proteins during proteolysis. In this respect, the major goal of this work is to investigate the proteolysis kinetics and conformational changes of the proteins during proteolysis.

In the present study, β -Casein and β -Lactoglobulin which are from bovine milk and serum albumin from bovine blood (BSA) will be utilized as substrates. Trypsin and α -chymotrypsin are applied as protease enzymes in various concentrations to digest the substrates so that the substrate-to-enzyme ratio will vary. Fourier Transform Infrared Spectroscopy (FT-IR) and Circular Dichroism Spectroscopy (CD) are used to confirm the results of the enzymatic reactions. Additionally, protein secondary structure will be analyzed using both spectroscopic methods.

It is expected that an intact protein will lose its secondary structure upon conversion to the free fragments of peptides during enzymatic attack. This perturbation alters the backbone of the native protein resulting in degrading of the intact protein.

To sum up, the study of proteolysis is an elaborated task due to the complex cleavage sites and conformational states of polypeptide chain. In this respect, we aim to get more information about the proteolysis model determining the changes of secondary protein structure and their kinetics.

II. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Proteins

K ₂ HPO ₄	Sigma
KH ₂ PO ₄	Sigma
Deuterium oxide (99.9 %)	Aldrich
Bovine Serum Albumin (BSA)	Sigma
β-Lactoglobulin	Sigma
β-Casein	Sigma
Trypsin	Sigma
α-Chymotrypsin	Fluka

H₂O/D₂O Buffers

For 10 mM potassium phosphate buffer, appropriate amounts of acid and base salts (K₂HPO₄ and KH₂PO₄) were mixed in distilled water (pH 8) and in deuterium oxide (pD 8.4) to obtain the desired pH. The final pH value of buffer was controlled with a pH-meter.

2.1.2 Substrates

Bovine Serum Albumin (BSA)

BSA is the main protein in the plasma and is one of the versatile proteins in mammals that binds to hormones, fatty acids, bilirubin, steroids, water, Ca²⁺, Na⁺, K⁺ and drugs. Serum albumin is the most abundant protein in the circulatory system, whose main biological function is to maintain the pH and to regulate the colloidal osmotic pressure of blood (Carter and Ho, 1994).

BSA is a single polypeptide chain with an average molecular weight of 66,430 Da which consists of 583 amino acid residues (Hirayama et al., 1990). It involves 99 acidic side chains with 41 Asp and 58 Glu residues, and 102 basic side chains with 26 Arg, 60 Lys and 16 His residues. The isoelectric point of BSA is 4.8 and the balance between negative and positive charges depends on the pH of the solution (Quiquampoix et al., 1992). The amino acid

sequences of BSA are homologous to that of human serum albumin (Hirayama et al., 1990), and their identity is about 76% (Carter and Ho, 1994). HSA consists of 70% α -helix with 34 helices [PDB]. The main difference is that BSA has a second tryptophan residue in position 131 whereas HSA has only one (Carter and Ho, 1994; Peters et al., 1985). The heart-shaped structure of serum albumin consists of three similar helical domains, each has two subdomains shown by a and b, and of eight pairs of double disulfide bridges. Deep hydrophobic pockets with positively charged entrances are found at similar positions in subdomains IIa and IIIa, and thought as site I and site II binding sites for various compounds (Sugio, 1999). BSA is a readily soluble protein in water and its secondary structure is predominantly α -helical (67%) and the rest is turns and extended or flexible conformations with no β -sheets (Carter and Ho, 1994). Figure 2.1 shows 3-D structure of BSA. In this study, BSA was chosen as substrate due to its commercially abundance and well-known structure.

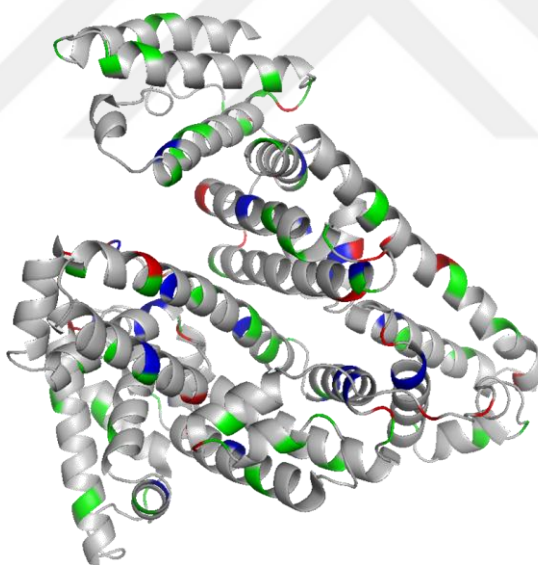


Figure 2.1: 3-D structure of predominantly α -helical BSA obtained from PDB (1AO6), representing residues of arginine (blue), lysine (green) and proline (red). Arginine and lysine residues are fit into the active site of trypsin enzyme to be cleaved, unless they are followed by the proline residue.

β - Lactoglobulin from Bovine Milk (β -Lg)

Proteins from milk, in particular the residual liquid in cheese production termed whey, are used as food ingredients due to their functional and nutritional properties. The major constituent of bovine whey proteins is the small globular β -lactoglobulin (55-60%) which is a water-soluble protein. It is a transport protein that binds to small hydrophobic molecules, such as retinol, into a hydrophobic cavity. β -Lg is composed of eight antiparallel β -strands; however, there is still some doubt about the physiological and biological roles of this protein. Whereas human milk does not contain β -lactoglobulin protein, the bovine β -Lg has genetically different variants of A and B (Farrell *et al.*, 2004; Hambling *et al.*, 1992).

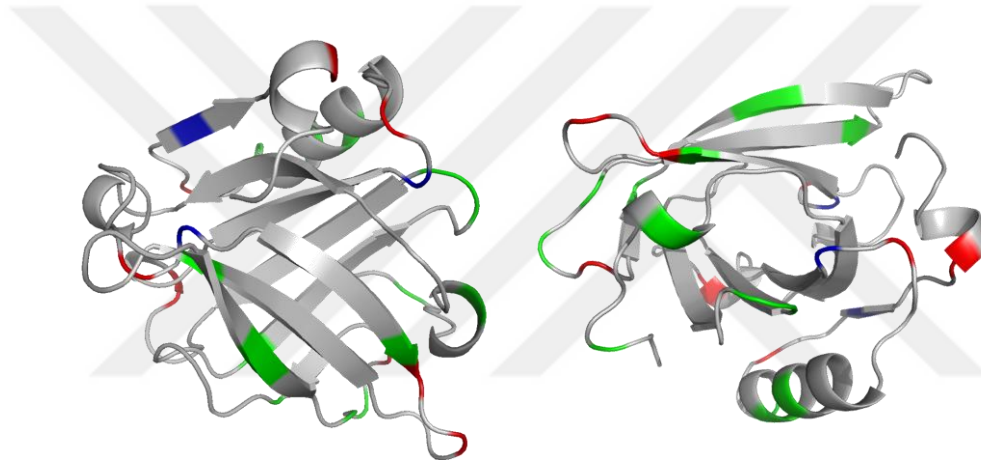


Figure 2.2: 3-D structure of predominantly β -sheet protein β -lactoglobulin seen from different sides, representing residues of arginine (blue), lysine (green) and proline (red).

Interestingly, the quaternary structure of the protein varies among monomers, dimers or oligomers depending on the temperature, pH and ionic strength (Casal *et al.*, 1988). For instance, it exists as a dimer with a concentration above 50 μ M at neutral pH (Hambling *et al.*, 1992). Above pH 6.5 and below pH 3.5, the dimer starts to dissociate into two identical monomers (Townend *et al.*, 1969). Additionally, it is proposed that dimeric lactoglobulin molecules exist in the open conformation at basic pH, whereas they exist in the closed conformation at acidic pH, after undergoing the Tanford transition around neutral pH due to loop movement (residues 85 to 90). Thereby, the hydrophobic pocket becomes inaccessible

at low pH (Vijayalakshmi et al., 2008). This loop acts as a lid which closes the protein interior/binding site below pH 7.3 and opens it at higher pH. The Tanford transition might include structural changes and causes a change in the relative orientation of monomers in the dimer (Sakurai and Goto, 2005; Taulier and Chalikian, 2001; Brownlow et al., 1997).

The monomeric β -Lg has a molecular weight of approximately 18.3 kDa which is made up of 162 amino acids and is stabilized by 2 disulfide bonds (Hambling et al., 1992). Its secondary structure was predicted with an algorithm method to comprise 15% α -helix, 50% β -sheet and 15-20% reverse turn (Creamer et al., 1983). According to the *Protein Data Bank* (2Q2M, PDB), β -Lg consists of 12% α -helix with 4 helices and 40% β -sheet with 10 strands. Additionally, the total number of negatively charged residues (Asp + Glu) is 26 while the total number of positively charged residues (Arg + Lys) is 18. Figure 2.2 shows 3-D structure of β -lactoglobulin. In this work, β -lactoglobulin was selected to study due to its ready availability and its usage in the food products and in the dairy industry.

β -Casein from Bovine Milk (β -CN)

The casein micelles are colloidal complexes of proteins and salts, and their primary biological function is to carry highly insoluble calcium and phosphate to the infants in a liquid form (Swaigood, 1982). The casein micellar structure dissociates into such submicelles in the absence of calcium. The casein amount is about 80% of bovine milk proteins (Creamer et al., 1982) and they have four major protein species, termed α_{s1} -, α_{s2} -, β - and κ -caseins. All caseins have little secondary structure to unfold. The bending of casein protein chains due to their high number of proline residues prevents the formation of close-packed, ordered secondary structure. Also, a tertiary structure of caseins is absent, because they do not contain disulfide bonds. Therefore, there is a considerable exposure of hydrophobic residues.

In this study, we are interested in β -casein protein (β -CN) due to its commercial availability and its industrial significance. β -CN is the most hydrophobic one within the group of caseins, which constitutes about 45% of the casein of bovine milk (Farrell et al., 2004). It is a 24 kDa single polypeptide chain which consists of 209 amino acids (Grosclaude et al., 1973) and it is a proline-rich (35 residues) protein. It is considered to have a little native secondary structure. β -casein does not have any cysteine residues. Particularly, β -casein has a

hydrophobic C-terminal region and a highly charged N-terminal region which contains hydrophilic amino acid residues within the first 50 residues, providing the net charge of -12 at neutral pH (Holt, 1992). The 3-D structure of β -CN from X-ray has not yet been presented; however, a computational 3-D model has been performed using a molecular modelling and secondary structure prediction algorithms (Kumosinski et al., 1993b). This model showed a loosely packed asymmetrical structure that has hydrophobic side chains which spread over C terminal and hydrophilic side chains at the N terminal. The hydrophilic part also has a large loop which can be easily exposed by water.

2.1.3 Enzymes

Trypsin from Bovine Pancreas

Trypsin is a single polypeptide chain with a molecular weight of approximately 23.8 kDa and consists of 223 amino acid residues. It is a member of the serine protease family, found in the digestive system, where it breaks up proteins into small fragments (Rawling and Barret, 1994; Walsh and Wilcox, 1970). Therefore, it is utilized for a great many of biotechnological processes. Trypsin has a catalytic triad (see fig. 1.8a) as active site, which involves histidine (base), serine (nucleophile) and aspartate (electrophile) amino acids residues (Polgar, 2005). These residues generate a charge relay which serves to make the active site serine nucleophilic. This is achieved by modifying the electrostatic environment of the serine. The aspartate in the catalytic triad is responsible for attracting and stabilizing positively charged lysine and arginine, and therefore this mechanism provides the specificity feature of the enzyme (Stryer, 1998). Trypsin predominantly cleaves peptides on the C-terminal of lysine and arginine amino acid residues (Walsh and Wilcox, 1970) unless they are followed by proline residues (see fig. 1.8b). Furthermore, if an acidic residue is on either side of the cleavage site, the rate of hydrolysis is slower. Trypsin has an optimal pH value about 8 and its optimum temperature value is about 37 °C (Alistair et al., 2000; Walsh and Wilcox, 1970).

α -Chymotrypsin from Bovine Pancreas

Like trypsin, chymotrypsin is a digestive enzyme from the protease family that can carry out proteolysis. Chymotrypsin, a 25-kDa enzyme, composed of three polypeptide chains connected by two disulfide bond also has a catalytic triad within its active site (see fig. 1.8a) which involves serine195-histidine57-aspartate102 residues (Fig. 2.3 and 2.4). The amino acid sequence around serine is the same for both enzymes. Chymotrypsin and trypsin have a similar amino acid sequence (about 40%) and have very similar 3-D structures, although their substrate specificities are different. Chymotrypsin has the preference to cut on the carboxyl end of peptide bonds with aromatic and large hydrophobic residues (tyrosine tryptophan, phenylalanine, methionine and leucine, unless followed by a proline residue) which fit into a hydrophobic pocket in the enzyme (Stryer, 1998). In other words, chymotrypsin attacks with serine residue, a strong nucleophile found in the enzyme's active site that it is bonded covalently to the substrate, generating an enzyme-substrate intermediate. Like trypsin, it is also sensitive to pH and temperature changes. For both enzymes, it is proposed that the autolysis (self-digestion) proceed with the highest velocity at pH 8 (Simon et al., 2001).

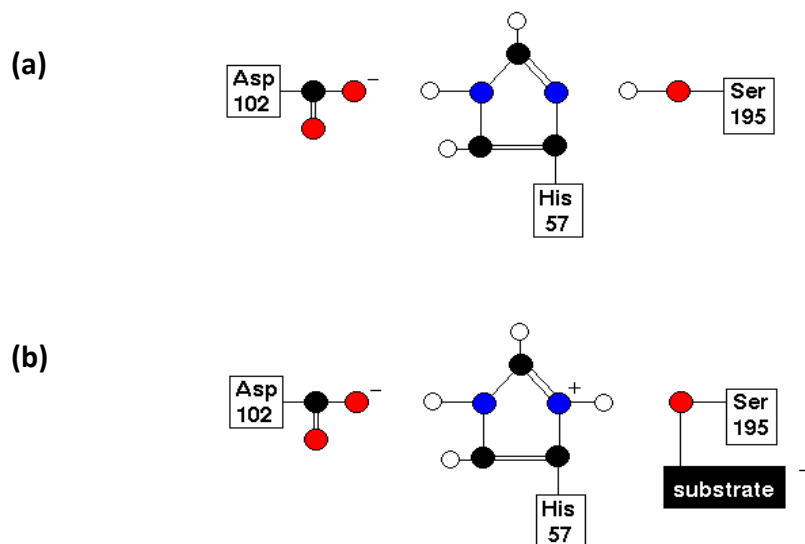


Figure 2.3: The role of catalytic triad in Chymotrypsin. (a) Enzyme alone; (b) a proton is transferred from serine195 to histidine57 upon addition of substrate; thereby a positively charged imidazole ring is oriented stabilized by electrostatic interaction with negatively charged aspartate102.

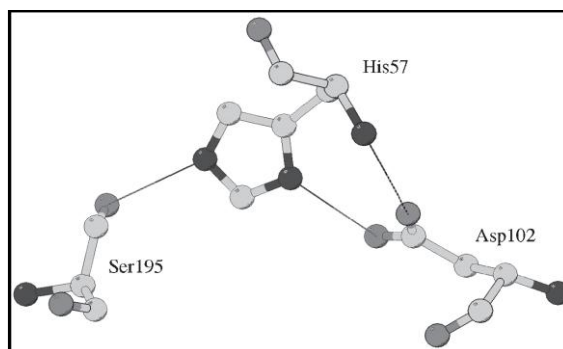


Figure 2.4: The catalytic triad of chymotrypsin (from reference Polgar, 2005; PDB code: 4CHA)

Catalytic Mechanism and Enzyme Kinetics

Serine proteases are secreted by the pancreas as inactive precursors (zymogens) that become activated by cleavage of a single peptide bond. Although these enzymes have nearly identical catalytic mechanisms, their substrate specificity is different. According to X-ray studies, these different specificities are because of quite small structural differences in the binding site (Stryer, 1998) and the active site location within the protease structure. The active site is generally found in the cavity of the protein. In this respect, a nonpolar pocket in the chymotrypsin serves as a niche for the aromatic or bulky nonpolar residues. However, in trypsin, a serine residue is replaced by an aspartate at the bottom of this pocket (Fig. 2.5). This negatively charged aspartate of trypsin generates electrostatic interactions with a positively charged lysine or arginine side chain of a substrate. The catalytic mechanism of serine peptidases is based on a nucleophile, a general base and an acid.

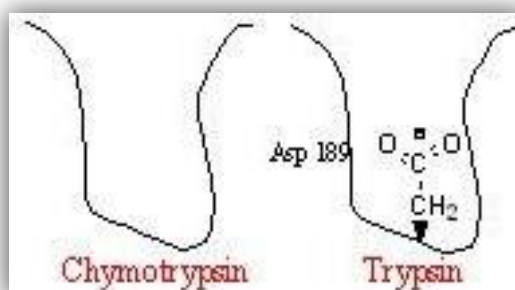


Figure 2.5: A simple representation of part of the substrate binding site in trypsin and chymotrypsin (reproduced from http://en.wikibooks.org/wiki/Structural_Biochemistry).

As it was maintained in the previous section (1.1.3 Enzymes), the enzyme's active site is in a unique three dimensional cleft with a unique pattern of electrostatic charge and hydrophobicity. Enzyme and its specific substrate combine temporarily at this site, establishing an enzyme-substrate complex, ESC (Fig. 1.7). After the reaction has happened, the product of the reaction departs from the enzyme without binding. Then the enzyme is capable of repeating the reaction. Precisely, the verification of how enzymes bind to substrates and convert them into products is described by the enzyme kinetic theory which utilizes basically the Michaelis-Menten equation. Taking the reaction mechanism in figure 1.7 into consideration, Michaelis-Menten equation is defined as

$$V_0 = \frac{V_{\max} [S]}{K_m + [S]} \quad (\text{Eq. 2.1})$$

where V_0 is the initial reaction velocity (the reaction rate), $V_{\max}$ is the maximum reaction rate, $[S]$ is substrate concentration and K_m is Michaelis-Menten constant which represents the substrate concentration when the reaction velocity is one half of the maximal velocity for the reaction (Fig. 2.6). This equation relates the initial reaction rate and substrate concentration and describes the rates of irreversible reactions.

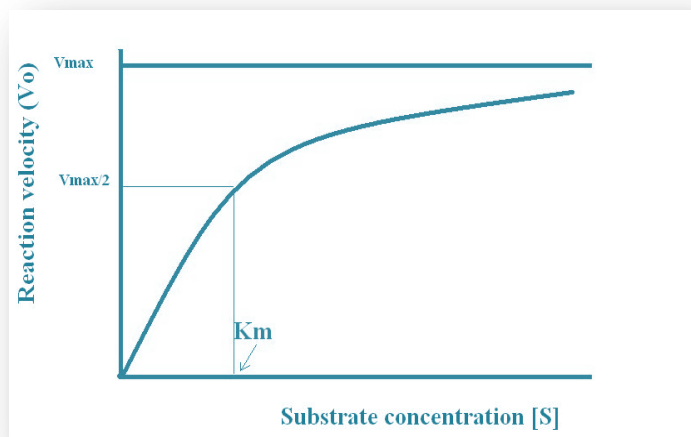


Figure 2.6: Michaelis-Menten plot relates the reaction rate V_0 to the substrate concentration $[S]$.

To determine the constants easier from the measured data, equation 2.1 can be also rewritten taking the reciprocal as shown equation 2.2 below.

$$\frac{1}{V_0} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (\text{Eq. 2.2})$$

There are several possible cases that equation 2.1 can be interpreted as:

- If substrate concentration is large compared to K_m ($[S] \gg K_m$), then the product formation rate becomes $V_{\max}$ and only depends on the enzyme concentration. If that is in the constant enzyme concentration, then the active sites of enzyme on the protein will be occupied as the reaction is proceeding. The enzymatic reaction is completed when the all active sites have been occupied.
- If substrate concentration is equal to K_m ($[S] = K_m$), then the product formation rate becomes $\frac{1}{2}V_{\max}$ and depends on the enzyme concentration.
- If substrate concentration is small compared to K_m ($[S] \ll K_m$), then the product formation rate becomes $\frac{V_{\max}[S]}{K_m}$ depends on the enzyme concentration as well as substrate concentration, forming very little enzyme-substrate complex.

2.2 METHODS

2.2.1 Sample Preparation

β -Lactoglobulins A & B $\geq$ 90 % (Sigma, L3908) and β -Casein $\geq$ 90 % (Sigma, C6905) from Bovine Milk; albumin from bovine serum $\geq$ 98 % (Sigma-Aldrich, A7906); the enzymes, trypsin 12500 units/mg (Sigma, T1426) and α -chymotrypsin 59 units/mg (Fluka, 27270) from bovine pancreas, were used without further treatment.

Solutions of all proteins were prepared by dissolving the lyophilized powder in 10 mM potassium phosphate buffer with H₂O at pH 8 and with D₂O at pD 8.4 (pD = 0.4 + pH) (Glasoe and Long, 1960). Deuterated samples equilibrated for 3 hours in D₂O buffer at +4⁰C. Enzymes were also prepared under the same conditions. Proteins (BSA, β -Lactoglobulin and β -Casein) to be used as substrates were adjusted to a concentration 50 mg/ml and the enzymes were 0.1 mg/ml, 0.25 mg/ml, 1 mg/ml and 5 mg/ml, respectively. Both substrate and enzyme were mixed at equal amount of volume so that their concentration would be at the desired ratios. Substrate to enzyme ratio was 500:1, 200:1, 50:1 or 10:1 (w/w). Also, blank solutions of substrates of 25 mg/ml and 50 mg/ml were prepared in the same way both in H₂O and D₂O buffers without enzyme addition. All solutions were used on the day that they were prepared.

2.2.2 Spectroscopic Techniques

In the present study, FTIR-Transmission spectroscopy and Circular Dichroism spectroscopy have been used as instruments. The same methods of sample-preparation, explained above, were carried out for both techniques.

FTIR-Transmission Spectroscopy

The IR-spectra were recorded with a Bruker VECTOR 22 FTIR spectrometer (Bruker, Germany) equipped with a DGTS detector.

The measurements were performed in a transmission mode using a demountable thin layer IR-cell with calcium fluoride (CaF₂) windows developed at our institute (Fabian and Măntele, 2002). In this study, 2.5 μ l of protein blank solution and protein-enzyme mixture were sandwiched between two CaF₂ windows with an optical path length of 9.2 or 9.6 μ m. These CaF₂ windows are transparent in the mid-IR region. Before sealing the cuvette, the outer ring of one of the windows was diligently coated by using approximately 2 μ l of silicon-oil to

prevent loss of sample by evaporation upon heating. The sample was mounted in a home built motor driven shuttle that provides recording of the background directly just before recording of each sample spectrum, taking the air spectrum as reference. In that way, water vapour contributions to the spectra are suppressed because the chamber is not opened for each spectrum. The sample temperature was controlled with a circulation water system through the cell holder and taken constant at 37 °C for all measurements. Temperature was controlled using a thermocouple sensor which was connected to the Haake C25 water-bath. After adjusting the temperature to 37 °C and waiting for the chamber to dry, spectra were recorded from 5 min to 5 h at various times. Each spectrum was recorded with 128 interferometer scans and a spectral resolution of 4 cm⁻¹. Besides, IR spectra of the buffer solution and blank enzyme solution were measured under the identical conditions. The spectra processing, visualization, and second derivative and difference spectrum calculations were mathematically performed by using the spectrometer OPUS software 4.2 (Bruker, Germany).

H₂O/D₂O Buffer Absorption

In this work, IR experiments of proteins were carried out in aqueous solutions of H₂O and D₂O (Deuterium oxide, heavy water or ²H₂O). It is clear that infrared studies of proteins in water solution are more complicated than in deuterium oxide solution. Water has strong absorption around 3400 cm⁻¹ due to stretching vibrations and around 2200 cm⁻¹ because of combination band of OH bending mode and vibration mode of associated water molecules (Figure 2.7). The H-O-H bending vibration of H₂O absorbs very strongly near 1640 cm⁻¹ and overlaps with the amide I region that it is the most useful region for the secondary structure determination. This can impair the analysis and interpretation of a spectrum. In order to overcome this problem, we have also used heavy water that leads to the isotope shifting upon ¹H₂ → ²H₂ substitution. In D₂O solution, amide hydrogen atoms in the peptide bonds of proteins are replaced by deuterium atoms by means of H/D exchange. The exchangeable hydrogens of proteins include the main-chain peptide group NH and the side-chain protons bound to N, O and S atoms of polar groups. In this respect, overlapping bands in ¹H₂O are greatly reduced in ²H₂O (D₂O). The ¹H₂O/²H₂O exchange causes small band shifts of the components in amide I region due to small contribution of N-H bending vibration. Also, the intensity of the amide II region (1480-1580 cm⁻¹) decreases to the lower wave numbers of

amide II' region ($1490\text{--}1450\text{ cm}^{-1}$) upon H/D exchange. Therefore, this brings out the side chain vibration bands of the protein such as tyrosine, arginine, aspartate, glutamate in the range of $1490\text{--}1614\text{ cm}^{-1}$. Additionally, amide II' band overlaps with HOD mode.

H/D exchange also allows a relatively low water absorbance between 1400 and 1800 cm^{-1} so that weak IR bands of proteins could be clearly observed. This shifting becomes due to exchange of protons with deuterons.

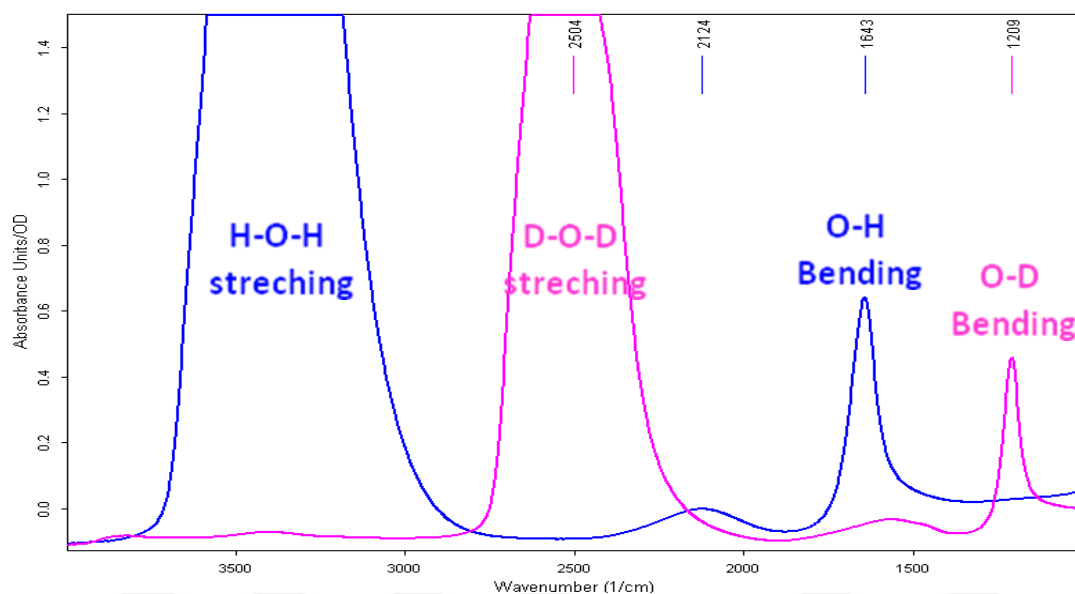


Figure 2.7: FTIR-Transmission spectra of H_2O (red) and D_2O (blue).

Buffer Subtraction from the Sample Spectra

In order to obtain the spectrum of proteins, if necessary, water subtraction was mathematically performed by taking the $1900\text{--}2400\text{ cm}^{-1}$ region as reference. It should be a straight line between these wave numbers for a perfect subtraction (Fabian and Mäntele, 2002). Besides, a low frequency band around 1210 cm^{-1} , originating from D-O-D bending, was taken into account for D_2O buffer subtraction. Precisely, to obtain an appropriate subtraction, the spectrum of the buffer was recorded under the same conditions with those of the sample spectrum such as number of scans, resolution, temperature, pH and ionic strength. Figure 2.8 shows the absorbance spectrum and second derivative spectrum of $\beta\text{-Lg}$ protein after subtraction of overlapping H_2O absorption. The second derivative spectrum needs to be smoothed in order to reduce the noise formed by derivatization. In this respect, a smoothing factor of 9 means that 9 nearby data points are averaged. This reduces the noise contribution in the resultant spectrum.

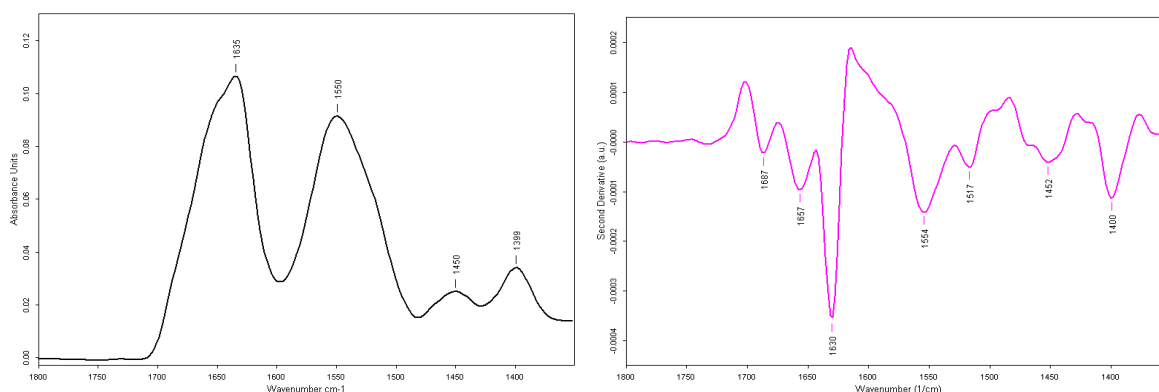


Figure 2.8: Left: FTIR absorbance spectra showing the amide I and amide II bands of β -lactoglobulin in H_2O buffer obtained after subtraction of the H_2O buffer absorption. β -Lg is a predominantly β -sheet protein.

Right: Second derivative spectrum of the absorbance spectrum calculated with a smoothing factor of 9 that it reveals the overlapping components in the absorbance spectrum of β -Lg.

Protein Secondary Structure Analysis by FTIR

The amide I region is frequently used to determine the secondary structure of proteins due to its conformation sensitivity. In this study, 2nd or 4th derivative, the Fourier self-deconvolution (FSD) and curve fitting of absorbance spectrum are used within the analysis methods. Both the differentiation and FSD refer to the band narrowing techniques that allow resolving of the overlapping components. Both mathematical procedures do not increase the instrumental resolution, but yield narrower component bands. Before these methods are performed, peaks arising from residual water must be carefully subtracted from the raw spectra.

The second derivative spectrum is the most commonly used mathematical technique which gives a negative peak for every band and shoulder in the absorbance spectrum of a protein. The line width of the second derivative of a band is smaller than that of the original absorbance band. Maxima of an absorbance spectrum are reflected as minima in the second derivative spectrum (Fig. 2.8Left) that gives the positions of the overlapping components. A simultaneous smoothing of the spectrum is necessary to diminish the noise originated by derivatization, averaging nearby data points. In the present study, a smoothing factor of 9 has been used while managing 2nd derivative of spectra. Also in some cases, maxima of 4th derivative have been utilized since it represents a better resolving than 2nd derivative to reveal strongly overlapped components in the amide I and amide II regions.

Fourier self-deconvolution (FSD) is another useful technique in order to resolve overlapping bands (Kauppinen et al., 1981). It reduces the band width of component bands revealing more distinguishable peaks. In principle, the Fourier transform of the original spectrum is multiplied by a line shape dependent function to amplify the amplitude of the spectrum in the desired region. After back transformation from interferogram into a spectrum, the component bands of deconvolved spectrum become sharper while relative ratios of peaks remain the same. As an example, figure 2.9 represents the Fourier self deconvoluted spectrum of β -lactoglobulin in H₂O buffer. FSD involves three independent parameters, i.e. line shape, line width and resolution enhancement factor. The line shape can be either chosen as Lorentzian or Gaussian function. Then, the line width and resolution enhancement factor are scaled to provide a spectrum with maximum resolution. In most cases, FSD and second derivative, which are two equivalent techniques, are used together to get peaks of the components bands for secondary structure analysis.

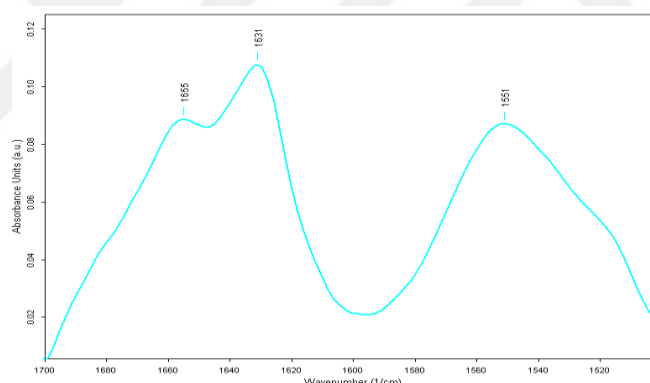


Figure 2.9: Amide I and amide II region of Fourier self deconvoluted spectrum of β -lactoglobulin at pH 8. For deconvolution, a Lorentz band shape was used with a noise reduction factor of 0.5 and with a deconvolution factor of 1500.

Curve fitting is the process of regenerating a measured spectrum by mathematically giving the number of components with their known peak positions obtained from 2nd derivative, 4th derivative or FSD. Curve fitting method is applied to estimate quantitatively the area of each component. In the present study, curve fitting method has been diligently performed using Levenberg-Marquardt algorithm for amide I and amide II region by utilizing the software OPUS 4.2 from Bruker Optik GmbH.

To start the curve fitting, first the number of components with their positions was determined by using band narrowing techniques from the buffer subtracted spectrum. There

are there more crucial parameters to manage the broad envelope band such as width, intensity and shape of the each component. Either Lorentzian or Gaussian function, or convolution of both (Voight function) can be chosen as the shape of the bands. A Lorentz function is defined as,

$$L(x; \gamma) = \frac{1}{\pi \gamma \left[1 + \left(\frac{x - x_0}{\gamma} \right)^2 \right]} \quad (\text{Eq. 2.3})$$

where x_0 represents the position of the peak and γ_0 is the half-width at half-maximum (HWHM). A Gaussian function is defined as,

$$G(x; \sigma) = \frac{1}{\sigma \sqrt{2\pi}} \exp \left(-\frac{(x - \mu)^2}{2\sigma^2} \right) \quad (\text{Eq. 2.4})$$

where μ_0 is the position of the peak and σ_0 is the represents the standard deviation. Additionally, a Voight function which is the convolution of Lorentzian or Gaussian function is defined as,

$$V(x; \sigma; \gamma) = \int_{-\infty}^{\infty} G(x'; \sigma) L(x - x'; \gamma) dx' \quad (\text{Eq. 2.5})$$

where x is the frequency of the line center. After the choice of the shape of bands, the programm changes these parameters until the difference between the original and fitted curve is a minimum. Figure 2.10 shows the amide I and amide II bands of BSA at PH 8 with original spectrum from experiment and regenerated spectrum from curve fitting. Second derivatives of both envelopes have been compared to be sure the accuracy of curve fitting because only the exact numbers and contents of the bands result in the same second derivative profiles. Finally, the fractional areas of the fitted components are used to calculate the percentages of individual bands and then different secondary structure elements (α -helix, β -sheet, turn, and random coils).

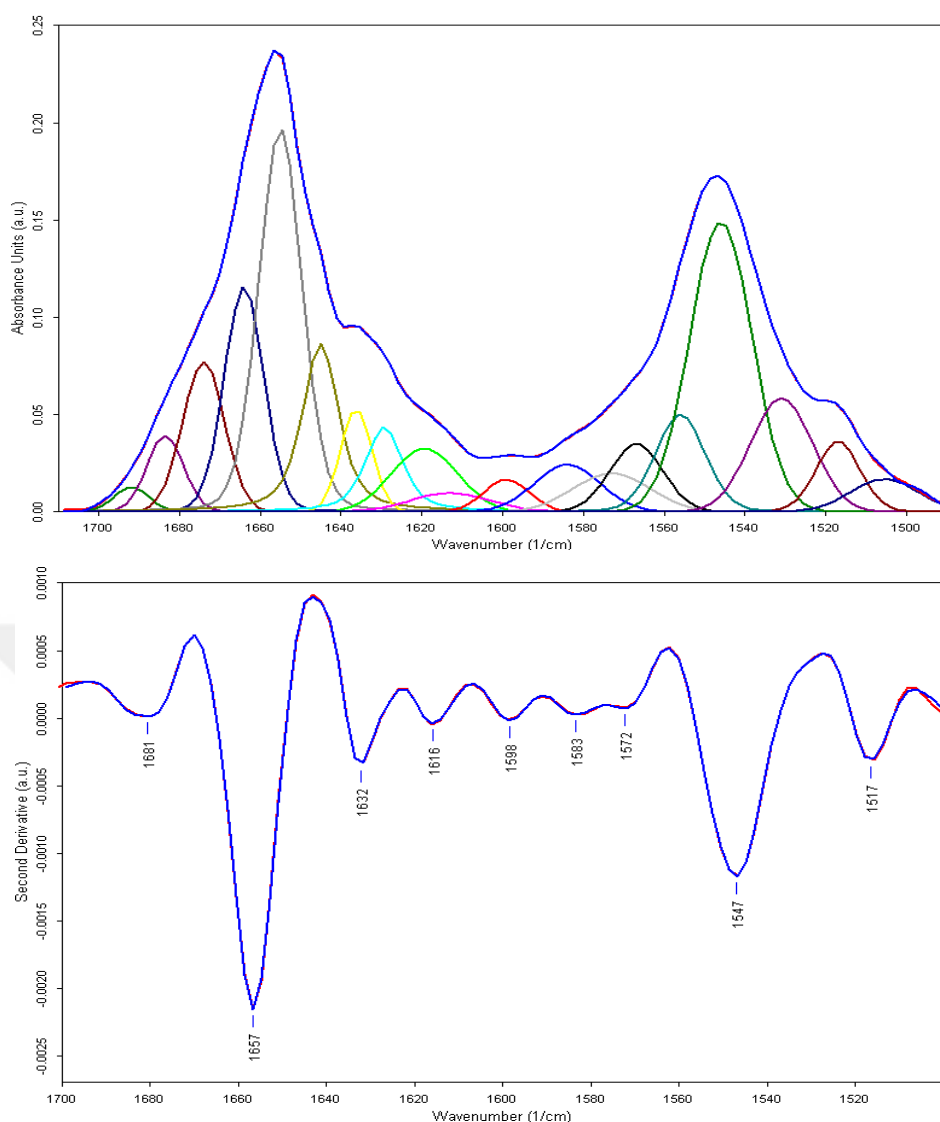


Figure 2.10:

Top: Amide I and amide II bands of BSA after applying Fourier self deconvolution at PH 8 with original (blue) and constructed (red) spectrum from curve fitting.

Bottom: Second derivatives of both envelopes have been compared in order to be sure the accuracy of fitting (residual RMS error: 3.2×10^{-4}).

CD-Spectroscopy

The CD spectra were recorded by using a JASCO J 720 spectropolarimeter in the range of 178-260 nm. The spectrometer was purged with liquid nitrogen at a rate of approximately 7 L/min. For each CD spectrum, the following parameters were used:

- band width: 1 nm
- response time: 1 sec

- resolution: 0.1 nm
- scan speed: 50 nm/min
- number of scans: 8
- pathlength of CaF₂ cell: 8.1 μ m

The same type of CaF₂ cell was used as in FTIR- transmission experiments. Again, before sealing the cuvette, the outer ring of one of the windows was diligently coated by using approximately 2 μ l silicon oil. The spectra were recorded up to 5 hours at various times. Additionally, the CD spectrum of the buffer and blank enzyme were also measured under the same conditions. All measurements were done in ²H₂O buffer; the concentration of both substrate and enzyme were taken the same as in FTIR-transmission measurements so that the results of both techniques can be compared. All data were analyzed using a spectrum analysis program of the instruments software, and also converted into text files to be used by Origin program 5.0.

The sample temperature was kept exactly at 37 °C during all measurements. The sample holder was heated by a circulating water-bath so that one could adjust and control the desired temperature just using the monitor of the water-bath.

Protein Secondary structure analysis by CD

The CD spectrum of a protein in the far UV is one of the most commonly used techniques for determining the protein secondary structure content since this region of the electromagnetic spectrum has a remarkable sensitivity to the backbone conformations of proteins. In this respect, various algorithms have been developed and improved in order to analyse protein secondary structures (Greenfield and Fasman, 1969; Johnson, 1999; Sreerama and Woody, 2000). The estimation of protein secondary structures from CD spectra is based on a linear combination of the spectra of individual secondary structure components.

In this study, the secondary structure analysis of proteins was performed from the CD-spectra by using a reference set of proteins whose 3-D structures are well known from X-ray diffraction studies. For that purpose, we have used three methods, so called CONTINLL, SELCON3, and CDSSTR. CDPro software which involves these programs is available online. The option for a given reference set is selected in the input file and then the program runs

for the analysis of CD spectra with a chosen reference set. Solutions are obtained using the singular value decomposition (SVD) algorithm. Although all three programs utilize different algorithms, their results are comparable. All three methods should be used taking the averages of the results, and a reference set which involves the highest number of proteins should be selected in order to improve the reliability of estimated secondary structural fractions. In addition to this, proteins in the chosen reference set should be measured at lower wavelengths for the analysis of CD spectra of a protein (Sreerama and Woody, 2000). The large protein databases are required for estimation of unknown proteins (Oberg et al., 2004).

The essential point is to select a proper reference set to be performed for analysis. The reference database differs in the number of reference proteins in a different wavelength region. For instance, a reference set of 48 proteins (43 proteins + 5 denaturated proteins) has a wavelength between 190-240 nm. Another reference set has only 29 reference protein between 178-260 nm which is the largest range because the Xenon-light source affects strongly below 180 nm.

According to CONTINLL, SELCON3, and CDSSTR methods, the analysis of a CD spectrum gives six secondary structural elements. These are regular and distorted α -helix, regular and distorted β -sheet, turns and unordered structures. On an average, four residues per α -helix and two residues per β -sheet can be considered as distorted in proteins (Sreerama et al., 1998). In this study, regular and distorted α -helix were summed to get the total α -helix content; regular and distorted β -sheet were also taken together to get total β -sheet content. For final result of the secondary structure content, averages of solutions of all three methods (CONTINLL, SELCON3, and CDSSTR) were taken in the range of 185-240 nm with 42 reference database for soluble proteins. Before running the programs, CD spectra were background corrected with buffer and enzyme spectra and then scaled to molar ellipticity. Additionally, there are a few important parameters before to start secondary structure analysis, i.e. concentration and molecular weight of protein, number of amino acid residues of protein to calculate mean residue weight and path length of used cells.

III. RESULTS AND DISCUSSION

3.1 BOVINE SERUM ALBUMIN (BSA)

Before we discuss the effect of the proteolysis on the BSA, we shall first mention the effects of H/D exchange to the band positions of BSA. Figure 3.1 shows the BSA spectra in H₂O buffer, pH 8 at 37 °C. As seen from the figure, the main component of amide I region is at 1657 cm⁻¹ and of amide II region is at 1547 cm⁻¹ which corresponds to the vibrations of α -helices or unordered structures in H₂O buffer (Fabian and Măntele, 2002; Goormaghtigh et al., 1994).

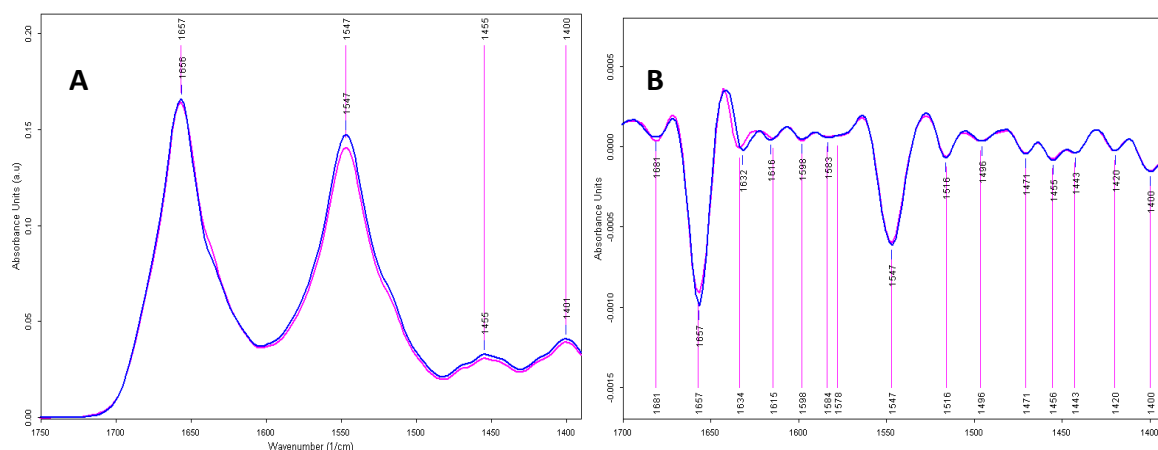


Figure 3.1: (A) The absorbance spectra of BSA (50 mg/ml) in H₂O buffer at pH 8. (B) The second derivatives of the absorbance spectra in (A). Blue colour indicates the spectrum at $t = 15$ min and magenta colour is the spectrum at $t = 5$ h during the incubation at $T = 37$ °C.

In the present study, it has been mainly studied with D₂O buffer in order to overcome the absorbance of the H₂O in the amide I and amide II region. H/D exchange helps to distinguish between the fast-exchanging protons of external amide bonds and the slow-exchanging protons found in the less accessible domains (Gregory and Lumby, 1985). Figure 3.2 A and B represent the BSA spectra in D₂O buffer at pH 8.4, 37 °C. For the deuterated BSA, the absorbance around 1543 cm⁻¹ in the amide II region is shifted to the amide II' region centred at 1454 cm⁻¹ because the N-H bending vibration is replaced by the N-D bending vibration. Moreover, the amide I region shifts towards lower wavenumbers by 2-10 cm⁻¹ upon H/D exchange. In this experiment, after 3 h deuteration time at +4 °C, the band at 1657 cm⁻¹ shifts towards 1654 cm⁻¹ which can be attributed to the solution-accessible α -helices

exposed to the D₂O buffer. The band at 1683 cm⁻¹ in H₂O buffer shifts towards 1679 cm⁻¹ in D₂O buffer. These bands are attributed to the β -turns or β -sheet structures because these structures can overlap (Fabian and Mäntele, 2002; Goormaghtigh et al., 1994). However, in contrast to β -sheet and α -helix structures, the large shifts towards lower wavenumbers are observed for turns in the amide I region. In this respect, the H/D exchange of BSA has been already studied that the 1683 cm⁻¹ band induces the fastest H/D exchange rate (Grdadolnik and Marechal, 2005). Since our experiment was performed at 37 °C, the H/D exchange rate will be faster as a consequence of a more flexible, dynamic conformation and diffusion at 37 °C (faster than +4 °C). A rise in temperature leads to weakening or breaking of the H-bonds found between the various protein groups, and therefore, the protein has more open conformation to the solvent at higher temperatures.

According to the difference spectra (Fig. 3.2C), the negative band indicates a decrease of the high frequency α -helices around 1660 cm⁻¹ while the positive band around 1640 cm⁻¹ appears. Then, less ordered alpha helices around 1660 cm⁻¹ shift further towards 1640 cm⁻¹ which can be attributed to the unordered/extended helices that occur due to the H/D exchange. This shifting is in agreement with the curve fit results (Table 3.1) that 1663 cm⁻¹ band in H₂O buffer disappears after deuteration.

Table 3.1: Assignment of amide I components and percentages of secondary structure elements of BSA in H₂O and D₂O buffers at 37°C (Spectra at t = 15 min).

Position ^a in H ₂ O (cm ⁻¹)	Proportion ^b (%) in H ₂ O	Position ^a in D ₂ O (cm ⁻¹)	Proportion ^b (%) in D ₂ O	Band Assignments
1620	7	1620	11	Extended/ β -sheet
1629	4	1628	7	Extended/ β -sheet
1636	15	1640	28	Disordered/extended α -helices
1655	40	1654	40	α -helix
1663	21	-	-	α -helix/unordered
1675	10	1668	12	Turn
1683	4	1680	3	β -sheet/turn
^a After deconvolution.				
^b Area percentages of secondary structure elements in the amide I region calculated after curve fitting.				

For α -helices, the frequency of amide I depends on the length of the helix. The main frequency of α -helices is found at 1652-1653 cm^{-1} in the amide I region (Nevskaya and Chirgadze, 1976). The extreme frequency of the α -helices locates between 1664 cm^{-1} and 1642 cm^{-1} (Goormaghtigh et al., 1994). For BSA, the band around 1630 cm^{-1} is ascribed to short segment chains connecting α -helical segments or solvated helical structure (Murayama and Tomida, 2004).

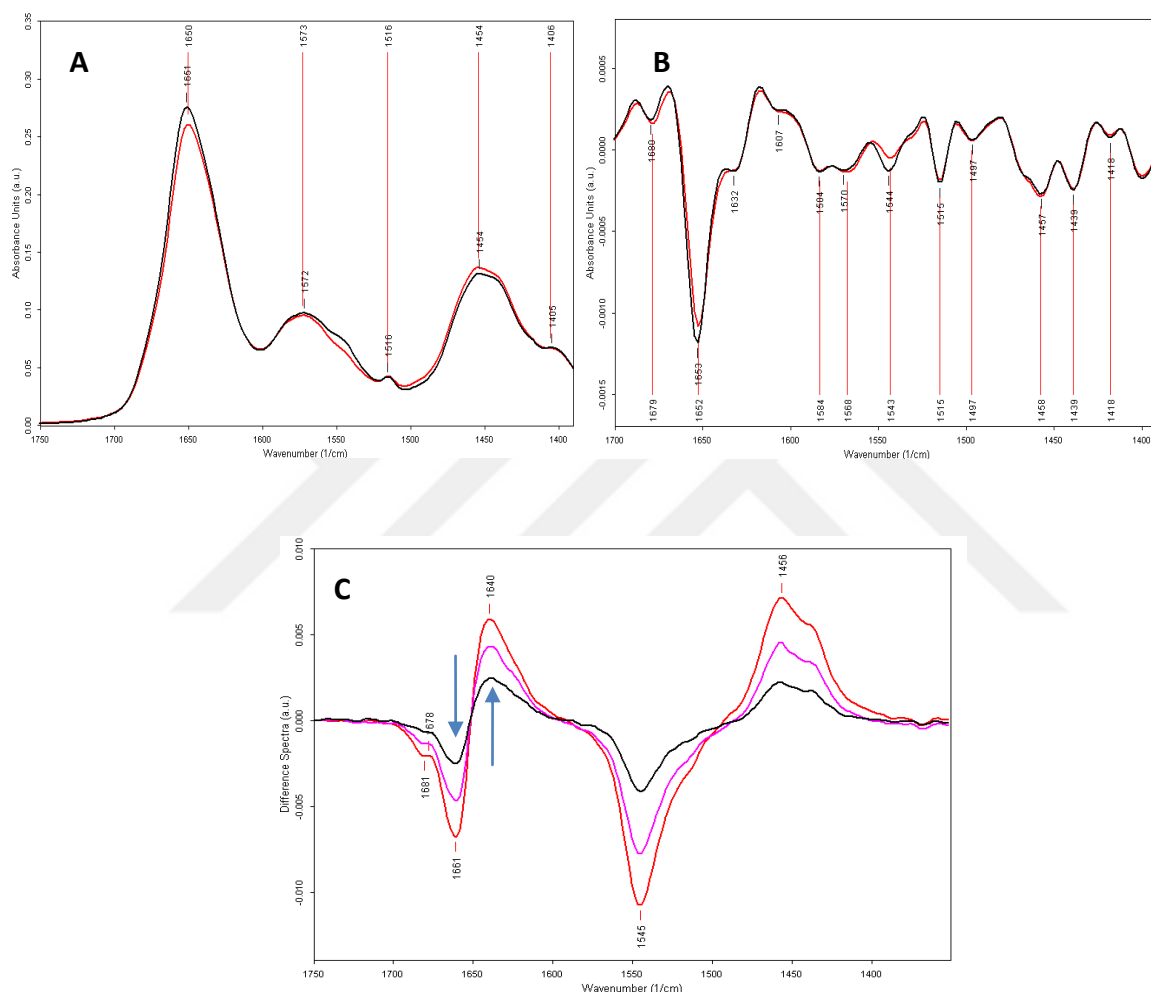


Figure 3.2: (A) The absorbance spectra of BSA (50 mg/ml) in D₂O buffer at PD 8.4, 37 °C. (B) The second derivative spectra of the absorbance spectra in (A). Black colour indicates the spectrum at t = 5 min and red colour represents the spectrum at t = 5 h. (C) Difference spectra of the absorbance spectra in (A). Difference spectra were obtained by subtraction of the first spectrum at t = 5 min from the each of the spectra in (A) taken during the incubation at T = 37 °C. The arrows indicate the amide I and amide II changes as a function of time. (Black colour at t = 1 h; magenta at t = 2 h; red at t = 5 h). To avoid the water vapour absorbance in the spectrum at t = 5 min, it was corrected with water vapour subtraction and smoothed by a factor 9.

We have also performed the temperature dependent experiment to understand the temperature stability of BSA around our working temperature, 37 °C. Figure 3.3 demonstrates the FT-IR spectra of BSA measured over a temperature range of 20-80 °C.

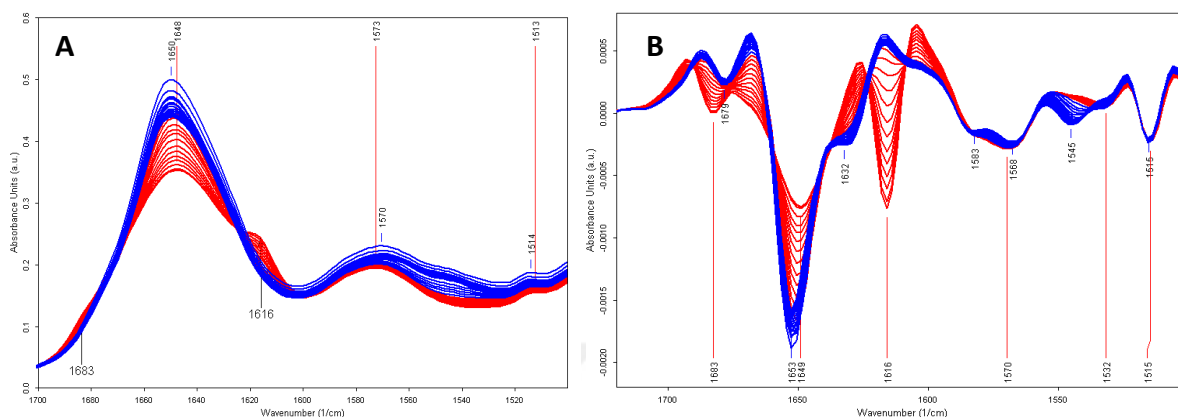


Figure 3.3: (A) The FTIR spectra of BSA collected at increasing temperatures. (B) The second derivatives of the absorbance spectra in (A) with the temperature-dependent peak positions. The spectra are shown in blue for the temperature range between 20 and 56 °C; in red between 58 and 80 °C.

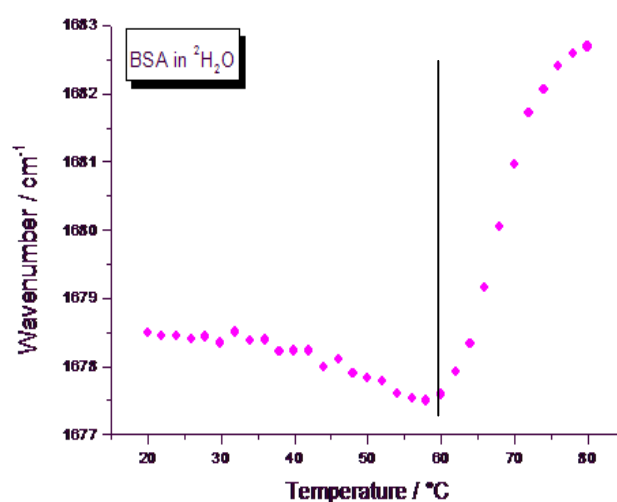


Figure 3.4: Temperature-dependent peak position of 1679 cm⁻¹ which is assign to the turns, from the second derivative profile of BSA. The spectra were recorded every 2 °C between 20 and 80 °C.

It is clear from figure 3.3 that amide I band intensity decreases and two new component increases with rising temperature. These 1683 cm^{-1} and 1616 cm^{-1} bands, which correspond to intermolecular β -sheets, appear due to the aggregation of the protein when the temperature is further increased. Moreover, the amide II band intensity decreases, representing an increase of deuteration and a loss of tertiary structure with higher temperature. According to the peak position profile in figure 3.4, BSA does not denature around $37\text{ }^{\circ}\text{C}$ which is our working temperature; however, the unfolding state progresses above $60\text{ }^{\circ}\text{C}$ and aggregation begins. The transition temperature between the folded and unfolded state is $68.4\text{ }^{\circ}\text{C}$ and the spectral changes happen sharply. Shifting of the peak positions up to $60\text{ }^{\circ}\text{C}$ may occur because of the further H/D exchange of molecules in BSA. Furthermore, it has been recently studied that the intermolecular β -sheet formation of BSA is an irreversible process above $70\text{ }^{\circ}\text{C}$ (Murayama and Tomida, 2004).

3.1.1 FTIR Studies: Proteolysis of BSA by α -Chymotrypsin

FT-IR spectroscopy has been used to characterize the proteolytic digestion of BSA by α -chymotrypsin. The conformational changes during the proteolysis process have been demonstrated below for the various ratios of substrate and enzyme (S:E = 10:1, 50:1, 200:1, 500:1).

BSA/ α -Chymotrypsin Ratio 10:1

Figure 3.5 displays the spectral changes of BSA in D_2O buffer during proteolysis by α -chymotrypsin at a ratio of 10:1 for 5 hours. It is clear from the figure that the most significant spectral changes occur in the amide I and amide II region. Precisely, the amide I region centred at 1654 cm^{-1} decreases and shifts towards lower wavenumbers while a broad band near 1593 cm^{-1} increases for all digestion times, which is attributed to the antisymmetric stretching of free carboxylates (Barth, 2000; Guido et al., 1999; Oomens and Steill, 2008). In other words, the content of the α -helices diminishes strongly whereas the free carboxylate groups of short fragments emerge due to the cleavage of peptide bond. Furthermore, the band around 1638 cm^{-1} and 1640 cm^{-1} broadens due to the effect of the proteolysis, which corresponds to the unordered or bent domains. Moreover, the short helices can show a band near 1640 cm^{-1} (Barth and Zscherp, 2002). This band also became

visible upon H/D exchange for the blank BSA measurement without adding an enzyme. Besides, it is clear from the second derivative spectra at 10:1 ratio (Fig. 3.5B) that the 1679 cm^{-1} band shifts towards 1675 cm^{-1} (turns) and becomes relatively broad. Therefore, this component may be thought as proteolysis product in addition to the free carboxylate groups. Turns are regular structures often exposed to the solvent, therefore, they can be considered as fundamental sites for enzymatic reactions. However, in the amide I region, β -turns can overlap strongly the high frequency component of the β -sheet structure and the α -helix domain. Yet, the contributions of the turns are often assigned at 1680 cm^{-1} (Bandekar and Krimm, 1980).

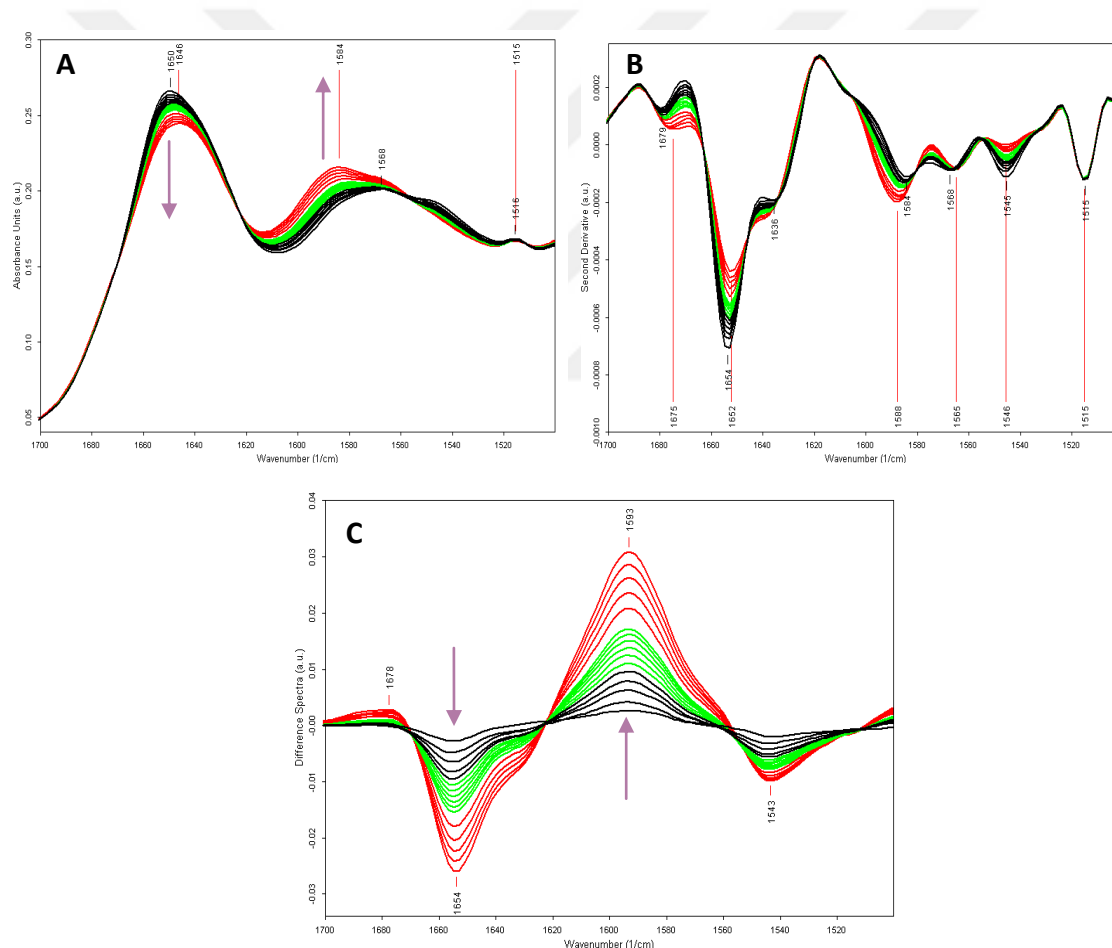


Figure 3.5: (A) The original FTIR spectra of BSA (50 mg/ml) taken during the enzymatic digestion by α -chymotrypsin (5 mg/ml) at a ratio of 10:1, at 37 °C, from 5 min to 5h. (B) The second derivative profiles of the absorbance spectra in (A). (C) Difference spectra of the absorbance spectra in (A). Difference spectra were obtained by subtraction of the first spectrum at $t = 5$ min from each of the spectra in (A) taken when the proteolysis proceeds. The arrows indicate the amide I and amide II changes as a function of time.

The difference spectra (Fig. 3.5C) represent the spectra of removed and produced part of the protein during digestion of BSA by the enzyme. BSA loses its alpha helical domains during digestion (negative band near 1654 cm^{-1}) whereas the free carboxylates increase strongly (positive band near 1593 cm^{-1}). In addition, a shoulder around 1640 cm^{-1} which arises from the disordered structure or short chains of digested polypeptide is clearly noticeable.

Table 3.2 gives the curve fitted secondary structure percentages of BSA during proteolysis by α -chymotrypsin for 5 hours, and figure 3.6A and 3.6B show the curve fitted spectra at $t = 5\text{ h}$. They also confirm that the content of α -helices decreases (1655 cm^{-1}) while turns and unordered structures increase. However, some bands remain still unresolved and overlap although Fourier self deconvolution has been performed. There is a striking decrease of the α -helix component seen in the absorbance, second derivative and difference spectra, yet this component is partially masked by the nearby random coil components in the curve fit results.

Table 3. 2: Secondary structure percentages of BSA during proteolysis by α -chymotrypsin.

	BSA	BSA + α -Chymotrypsin (10:1 ratio)	
Amide I components	5 h	3h	5 h
1614 cm^{-1} (intermolecular β -sheet)	-	1	6
$1628\text{-}1626\text{ cm}^{-1}$ (β -sheet/extended)	18	15	11
$1639\text{-}1636\text{ cm}^{-1}$ (Disordered/extended α -helices)	22	21	17
1645 cm^{-1} (α -helix)	-	17	19
$1653\text{-}1652\text{ cm}^{-1}$ (α -helix)	50	19	13
1660 cm^{-1} (Disordered/turn)	-	11	16
$1667\text{-}1666\text{ cm}^{-1}$ (Turn)	5	7	-
$1676\text{-}1672\text{ cm}^{-1}$ (Turn)	6	7	17
1682 cm^{-1} (β -sheet)	-	2	-
Band narrowing techniques were used to determine the positions. Fourier self deconvolution was applied using Lorentzian band shape with 0.5 noise reduction factor. Deconvolution factor was chosen according to the individual spectrum.			

In figure 3.6 A and B, there is a strong absorbance reduction of the amide I region centred at 1653 cm^{-1} while a broad band near 1592 cm^{-1} increases which is attributed to the antisymmetric stretching of free carboxylates of short fragments arises due to the digestion.

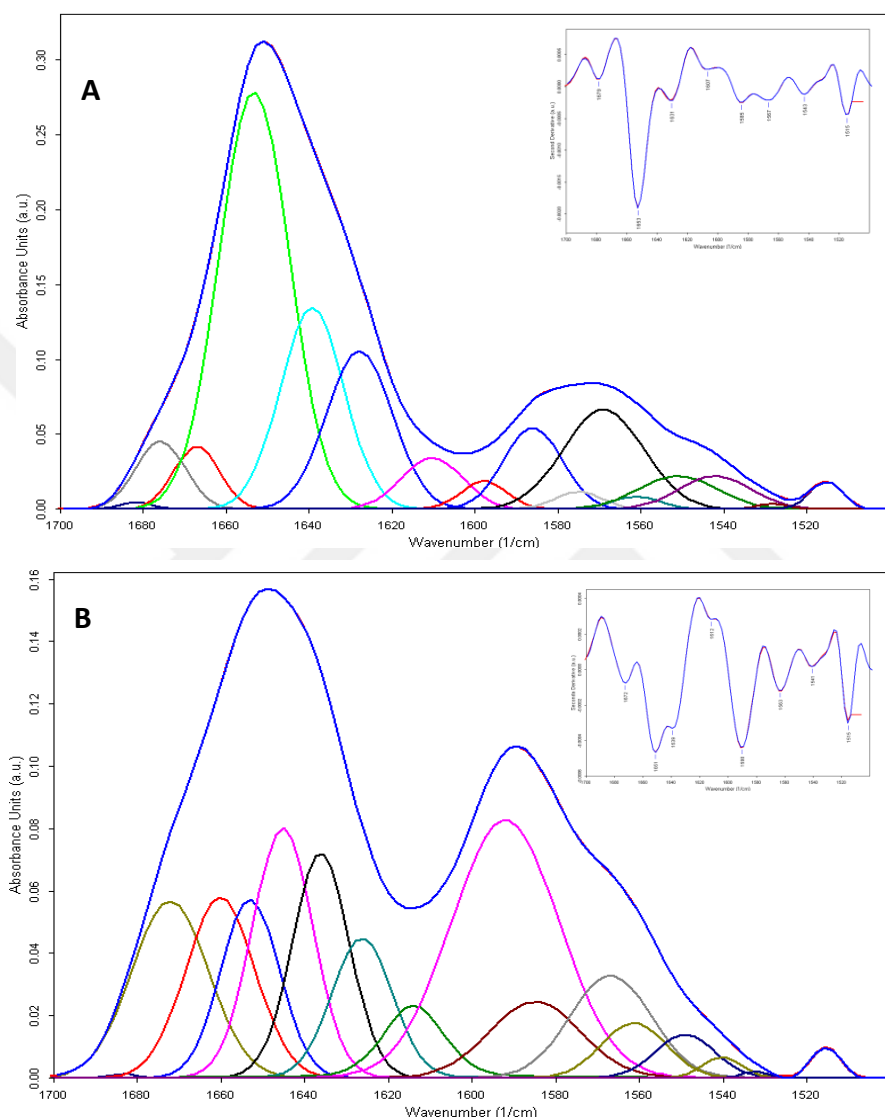


Figure 3.6: Curve fit analysis after the band narrowing techniques for amide I and amide II regions at $t = 5\text{ h}$. **(A)** Blank BSA (50 mg/ml). **(B)** Digested BSA ($10/1$ ratio (w/w)). Both envelope bands formed by the fitted bands are shown in red and the original spectra are shown in blue. Insets are the second derivatives of the original spectra and the fitted envelope in order to compare the success of the curve fit. Before curve fitting, buffer was subtracted from the blank BSA, and enzyme (5 mg/ml) was subtracted from the digested BSA.

BSA/ α -Chymotrypsin Ratio 50:1

Figure 3.7 represents the FTIR spectra of the digested BSA by α -chymotrypsin for 5 h at a ratio of 50:1. It is obvious from the figure that the spectral changes due to proteolysis take place in the amide I and amide II regions, in a same manner with the case of 10:1 ratio. In this respect, the antisymmetric stretching of free carboxylates around 1593 cm^{-1} increases during digestion while alpha helices diminish and shift towards lower wavenumbers. In contrast to the 10:1 ratio, the proteolysis at 50:1 ratio leads to relatively less effect to the spectral changes and degrading of the protein. The amide modes around 1675 cm^{-1} ascribed to the turns becomes broader as also seen at 10:1 ratio. According to the difference spectra (Fig. 3.7C), a band near $1636\text{--}1640\text{ cm}^{-1}$ also increases during digestion, suggesting an increment in the unordered structures/short helices.

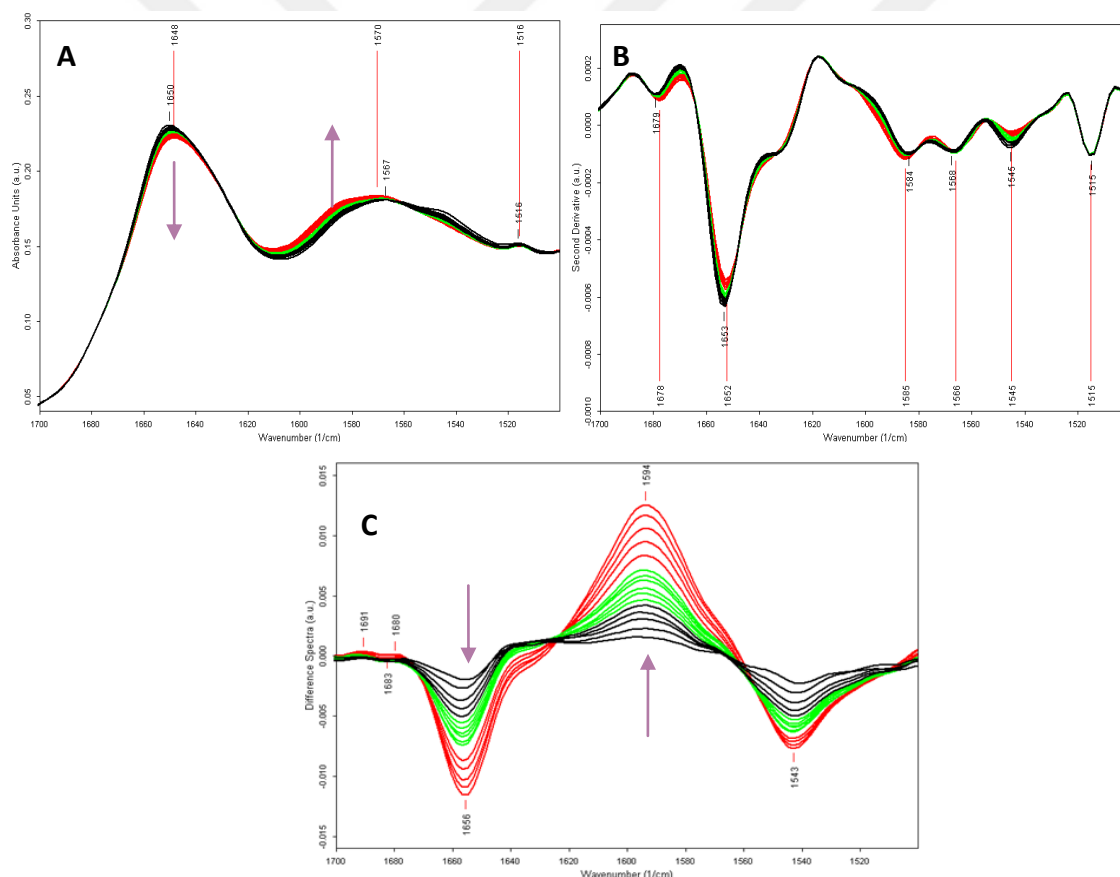


Figure 3.7: (A) The original FT-IR spectra of BSA taken during digestion by α -chymotrypsin at a ratio of 50:1, at $37\text{ }^{\circ}\text{C}$ from 5 min to 5h. (B) The second derivative spectra of the absorbance spectra in (A). (C) Difference spectra of the absorbance spectra in (A). Difference spectra were obtained by the subtraction of first spectrum at $t = 5\text{ min}$ from each of the spectra in (A) taken during proteolysis. The arrows indicate the amide I and amide II intensity changes as a function of time.

BSA/ α -Chymotrypsin Ratio 200:1 and 500:1

The spectral changes due to proteolysis for the 200:1 and 500:1 ratios are much lower than the other ratios described above (Fig. 3.8). These spectra have similar but less detectable changes when they are compared to the 10:1 and 50:1 ratios. Besides, there are some small changes for the 200:1 ratio between 1600 cm^{-1} and 1585 cm^{-1} (Fig. 3.9) which corresponds to the side chains and free carboxylates (Barth, 2000). Such a spectral change has not been observed for the blank BSA (Fig. 3.2).

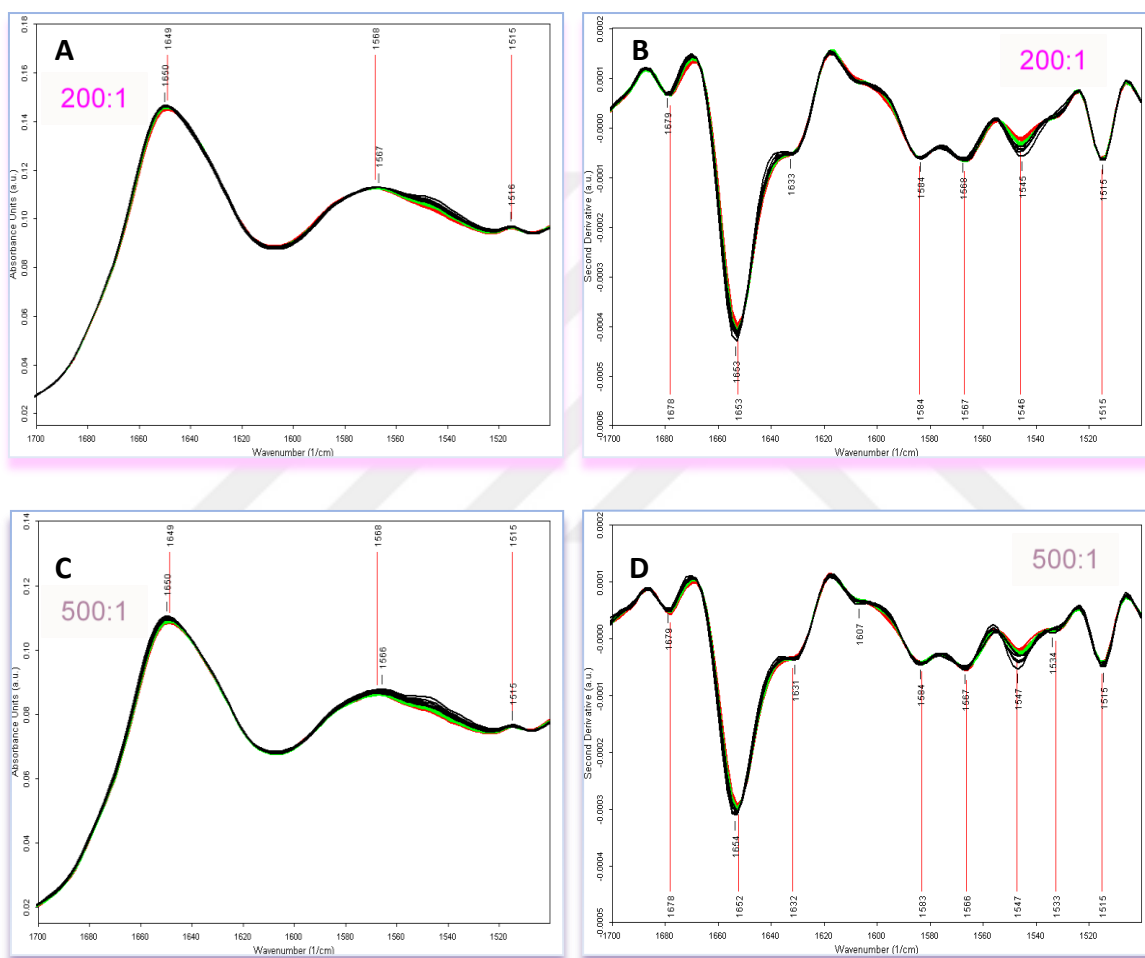


Figure 3. 8: (A) and (C) are the original FT-IR spectra of BSA taken during digestion by α -chymotrypsin at a ratio of 200:1 (0.25 mg/ml enzyme) and 500:1 (0.1 mg/ml enzyme), respectively, at 37°C from 5 min to 5h. (B) and (D) are the second derivatives of the absorbance spectra in (A) and (C), respectively.

It is obvious that the low concentrations of the enzyme are not enough to degrade the whole BSA because the active sites of the enzyme has already occupied as the enzymatic reaction proceeding. As it was mentioned in the materials and methods section (2.3.1), α -

chymotrypsin cleaves on the carboxyl end of peptide bonds with aromatic and large hydrophobic residues (tyrosine tryptophan, phenylalanine, methionine and leucine, unless followed by a proline residue). When the enzyme concentration is large at constant substrate concentration (50 mg/ml), there will be more noteworthy structural changes. It is clear from the difference spectra that the same component bands are considerably shifted for all ratios of substrate and enzyme, but the strength of the shifting differs on enzyme concentration (Fig. 3.9Top). The biggest spectral changes for the amide bands are observed for a 10:1 ratio while for the 500:1 ratio there is no significant change on BSA structure.

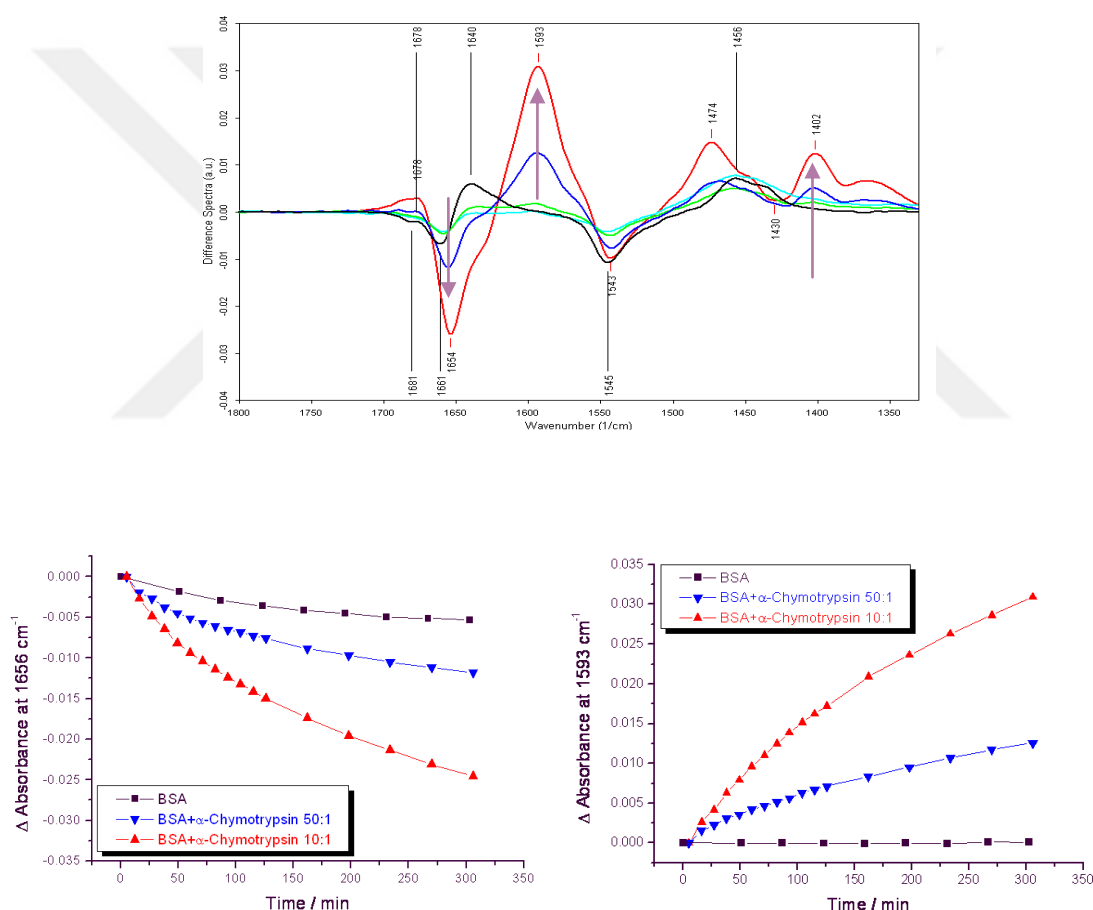


Figure 3.9: Top: The difference spectra of BSA and α -chymotrypsin for 10:1 (red); for 50:1 (blue); for 200:1 (green) and for 500:1 (cyan) ratios. Difference spectra were obtained by the subtraction of the first spectrum at $t = 5$ min from the last spectrum at $t = 5$ h.

Bottom: The intensity changes of α -helix content (1656 cm^{-1}) and of antisymmetric stretching of free carboxylates content (1593 cm^{-1}) as a function of time during proteolysis with various ratios. Intensities were obtained from the difference spectra in fig. 3.5C and fig. 3.7C.

The enzyme induces a decrease of the α -helix content while it causes an increase of the 1593 cm^{-1} and 1405 cm^{-1} bands as the product of the digestion process, which are attributed to the antisymmetric and symmetric stretching of free carboxylates, respectively. For the all ratios of substrate and enzyme, there is a strong absorbance reduction of the amide I region and an increase of the amide II region during digestion. In all cases, there is no considerable aggregation as the proteolysis proceeds.

Figure 3.9 (Bottom) obtained from difference spectra illustrates the intensity changes of BSA with various ratios of substrate and enzyme during proteolysis. It reveals that when the enzyme concentration is increased at a constant substrate concentration, trypsin causes faster and more degraded BSA. The α -helix intensity at 1656 cm^{-1} for blank BSA diminishes during incubation at 37 °C because this band shifts towards lower wavenumbers due to the further H/D exchange. However, such a decrease in α -helices is considerably slower than the digested BSA. This indicates that such intensity changes occur only because of H/D exchange for the blank BSA. The H/D exchange also affects the intensity of the alpha helix of the digested BSA but the intensity changes for 10:1 and 50:1 ratios are significantly much larger than for blank BSA. Then, such a major difference is the evidence of the enzymatic attack. Since BSA is a protein rich in alpha helices, it loses mainly this component during enzymatic digestion. Moreover, the 1593 cm^{-1} band does not increase for the blank BSA while it rises strongly for the digested BSA that it refers to the proteolysis products.

3.1.2 FTIR Studies: Proteolysis of BSA by Trypsin

BSA/Trypsin Ratio 10:1

Figure 3.10 shows the amide I and amide II intensity changes of BSA (50 mg/ml) occurring during proteolysis by trypsin (5 mg/ml) for 5 hours. The intensity of the band at 1654 cm^{-1} attributed to the alpha helix structure is drastically reduced over time and at the same time the peak shifts to lower wavenumbers (1652 cm^{-1}), while the intensity of the carboxylate groups (1594 cm^{-1}) increases, as in the case of proteolysis with α -chymotrypsin. There are similar but less spectral changes of BSA for the trypsin attack than for the α -chymotrypsin although the ratio is the same. Because trypsin predominantly cleaves peptides on the C-terminal of lysine and arginine amino acid residues which are polar groups except if followed by the proline residue. The difference spectra (Fig. 3.10C) reveal that a positive intensity

change near $1636\text{--}1640\text{ cm}^{-1}$ also appears as a shoulder during digestion. Additionally, a band around 1678 cm^{-1} shows small detectable changes.

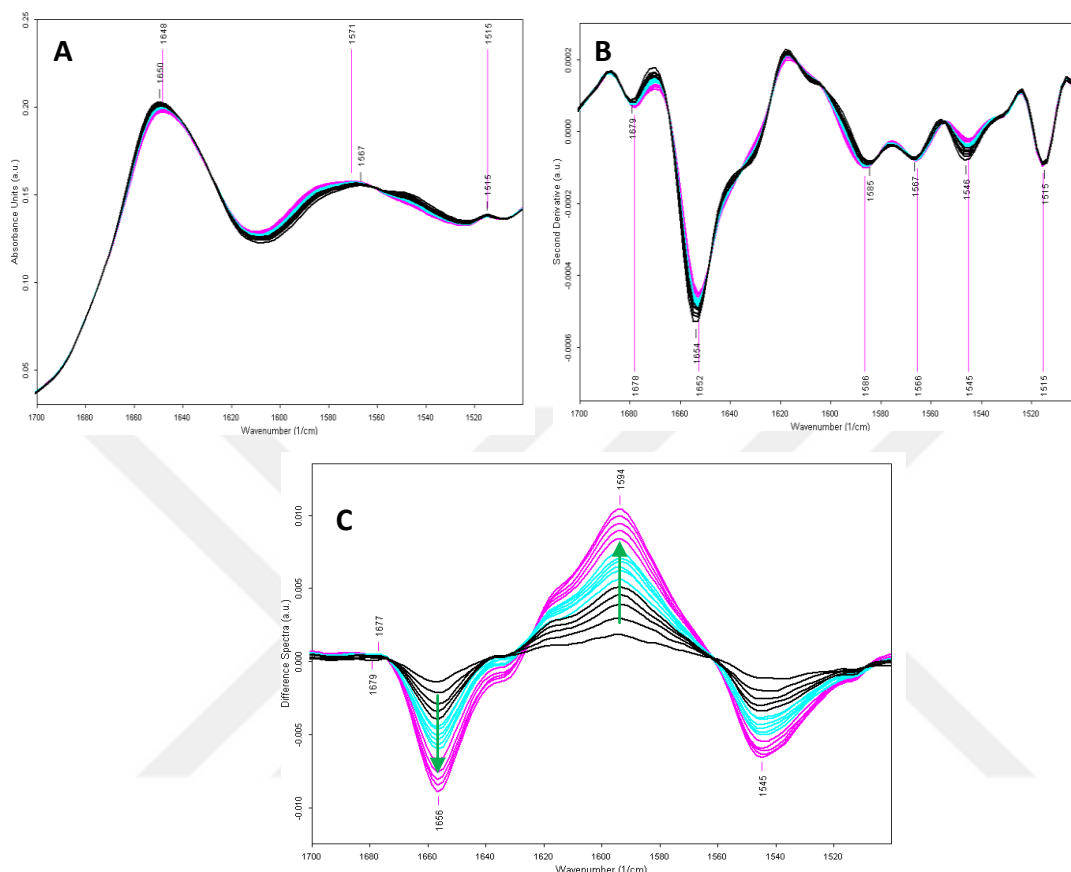


Figure 3.10: (A) The original FT-IR spectra of BSA taken during digestion by trypsin with a ratio of 10:1, at $37\text{ }^{\circ}\text{C}$ from 5 min to 5h. (B) The second derivative profiles of the absorbance spectra in (A). (C) The difference spectra of the absorbance spectra in (A).

BSA/Trypsin Ratio 50:1

The intensity changes of BSA (50 mg/ml) due to proteolysis by trypsin (1 mg/ml) for the 50:1 ratio are much lower than for the 10:1 ratio described above (Fig. 3.11). However, the same characteristic bands show a similar tendency: the intensity of alpha helices (1654 cm^{-1}) diminishes, converting into the short fragments (1640 cm^{-1}) and into the products (1593 cm^{-1}) as a result of the breakdown of BSA by trypsin. Besides, the intensity around the 1545 cm^{-1} band decreases for all ratios of substrate and enzyme as the proteolysis proceeds, indicating

an increase of deuteration, because more residues become accessible to the D₂O and the N-H bending vibration is replaced by the N-D bending vibration as an evidence of the digestion.

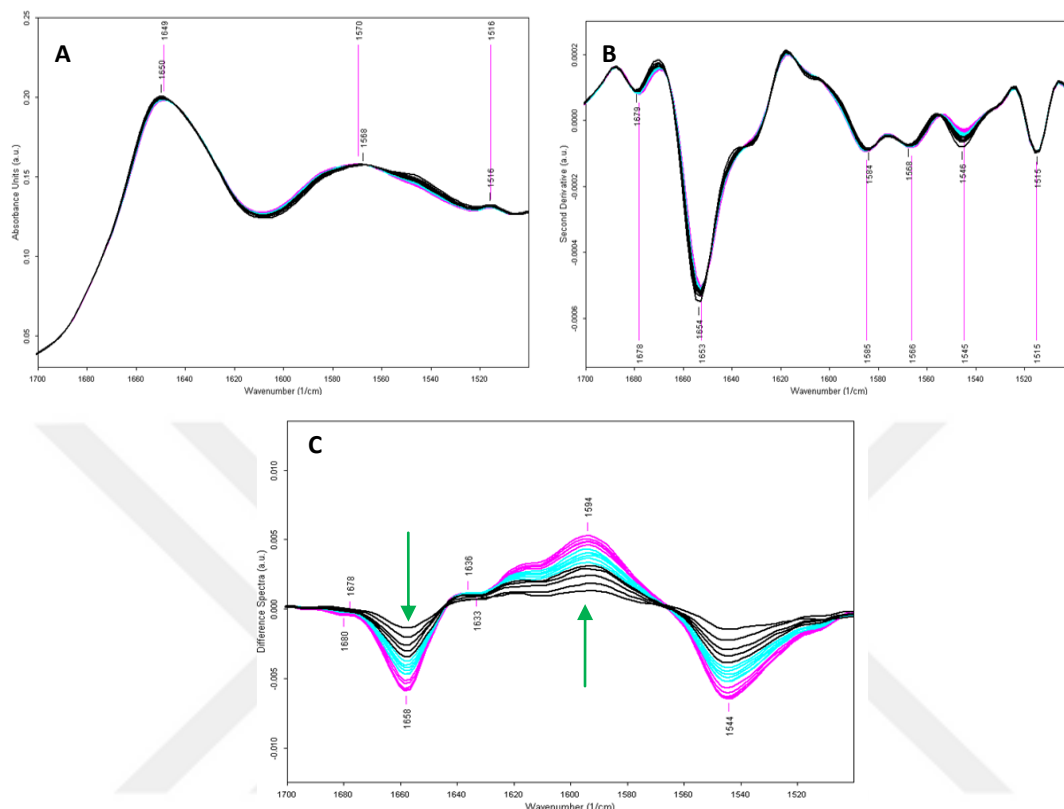


Figure 3.11: (A) The original FTIR spectra of BSA taken during tryptic digestion with a ratio of 50:1, at 37 °C for 5h. (B) The second derivative profiles of the absorbance spectra in (A). (C) The difference spectra of the absorbance spectra in (A).

Figure 3.12 (Bottom) shows the intensity changes of BSA with various ratios of substrate and enzyme as the proteolysis proceeds. It is clear that when the S/E ratio is 10:1 (w/w) at a constant concentration of substrate, the enzymatic attack is larger and faster than the 50:1 ratio, resulting in more degraded BSA. The α -helix intensity at 1656 cm⁻¹ for the blank BSA decreases slower than the digested BSA due to H/D exchange. However, for the digested BSA, the intensity changes of the α -helix for 10:1 and 50:1 ratios are considerably larger than the blank BSA. Additionally, the 1593 cm⁻¹ band (proteolysis products) does not change for the blank BSA while it increases remarkably for the digested BSA giving a confirmation for the proteolysis process. According to the common difference spectra (Fig. 3.12Top), even

though the same amide bands change for the all ratios of substrate and enzyme, the strength of the shifting differs due to enzyme concentration. Here, also 1593 cm^{-1} and 1405 cm^{-1} bands increase ascribed to the antisymmetric and symmetric stretching of free carboxylates, respectively, while these bands do not grow for the blank BSA.

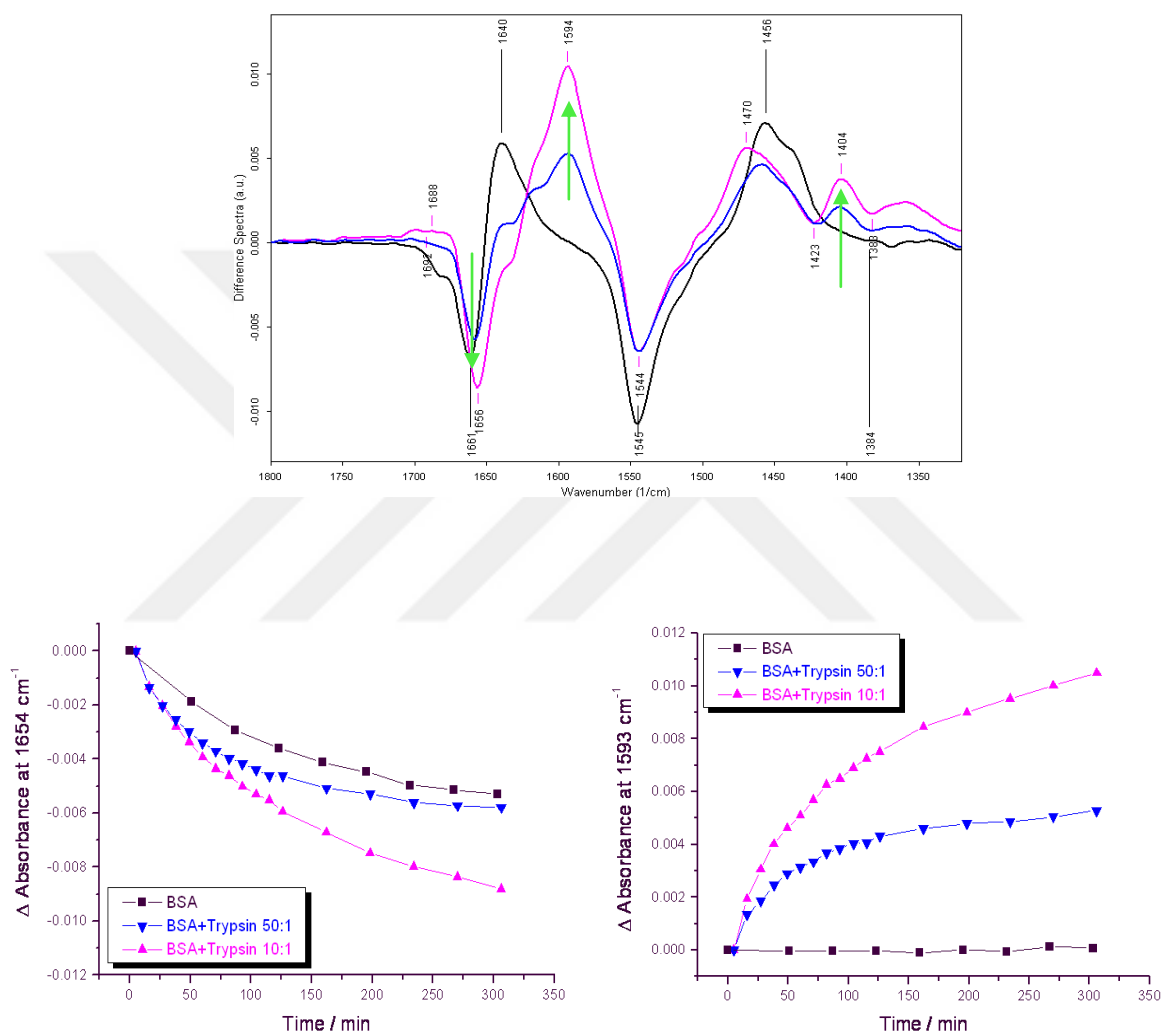


Figure 3.12: Top: The difference spectra of blank BSA (black) and digested BSA by trypsin for 10:1 (cyan); for 50:1 (red) ratios. The difference spectra were obtained by the subtraction of first spectrum at $t = 5$ min from the last spectrum at $t = 5$ h. The arrows indicate the amide changes as a function of time.

Bottom: The intensity changes of the α -helix content (1654 cm^{-1}) and of antisymmetric stretching of free carboxylates at (1593 cm^{-1}) as a function of time during proteolysis by trypsin with various ratios. The intensities were obtained from the difference spectra.

3.1.3 CD Studies: Proteolysis of BSA by α -Chymotrypsin and by Trypsin

The far-UV CD studies were performed to evaluate the influence of the enzymatic attack on the BSA (Fig. 3.13 and 3.14). The CD spectra were background corrected, and were scaled to the mean residue ellipticity based on the protein concentration and the number of amino acid residues of the sample. The spectra of the blank BSA reveal two minima at 209 nm and 222 nm, and a positive maximum at 190-195 nm, which is a typical feature for a protein rich in α -helices (Greenfield and Fasman, 1969). During the proteolysis of BSA by α -chymotrypsin or trypsin, the band intensity at all wavelengths diminishes without any significant shift of the peaks. Particularly, the intensity at 190-195 nm markedly decreases, indicating a loss of α -helical structure. Such intensity alteration around this band has the same manner for the all ratios of substrate and enzyme (10:1 and 50:1) but the strength of the intensity change differs because of the enzyme concentration. Since a typical spectrum for the unordered structure due to denaturation has not been observed (a negative band around 200 nm and a positive band around 220 nm) during proteolysis, then the intact BSA do not aggregate during enzymatic attack although the polypeptide of BSA were broken down into smaller fragments by the enzyme. Moreover, tryptic perturbation on BSA causes larger diminishes in the α -helical structure for the S/E ratio of 10:1 than for the S/E ratio of 50:1 (Fig. 3.13 and 3.14).

As it was explained in the materials and methods section (2.2.2 Spectroscopic Techniques), the assignments of the secondary structures were obtained by a software programme, CDPro, which contains SELCON3, CONTINLL and CDSSTR methods (Sreerama and Woody, 2000). These three methods contain the reference sets of proteins, whose 3-D structures are well known from the X-ray diffraction studies. The 37-soluble protein reference set of those databases was performed for each spectrum between 185 and 240 nm. After that, the estimated values from these three methods were averaged.

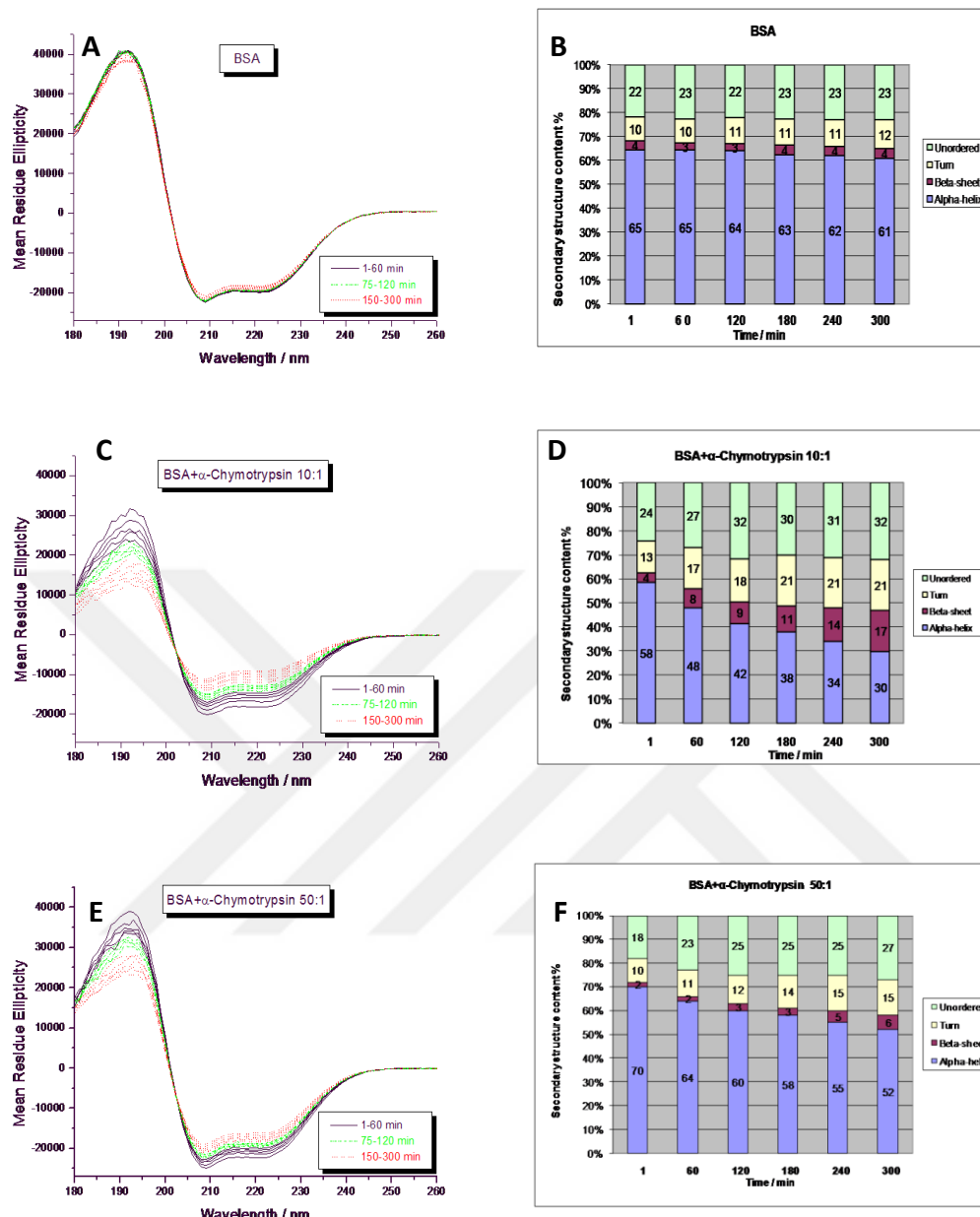


Figure 3.13: The buffer corrected CD spectra without smoothing and the assignment (%) of the secondary structures of: **(A, B)** Blank BSA at 25 mg/ml. **(C, D)** BSA with α -Chymotrypsin at 10:1 ratio. **(E, F)** BSA with α -chymotrypsin at 50:1 ratio. All spectra were recorded at pD 8.4, 37 °C. The assignments of the secondary structures are according to the 37-soluble protein reference set for the range of 185-240 nm (Sreerama and Woody, 2000).

Precisely, this reference set gives 6 secondary structural classes: turns, unordered, and the sub-groups for α -helix and β -strands which are split into regular and distorted classes. The distorted class consists of four residues per α -helix and of two residues per β -strand

(Sreerama and Woody, 2000). In order to make a more accurate prediction, the fractions of the sub-groups were added, and the resulting totals were used for the prediction of the assignments. The increasing number of proteins in the database is a significant factor to improve the estimation of the secondary structure of a protein. As a result, the blank BSA (25 mg/ml) consists of 65 % α -helix, 4 % β -strand, 10 % β -turn and 22 % unordered structure. It is a quite stable protein at 37 °C for 5 hours. Interestingly, the mixture of BSA with α -chymotrypsin or trypsin, each at 10:1 and 50:1 ratios, induces a decrease in α -helix content with a parallel increase in the remaining secondary structures.

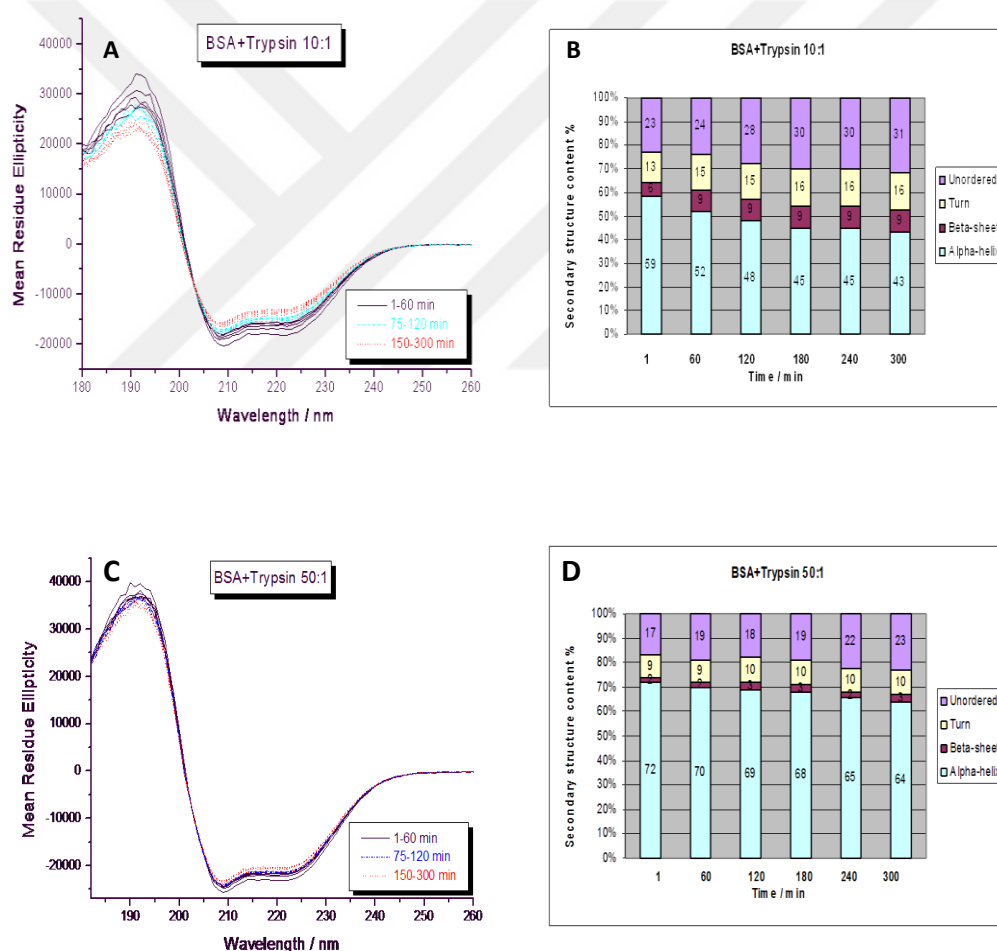


Figure 3.14: The buffer corrected CD spectra without smoothing and the assignment (%) of the secondary structures of: (A, B) BSA with trypsin at 10:1 ratio. (C, D) BSA with trypsin at 50:1 ratio. The spectra were recorded at pD 8.4, 37 °C.

The enzymatic perturbation on the secondary structure of BSA becomes more visible with an increasing amount of the enzyme. This is valid for both α -chymotrypsin and trypsin. For the large amounts of the enzymes (5 mg/ml α -chymotrypsin or trypsin for the 10:1 ratio), the helix content diminishes marginally while turns, β -sheets and unordered structures of BSA (50 mg/ml) increase at the same time.

3.1.4 Comparison of FTIR and CD Spectroscopies

The proteolysis experiments on BSA have been carried out in parallel using FTIR and CD spectroscopies (same timing, same temperature and same deuteration time). These spectroscopic methods are the most widely used tools to investigate the secondary structure and the conformational changes of a protein.

The results of the FTIR and CD studies showed very similar spectral changes during the digestion of BSA by α -chymotrypsin. According to the FTIR-spectra, the most significant spectral changes occur in the intensity of the amide I and amide II regions for the all ratios of substrate and enzyme. In this respect, the content of the α -helices diminishes strongly whereas turns and unordered structures increase concomitant with releasing of the free carboxylates due to the cleavage of peptide bond. According to the CD spectra, the content of α -helix decreases marginally while turns, β -sheets and unordered structures of digested BSA increase. Turns can be considered as a proteolysis product in addition to the free carboxylate groups. Both spectroscopic studies reveal that the enzyme induces a loss in the content of α -helices and leads to an increase in the remaining structure of BSA. Additionally, the curve fit results (Table 3.2) for the secondary structure changes of BSA during proteolysis confirm that the content of α -helices decreases while the content of turns and unordered structures increase. Then, these results are in agreement with both FTIR and CD results, revealing degradation of BSA.

Proteolytic degradation of BSA by trypsin shows similar results as in the case of the proteolysis by α -chymotrypsin. Here, the FTIR results are also in agreement with the CD spectra. In both studies, trypsin causes a loss in α -helices and an increment in the remaining secondary structure. In this process, FTIR results also reveal that the free carboxylates are released rapidly because of the tryptic attack.

The enzymatic perturbation on the secondary structure of BSA becomes more visible with an increased amount of the α -chymotrypsin and trypsin. Yet, degradation of BSA is less affected by trypsin than by α -chymotrypsin. Because trypsin predominantly cleaves peptides on the C-terminal of lysine and arginine amino acid residues which are polar groups except if followed by the proline residue. However, α -chymotrypsin hydrolyses on the carboxyl end of the peptide bonds with aromatic and large hydrophobic residues (tyrosine tryptophan, phenylalanine, methionine and leucine, unless followed by a proline residue). According to figure 3.9 and 3.12, which are plotted for the intensity changes of the α -helices (1656 cm^{-1}) and of antisymmetric stretching of the free carboxylate groups (1593 cm^{-1}) as a function of time during digestion, α -chymotrypsin induces a larger reduction in the α -helix intensity and more increment in the intensity of free carboxylates than the tryptic digestion. Thereby, it can be interpreted that after the enzyme cleaves at the specific residues of the peptide, α -chymotrypsin can reach through the hydrophobic residues and then towards the core of BSA, causing more degraded BSA structure. However, since trypsin cleaves the hydrophilic residues of BSA, it can access only to the solvent-exposed residues of BSA. Therefore, trypsin can not reach to the core of BSA.

The 10:1 and 50:1 ratios lead to the significant structural changes according to the FTIR and CD spectra. Figure 3.15 illustrates the ellipticity changes of BSA at 222 nm as a function of time during proteolysis by α -chymotrypsin (left) and by trypsin (right). It is clear from the figure that if the substrate concentration is increased and the concentration of BSA remains same, the enzymatic perturbation causes rapidly more degraded protein. This statement is also confirmed by the FTIR results of proteolysis by α -chymotrypsin and by trypsin (Fig. 3.9Bottom and Fig. 3.12Bottom). According to the both FTIR and CD results, the most remarkable alterations occur in the digestion of BSA by α -chymotrypsin at 10:1 ratio. On the other hand, the least structural changes of BSA occur during digestion by trypsin at 50:1 ratio.

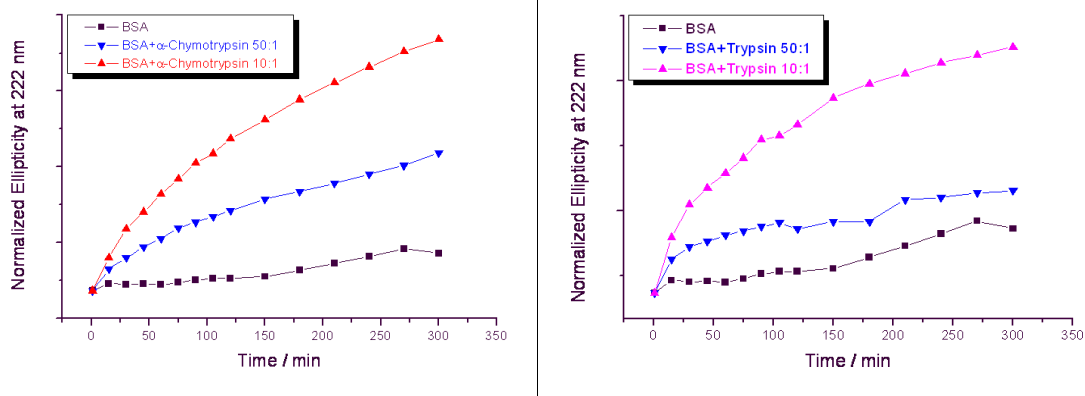


Figure 3.15: The ellipticity changes of BSA at 222 nm as a function of time during proteolysis by α -chymotrypsin (left) and by trypsin (right). The ellipticity changes were taken from the CD spectra which are shown in the figure 3.13 for digestion by α -chymotrypsin and in 3.14 for digestion by trypsin.

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAE₆₀
 NCDKSLHTLFGDKKLCTVATLRETYGEMADCCAKQEPRNECFLQHKDDN₁₀₉PNLPRLVRPE₁₂₀
 DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKA₁₈₀AFTECCQAADKAACLLP₁₈₀
KLDELRDEGKASSAKQRLKCASLQKFERAFKAWAVARLSQRFPKA₂₄₀EFAEVSKLVTDLTK₂₄₀
VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPA₃₀₀
 DLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKC₃₆₀
 CAAADPHECYAKVFDEFKPLVVEPQNLIKQNC₄₂₀ELFEQLGEYKFQNALLVRYTKKVPQVST₄₂₀
PTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTES₄₈₀
 LVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKAT₅₄₀
KEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAAASQAALGL₅₈₅

Figure 3.16: The amino acid sequence of HSA which is homologous to BSA (PDB ID: HSA, 1AO6). The number of proline (P) residues is 24, of lysine (K) is 60 and of arginine (R) is 24. The estimated undigested parts of the residues were marked with yellow. The underlined residues indicate the possible digestable parts of BSA.

The amino acid sequence of HSA reproduced from PDB bank (PDB ID: 1AO6) is identical to primary sequence of BSA (Figure 3.16). There is a homologous among the sequences in the whole region (Hirayama et al., 1990). Arginine and lysine residues are the specific sites of the intact BSA to be cleaved by trypsin. Yet, when a proline residue is on the right side of these basic residues, trypsin is not able to catalyze such residues due to hydrophobic feature of the proline residues (yellow marked residues in the figure 3.16). If we take into account the 3-D structure and the primary sequence of BSA (Figure 2.1 and Figure 3.16), the region of (...3-21...) in the N-terminal region and the region of (...538-575...) in the C-terminal may be susceptible to the tryptic attack and may be easily degraded into the short fragments because this part does not include proline residues. On the other hand, the region (...110-118...) of BSA is not accessible to the tryptic digestion due to the nearby apolar proline residues. According to the primary structure of BSA, there are many trypsin-sensitive peptide bonds (underlined residues in figure 3.16), but hydrolysis of these bonds is related to the accessibility of peptide bonds of BSA to the trypsin and to the structural stability of the protein. The accessible or solvent exposed α -helices are hydrolyzed by trypsin and then converted into the distorted or short helices, namely short fragments; after that, the liberated free carboxylate groups appear.

3.2 β -LACTOGLOBULIN (β -Lg)

Figure 3.17 represents the amide I and amide II bands of β -Lactoglobulin in H_2O buffer at 37 °C. It is clear from the figure that β -Lg is mainly dominated by the β -sheet structures around 1634 cm^{-1} (53 %, Table 3.3). Additionally, the amide I region is composed of the signals at 1656 cm^{-1} (overlap of signals from α -helix and random segments) and at 1687 cm^{-1} (antiparallel β -sheet or turns). According to the FTIR spectra, there is no considerable alteration on the β -Lg structure during incubation at 37 °C.

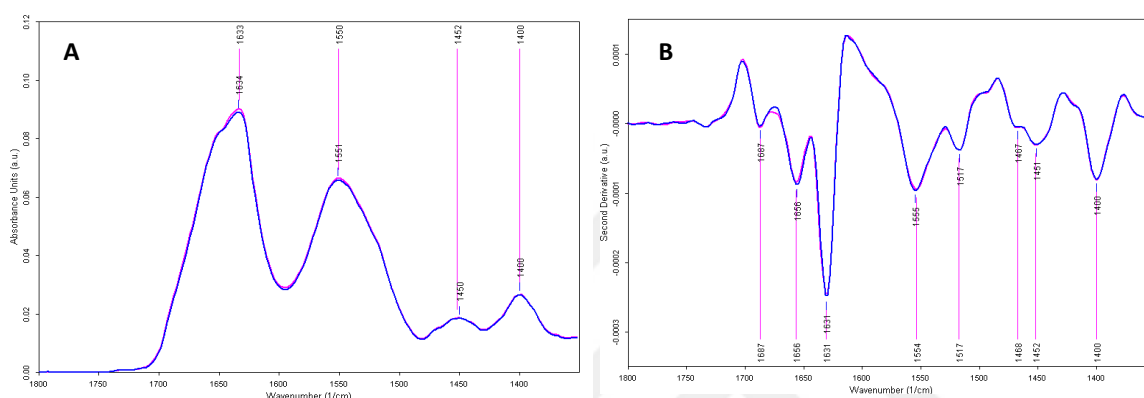


Figure 3.17: (A) The FTIR spectra of β -Lactoglobulin (50 mg/ml) in H_2O buffer at pH 8. (B) The second derivatives of the absorbance spectra in (A). Blue colour indicates the spectrum at $t = 15$ min and magenta colour is the spectrum at $t = 5$ h during the incubation at $T = 37$ °C.

Furthermore, β -lactoglobulin was prepared in D_2O and equilibrated 3 h at +4 °C. After that, the deuterated β -Lg sample was recorded at 37 °C. Figure 3.18 shows the infrared spectra of β -lactoglobulin amide I and amide II regions. According to the spectra, the amide I band is mainly composed of a maximum peak at 1634 cm^{-1} which is attributed to the β -sheet structure. Besides, the amide I region includes the signals at 1622 cm^{-1} (β -sheet) as a shoulder, 1649 cm^{-1} (overlap of signals from α -helix and random segments), 1664 cm^{-1} (turns), 1677 cm^{-1} (antiparallel β -sheet) and 1692 cm^{-1} (protonated carboxylate groups). The band at 1622 cm^{-1} is also ascribed to the exposed β -sheets that are not part of the core of the β -sheet (Arrondo, Young and Mantsch, 1988) or it is ascribed as a signal from the mixture of dimers and monomers because it locates as a shoulder in the spectra.

Upon H/D exchange, the absorbance around 1551 cm^{-1} in the amide II region shifts towards the amide II' region centred at 1453 cm^{-1} due to replacing of the N-H bending vibration from

the protein backbone by the N-D bending vibration. Furthermore, the signal at 1656 cm^{-1} in H_2O buffer shifts towards 1649 cm^{-1} in D_2O buffer which can be attributed to the solution-accessible α -helices/short helices and/or disordered structures exposed to the D_2O buffer. Moreover, the peak at 1687 cm^{-1} shifts towards 1677 cm^{-1} (β -turn or β -sheet) and 1663 cm^{-1} (turns) upon H/D exchange.

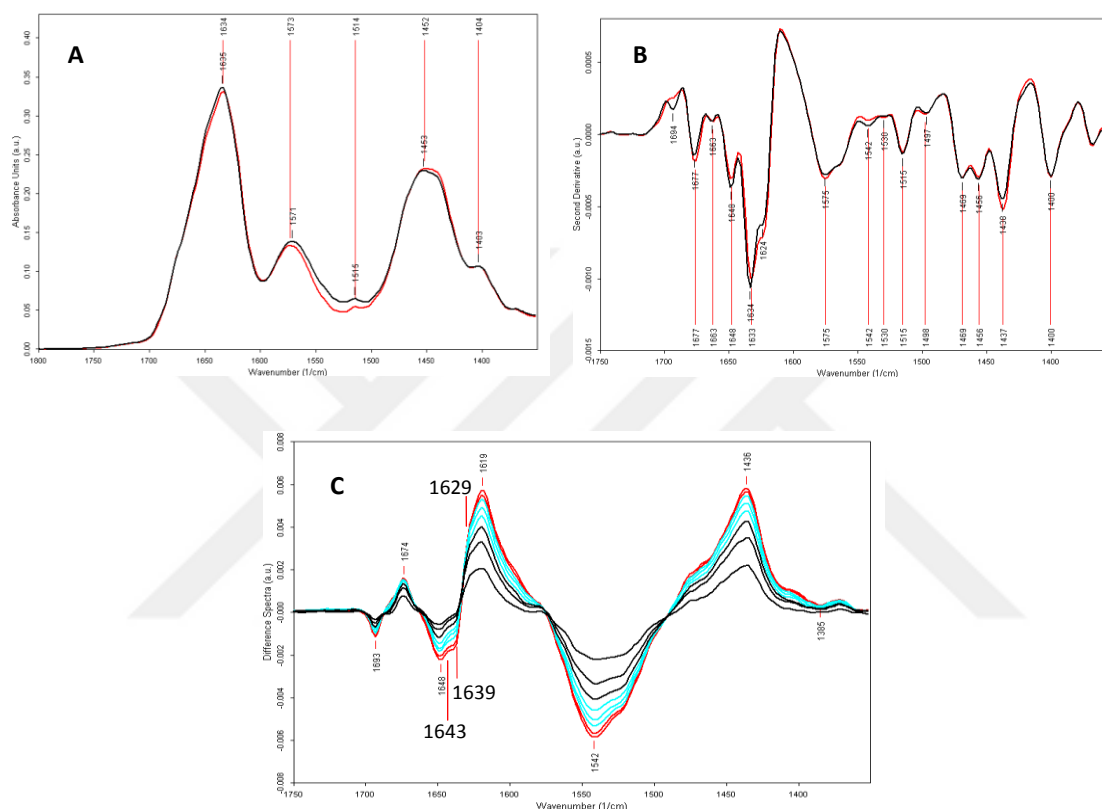


Figure 3.18: (A) The FTIR spectra of β -lactoglobulin (50 mg/ml) in D_2O buffer at pH 8.4, 37°C . (B) The second derivatives of the absorbance spectra in (A). Black colour indicates the spectrum at $t = 15\text{ min}$ and red colour represents the spectrum at $t = 5\text{ h}$. (C) The difference spectra of the absorbance spectra in (A). Difference spectra were obtained by subtraction of the first spectrum at $t = 5\text{ min}$ from the each of the spectra in (A) taken during the incubation at $T = 37^\circ\text{C}$ [$\Delta\text{Absorbance} = \{\beta\text{-Lg}(t)\} - \{\beta\text{-Lg}(t=5\text{min})\}$]. In order to avoid the water vapour absorbance for the spectrum at $t = 5\text{ min}$, it was corrected with water vapour subtraction and was smoothed by a factor of 9.

According to the difference spectra (Figure 3.18C), the effects of H/D exchange continue further during incubation at 37°C because the H/D exchange at 37°C is faster than at 4°C . Thereby, the more flexible and dynamic conformations of β -Lg shift towards the lower

wavenumbers. In this respect, the intensity at 1648 cm^{-1} decreases whereas the intensity at the 1643 cm^{-1} band appears as a shoulder, indicating the D_2O exposed unordered structure. Additionally, the signal at 1639 cm^{-1} diminishes while the intensity at 1629 cm^{-1} increases as a shoulder, revealing D_2O exposed β -sheet structure. Altogether indicates the access of D_2O through the accessible parts of the protein. Additionally, a positive peak around the 1619 cm^{-1} band increases which corresponds to the D_2O exposed or intermolecular β -sheet structure upon heating.

For a quantitative analysis, the peak positions were determined by the band narrowing techniques and were curve fitted for the amide I and amide II regions of the spectra. This allowed us to calculate the percentages of the secondary structure elements of β -Lg (Table 3.3). According to the table, β -Lg is a protein rich in β -sheets (53%). It is also composed of turns (18%) and α -helix+random structures (29%). The band narrowing techniques allowed us to obtain the resolved bands in the amide I and amide II region. However, there were still underlying components in the spectra. For instance, the signals of α -helix and random structures are indistinguishable in their positions although Fourier self deconvolution and derivatives of the spectra have been diligently performed. Therefore, we take together the percentages of the α -helix and random structures at the 1649 cm^{-1} band.

Table 3.3: Assignment of amide I components and percentages (%) of secondary structure elements of β -Lg in H_2O and in D_2O buffers at 37°C (Spectra at $t = 15\text{ min}$).

Position ^a in H_2O (cm^{-1})	Proportion ^b (%) in H_2O	Position ^a in D_2O (cm^{-1})	Proportion ^b (%) in D_2O	Band Assignments
1629	33	1622	20	β -sheet
1641	16	1634	33	β -sheet
1655	23	1649	29	α -helix+random
1662	13	-	-	α -helix/random, β -turn
1672	5	1664	10	β -turn, β -sheet
1683	9	1677	8	β -turn, β -sheet
^a After deconvolution.				
^b Area percentages of secondary structure elements in the amide I region calculated after curve fitting.				

In the previous studies, it has been shown that the existence of two components around 1634 cm^{-1} and 1622 cm^{-1} is the characteristic of β -Lg and this is associated with dimeric forms while appearance of only one component around 1630 cm^{-1} is associated with the monomeric form of β -Lg (Lefevre and Subirade, 1999). Additionally, β -Lg shows a number of pH induced, local and global structural alterations between pH 2 and pH 13. Above pH 6.5, β -Lg undergoes the Tanford transition which involves the displacement of a loop (residues 85 to 90). This loop acts as a lid which closes the protein interior/binding site below pH 7.3 and opens it at higher pH. The Tanford transition may involve some structural alterations and leads to a change in the relative orientation of monomers in the dimer (Sakurai and Goto, 2005; Taulier and Chalikian, 2001; Brownlow et al., 1997). Besides, the dimer starts to dissociate into monomeric form above pH 6.5 (Townend, Herskovits, Timasheff, and Gorbunoff, 1969).

In our FTIR measurements, the blank solution of the β -Lg (50 mg/ml) in D_2O buffer has a signal at 1622 cm^{-1} which can be attributed to the exposed β -strands because this band does not exist in H_2O buffer, or this band can be a signal from the β -sheet structure of dimeric formation due to high concentration of the protein solution. Such a low frequency of β -sheet structure indicates that strong hydrogen bonds arise between the peptide linkages. Alternatively, this component is usually attributed to the strongly bonded, distorted or solvent-exposed strands (Jackson and Mantsch, 1992).

We have also performed the temperature dependent experiment in order to investigate the temperature stability of the β -Lg around our working temperature, 37°C . Figure 3.19 represents the temperature-induced structural changes of β -Lg in D_2O buffer. The FTIR spectra were recorded at increasing temperatures from 20 to 94°C in intervals of 2°C . It is clear from the spectra (Figure 3.19 A and B) that the intensity in the amide I band decreases and two new components (1682 and 1617 cm^{-1}) increase with a rising temperature. The spectra at and above 76°C show a typical pattern of the thermally denaturated protein with 1682 and 1617 cm^{-1} bands, indicating formation of the intermolecular β -sheet structures (Arrondo et al., 1994; Sokolowski and Naumann, 2005). Additionally, the amide II band diminishes dramatically upon increasing temperature, indicating an increase of deuteration and a loss in the tertiary structure. The component located near 1623 cm^{-1} starts to broaden above 40°C and disappears above 50°C , indicating the dissociation of the dimer into monomers upon heating. This band can be a contact between two monomers of β -Lg.

Besides, the band around 1664 cm^{-1} (turn) and 1648 cm^{-1} (α -helices/disordered structures) broaden at the higher temperatures.

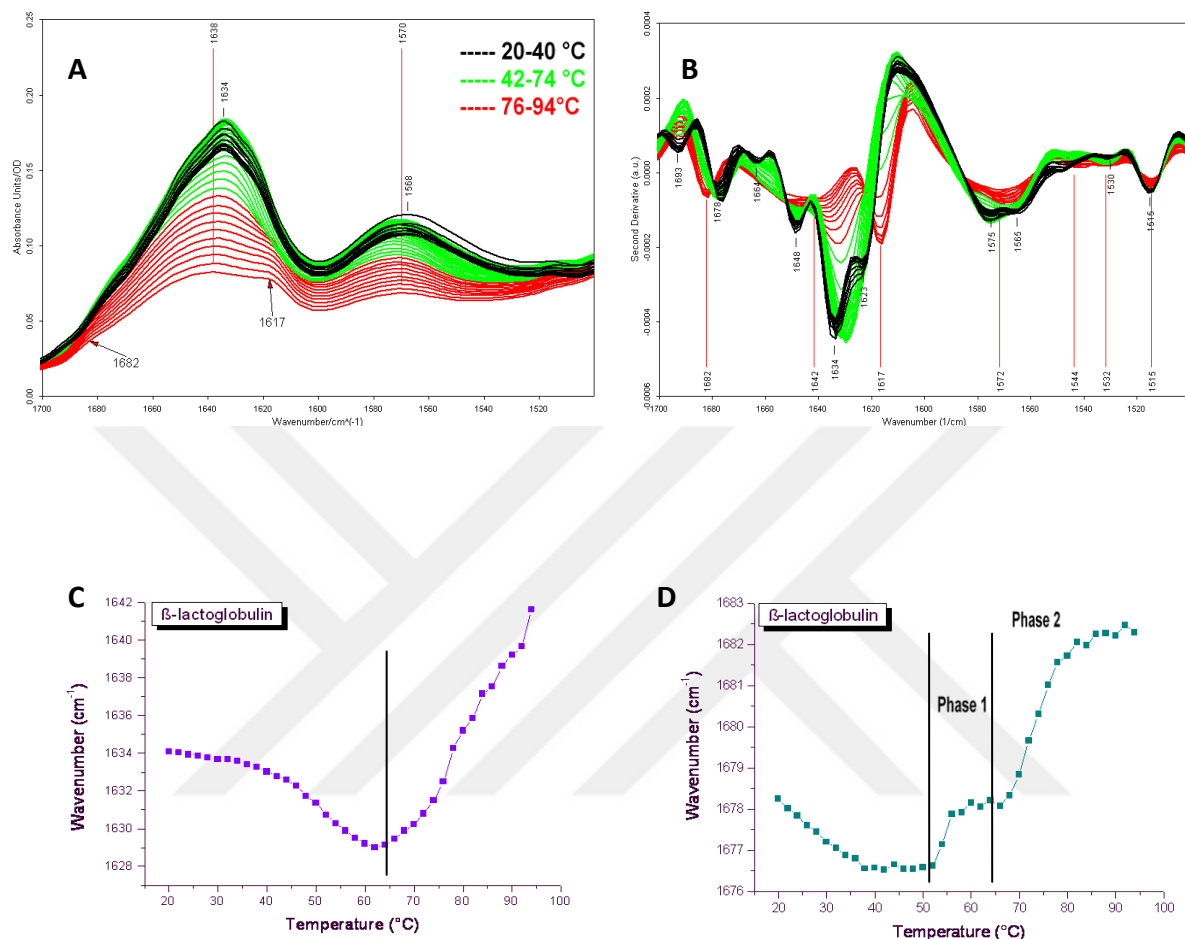


Figure 3.19: (A) The FTIR spectra of β -lactoglobulin (50 mg/ml) in D_2O collected at increasing temperatures. (B) The second derivatives of the absorbance spectra in (A) with the temperature-dependent peak positions. The spectra are shown in black for the temperature range 20-40 $^{\circ}\text{C}$; in green for 42-74 $^{\circ}\text{C}$; in red for 76-94 $^{\circ}\text{C}$. (C) and (D) show the temperature dependent peak positions of 1632 cm^{-1} (β -sheet) and 1678 cm^{-1} (turns or β -sheets), from the second derivative profile of β -Lg.

Figure 3.19 C and D show the temperature dependence of the peak positions at 1632 cm^{-1} (β -sheet) and at 1678 cm^{-1} (turns/ β -sheet) taken from second derivative spectra, by following the degree of shift with respect to temperature. The 1632 cm^{-1} band shifts towards lower wavenumber up to 65 $^{\circ}\text{C}$, indicating stronger hydrogen bonding in this part. Also, shifting of this band can occur due to protein flexibility and dynamics upon heating and upon

further H/D exchange. After that, such component shifts towards higher wavenumbers, converting into unordered structure. Thereby, the unfolding state starts above 66 °C. Similarly, the 1678 cm⁻¹ component shifts towards lower wavenumbers up to 40 °C due to flexibility of the protein. However, the thermal induced changes of this band occur in two stages, between 50-66 °C (phase 1) and above 66 °C (phase 2). Phase 1 corresponds to the monomerisation of the protein while phase 2 reveals the unfolding state of β -Lg at higher temperatures. Therefore, 1678 cm⁻¹ component can be a contact between two monomers of the protein. It was previously studied that dimeric β -Lg at pH ~ 7 dissociates into monomers around 60 °C (Fessas et al., 2001; Roefs et al., 1994).

3.2.1 FTIR Studies of Proteolysis by Trypsin

During this work, β -lactoglobulin was digested by trypsin under the two protein/protease ratios of 10/1 and 50/1 (w/w) in D₂O buffer during 5 hours at 37 °C. The FTIR spectra were recorded at a resolution of 4 cm⁻¹ with 128 scans after the protein equilibrated 3 h at +4 °C.

β -Lactoglobulin/Trypsin Ratio 10/1

Figure 3.20A represents the original FT-IR spectra of β -Lg (50 mg/ml) during digestion by trypsin (5 mg/ml) at a ratio of 10:1 at 37 °C. The spectra show a decrease at 1636 cm⁻¹ as well as an increase around 1594 cm⁻¹, suggesting a diminution in β -sheet structure and an increment in the antisymmetric stretching of free carboxylates as a digestion product. Figure 3.20B shows the second derivative profiles of the absorbance spectra. It reveals further that the major conformational changes occur in the amide I and amide II region during digestion. The amide I band decreases upon digestion, indicating the loss of peptide bonds. The intensity at 1634 cm⁻¹ becomes broader and shifts towards 1638 cm⁻¹, indicating the unordered structure upon digestion. Moreover, the intensity at 1664 cm⁻¹ increases and becomes broader. Therefore, this band may be also considered as a proteolysis product.

The difference spectrum of a protein represents precisely small and subtle conformational changes in the structure of a protein. Figure 3.20C reveals exact information during digestion of β -Lg by trypsin because the difference spectra indicate the spectra of removed and produced part of the β -Lg during digestion. In this respect, the intact β -Lg loses dramatically its β -sheet content due to tryptic digestion (negative band around 1634 cm⁻¹) while the free

carboxylate groups increase drastically (positive band around 1593 cm^{-1}). Additionally, there is a shoulder around 1643 cm^{-1} which arises from the disordered structure and/or from the short chains of the digested polypeptide.

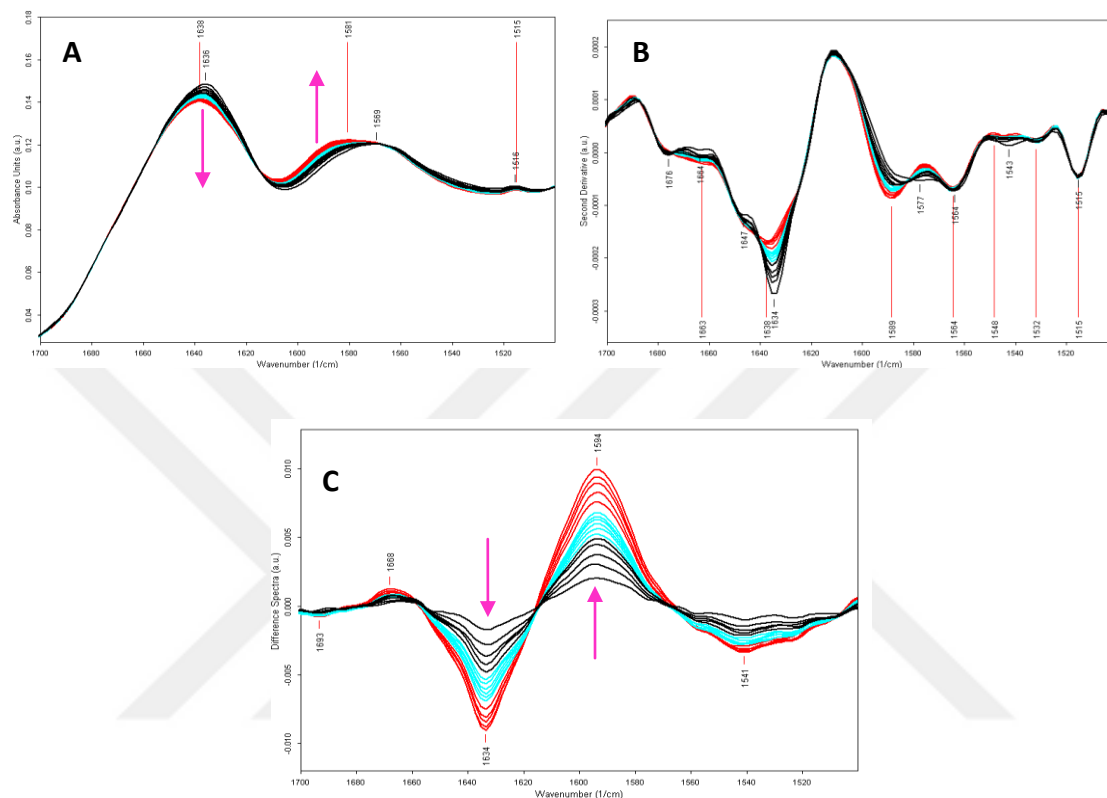


Figure 3.20: (A) The original FT-IR spectra of β -lactoglobulin (50 mg/ml) were taken during digestion by trypsin (5 mg/ml) at a ratio of 10:1, at 37°C , from 5 min to 5h. (B) The second derivative profiles of the absorbance spectra in (A). (C) Difference spectra of the absorbance spectra in (A). Difference spectra were obtained by subtraction of the first spectrum at $t = 5\text{ min}$ from each of the spectra in (A) taken when the proteolysis proceeds [$\Delta\text{Absorbance} = \{\text{Digested } \beta\text{-Lg (t)}\} - \{\text{Digested } \beta\text{-Lg (t=5min)}\}$]. The arrows indicate the amide I and amide II changes as a function of time.

Table 3.4 gives the curve fitted secondary structure percentages of β -Lg during proteolysis by trypsin for 5 hours. The content of the band at 1623 cm^{-1} decreases in the beginning of the proteolysis (at $t = 1\text{h}$), but its content increases for the digested β -Lg at $t = 5\text{h}$. This suggests that trypsin cleaves the β -sheets which have a signal at this band, but after that, the intermolecular β -sheet structures are formed between the short fragments. Additionally, the

presence of the band at 1682 cm^{-1} suggests formation of the intermolecular β -sheets due to digestion. It is also clear that the content of β -sheets (1634 cm^{-1}) and of turns ($1662\text{-}1667\text{ cm}^{-1}$) decrease upon digestion whereas the unordered structure increases (1649 cm^{-1}).

Table 3.4: Positions and assignment of the amide I components and the secondary structure percentages (%) of β -lactoglobulin/trypsin (10/1).

β -lactoglobulin without trypsin		β -lactoglobulin+Trypsin 10:1		
Position ^a (cm^{-1}) and Amide I components	% ^b at t = 5h	Position ^a (cm^{-1}) and Amide I components	% ^b at t = 1h	% ^b at t = 5h
1621 (β -sheet)	22	1623 (β -sheet)	16	22
1634 (β -sheet)	34	1634 ^c - 1636 ^d (β -sheet/unordered)	28	23
1648 (α -helix+unordered)	20	1649 (α -helix+unordered)	34	32
1662 (Turn)	17	1662 ^c -1667 ^{c,d} (turn)	15	11
1677 (β -sheet, turn)	7	1676 ^c -1672 ^d (turn/ β -sheet)	6	10
-	-	1682 (intermolecular β -sheet)	-	3
^a After deconvolution. Band narrowing techniques were used to determine the positions. Fourier self deconvolution was applied using Lorentzian band shape with 0.5 noise reduction factor. Deconvolution factor was chosen according to the individual spectrum. ^b Area percentages of secondary structure elements in the amide I region calculated after curve fitting. ^c The band positions for the digested β -Lg at t = 1h. ^d The band positions for the digested β -Lg at t = 5h.				

Figures 3.21A and 3.21B show the curve fitted spectra of native and digested β -Lg at t = 5 h. They also confirm how the β -sheet (1634 cm^{-1}) structures decrease and how the free carboxylate groups increase (1593 cm^{-1}) for the digested β -lg. Also it is clear that the amide I region becomes broader, indicating loss of the peptide structure upon digestion. Although the band narrowing techniques have been performed, there were still unresolved components in the both spectra. The random structures overlap with α -helix at 1649 cm^{-1} and with β -sheet structures at 1636 cm^{-1} .

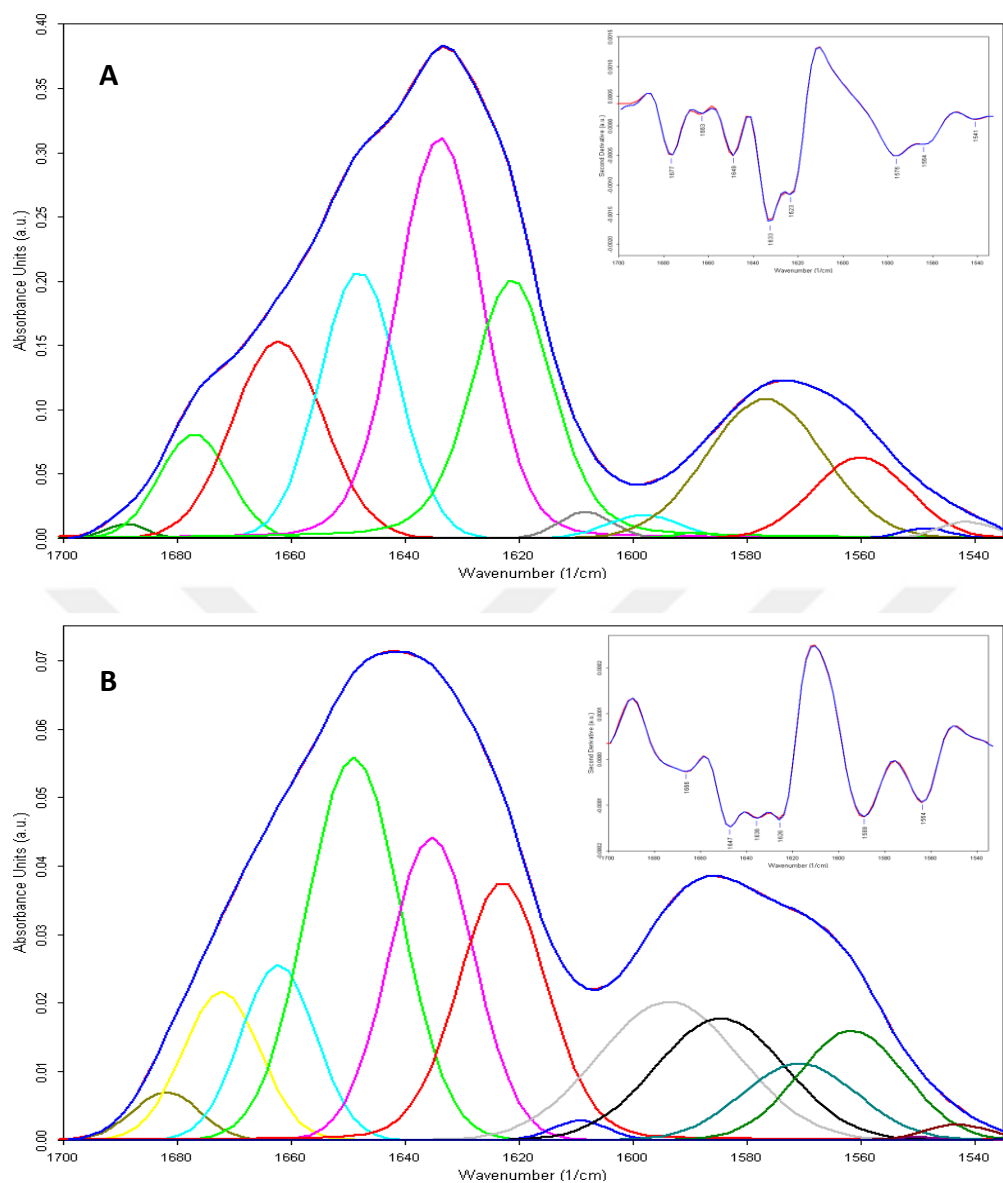


Fig 3. 21 : Curve fit analysis after deconvolution for amide I and amide II regions at t = 5 h.

(A) Native β -Lg (50 mg/ml). **(B)** β -Lg digested by trypsin (10:1). The envelope bands which are formed by curve fitting are shown in red and the envelope bands of the original spectra are shown in blue. Insets represent the second derivatives of the original spectra and the fitted envelope in order to compare the success of the curve fit. Before curve fitting, buffer was subtracted from the blank β -Lg, and the enzyme (5 mg/ml) was subtracted from the digested β -Lg.

β -Lactoglobulin/Trypsin Ratio 50/1

Figure 3.22 shows the spectral changes of the intact β -Lg (50 mg/ml) upon proteolysis by trypsin (1 mg/ml). The significant spectral changes which arise from digestion take place in the amide I and amide II regions, in a same manner with the case of 10:1 ratio. The amide I intensity decrease and becomes broad upon tryptic attack, suggesting a loss in the β -Lg peptide bonds and an increase of unordered structure. Also, the second derivative profile of the absorbance spectra confirms that the intensity at 1634 cm^{-1} (β -sheets) decreases and broadens, resulting in the unordered structure. Additionally, turns (1662 cm^{-1}) and free carboxylates (around 1593 cm^{-1}) increase and broaden during digestion of the protein.

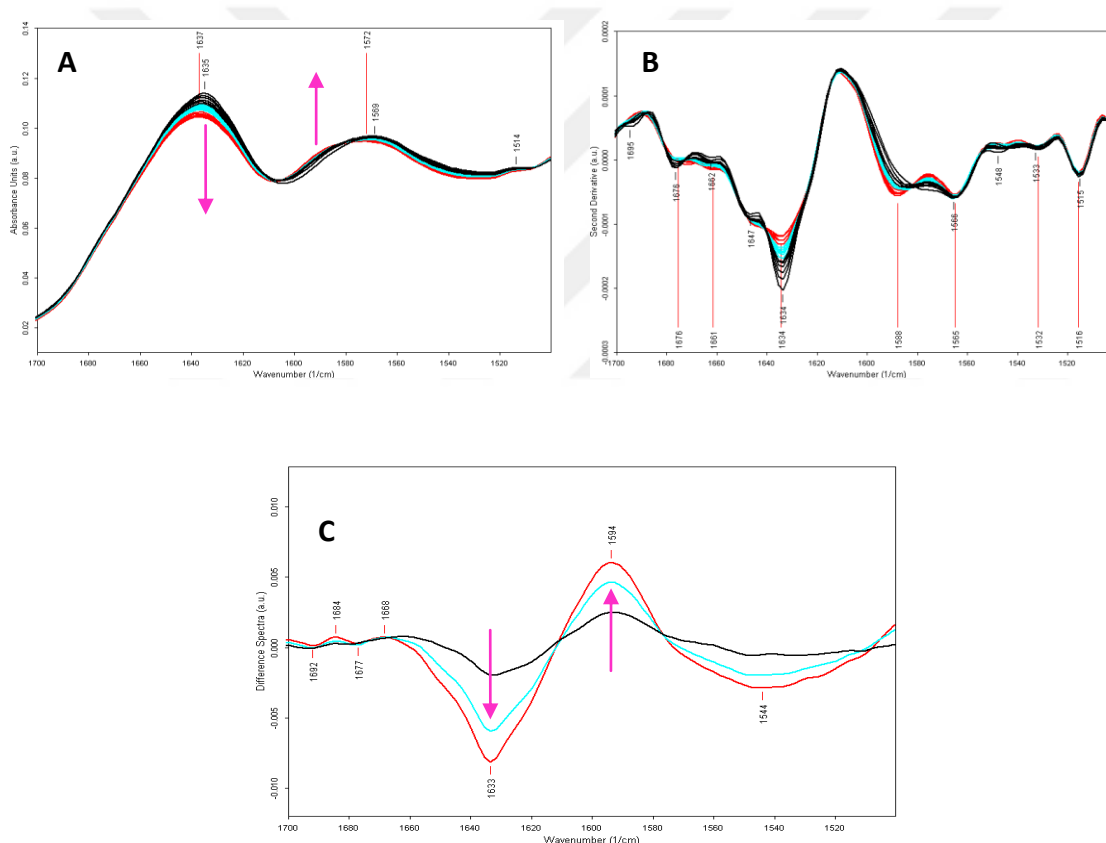


Figure 3.22: The original FT-IR spectra of β -Lg taken during digestion by trypsin at a ratio of 50:1, at 37°C from 5 min to 5h. **(B)** The second derivatives of the absorbance spectra in (A). **(C)** Difference spectra of the absorbance spectra in (A). Difference spectra were obtained by the subtraction of first spectrum at 5 min from each of the spectra in (A) taken during proteolysis. The black colour shows the difference spectrum at $t = 27$ min, cyan is at $t = 2$ h and red is at $t = 5$ h. The arrows indicate the amide I and amide II intensity changes as a function of time.

According to the difference spectra (Figure 3.22C) it is also confirmed that, β -Lg loses its β -sheet structure upon digestion (negative band around 1633 cm^{-1}) whereas the free carboxylates increase strongly (positive band around 1594 cm^{-1}).

Figure 3.23 (Top) shows the difference spectra for the blank β -Lg and for the digested β -Lg with various ratios. Although the same amide positions change for the different ratios of substrate and enzyme, the strength of degradation of the intact protein differs with respect to the enzyme concentration. The intensity of 1593 cm^{-1} and 1406 cm^{-1} bands increase, which are ascribed to the antisymmetric and symmetric stretching of free carboxylates, respectively.

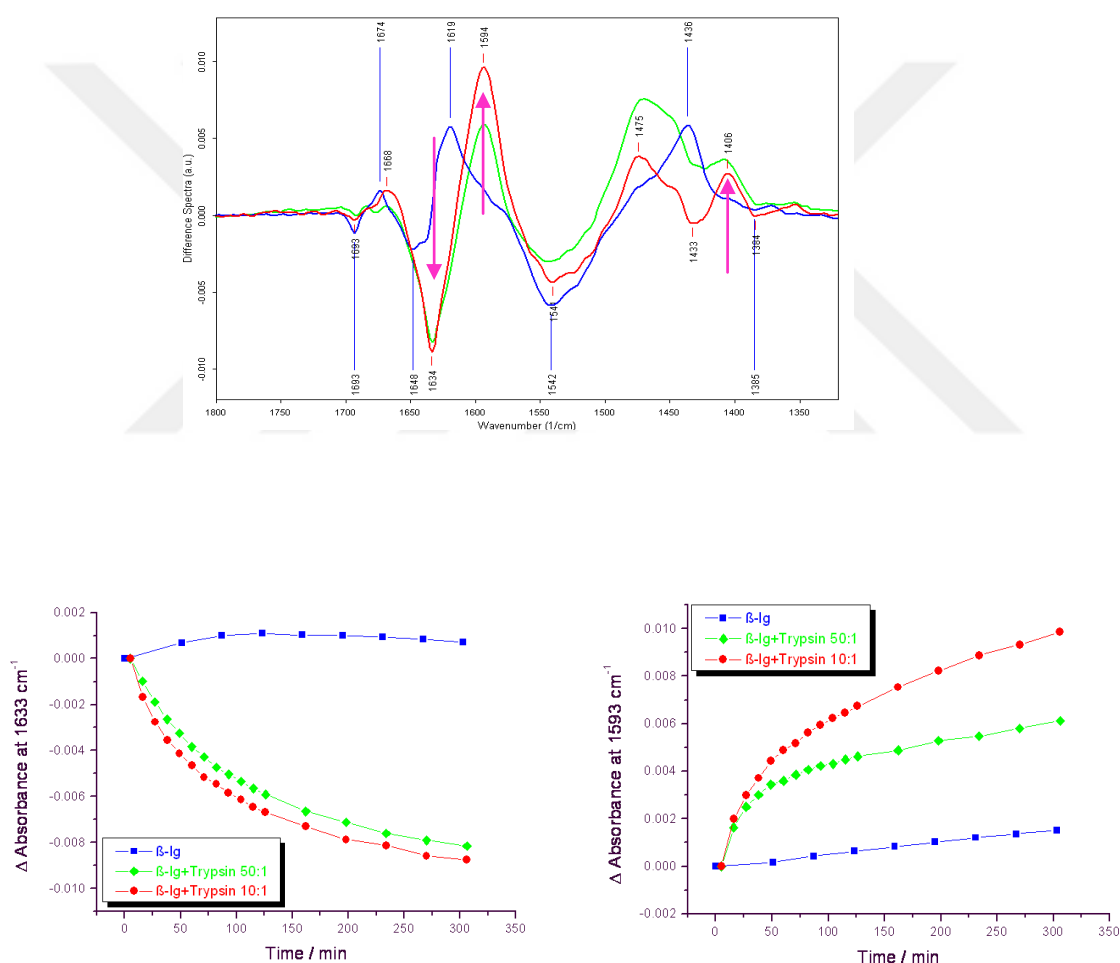


Figure 3.23: Top: Difference spectra of the blank β -Lg (blue) and digested β -Lg by trypsin for 10:1 (red); for 50:1 (green) ratios. The difference spectra were obtained by the subtraction of the first spectrum at 5 min from the last spectrum at 5 h. The arrows indicate the intensity changes as a function of time.

Bottom: The intensity changes of β -sheet content (1633 cm^{-1}) and of antisymmetric stretching of free carboxylates content (1593 cm^{-1}) as a function of time during proteolysis with various ratios. The intensities were obtained from the difference spectra in figures 3.20C and 3.22C.

However, these bands (1593 cm^{-1} and 1406 cm^{-1}) do not increase for the blank β -Lg, revealing an evidence of the digestion. Furthermore, there is an outstanding outcome of tryptic digestion that a decrease in β -sheet structure (negative band around 1634 cm^{-1}) is almost same for both ratios. This implies that trypsin in the both ratios can reach towards almost similar accessible parts of the β -Lg.

Figure 3.23 (Bottom) represents the intensity changes of β -Lg with various ratios of substrate and enzyme as the proteolysis proceeds. If the S/E ratio is 10:1 (w/w) at a constant concentration of the substrate, the enzymatic digestion is faster and larger than the 50:1 ratio, resulting in more degraded β -Lg structure, because the 10:1 ratio gives rise to the more free carboxylates groups than in the case of 50:1 ratio.

3.2.2 CD Studies of Proteolysis by Trypsin

In order to investigate the effect of tryptic digestion on β -lactoglobulin, the far-UV CD spectra were recorded for the range 185-260 nm during 5 hours at pH 8.4, 37°C . The CD spectra were background corrected, and were scaled to the mean residue ellipticity based on the protein concentration and the number of amino acid residues of the sample.

Figure 3.24 shows the effect of proteolysis on β -Lg CD spectra at two different ratios of substrate and enzyme. All spectra were smoothed with a smoothing factor of 9. Figure 3.24A and 3.24B represent the CD spectra of the blank β -Lg and its secondary structure changes. As seen from the figure, the spectra have a maximum around 196 nm and have a deep minimum around 217 nm which is ascribed to the typical β -sheet configuration. After 5 h, the β -sheet content of β -Lg is about 38% and the rest content involves 31% unordered, 21% turns, 7% α -helix structure. There is no significant secondary structure alteration of the blank sample during 5 h measurement at 37°C . In this respect, β -sheet content decreases only 4% while unordered structure increases only 3%. Also, the shape of the CD spectra of blank β -Lg also shows no significant change for 5 hours, at 37°C .

Figure 3.24C and 3.24D show the CD spectra of the digested β -Lg by trypsin at 10/1 (substrate/enzyme) ratio and show its secondary structure alterations during proteolysis. There are markedly structural changes at this ratio, indicating the effect of the tryptic attack on β -Lg. The addition of trypsin has resulted in a dramatic change in the shape of the spectra, with a peak shift in the negative minima around 217 nm towards 204 nm together

with a more negative ellipticity. Additionally, the minimum at 217 nm is replaced by a less negative shoulder, revealing an increase in unordered structure whereas β -sheet structure diminishes. An increase in the unordered structure concomitant with a decrease in the remaining structure suggests the breakdown of β -Lg polypeptide into smaller fragments due to the enzyme. The secondary structure content of the digested β -Lg at $t = 5$ h involves 24% β -sheet, 54% unordered, 16% turns, 5% α -helix structure.

Figure 3.24E and 3.24F show the CD spectra of the digested β -Lg at 50/1 (substrate/enzyme) ratio and the secondary structure changes during 5 hours. The positive band around 190-200 nm is drastically reduced over time and at the same time the peak at 217 nm is blue shifted towards lower wavelength at 204 nm, and the minimum at 217 nm is replaced by a less negative shoulder, as in the case of the 10/1 ratio explained above. The tryptic perturbation on β -Lg causes a large decrease in the β -sheet structure for 10:1 and for 50:1 ratios. According to the CD spectra, both ratios reveal almost the same intensity changes, indicating similar strength of tryptic digestion. Essentially, there is no large difference between two ratios on degradation of β -Lg. For 50/1 ratio, the secondary structure content of the digested β -Lg at $t = 5$ h is composed of 27% β -sheet, 47% unordered, 16% turns, 6% α -helix structure. Then, the unordered structure increases while the remaining structure diminishes, suggesting degrading of β -Lg and loss of its secondary structure.

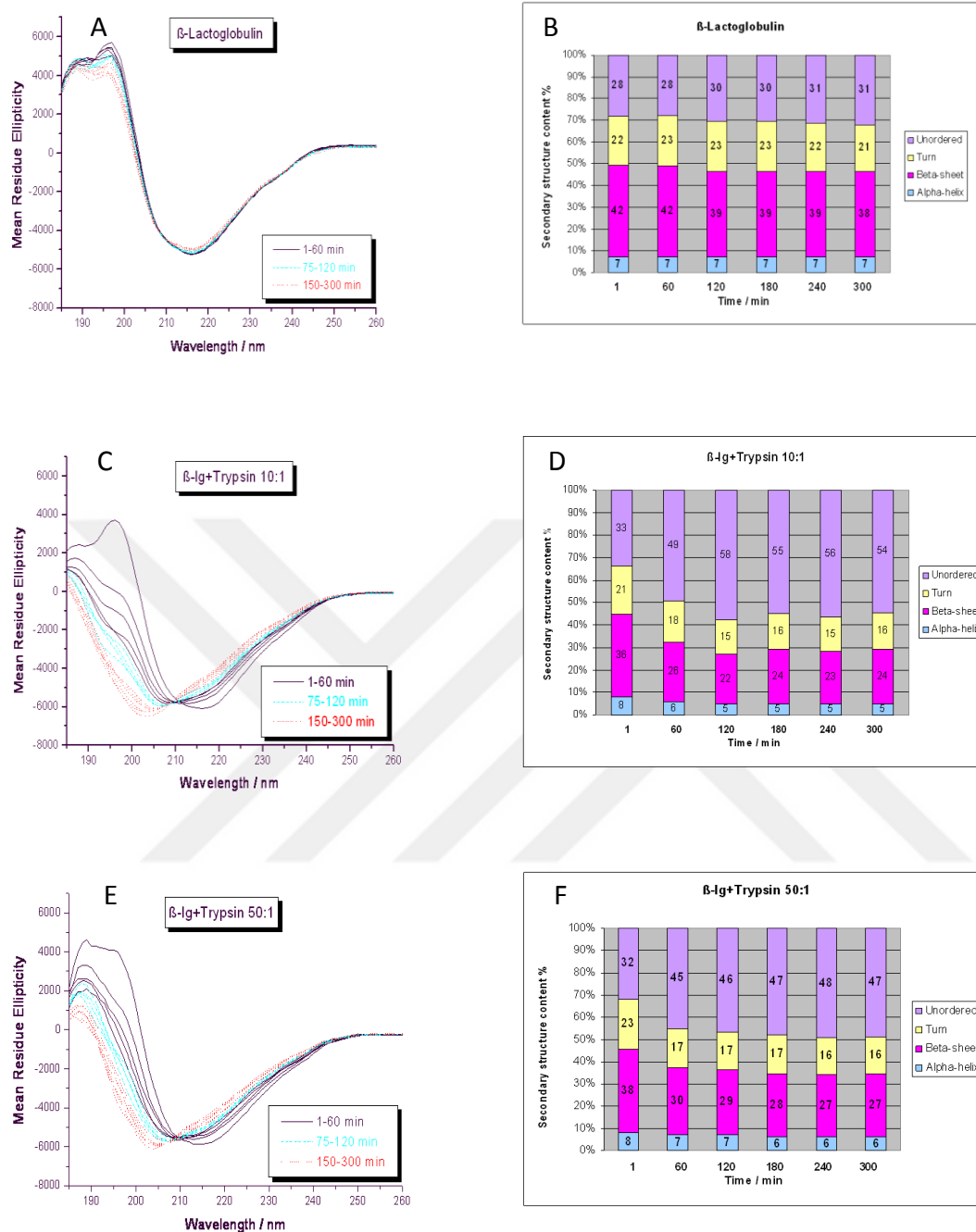


Figure 3.24: The buffer corrected and smoothed far-UV CD spectra with the assignment (%) of the secondary structures of: **(A, B)** Blank β -Lg at 25 mg/ml. **(C, D)** β -Lg digested by trypsin at 10:1 ratio. **(E, F)** β -Lg digested by trypsin at 50:1 ratio. All spectra were recorded at pD 8.4, 37 °C for 5 hours. The assignments of the secondary structures were calculated according to the 37-soluble protein reference set for the range of 185-240 nm (Sreerama and Woody, 2000).

3.2.3 Comparison FTIR and CD Spectroscopies

Both FTIR and CD results reveal that trypsin induces a decrease in β -sheet, turns and α -helix structures concomitant with an increment in the unordered structure. This indicates that the β -Lg peptides are digested by trypsin and converted into smaller fragments, resulting in accumulation of the unordered structures.

According to the both FTIR and CD spectra, the 10:1 and 50:1 ratios induce significant structural changes. Yet, they show almost the same intensity changes, suggesting similar strength of tryptic digestion. In this respect, the mean residue ellipticity at 205 nm from the CD spectra was plotted versus time (Fig. 3.25). In the CD measurements, the amount of the ellipticity alterations is almost the same as in the FTIR measurements (Fig. 3.23 Bottom). Then, both ratios degrade the β -Lg with a similar strength, but the 10:1 ratio causes more product than 50:1 ratio during proteolysis. This is also related to the accessibility of the protein to the enzyme.

According to the FTIR spectra, the most significant conformational changes occur in the amide I and amide II region during proteolysis. The intensity of β -sheets at 1634 cm^{-1} decreases and the unordered structure increases upon digestion. Thereby, the results are in agreement with the CD results.

Figure 3.26 shows the amino acid sequence of β -Lg taken from Protein Data Bank (PDB ID: 2Q2M). As mentioned previous, trypsin predominantly cleaves peptides on the C-terminal of lysine and arginine residues except followed by the proline residue. Proline (red), lysine (green) and arginine (blue) residues were colour coded in figure 3.26. β -lactoglobulin is a relatively small protein with 162 amino acid residues. Although only one lysine (Lys47) is followed by proline (Pro48) among the trypsin-sensitive peptide bonds, the cleavage of the peptide bonds is related to the accessibility of these bonds to the trypsin as well as structural stability of β -Lg. Then, the yellow highlighted point in figure 3.26 was not digested by trypsin because proline is a hydrophobic amino acid and this bond locates within the β -sheet structure of β -Lg.

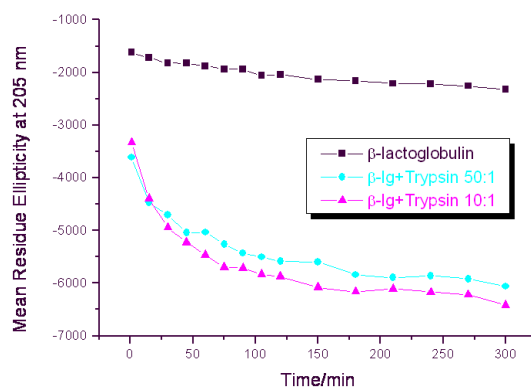


Figure 3.25: The ellipticity changes of β -Lg at 205 nm as a function of time during proteolysis by trypsin.

According to the 3-D structure and the primary structure of β -Lg (Figure 2.2 and Figure 3.26), the region of (...8-14...) in the N-terminal region is susceptible to the digestion and can be easily degraded into the short fragments. Because this part does not include proline residues and it locates within the solvent exposed part of the β -Lg. Then, the accessible and solvent exposed bonds are hydrolyzed by trypsin, and the digested secondary structures are converted into the short fragments and unordered structures, concomitant with releasing of the free carboxylate groups.

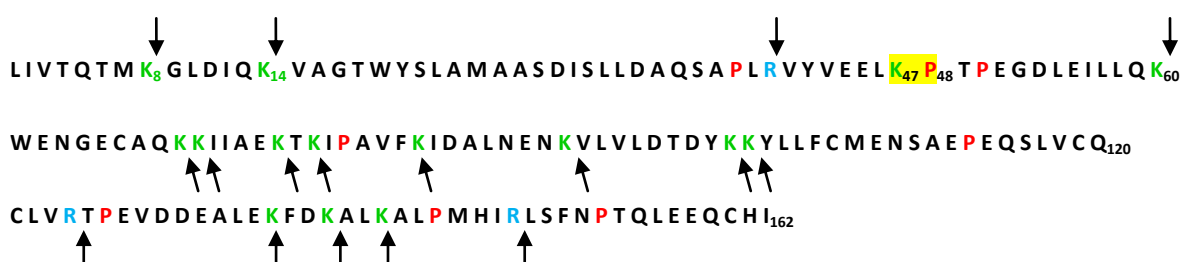


Figure 3.26: The amino acid sequence of β -lactoglobulin (reproduced from PDB code: 2Q2M). The number of proline (**P**) residues is 8, of lysine (**K**) is 15 and of arginine (**R**) is 3. The hydrolysis points (cleaved peptide bonds) are shown by means of black arrows. The estimated undigested residue was highlighted by yellow colour.

3.3 β -CASEIN (β -CN)

Before we discuss the proteolysis of β -casein by trypsin, we shall first mention the effects of H/D exchange to the band positions of β -casein. Figure 3.27 represents the FTIR spectra of β -casein in H₂O buffer, pH 8 at 37 °C. The amide I peak of β -casein has a main component around 1653 cm⁻¹ and the amide II band has a peak around 1549 cm⁻¹. These bands are ascribed to the overlap of α -helices and unordered structures in H₂O buffer. Additionally, the 1653 cm⁻¹ band has two shoulders located at 1674 and 1627 cm⁻¹ which correspond to the turns and β -sheets, respectively. Alternatively, these 1674 and 1627 cm⁻¹ peaks are attributed to the vibrations of $\nu(\text{C}=\text{O})$ and $\nu_{\text{as}}(\text{CN}_3\text{NH}_5^+)$ arising from side chains (Asn, Gln and Arg), and to the vibration of the $\delta_{\text{as}}(\text{NH}_3^+)$ arising from Lys, respectively, in H₂O (Barth, 2000). According to the FTIR spectra, there is no considerable alteration on the β -casein structure during incubation at 37 °C. The peaks located at 1674, 1652 and 1627 cm⁻¹ in the amide I region show some intensity changes and shift towards the lower wavenumbers due to weakening of H-bonds upon heating at 37 °C for 5 h.

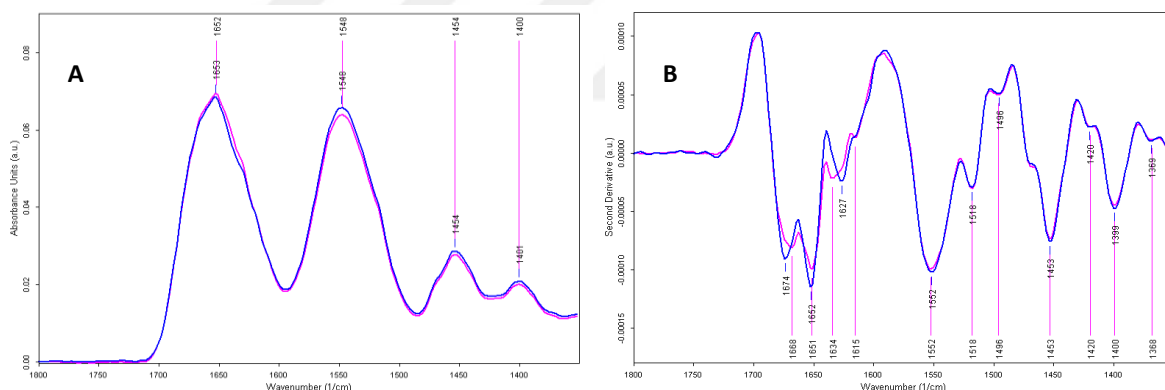


Figure 3.27: (A) The FTIR spectra of β -casein (50 mg/ml) in H₂O buffer at pH 8. (B) The second derivatives of the absorbance spectra in (A). Blue colour indicates the spectrum at $t = 15$ min and magenta colour is the spectrum at $t = 5$ h during the incubation at $T = 37$ °C.

Moreover, β -casein was prepared in D₂O buffer and equilibrated 3 h at +4 °C. The deuterated β -casein sample was recorded at 37 °C for 5 h. Figure 3.28 represents the FTIR spectra of β -casein the amide I and amide II regions. A peak at 1653 cm⁻¹ in H₂O buffer shifts towards 1643 cm⁻¹ upon H/D exchange. The amide I band is mainly composed of a maximum peak at 1643 cm⁻¹ which is ascribed to the overlap of signals from α -helix and unordered structures. Additionally, the signals at 1674 and 1627 cm⁻¹ in H₂O buffer shift towards lower

wavenumbers upon H/D exchange. Thereby, there is a broad shoulder located around 1669 cm^{-1} which corresponds to the deuterated turns or side chains. According to the second derivative profile of β -casein, the amide II band has maxima at 1565 and 1585 cm^{-1} which are attributed to the deuterated side chains (Glu, Asp, Arg). The intensity around 1552 cm^{-1} in the amide II region shifts towards the amide II' region centred at 1451 cm^{-1} upon H/D exchange because the N-H bending vibration is replaced by the N-D bending vibration.

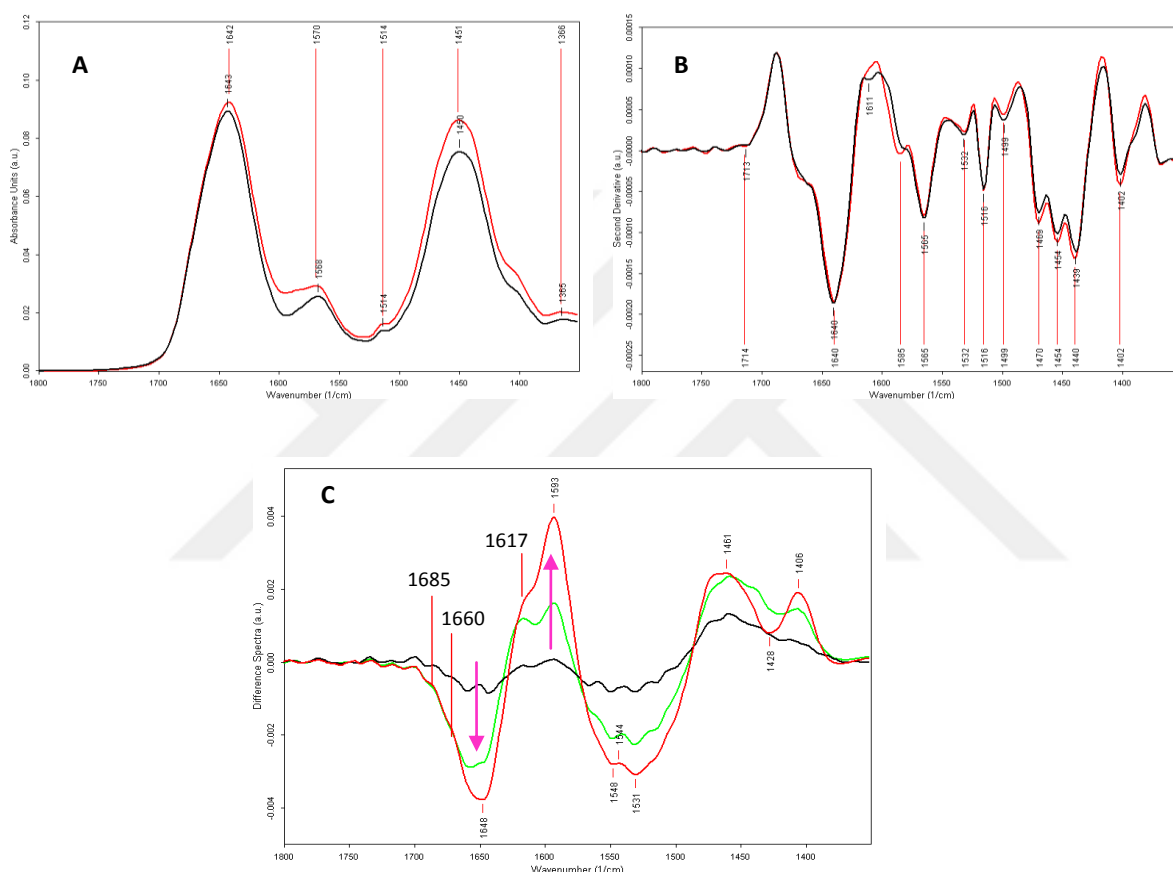


Figure 3.28: (A) The FTIR spectra of β -casein (50 mg/ml) in D_2O buffer at PD 8.4, 37 $^{\circ}\text{C}$. (B) The second derivatives of the absorbance spectra in (A). Black colour indicates the spectrum at $t = 15$ min and red colour represents the spectrum at $t = 5$ h. (C) The difference spectra of the absorbance spectra in (A). Black colour shows the difference spectrum at $t = 38$ min, green is at $t = 2$ h and red is at $t = 5$ h. The arrows indicate the amide I and amide II intensity changes as a function of time. Difference spectra were obtained by subtraction of the first spectrum at $t = 5$ min from the each of the spectra in (A) taken during the incubation at $T = 37$ $^{\circ}\text{C}$ [$\Delta\text{Absorbance} = \{\beta\text{-casein}(t)\} - \{\beta\text{-casein}(t=5\text{min})\}$].

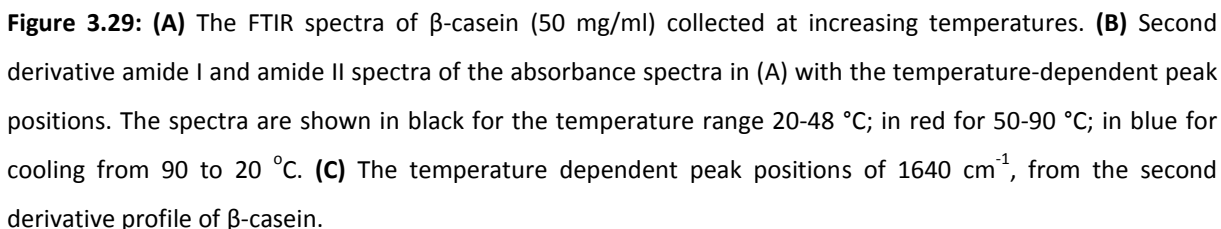
The difference spectra of β -casein (Fig. 3.28C) shows still the effects of H/D exchange during incubation at 37 °C because the H/D exchange rate is faster at 37 °C than at 4 °C as a result of a more flexible, dynamic conformation and diffusion at 37 °C. In this respect, the signal at the 1648 cm^{-1} band decreases (α -helix) and shifts to lower wavenumbers, indicating shifting of the unordered structure exposed to D_2O . According to the curve fit results for the spectra at $t = 5\text{h}$, this band is partially masked by nearby random coils although the band narrowing techniques have been diligently performed (Table 3.6). Additionally, the intensity at the 1593 cm^{-1} band increases, suggesting the D_2O exposed-side chains. In the difference spectra, a positive shoulder around 1660 cm^{-1} appears, revealing increasing of turns. The positive shoulders at 1685 and 1617 cm^{-1} , nevertheless, increase which corresponds to the typical aggregation bands upon heating. This is also accounted for the aggregation of some of the flexible structures. Since β -casein has very little structure to unfold due to high number of proline residues, the solution-accessible residues (hydrophilic amino acid residues within the N-terminal region) might be affected by heating.

Table 3.5 gives the percentages of secondary structure elements of β -casein in H_2O and D_2O buffers. The band positions were determined by the band narrowing techniques and were curve fitted for the amide I and amide II regions of the spectra. According to the table, the signals at 1652 cm^{-1} shifts towards 1651 cm^{-1} which is assigned to the α -helix structure. Additionally, the signal at 1643 cm^{-1} appears upon H/D exchange which corresponds to the unordered structures. The bands between 1667 and 1691 cm^{-1} in H_2O buffer shift towards lower wavenumbers which are attributed to the turns. Then, the secondary structure content of β -casein is composed of 20% β -sheet, 28% turn, 16% α -helix and 36% unordered structure.

Table 3.5: Assignment of amide I components and percentages (%) of secondary structure elements of β -casein in H_2O and in D_2O buffers at 37°C (Spectra at t = 15 min).

Position ^a in H_2O (cm^{-1})	Proportion ^b (%) in H_2O	Position ^a in D_2O (cm^{-1})	Proportion ^b (%) in D_2O	Band Assignments
1619	6	-	-	β -sheet
1629 ^{c,e}	12	1627	20	β -sheet/extended
1638 ^{c,e}	4	1636	20	β - sheet/extended/Random
-	-	1643	16	Random coil
1652	49	1651	16	α -helix/random
-	-	1660	12	Turn/loop
1667	5	1665	9	Turn
1673 ^d	12	1673	5	Turn
1681	9	1680	2	Turn
1691	3	-	-	Turn
^a After deconvolution. ^b Area percentages of secondary structure elements in the amide I region calculated after curve fitting. ^c This band contains elements of amide side chains (Arg, Lys). ^d This band contains elements of amide side chains (Asn, Gln). ^e This band includes stranded (H-bonded) sheet and unstranded (extended) sheet (Kumosinski & Unruh, 1996).				

Figure 3.29 demonstrates the temperature-induced structural changes of β -casein in D_2O buffer. The FTIR spectra were recorded at increasing temperatures from 20 to 90 °C and then were cooled back to 20 °C in intervals of 2 °C. It is clear from the figure that the intensity of the amide I and amide II bands decreases with rising temperature. Additionally, the signal at 1640 cm^{-1} (unordered structure) shifts towards 1646 cm^{-1} with rising temperature up to 90 °C; indicating an increase in polyproline II structure (Qi et al., 2004). This could be a consequence of the movement of polyproline II model peptide from aqueous to a more hydrophobic environment. However, when it is cooled back to 20 °C, this peak position returns to again 1640 cm^{-1} , indicating the reversible changes of β -casein upon heating up to 90 °C. The bands between 1660 and 1672 cm^{-1} broaden with rising temperature but become again narrow when the sample was cooled. This might results from the flexibility of the protein upon heating. According to the peak position profile (Figure



In this study, β -casein was digested by trypsin under the two protein/protease ratios of 10/1 and 50/1 (w/w) in D₂O buffer during 5 hours at 37 °C. The FTIR spectra were recorded at a resolution of 4 cm⁻¹ with 128 scans after the protein was equilibrated 3 h at +4 °C.

Figure 3.30 demonstrates the original FTIR spectra of β -casein (50 mg/ml) during proteolysis by trypsin (5 mg/ml) at a ratio of 10:1. First of all, it is important to mention that when trypsin was added into the β -casein sample just before the measurement, the sample had a

translucent colour, suggesting precipitation of the β -casein and breakdown of the peptide bonds due to tryptic digestion. Therefore, some of the structural changes of β -casein might be probably triggered by trypsin before the recording of the FTIR spectra.

According to figures 3.30A and 3.30B, the intensity at 1641 cm^{-1} increases due to effect of trypsin. Such an alteration does not exist for the blank β -casein in fig 3.28. Additionally, the intensity at 1591 cm^{-1} increases whereas the intensity between 1615 cm^{-1} and 1623 cm^{-1} decreases upon digestion. Furthermore, the intensities around 1670 and 1677 cm^{-1} (turns) shift towards lower wavenumbers.

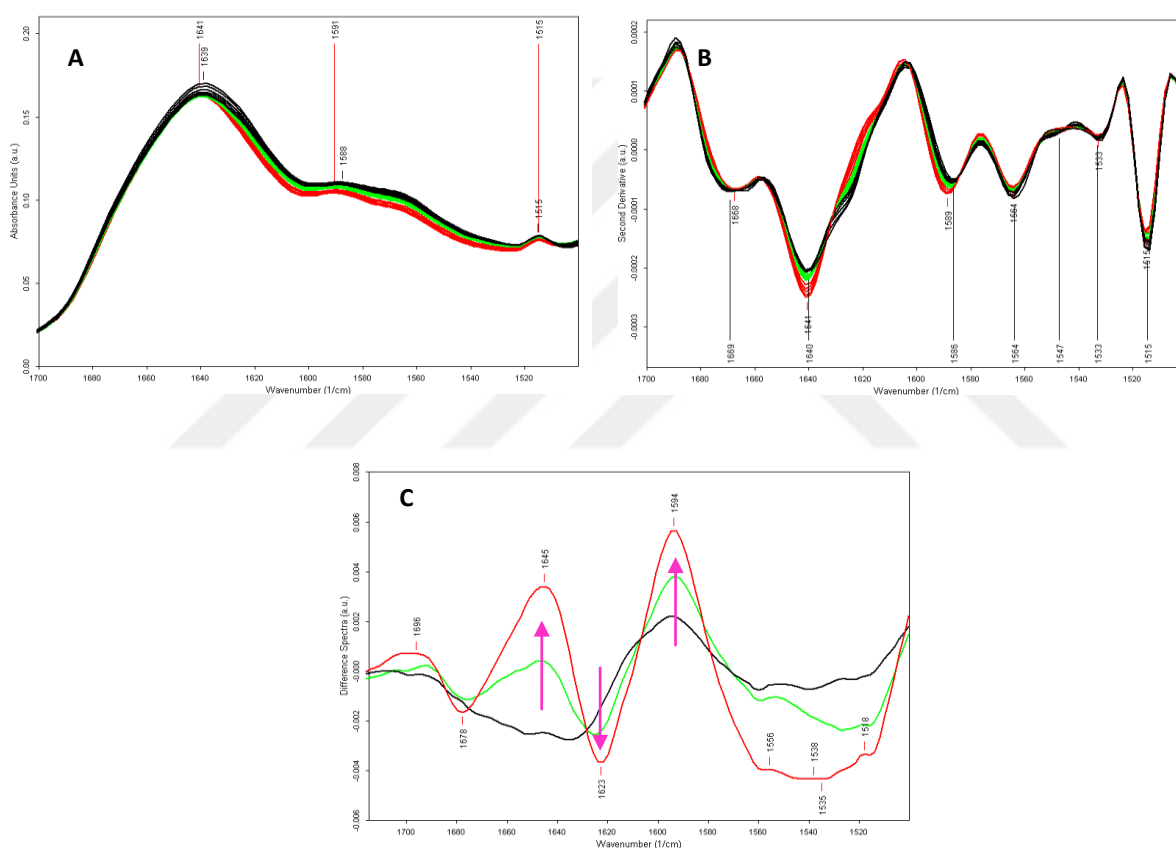


Figure 3.30: (A) The original FT-IR spectra of β -casein (50 mg/ml) were taken during digestion by trypsin (5 mg/ml) at a ratio of 10:1, at 37°C , from 5 min to 5h. (B) The second derivative profiles of the absorbance spectra in (A). (C) Difference spectra of the absorbance spectra in (A). The black colour shows the difference spectrum at $t = 38\text{ min}$, green is at $t = 2\text{h}$ and red is at $t = 5\text{h}$. The arrows indicate the amide I and amide II intensity changes as a function of time. Difference spectra were obtained by subtraction of the first spectrum at $t = 5\text{ min}$ from each of the spectra in (A) taken during the incubation at $T = 37^\circ\text{C}$

$$[\Delta\text{Absorbance} = \{\beta\text{-casein}(t)\} - \{\beta\text{-casein}(t=5\text{min})\}].$$

Difference spectra (Figure 3.30C) reveal a decrease at 1623 cm^{-1} as well as an increase around 1594 cm^{-1} , suggesting a decrease in β -sheet/unstranded sheet (extended) and an increment in the antisymmetric stretching of free carboxylate groups. Additionally, the intensity at 1678 cm^{-1} diminishes due to the digestion and/or further H/D exchange effects. Interestingly, the intensity at the 1645 cm^{-1} band increases during digestion. It seems as if the content of α -helices/unordered structure increased. This band might arise from the aggregated C-terminal region of β -casein because this part is rich in proline residues and the apolar tail probably forms some ordered secondary structure. However, the N-terminal region of β -casein is composed of hydrophilic and charged amino acid residues within the first 50 residues. Thereby the trypsin-sensitive peptide bonds within the N-terminal part are first digested by trypsin, causing aggregation of the residual part (C-terminal). After the N-terminal region was digested, the C-terminal region of β -casein is probably converted into a tightly packed structure due to hydrophobic residues. Alternatively, the 1645 cm^{-1} band might arise from polyproline II (PPII) structure (left-handed 3_1 -helix) due to the movement of polyproline II structure from aqueous to a more apolar environment (Qi et al., 2004). It was studied by vibrational CD spectroscopy and reported that such structures (3-6 residues) would yield FTIR spectra with maxima at 1656 and 1620 cm^{-1} in D_2O . The polyproline residues composed of longer chains ($n > 8$) show a peak at 1620 cm^{-1} (Dukor and Keiderling, 1991). According to Adzhubahi and Sternberg (1993), polyproline II-helices represent an exposed, mobile structure, mostly lie on the protein surface (hydrophilic) and hydrogen bonded with water molecules. Polyproline II-helices have a preference for proline amino acids, although this not necessarily occurs in all PPII-helices.

Table 3.6 gives a summation of the FTIR results showing areas and peak positions of β -casein during proteolysis by trypsin at 37°C . It is also confirmed that the content of the turns at 1661 cm^{-1} increase while the band at 1666 cm^{-1} disappears. Moreover, the content of the 1623 cm^{-1} band (β -sheet/extended) decreases upon proteolysis. For the digested β -casein at $t = 5\text{ h}$, the content of the α -helices became 9% whereas it was 8% for the blank β -casein. Additionally, the content of the unordered structure at 1643 cm^{-1} decreases slightly during digestion; there is no significant change in this structure.

Table 3.6: Positions and assignment of the amide I components and secondary structure percentages (%) of β -casein/trypsin (10/1).

Blank β -Casein			β -Casein+Trypsin 10:1			
Position ^a (cm ⁻¹) and Amide I components	% ^b at t = 15 min	% ^b at t = 5h	Position ^a (cm ⁻¹) and Amide I components	% ^b at t = 5 min	% ^b at t = 1h	% ^b at t = 5h
1620 (β -sheet)	-	-	1613 (intermolecular β -sheet)	-	9	9
1627 (β -sheet/extended)	19	29	1623 ^c -1625 ^d (β -sheet/extended)	18	17	17
1636-1639 ^e (β -sheet/extended)	20	27	1636 (β -sheet/extended)	27	17	21
1643 (unordered)	16	-	1643 (unordered)	15	13	15
1651-1647 ^e (α -helix)	16	8	1651 ^e (α -helix)	8	16	9
1660 (turn)	12	29	1661 (turn)	18	14	18
1666-1668 (turn)	10	2	-	-	-	-
1674 (turn)	5	4	1674-1671 (turn)	14	12	10
1680 (turn)	2	2	1682 (turn)	-	2	2

^aAfter deconvolution. Band narrowing techniques were used to determine the positions. Fourier self deconvolution was applied 1500 using a Lorentzian band shape with 0.5 noise reduction factor for the all spectra.

^bArea percentages of secondary structure elements in the amide I region calculated after curve fitting.

^cThe band positions for the digested β -casein at t = 5 and t = 1h.

^dThe band positions for the digested β -casein at t = 5h.

^eThese bands are partially masked by the nearby random coil components.

Figures 3.31A and 3.31B demonstrate the curve fitted spectra of the native and digested β -casein at $t = 5$ h. It is clear that the digested β -casein shows a higher increment in the intensity of 1593 cm^{-1} than the blank β -casein. This band which is attributed to the antisymmetric stretching of free carboxylates of short fragments is a product of the proteolysis process.

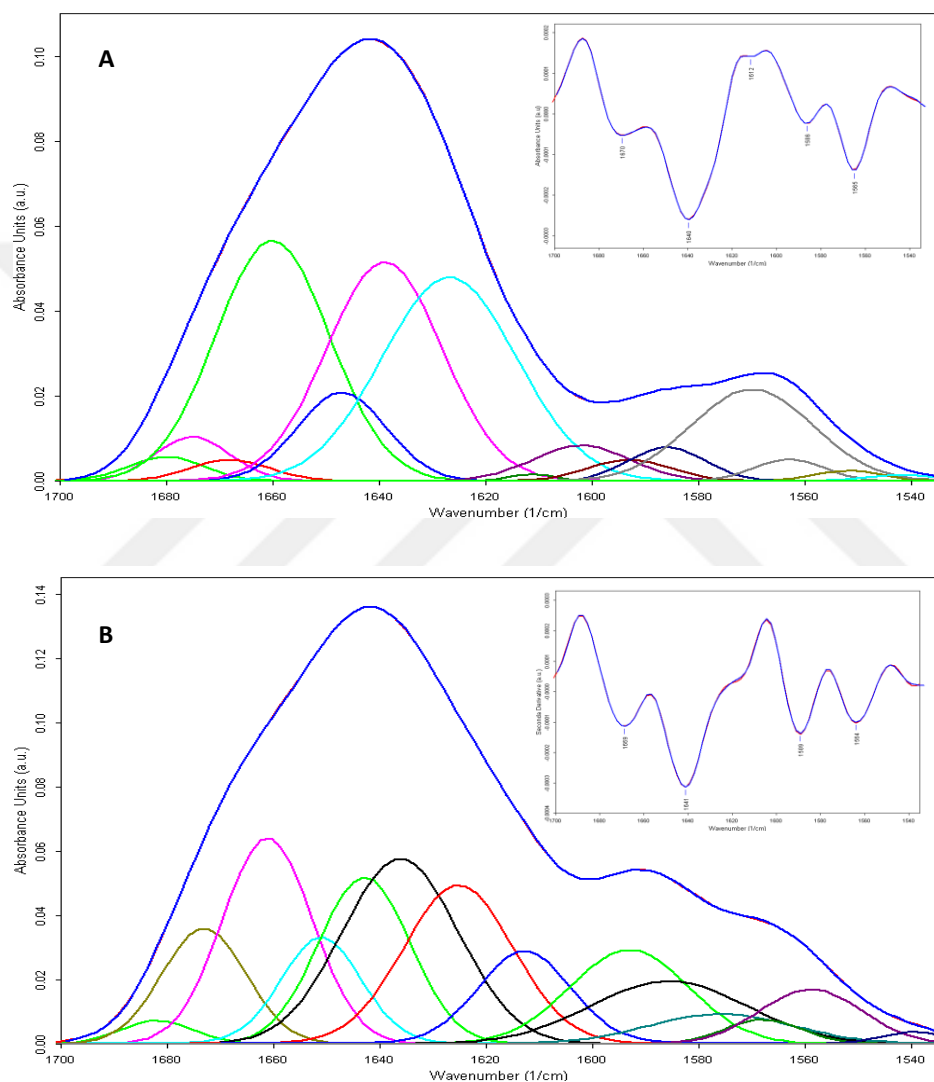


Figure 3.31: Curve fit analysis after deconvolution for amide I and amide II regions at $t = 5$ h.

(A) Native β -casein (50 mg/ml). **(B)** β -casein digested by trypsin (10:1). The envelope bands which are formed by curve fitting are shown in red and the envelope bands of the original spectra are shown in blue. Insets represent the second derivatives of the original spectra and the fitted envelope in order to compare the success of the curve fit. Before curve fitting, buffer was subtracted from the blank β -casein, and the enzyme (5 mg/ml) was subtracted from the digested β -casein.

β -Casein/Trypsin Ratio 50/1

Figure 3.32 demonstrates the original FTIR spectra of β -casein (50 mg/ml) upon proteolysis by trypsin (1 mg/ml) at 50:1 ratio. There are no significant changes in the absorbance and second derivative spectra of the digested β -casein at that ratio in contrast to 10:1 ratio. However, the difference spectra of digested β -casein (Fig. 3.32C) by trypsin at 50:1 ratio are different from the difference spectra of the blank β -casein (Figure 3.28C). Probably, the spectral changes for digested β -casein have already started before the measurements.

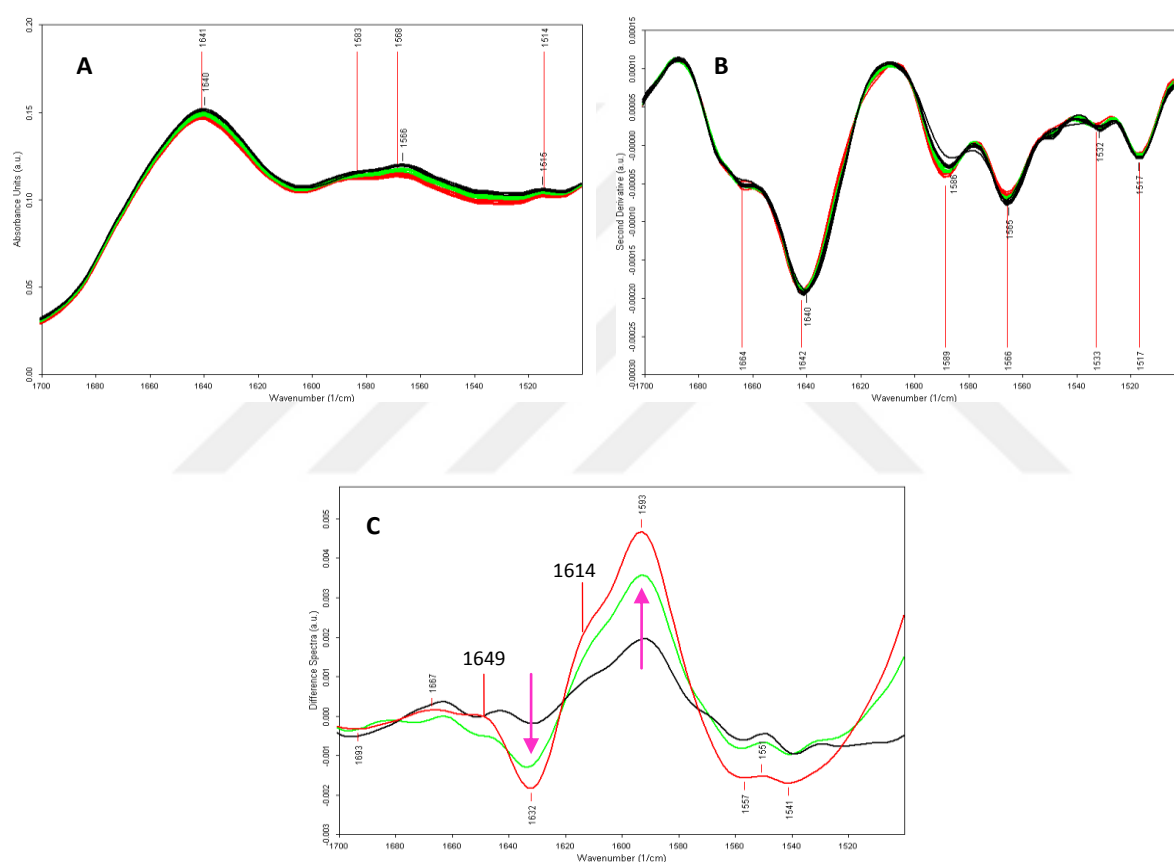


Figure 3.32: (A) The original FT-IR spectra of β -casein taken during digestion by trypsin at a ratio of 50:1, at 37 °C from 5 min to 5h. (B) The second derivatives of the absorbance spectra in (A). (C) Difference spectra of the absorbance spectra in (A). Difference spectra were obtained by the subtraction of first spectrum at 5 min from each of the spectra in (A) taken during proteolysis. The black colour shows the difference spectrum at t = 38 min, green is at t = 2h and red is at t = 5h. The arrows indicate the amide I and amide II intensity changes as a function of time.

According to figure 3.32C, the intensity at 1632 cm^{-1} diminishes while the signal at 1593 cm^{-1} increases, indicating a decrease in β -sheet/unstranded extended (1632 cm^{-1}) and an increment in the antisymmetric stretching of free carboxylates (1593 cm^{-1}) as in the case of the proteolysis at 10:1 ratio. Besides, two shoulders at 1614 and 1649 cm^{-1} are observable in the difference spectra. In this respect, a shoulder at 1614 cm^{-1} may arise from the formation of intermolecular β -sheets. A shoulder at 1649 cm^{-1} might result from the segments of polyproline II (left-handed 3_1 -helix) as previously mentioned in the section of β -casein/trypsin ratio 10/1.

Figure 3.33A represents the difference spectra for the blank β -casein and digested β -casein with various ratios. It is clear from the difference spectra that the same band signals are shifted for all ratios of substrate and enzyme. However, the strength of the shifting differs on enzyme concentration. The signal at 1593 cm^{-1} continuously increases not only for the digested β -casein but also for the blank protein. This may also arise from side chains upon D_2O exposure. For instance, arginine signals shift towards 1608 cm^{-1} and 1586 cm^{-1} upon H/D exchange. Furthermore, Asp and Glu COO^- signals shift towards 1584 and 1567 cm^{-1} , respectively, due to D_2O exposure (Barth, 2000). Interestingly, the intensity around the 1645 cm^{-1} band shows an increment for both 50:1 and 10:1 ratios during digestion. This band may arise from polyproline II (left-handed 3_1 -helix) structure. Additionally, the intensity at the 1632 cm^{-1} band decreases for the 50:1 ratio while the signals at 1623 cm^{-1} decrease for the 10:1 ratio.

Figure 3.33B shows the intensity changes of antisymmetric stretching of free carboxylates (1593 cm^{-1}) as a function of time during proteolysis with various ratios. The appearance of the liberated short fragments for the 10:1 ratio is rapid and is sharper than for the 50:1 ratio. The intensity of these free carboxylate groups for the blank β -casein is constant up to 40 min, and then increases during incubation at 37°C .

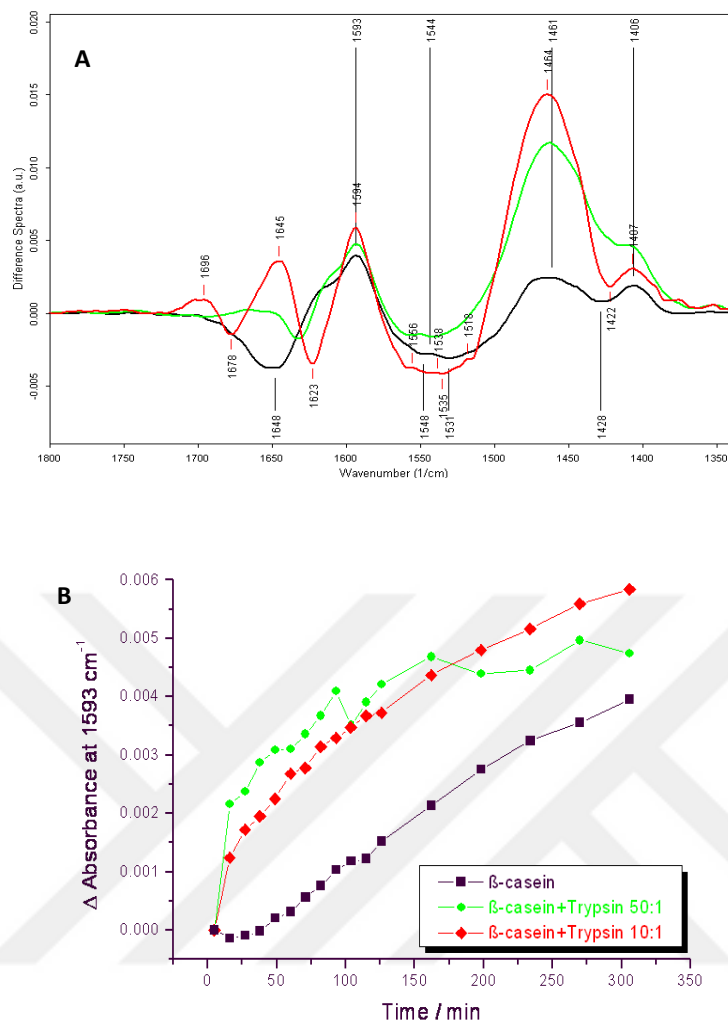


Figure 3.33: (A) Difference spectra of the blank β -casein (black) and β -casein digested by trypsin for 10:1 (red); for 50:1 (green) ratios. The difference spectra were obtained by the subtraction of the first spectrum at 5 min from the last spectrum at 5 h. (B) The intensity changes of antisymmetric stretching of free carboxylates (1593 cm^{-1}) as a function of time during proteolysis with various ratios. The intensities were obtained from the difference spectra in figures 3.28C, 3.30C and 3.32C.

3.3.2 CD Studies for Proteolysis of β -Casein by Trypsin

Far-UV CD spectroscopy was used to evaluate the secondary structure of β -casein upon proteolysis. The CD spectra (185-260 nm) were recorded at pD 8.4 for 5 h at 37°C . The CD spectrum of the buffer and blank enzyme were also measured under the same conditions. Thereby, the CD spectra were corrected using corresponding background as mentioned in the materials and methods section (2.2.2 Spectroscopic Techniques), and were scaled to the mean residue ellipticity based on the protein concentration and the number of amino acid

residues of the sample. Figure 3.34 demonstrates the effect of tryptic digestion on β -casein at two different ratios of substrate and enzyme. All spectra were smoothed with a smoothing factor of 9.

Figure 3.34A and 3.34B show the CD spectra of blank β -casein and its secondary structure alterations for 5 h. It is clear from this figure that blank β -casein has a deep negative band around 200 nm and has a low ellipticities in the region 210-230 nm. This suggests that β -casein is primarily a random coil conformation and has very little periodic structure. During incubation at 37 °C, the intensity at 190-195 nm slightly decreases, indicating a loss of α -helical structure. Additionally, the ellipticities around 210-230 nm becomes less negative, suggesting occurrence of the some structural changes in the remaining structural elements. Besides, for the CD spectra of blank β -casein, there is a trough around 224 nm arising from some sheet or turn as well as PPII-like structure (Farrell et al., 2001; Siligardi et al., 1991; Makarov et al., 1984).

The analysis of the secondary structures of β -casein indicates the presence of 50% unordered, 23% β -sheet, 15% turns, 12% α -helical structure. There is no significant secondary structure alteration of the blank sample during incubation at 37 °C for 5 h. For the blank β -casein spectra at $t = 5$ h, the content of unordered structure increases by only 7% whereas helices decreases by only 4% and the remaining elements show no remarkable changes (only 1-2%).

This indicates that it is a quite stable protein at 37 °C for 5 h.

Figure 3.34C and 3.34D show the CD spectra of the digested β -casein by trypsin at 10/1 (substrate/enzyme) ratio and give its secondary structure alterations upon digestion. There are pronounced changes in the shape of the spectra at this ratio, indicating the effect of trypsin on β -casein. A negative peak at 201 nm shifts towards 203 nm (red shift) together with an appearance of the more negative ellipticity. Additionally, the ellipticity between 185-200 nm increases and a downward shift appears between 210-230 nm upon digestion. Besides, the secondary structure analysis of digested β -casein at $t = 5$ h reveals 40 % unordered, 24 % β -sheet, 19 % turns, 18 % α -helical structure. Then, there is a decrease in the unordered structure concomitant with an increase in the α -helical and turn structures and no change for β -sheet. Altogether this suggests that an apparent increase in the helices can be accounted for polyproline II structure.

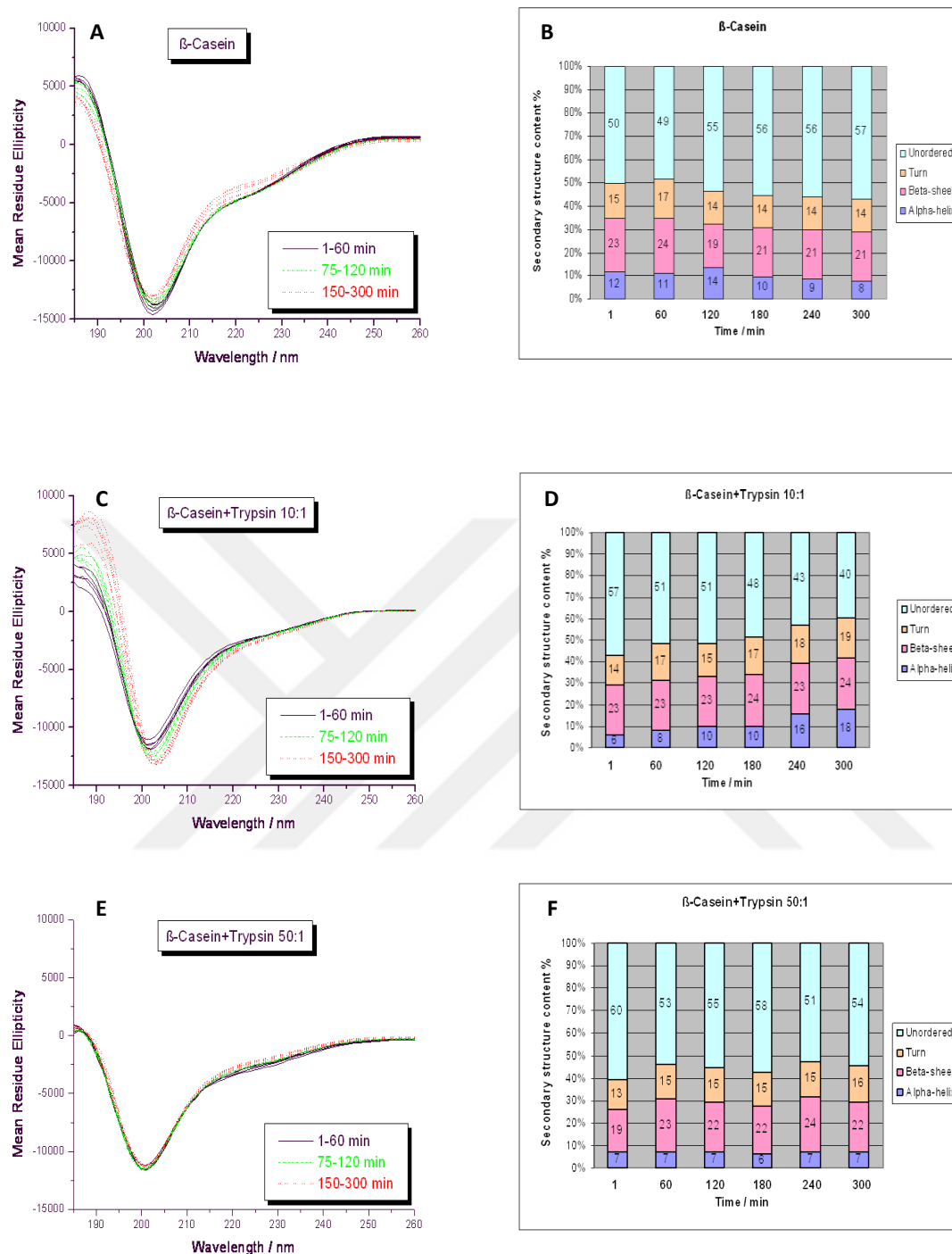


Figure 3.34: The buffer-corrected and smoothed far-UV CD spectra with the assignment (%) of the secondary structures of: **(A, B)** Blank β -casein at 25 mg/ml. **(C, D)** β -casein digested by trypsin at 10:1 ratio. **(E, F)** β -casein digested by trypsin at 50:1 ratio. All spectra were recorded at pD 8.4, 37 °C for 5 hours. The assignments of the secondary structures were calculated according to the 37-soluble protein reference set for the range of 185-240 nm (Sreerama and Woody, 2000).

Figure 3.34E and 3.34F show the CD spectra of digested β -casein by trypsin at 50/1 (substrate/enzyme) ratio and the secondary structure changes for 5 h. The shape of the spectra for digested β -casein at 50:1 ratio is different from figure 3.34C (10:1 ratio). There is no shifting in the band positions of the CD spectra upon proteolysis at 50:1 ratio when compared to the 10:1 ratio. However, it is observable that the ellipticities around 210-230 nm becomes less negative, suggesting structural changes in the secondary structure elements. Furthermore, the secondary structure analysis of digested β -casein at $t = 5$ h shows 54% unordered, 22% β -sheet, 16% turns, 7% α -helical structure. Then, there is a decrease in the α -helical and unordered structure concomitant with an increase in turns and β -sheet structures upon digestion.

3.3.3 Comparison of FTIR and CD Spectroscopies

The FTIR and CD spectra for the 50:1 ratio of β -casein and trypsin show very similar changes in their intensities upon digestion. Some of the alteration has triggered before the spectra were recorded and during the recording time there is no remarkable changes in the FTIR and CD spectra of β -casein due to digestion. The FTIR and CD spectra for the 10:1 ratio of substrate and enzyme also show similar kind of spectral changes in their intensities during digestion. Yet, in both spectra, the breakdown of the intact protein induced by trypsin has already started before the recording of the sample.

In the FTIR analysis, it is also confirmed that the content of the turns at 1661 cm^{-1} increases due to proteolysis for the 10:1 and 50:1 ratios. Additionally, the content of the 1623 and 1632 cm^{-1} band (β -sheet/extended) decreases for the 10:1 and 50:1 ratios, respectively, upon proteolysis. The intensity around the 1645 - 1649 cm^{-1} band shows an increment for both 50:1 and 10:1 ratios during digestion. However, the CD results reveal that trypsin causes a diminution in the unordered structure concomitant with an increment in the turns and α -helical structures, and there is no significant change in β -sheets. Since the extended structures do not have an ordered structure, probably, the 1623 and 1632 cm^{-1} bands would be sensed by FTIR, but not sensed by CD spectroscopy. UV-CD spectroscopy for the secondary structure analysis may predict these structures as unordered structure. Alternatively, this band may arise from the longer chains ($n > 8$) of the polyproline II (left-handed 3_1 -helix) residues which have a signal at 1620 cm^{-1} in D_2O (Dukor and Keiderling,

1991). According to Drake and co-workers (1988), random coils can be considered as a two-state conformational equilibrium including a left-handed polyproline II extended helix.

The existence of the polyproline II and turns in the native 1-25 peptide of the N-terminal region has been already revealed (Farrell et al., 2002). In our study, the CD spectra for the 10:1 ratio (Fig. 3.34C) demonstrate a red-shift from 201 nm to 203 nm together with a more negative ellipticity, and the band intensity between 210-230 nm becomes more negative during proteolysis with an apparent increase in the α -helical content. However, a strong negative band at 204-206 nm is a spectral feature of the polyproline II peptide (Rabanal et al., 1993). Then, such an increment in the content of the helices for the 10:1 ratio may arise from the polyproline II structure or from more ordered structure due to digestion-induced hydrophobic interactions.

Fig. 3.35 shows the mean residue ellipticity at 203 nm versus time. The β -casein/trypsin ratio of 10:1 reveals rapidly more negative ellipticity at this wavelength than the 50:1 ratio. In this respect, the CD analysis is in agreement with the FTIR analysis. As the protein is digested, the increment of the released short fragments for the 10:1 ratio is rapidly and is sharper than the 50:1 ratio (Fig. 3.35B). In both studies, digestion at the 50:1 ratio comes to equilibrium.

Figure 3.36 demonstrates the amino acid sequence of β -casein (reproduced from Vorob'ev et al., 2000 and Farrell et al., 2001). As mentioned previously, trypsin has a preference to cleave the peptide bonds which contains Arg and Lys residues. However, if these positively charged amino acid residues are followed by a proline residue, there would be a masking effect which prevents the digestion of the peptide bonds. In figure 3.36, proline (red), lysine (green) and arginine (blue) residues were colour coded. β -casein is a relatively small protein with 209 amino acid residues and is the most hydrophobic protein with 35 proline residues among the other type of caseins. According to the primary structure, β -casein has highly charged N-terminal region and a hydrophobic C-terminal region. Caseins are known to be easy hydrolysed by proteases due to their conformational flexibility and the abundance of the enzyme-accessible peptide bonds.

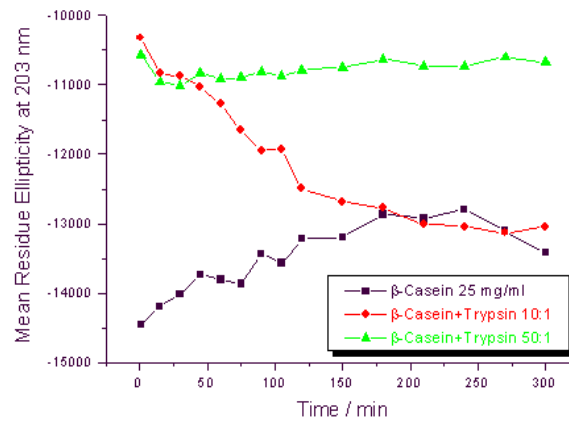


Figure 3.35: The ellipticity changes of β -casein at 203 nm as a function of time during proteolysis by trypsin.

Tryptic digestion of β -casein induces the rapid release of the polar part (intermediate products) of the protein during the early stages of proteolysis, and more hydrophobic intermediate fragments are formed whose some parts are more hydrophobic than whole protein (Leonil et al., 1988). Then, trypsin may cleave the intact β -casein from the specific sites (Arg and Lys) in the hydrophilic region (N-terminal), inducing the formation of the hydrophobic intermediates which may precipitate. This leads to the degradation of the intact protein and to the precipitation of fragments of the hydrophobic region (C-terminal), forming a more compact and tightly packed structural part in β -casein.

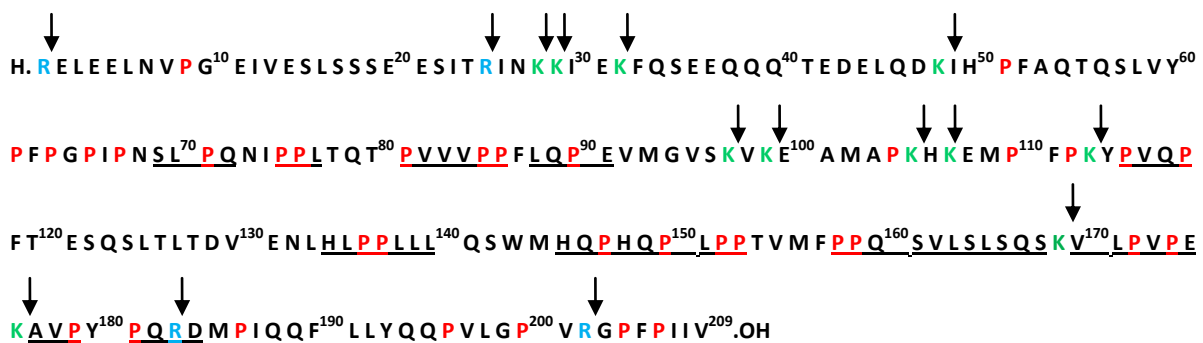


Figure 3.36: The amino acid sequence of β -casein (reproduced from Vorob'ev et al., 2000; Farrell et al., 2001). The number of proline (P) residues is 35, of lysine (K) is 11 and of arginine (R) is 4. The hydrolysis points (cleaved peptide bonds) are shown by means of black arrows. The underlined sequence parts indicate the polypyrrole II predictions of Adzhubahi and Sternberg (1993).

3.4 COMPARISON OF SECONDARY STRUCTURE ANALYSIS BY FTIR AND CD SPECTROSCOPIES

Proteolysis of BSA by α -Chymotrypsin

The secondary structural elements of blank BSA (Table 3.7) and of digested BSA by α -chymotrypsin at 10:1 (Table 3.8) and at 50:1 ratios (Table 3.9) were found using the curve-fitting method and band narrowing techniques as explained in the materials and methods section (2.2.2 Spectroscopic Techniques). In order to calculate the percentage of the different types of conformations to the area of all the components, bands assigned to a given conformation were summed and divided by the total amide I band area.

Table 3.7: Secondary structure percentages (%) of blank BSA in D₂O buffer at 37 °C, obtained by FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -sheet/Extended ^b %	Turn ^c %
15	68	20	12
60	67	20	13
126	69	18	13
162	69	17	14
234	68	17	15
306	71	18	11
^a α -helix (1639-1654 cm ⁻¹) ^b β -Sheet/extended (1620-1628 cm ⁻¹ ; 1682 cm ⁻¹) ^c Turn (1666-1676 cm ⁻¹)			

The curve fitting results of the FTIR spectra of blank BSA showed a strong peak at 1653 cm⁻¹, which is assigned to α -helix, and other peaks at 1620, 1628, 1639, 1667-1676 and 1682 cm⁻¹ corresponding to β -sheet, extended strand, random coil, turn and antiparallel β -sheet, respectively (Fabian and Mäntele, 2002; Goormaghtigh et al., 1994).

According to curve fit results of the FTIR spectra, BSA digested by α -chymotrypsin at the 10:1ratio showed peaks at 1614, 1627, 1637, 1645, 1653, 1660, 1667-1674 and 1682 cm⁻¹, which are assigned to intermolecular β -sheet, extended/ β -sheet, random coil, D₂O exposed α -helix/random coil, regular α -helix, distorted α -helix/random coil, turn and antiparallel β -sheet, respectively. In table 3.7, blank BSA does not show a significant change during incubation at 37 °C. The percentage of α -helices is 68-71%, of β -sheet/extended is 18-20% and of turns is 12-15%. However, the curve-fitting procedure indicates that the content of α -helices is 49% at t = 5 min and is 32% at t = 5h for BSA digested by α -chymotrypsin at 10:1

ratio (Table 3.8). Remarkably, for a 10:1 ratio, the content of turns increases from 9% to 18% and random coils increase from 23% to 33% due to digestion for 5 h.

Table 3.8: Secondary structure percentages (%) of BSA digested by α -chymotrypsin (10:1 ratio) in D₂O buffer at 37 °C, from FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -Sheet/Extended ^b %	Turn ^c %	Random Coil ^d %
5	49	19	9	23
60	41	13	10	36
126	41	7	13	39
162	36	18	14	32
234	31	15	10	44
306	32	17	18	33
^a α -helix (1644-1654 cm ⁻¹) ^b β -Sheet/extended (1614-1627 cm ⁻¹ ; 1682 cm ⁻¹) ^c Turn (1667-1676 cm ⁻¹) ^d Random coil (1637-1644 cm ⁻¹ , 1660 cm ⁻¹)				

According to table 3.9, the percentage of α -helices is 54% at t = 5 min and 48% at t = 5h for BSA digested by α -chymotrypsin at the 50:1 ratio. Furthermore, the content of turns increases from 15% to 19% and random coils increase from 15% to 21% due to proteolysis. Such a decrease in the helices and increase in turns and random coils are the effects of digestion, but these alterations are more remarkable for the 10:1 ratio than for the 50:1 ratio of substrate and enzyme. We have considered the signal at 1660 cm⁻¹ as distorted α -helix/random coil which arises from digestion. Therefore, the content of this band was taken into percentage of the random coil content.

Table 3.9: Secondary structure percentages (%) of BSA digested by α -chymotrypsin (50:1 ratio) in D₂O buffer at 37 °C, obtained from FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -Sheet/Extended ^b %	Turn ^c %	Random Coil ^d %
5	54	16	15	15
60	48	6	18	28
126	51	7	19	23
162	47	13	21	19
234	52	14	18	16
306	48	12	19	21
^a α -helix (1643-1654 cm ⁻¹) ^b β -Sheet/extended (1613-1627 cm ⁻¹ ; 1682 cm ⁻¹) ^c Turn (1665-1676 cm ⁻¹) ^d Random coil (1637-1643 cm ⁻¹)				

In order to compare the results of FTIR and CD spectroscopy, the content of α -helix for native BSA and for digested BSA (10:1 and 50:1 ratios) was plotted against time for 5 hours (Fig. 3.37). For FTIR spectroscopy, the percentages of helices were taken from tables 3.7, 3.8 and 3.9 while figure 3.13 was utilized for the estimated percentages of helices obtained from CD spectroscopy. According to figure 3.37, a drastic reduction observed in α -helix conformation due to proteolysis is consistent with both spectroscopic techniques. The secondary structure analysis of digested BSA studied by FTIR spectroscopy shows some difficulties to estimate the α -helix proportion. This component is partially masked by the nearby random coil and turn components in the curve-fitting results although Fourier self deconvolution has been diligently performed.

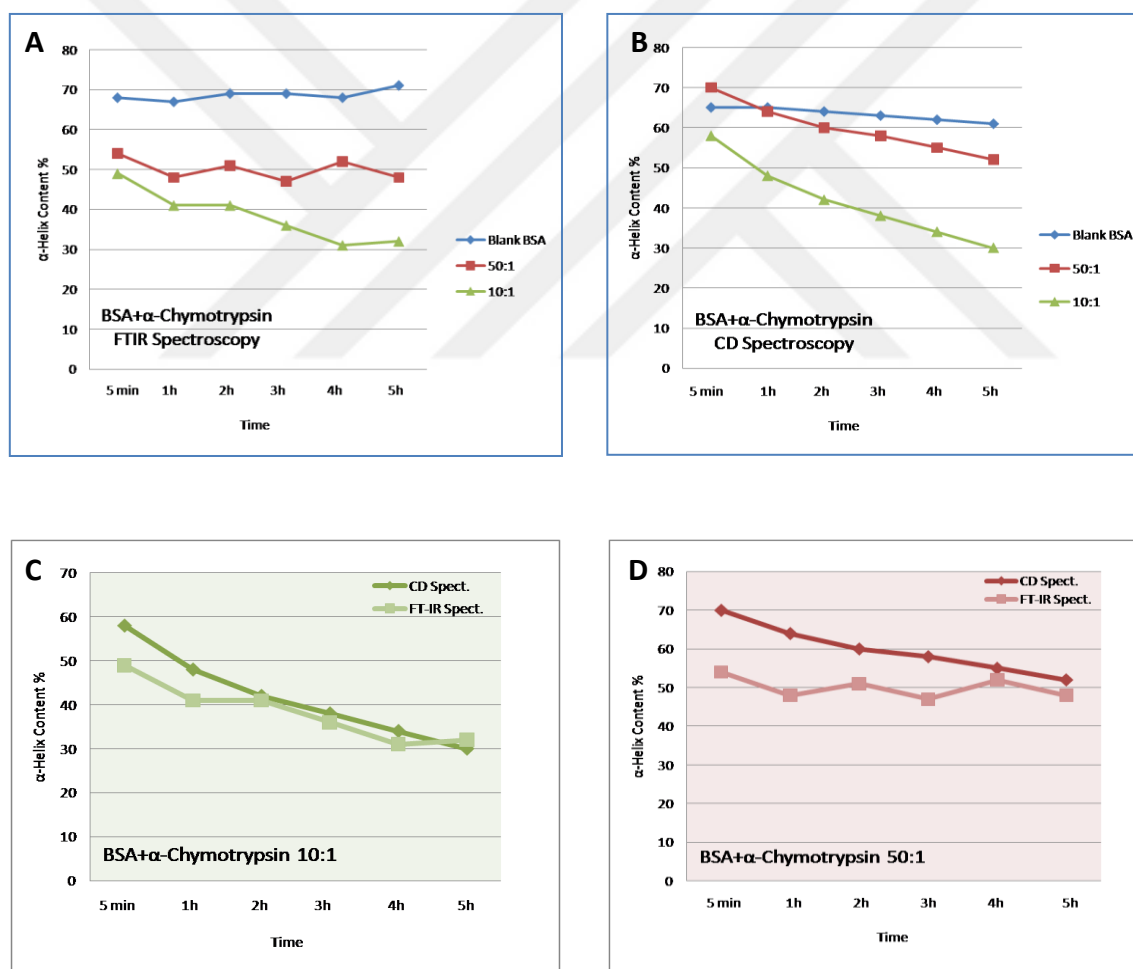


Figure 3.37: The content of α -helix for blank BSA and for BSA digested by α -chymotrypsin (10:1 and 50:1 ratios) obtained from FTIR spectroscopy (A) and from CD spectroscopy (B). Comparison of FTIR and CD spectroscopy: the content of α -helix against time for digested BSA at 10:1 ratio (C) and at 50:1 ratio (D).

Proteolysis of BSA by Trypsin

Tables 3.10 and 3.11 show estimated secondary structure proportion of BSA digested by trypsin at 10:1 and at 50:1 ratios, respectively. The FTIR-curve fit results of digested BSA by trypsin at 10:1 ratio represents peaks at 1616, 1628, 1638, 1645, 1654, 1662-1668-1676 and 1682 cm^{-1} , which are assigned to intermolecular β -sheet, extended/ β -sheet, random coil, D_2O exposed α -helix/random coil, regular α -helix, turn and antiparallel β -sheet, respectively (Table 3.10). The content of α -helix is 54 % at $t = 5$ min and is 41 % at $t = 5$ h for BSA digested by trypsin at 10:1. It is obvious that the α -helices show a strong decrease during proteolysis whereas the content of turns, β -sheets and random coils increase as it is shown in table 3.10.

Table 3.10: Secondary structure percentages (%) of BSA digested by trypsin (10:1 ratio) in D_2O buffer at 37 °C, from FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -Sheet/Extended ^b %	Turn ^c %	Random Coil ^d %
5	54	15	15	16
60	46	20	20	14
126	47	19	22	12
162	47	18	17	18
234	44	16	23	17
306	41	17	26	16
^a α -helix (1643-1654 cm^{-1}) ^b β -Sheet/extended (1616-1628 cm^{-1} ; 1681-1683 cm^{-1}) ^c Turn (1662-1677 cm^{-1}) ^d Random coil (1637-1638 cm^{-1})				

The curve fit results of BSA digested by trypsin at 50:1 ratio show almost the same peaks for the FTIR spectra (Table 3.11). The proportion of the α -helical structure is 64 % at $t = 5$ min and is 47 % at $t = 5$ h. Moreover, turns, β -sheets and random coils increase during digestion as it is shown in table 3.11. Then, the tryptic digestion leads to a drastic diminution in α -helices and an increment in the remaining structure of BSA.

Table 3.11: Secondary structure percentages (%) of BSA digested by trypsin (50:1 ratio) in D₂O buffer at 37 °C, obtained from FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -Sheet/Extended ^b %	Turn ^c %	Random Coil ^d %
5	64	15	9	12
60	54	19	12	15
126	54	20	12	14
162	52	17	20	11
234	49	9	22	20
306	47	20	16	17

^a α -helix (1643-1654 cm⁻¹)
^b β -Sheet/extended (1616-1628 cm⁻¹; 1682 cm⁻¹)
^c Turn (1665-1676 cm⁻¹)
^d Random coil (1638-1639 cm⁻¹)

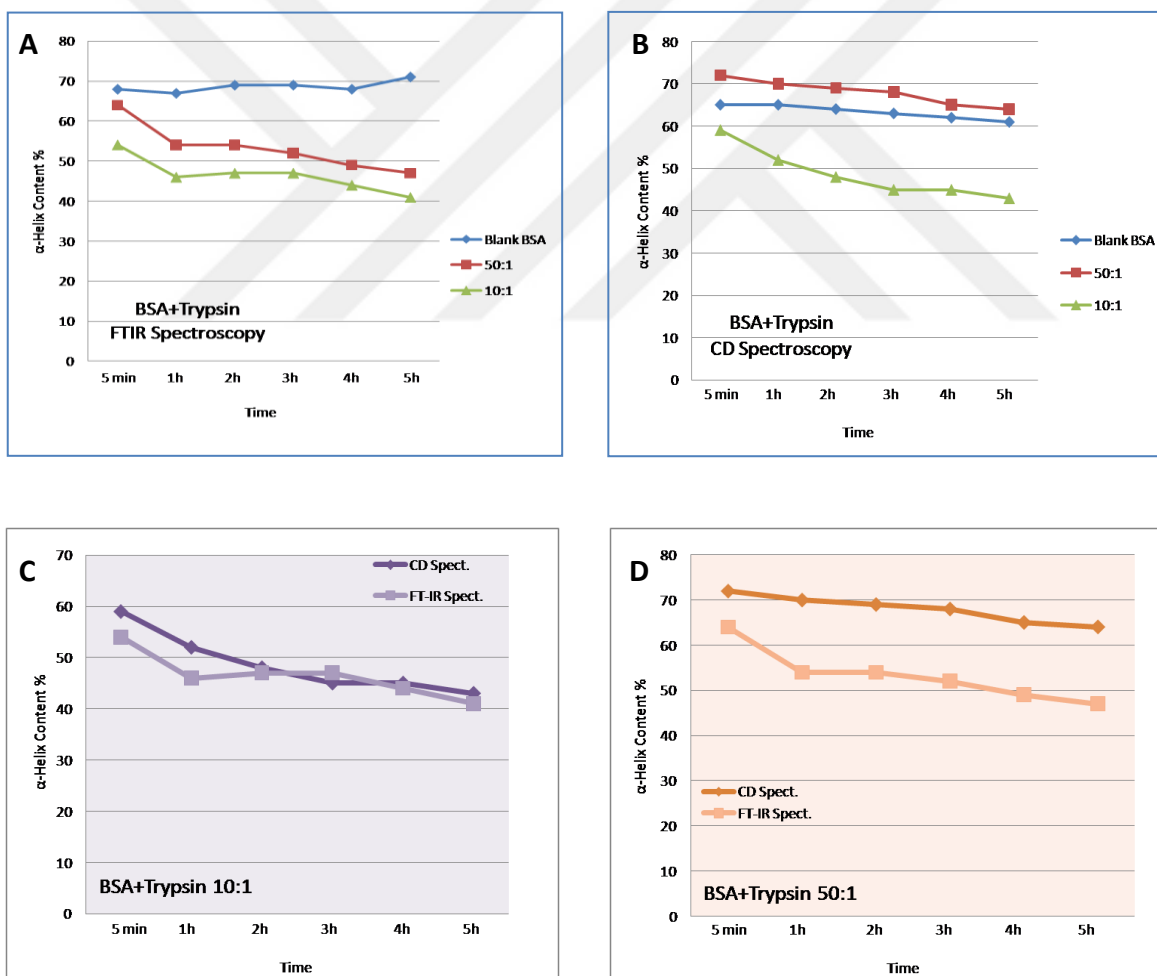


Figure 3.38: The content of α -helix for blank BSA and for BSA digested by trypsin (10:1 and 50:1 ratios) obtained from FTIR spectroscopy (**A**) and from CD spectroscopy (**B**). Comparison of FTIR and CD spectroscopy: proportion of α -helix against time for digested BSA at 10:1 ratio (**C**) and at 50:1 ratio (**D**).

Figure 3.38 demonstrates the content of the α -helical structure for blank (native) BSA and for digested BSA by trypsin (at 10:1 and 50:1 ratios), plotted versus time for 5 hours. Figure 3.14 obtained from CD spectroscopy was used for the proportion of α -helices, and the percentages of helices obtained from FTIR spectroscopy were taken from tables 3.7, 3.10 and 3.11. In figure 3.38, a striking decrease observed in the α -helix conformation due to tryptic digestion is almost in agreement with both spectroscopic techniques (Fig. 3.38 C and D) because this band overlaps with nearby components in the curve-fitting procedures. Since there is no significant change in the α -helix content of blank BSA, such diminution in α -helices is an evidence of degraded BSA arising from digestion by trypsin (Fig. 3.38 A and B).

Proteolysis of β -Lactoglobulin (β -Lg) by Trypsin

The secondary structure proportion of blank β -lactoglobulin (Table 3.12) and of digested β -Lg by trypsin at 10:1 (Table 3.13) and at 50:1 ratios (Table 3.14) were calculated using the FTIR- curve fit method and band narrowing techniques as explained in the materials and methods section (2.2.2 Spectroscopic Techniques). The FTIR curve-fitting results of blank β -Lg showed a strong signal at 1634 cm^{-1} , which is assigned to β -sheet, and other peaks at 1621 , 1649 , 1662 - 1677 cm^{-1} corresponding to β -sheet, α -helix+random coil and turn, respectively. As shown in table 3.12, blank β -Lg does not show a significant alteration during incubation at $37\text{ }^{\circ}\text{C}$. The percentage of β -sheets is 53-56%, of α -helix+random coil is 29-20% and of turns is 18-24%. The contents of random coils and α -helices were summed because these bands overlap at 1649 cm^{-1} .

Table 3.12: Secondary structure percentages (%) of β -lactoglobulin in D_2O buffer at $37\text{ }^{\circ}\text{C}$, obtained from FTIR spectroscopy.

Time (min)	α -Helix+RC ^a %	β -Sheet ^b %	Turn ^c %
15	29	53	18
60	21	58	21
126	21	55	24
162	20	56	24
234	19	57	24
306	20	56	24
^a α -helix+random coil (1649 - 1648 cm^{-1}) ^b β -Sheet (1621 - 1634 cm^{-1}) ^c Turn (1662 - 1677 cm^{-1})			

Table 3.13 gives the estimated secondary structure proportion of β -Lg digested by trypsin at a ratio of 10:1. According to the FTIR curve fit results of digested β -Lg reveals peaks at 1621-1634, 1645-1649-1651, 1662-1666-1677 and 1682 cm^{-1} corresponding to β -sheet, random coil/ α -helix, turn and intermolecular β -sheet, respectively. The content of β -sheet is 53% at $t = 5$ min and is 24% at $t = 5$ h for the digested protein. Therefore, it is clear that β -sheets show a decrease during digestion whereas the content of the random coil structure increase as it is shown in table 3.13. Furthermore, the proportion of turns diminishes due to digestion.

Table 3.13: Secondary structure percentages (%) of β -lactoglobulin digested by trypsin (10:1 ratio) in D_2O buffer at 37 $^{\circ}\text{C}$, obtained from FTIR spectroscopy.

Time (min)	α -Helix+RC ^a %	β -Sheet ^b %	Turn ^c %
5	24	53	23
60	34	45	21
126	56	18	26
162	55	25	20
234	56	21	23
306	55	24	21
^a α -helix+random coil (1635-1652 cm^{-1}). Since the signal of β -sheets overlaps with random coils at 1635 cm^{-1} , this band was taken into the content of random coils. Therefore, the content of β -sheet at $t=126$ min reveals less proportion than at the times up to 5h. ^b β -Sheet (1621-1635 cm^{-1} , 1682 cm^{-1}) ^c Turn (1661-1676 cm^{-1})			

Table 3.14: Secondary structure percentages (%) of β -lactoglobulin digested by trypsin (50:1 ratio) in D_2O buffer at 37 $^{\circ}\text{C}$, obtained from FTIR spectroscopy.

Time (min)	α -Helix+RC ^a %	β -Sheet ^b %	Turn ^c %
5	31	49	20
60	32	46	22
126	37	40	23
162	38	39	23
234	42	41	17
306	37	40	23
^a α -helix+random coil (1648-1660 cm^{-1}) ^b β -Sheet (1621-1634 cm^{-1} , 1684 cm^{-1}) ^c Turn (1665-1679 cm^{-1})			

Table 3.14 demonstrates the curve fit results of digested β -Lg by trypsin at the 50:1 ratio. The curve fit results of digested β -Lg reveals peaks at 1622-1634, 1646-1648-1651-1660, 1665-1677 and 1684 cm^{-1} which are assigned to β -sheet, random coil/ α -helix, turn and intermolecular β -sheet, respectively. The proportion of β -sheet is 49% at $t = 5$ min and is 40% at $t = 5$ h for the digested β -Lg. Then, the digestion of β -Lg at the 50:1 ratio induces a diminution in β -sheet and an increment in the unordered structure.

Figure 3.39 demonstrates the content of β -sheets for blank (native) β -Lg and for digested β -Lg by trypsin (at 10:1 and 50:1 ratios), plotted against time for 5 hours. Figure 3.24 obtained from CD spectroscopy was used for the percentages of β -sheets, and the contents of β -sheets obtained from FTIR spectroscopy were adopted from tables 3.12, 3.13 and 3.14. According to figure 3.39, the β -sheet conformation decreases drastically upon digestion and such a decrease is almost in agreement with both spectroscopic techniques (Fig. 3.39 C and D). Since the signal of β -sheet overlaps with random coils at 1635 cm^{-1} , this band was included into the content of the random coil structure. Therefore, the content of β -sheet at $t = 2$ h reveals less percentage than at the rest times (Fig. 3.39 A and C). However, figure 3.39D plotted for the 50:1 ratio shows a decrease in β -sheets with a similar tendency for both FTIR and CD spectroscopy. Additionally, figure 3.39B is in good agreement with figure 3.23 (Bottom, left) in terms of a decrease in the β -sheet content. In other words, both ratios (10:1 and 50:1) of substrate (β -Lg) and enzyme (trypsin) cause a close reduction in β -sheets.

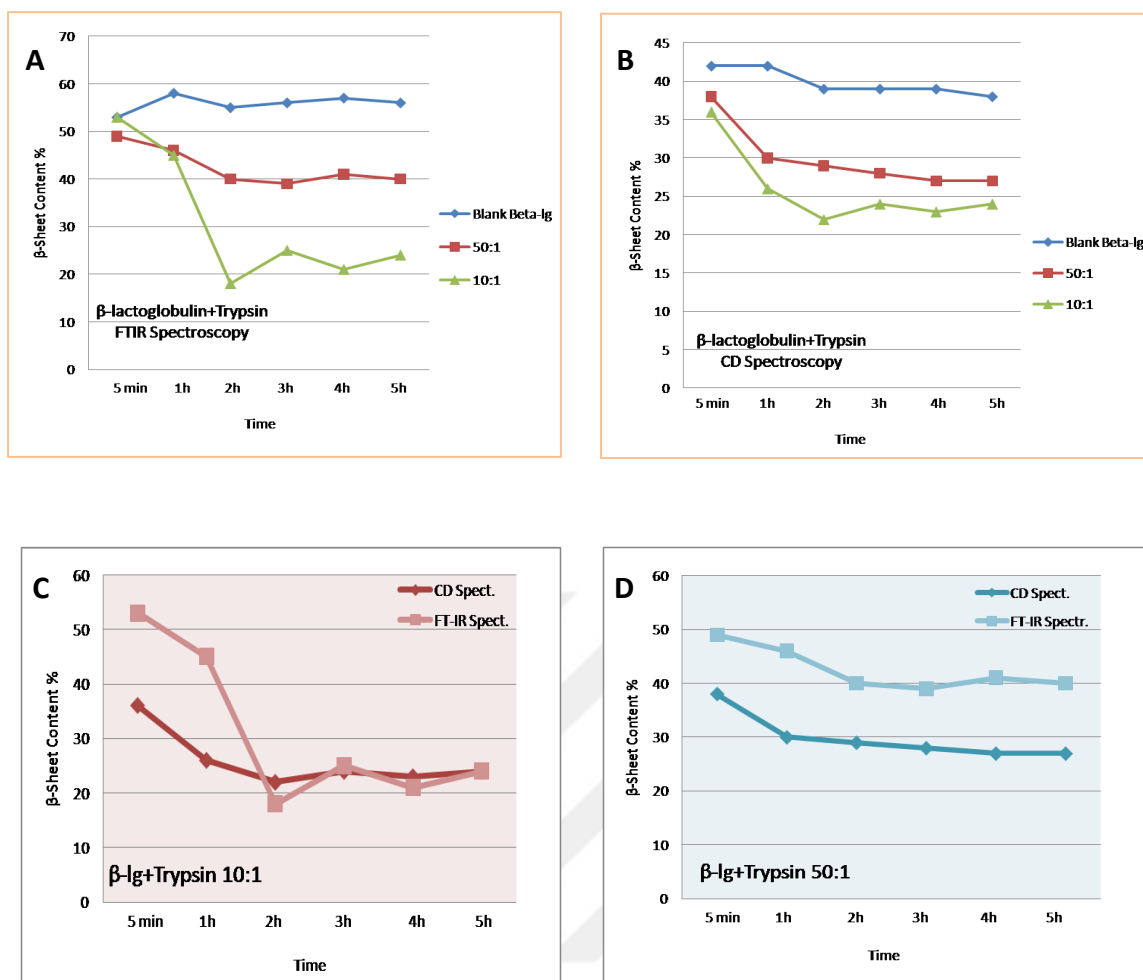


Figure 3.39: The content of β -sheet for blank β -Lg and for β -Lg digested by trypsin (S:E = 10:1 and 50:1 ratios) obtained from FTIR spectroscopy (A) and from CD spectroscopy (B). Comparison of FTIR and CD spectroscopy: proportion of β -sheet against time for digested β -Lg at 10:1 ratio (C) and at 50:1 ratio (D).

Proteolysis of β -Casein by Trypsin

The FTIR-curve fit method and band narrowing techniques were performed to the amide I and amide II bands of β -casein, as mentioned in the materials and methods section (2.2.2 Spectroscopic Techniques). The amide I band ($1610\text{--}1700\text{ cm}^{-1}$) was used in order to estimate the percentages of the secondary structural elements of blank β -casein (Table 3.15) and of digested β -casein by trypsin at 10:1 (Table 3.16) and at 50:1 ratios (Table 3.17).

The curve-fitting results of blank β -casein reveal a mixed distribution of secondary structural elements which is reminiscent of the globular proteins. The FTIR spectrum of deuterated blank (native) β -casein at 37°C showed peaks at 1627 , 1637 , 1643 , 1651 and $1660\text{--}1680\text{ cm}^{-1}$

attributed to β -sheet, extended/random coil, random coil, α -helix and turns, respectively (Fabian and Mäntele, 2002; Goormaghtigh et al., 1994). As it was shown in table 3.15, blank β -casein shows a flexible conformation during incubation at 37 °C because it does not have a close-packed, ordered secondary structure due to their high number of proline residues. In addition to this, such flexibility arises from its lack of tertiary structure because they do not contain disulfide bonds. Therefore, perturbations such as heating and deuteration affect the dynamics of the protein.

Table 3.15: Secondary structure percentages (%) of native β -casein in D₂O buffer at 37 °C, obtained from FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -Sheet ^b %	Turn ^c %	Random Coil ^d %
5	16	20	28	36
60	22	27	27	24
126	11	22	30	37
162	16	16	28	40
234	13	17	28	42
306	8	29	36	27
^a α -helix (1647-1651 cm ⁻¹) ^b β -Sheet (1620-1628 cm ⁻¹) ^c Turn (1660-1682 cm ⁻¹) ^d Random coil (1636-1644 cm ⁻¹)				

Table 3.15 demonstrates that, blank β -casein comprises around 16% α -helix, 20% β -sheet, 28% turn and 36% random coil. The random coils increase in both techniques except for the FTIR spectrum at t= 5h. This might arise from overlap of turns/loops at 1660 cm⁻¹ with unordered and helix structures. Turns might shift towards lower wavenumbers due to heating and deuteration. An increment of this band (1660 cm⁻¹) is also proved by the difference spectrum of blank β -casein (Fig. 3.28C). Figure 3.40 shows that the α -helical content decreases towards t = 5h according to both FTIR and CD measurements (see Fig. 3.34B for the CD spectroscopy results). However, the FTIR spectrum at t = 1h gives high proportion of α -helix structure at 1651 cm⁻¹ which might result from shifting of signals due to deuteration.

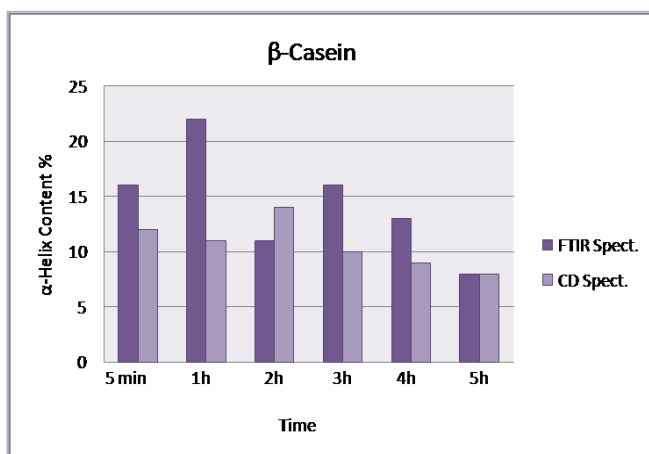


Figure 3.40: The content of α -helix for native β -casein for 5 h, obtained from FTIR spectroscopy and from CD spectroscopy.

Table 3.16 demonstrates the estimated secondary structure proportion of tryptic-digested β -casein at the 10:1 ratio. The FTIR-curve fit results show that digested β -casein has peaks at 1623, 1637, 1642, 1650 and 1661-1674-1680 cm^{-1} attributed to β -sheet, extended/random coil, random coil, α -helix and turns, respectively. Digested β -casein contains 8% α -helix at $t = 5$ min and 21% α -helix at $t = 162$ min, indicating an increment in the α -helical content. Besides, both FTIR (Table 3.16) and CD spectroscopy (Fig. 3.34D) results show that the content of random coils decreases. Therefore, it is obvious that random coils show a decrease during digestion whereas the content of the α -helical structure increases as it is shown in table 3.13. This suggests that polyproline II or tightly packed structures are constituted due to digestion.

Table 3. 16: Secondary structure percentages (%) of β -casein digested by trypsin (10:1 ratio) in D_2O buffer at 37 $^{\circ}\text{C}$, obtained from FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -Sheet ^b %	Turn ^c %	Random Coil ^d %
5	8	18	32	42
60	16	26	28	30
126	19	27	19	35
162	21	22	23	34
234	14	20	27	39
306	9	25	30	36
^a α -helix (1650-1652 cm^{-1})				
^b β -Sheet (1612-1625 cm^{-1})				
^c Turn (1660-1682 cm^{-1})				
^d Random coil (1636-1643 cm^{-1})				

Figure 3.41A was plotted for β -casein digested by trypsin at the 10:1 ratio in order to compare both FTIR and CD spectroscopy results. The α -helical content increases for all times during digestion according to the CD spectroscopy results. Similarly, it increases for the FTIR spectroscopy measurements; however, the FTIR spectroscopy results reveal that the proportion of α -helix decreases for the spectra at $t = 4\text{h}$ and 5h . This might arise from shifting of signals due to deuteration which is not sensed by CD spectroscopy.

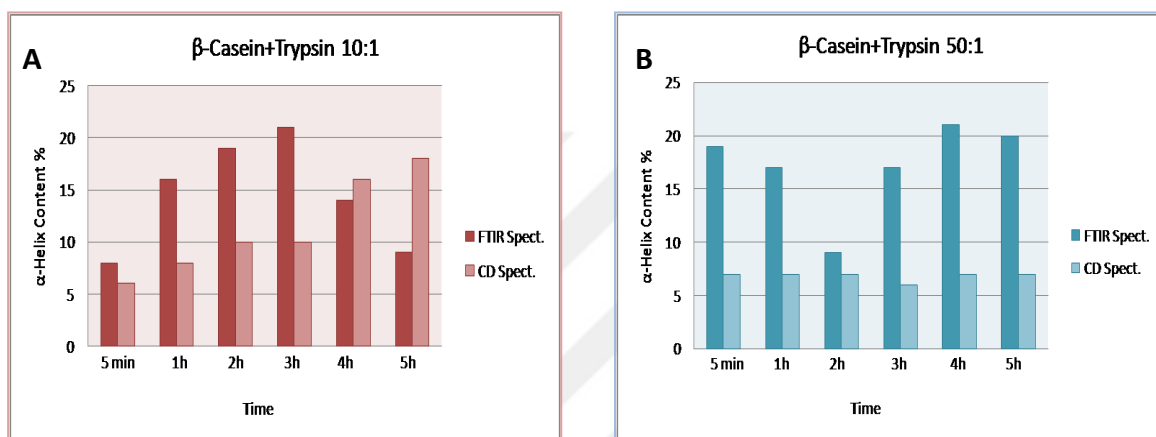


Figure 3.41: The content of α -helix for β -casein digested by trypsin at the 10:1ratio **(A)** and at the 50:1 ratio **(B)** obtained from FTIR spectroscopy and CD spectroscopy.

Table 3.17: Secondary structure percentages (%) of β -casein digested by trypsin (50:1 ratio) in D_2O buffer at 37°C , obtained from FTIR spectroscopy.

Time (min)	α -Helix ^a %	β -Sheet ^b %	Turn ^c %	Random Coil ^d %
5	19	28	32	21
60	17	25	31	27
126	9	30	39	22
162	17	26	33	24
234	21	25	28	26
306	20	28	30	22
^a α -helix ($1648\text{-}1651\text{ cm}^{-1}$)				
^b β -sheet ($1613\text{-}1629\text{ cm}^{-1}$)				
^c Turn ($1658\text{-}1683\text{ cm}^{-1}$)				
^d Random coil ($1638\text{-}1642\text{ cm}^{-1}$)				

Table 3.17 represents the curve fit results of β -casein digested by trypsin at the 50:1 ratio. The curve fit results of digested β -casein shows peaks at 1617, 1628, 1640, 1650 and 1660-1681 cm^{-1} attributed to intermolecular β -sheet, extended/ β -sheet, random coil, α -helix and turns, respectively. The area of α -helix is 19% at $t = 5$ min and is 20% at $t = 5$ h for digested β -casein. If the α -helical content is compared for both FTIR and CD spectroscopy results, it does not show a regular tendency for the FTIR spectroscopy. This might arise from protein flexibility due to heating or deuteration (Fig. 3.41B). Furthermore, other secondary structural elements also show some alterations as shown in table 3.17 but they are not noteworthy at that ratio in contrast to the 10:1 ratio. Such alterations are also different from the blank protein. Probably, breakdown of β -casein due to trypsin have already triggered before the measurements and most of the tryptic effects might complete in that interval. Additionally, formation of the intermolecular β -sheets is an outstanding result of the proteolysis at the 50:1 ratio, which is not striking outcome for the 10:1 ratio.

IV. CONCLUSION

Proteolysis induces degradation of food proteins in digestion and nutrition processes. Yet, the complexity of the enzyme specificity upon peptide bond is still far from being solved and it is not yet clear to figure out the liberated patterns of fragments and the secondary structure alterations of proteins during proteolysis. In the present work, the conformational changes of three proteins (BSA, β -lactoglobulin and β -casein), which are used as substrate, have been studied during proteolysis. For this purpose, trypsin and α -chymotrypsin were utilized as protease enzymes in various concentrations to digest the substrates. In this way, enzymatic-induced degradation of such proteins was identified. Fourier Transform Infrared Spectroscopy (FT-IR) and Circular Dichroism Spectroscopy (CD) were performed to study the structural changes and to confirm the results of the enzymatic reactions.

Temperature-profiling of BSA studied by FTIR spectroscopy revealed that the unfolding state progresses above 60 °C and then aggregation of the protein starts. The transition temperature between the folded and unfolded state was found 68.4 °C. Hence, this property does not affect the proteolysis process at our working temperature of 37 °C.

Digestion of bovine serum albumin by trypsin and α -chymotrypsin at 37 °C in D₂O buffer has been performed. In the FTIR studies, native BSA was found to have 68% α -helix, 20% β -sheet/extended, 12% β -turn in D₂O solution whereas digested BSA by α -chymotrypsin at 10:1 ratio (harsher proteolysis condition) exhibited 32% α -helix, 17% β -sheet/extended, 18% β -turn and 33% random coil structure after 5h proteolysis, and the most significant spectral changes occur in the amide I and amide II regions for the all ratios of substrate and enzyme. In the CD studies, blank BSA consists of 65% α -helix, 4% β -sheet, 10% β -turn and 22% random coil while chymotryptic-digested BSA at 10:1 ratio shows 30% α -helix, 17% β -sheet/extended, 21% β -turn and 32% random coil structure after 5h hydrolysis. Estimation of changes in secondary structural elements by CD and FTIR studies clearly demonstrates that the content of the α -helices diminishes strongly whereas turns and unordered structures increase concomitant with a release of the free carboxylates (absorbing at $\sim 1593\text{ cm}^{-1}$) due to the cleavage of the peptide bonds. It can be interpreted that the accessible α -helices are digested by enzyme and converted into the distorted or short helices (short fragments) and finally, the liberated free carboxylate groups appear as a proteolysis product. Thereby, both spectroscopies are in agreement to confirm the enzymatically-induced secondary structural changes. It is also concluded that the enzymatic breakdown of the

secondary structural elements of BSA becomes more visible with an increasing amount of the enzyme, causing a more degraded BSA. Since trypsin predominantly cleaves the hydrophilic part of the intact protein (from the polar groups of lysine and arginine) and α -chymotrypsin adversely hydrolyses the substrate from the aromatic and large hydrophobic residues (tyrosine tryptophan, phenylalanine, methionine and leucine), degradation of BSA is less affected by trypsin than by α -chymotrypsin. Therefore, even a low concentration of α -chymotrypsin (E:S = 50:1 ratio) caused a strong decrease in the content of α -helix structure of BSA concomitant with an increment in turns and random coils.

Temperature-profiling of β -lactoglobulin indicated that thermally induced changes of the 1678 cm^{-1} band occur in two stages, between $50\text{--}66\text{ }^{\circ}\text{C}$ (phase 1) and above $66\text{ }^{\circ}\text{C}$ (phase 2). Phase 1 exhibits monomerisation of the protein, suggesting a contact between two monomers of the protein. Phase 2 shows the unfolding state of β -Lg at higher temperatures. Temperature-profiling of 1632 cm^{-1} also reveals that the unfolding state starts above $66\text{ }^{\circ}\text{C}$. Hydrolysis of β -lactoglobulin by trypsin at $37\text{ }^{\circ}\text{C}$ in D_2O buffer was carried out using FTIR and CD spectroscopies. FTIR studies revealed that native β -Lg involves 53% β -sheet, 18% β -turn and 29% α -helix+random coil while digested β -Lg by trypsin at 10:1 ratio was found to have 24% β -sheet, 21% β -turn, 55% α -helix+random coil structure after 5h proteolysis. Furthermore, in the FTIR spectra of digested protein, the intensity of the 1593 cm^{-1} and 1406 cm^{-1} bands increases attributed to the antisymmetric and symmetric stretching of free carboxylates, respectively, as proteolysis products. According to the CD studies, native β -Lg consists of 42% β -sheet, 22% β -turn, 7% α -helix and 28% random coil structure whereas tryptic-digested β -Lg at the 10:1 ratio has 24% β -sheet, 16% β -turn, 5% α -helix and 54% random coil after 5h proteolysis. Thereby, the results of both spectroscopes clearly reveal that the content of β -sheet decreases marginally with a parallel increase in the unordered structures, causing breakdown of β -Lg into smaller fragments during digestion. Additionally, it was exhibited that the 10:1 and 50:1 ratios (S:E) degrade the β -Lg with a very close strength, but the 10:1 ratio induces more product than 50:1 ratio during digestion. This could be related to the accessibility of peptide bonds to the trypsin and to the structural stability of β -Lg.

Temperature-profiling of β -casein performed by FTIR spectroscopy exhibited that some structural changes occur without unfolding upon raising the temperature but these continuous changes are reversible up to $90\text{ }^{\circ}\text{C}$. Moreover, the 1640 cm^{-1} band (random coil)

shifts towards 1646 cm^{-1} up to $90\text{ }^{\circ}\text{C}$, suggesting movement of polyproline II structure from aqueous to a more hydrophobic environment.

Digestion of β -casein by trypsin at $37\text{ }^{\circ}\text{C}$ in D_2O buffer was performed by FTIR and CD spectroscopies. According to the FTIR studies, native β -casein was found to have 20% β -sheet, 28% β -turn, 16% α -helix and 36% random coil whereas degraded β -casein by trypsin at 10:1 ratio involves 20% β -sheet, 27% β -turn, 14% α -helix and 39% random coil after 4h proteolysis. Remarkably, the α -helix content of the digested protein is only 8% at $t = 5\text{ min}$. In the CD studies, native β -casein comprises 23% β -sheet, 15% β -turn, 12% α -helix and 50% random coil structures while tryptic-hydrolysed β -casein (10:1 ratio) involves 23% β -sheet, 18% β -turn, 16% α -helix and 43% random coils after 4h proteolysis. Thereby, there is a decrease in random coil structure concomitant with an increase in α -helices. According to the FTIR results, the intensity around the $1645\text{-}1649\text{ cm}^{-1}$ band exhibits an increment for both 10:1 and 50:1 ratios during proteolysis. Altogether these suggest that an apparent increment in the α -helix structure and a diminution in random coils might be accounted for the polyproline II structure, or alternatively, tightly packed structures are formed due to digestion.

To sum up, CD and FTIR spectroscopies have allowed us the detection of subtle and significant structural changes observed during enzymatic digestion. This study reveals that such substrates (BSA, β -lactoglobulin and β -casein) lose their secondary structures upon conversion to the free fragments of peptides without aggregation during enzymatic attack. This perturbation alters the backbone of the native protein resulting in degradation of the intact protein. It is also concluded that the rate of proteolysis of a substrate is related to the overall structural stability and hydrophobicity of proteins as well as enzyme specificity because peptide bonds buried in the hydrophobic region of protein are more resistant to enzymatic hydrolysis due to the masking effect.

V. ZUSAMMENFASSUNG

Bei der Verdauung und Nahrungsmittelaufnahme werden Proteine aus Lebensmitteln durch proteolytische Spaltung abgebaut. Die Mechanismen, die zur Erkennung von spezifischen Substraten und deren Spaltungsstellen dienen, sowie die anschließenden strukturellen Umlagerungen, die zur Spaltung führen, sind bis jetzt nicht bekannt und noch völlig unverstanden. In der vorliegenden Arbeit wurden die Konformationsänderungen von drei Proteinen (BSA, β -Lactoglobulin und β -Casein), die als Substrate dienten, während der Proteolyse untersucht. Um die Substrate zu verdauen, kamen als Proteasen Trypsin und α -Chymotrypsin in verschiedenen Konzentrationen zum Einsatz. Zur Analyse der strukturellen Umlagerungen und zur Bestätigung des enzymatischen Abbaus kamen die Fourier-Transformation-IR-Spektroskopie (FTIR) und die UV-Circulardichroismus-Spektroskopie (CD) zum Einsatz.

Zunächst wurde bei BSA die Temperaturabhängigkeit der korrekten Faltung untersucht und es zeigte sich, dass die Entfaltung des Proteins ab 60°C beginnt, wobei es zur Bildung von Proteinaggregaten kommt. Die Übertemperatur vom gefalteten in den ungefalteten Zustand liegt bei 68.4°C, wodurch sichergestellt ist, dass diese Effekte nicht bei der Temperatur, bei der die Proteolyse-Experimente ablaufen (37°C), zu erwarten sind.

Der enzymatische Verdau des Rinderserumalbumins mit Trypsin und α -Chymotrypsin wurde bei 37°C in D₂O Puffer angesetzt. Mit Hilfe von FTIR-Studien wurde herausgefunden, dass natives BSA in D₂O 68% α -Helix-, 20% β -Faltblatt- und 12% β -Schleife-Strukturen beinhaltet. Dagegen waren nach der Behandlung mit α -Chymotrypsin in einem Verhältnis von 10:1 für 5h 32% α -Helix-, 17% β -Faltblatt-, 18% β -Schleife und 33% ungeordnet Strukturen vorhanden. Die größten Unterschiede in den Spektren für alle getesteten Enzym-Substrat-Verhältnisse konnten in den Amid I- und Amid II-Regionen nachgewiesen werden. In den CD-Studien besteht BSA zu 65% aus α -Helix-, 4% β -Faltblatt-, 10% β -Schleife und zu 22% aus ungeordnet Strukturen. Nach 5-stündigem chymotryptischem Verdau im Verhältnis von 10:1 sind 30% α -Helix-, 17% β -Faltblatt-, 21% β -Schleife und 32% ungeordnete Strukturen vorhanden. Die Berechnungen der Veränderungen der Sekundärstrukturen mittels FTIR- und CD-Studien zeigt, dass der Anteil an α -Helices stark abnimmt, wogegen Schleifen und ungeordnete Strukturen zunehmen, was mit einem Anstieg an Carboxylaten einhergeht ($\sim 1593\text{ cm}^{-1}$), die bei der Spaltung von Peptidbindungen freigesetzt werden. Wir folgern, dass frei zugängliche α -Helices enzymatisch abgebaut werden und in kürzere Helices bzw.

ungeordnete Strukturen umgewandelt werden. Bei diesem Vorgang entstehen die zuvor genannten Carboxylatgruppen als Proteolyse-Produkte. Folglich konnten über die beiden verwendeten Spektroskopie-Verfahren die Veränderungen in den Sekundärstrukturen bei der enzymatischen Behandlung bestätigt werden.

Zusätzlich zeigt sich, dass eine höhere Enzymkonzentration zu einer stärkeren Degradierung und Auflösung von Sekundärstrukturen des BSA führt. Da Trypsin hauptsächlich an hydrophilen Abschnitten, mit polaren Gruppen von Lysin und Arginin, und α -Chymotrypsin umgekehrt an hydrophoben Resten (Tyrosin, Tryptophan, Phenylalanin, Methionin und Leucin) schneidet, ist der Abbau durch Trypsin schwächer als durch α -Chymotrypsin. Bei α -Chymotrypsin genügen bereits geringe Konzentrationen (E:S = 50:1), um einen starken Abbau von α -Helixstrukturen vom BSA zu induzieren und damit einhergehend den Anteil an ungeordneten Strukturen zu erhöhen.

Die Analyse der Temperaturabhängigkeit des β -Lactoglobulins zeigt Veränderungen der Bande bei 1678 cm^{-1} in zwei Stufen. Die erste Phase liegt zwischen $50\text{--}66^\circ\text{C}$ und die zweite Phase oberhalb von 66°C . Phase 1 ist durch Monomerisierung des Proteins charakterisiert, was auf eine Wechselwirkung zwischen zwei Monomeren schließen lässt. Bei höheren Temperaturen in der Phase 2 zeigt sich die Entfaltung des β -Lactoglobulin Proteins. Die Temperaturabhängigkeit von 1632 cm^{-1} zeigt ebenfalls den ungefalteten Zustand oberhalb von 66°C .

Die Hydrolyse von β -Lactoglobulin durch Trypsin wurde bei 37°C in D_2O Puffer mit FTIR- und CD-Spektroskopie durchgeführt. FTIR Studien zeigten, dass das native β -Lactoglobulin Protein aus 53% β -Faltblatt-, 18% β -Schleife-, sowie 29% α -Helixstrukturen und ungeordneten Strukturen besteht. Dagegen besteht das 10:1 über 5h enzymatisch verdaute Protein aus 24% β -Faltblatt-, 21% β -Schleifen-, sowie 55% α -Helixstrukturen und ungeordneten Strukturen. Zusätzlich wurden in den FTIR Spektren des abgebauten Proteins verstärkte 1593 cm^{-1} und 1406 cm^{-1} Banden gefunden, welche der antisymmetrischen und symmetrischen Streckenschwingungen der freien Carboxylatgruppen zugeschrieben werden, die als Proteolyse Produkte entstehen. Bei den CD Studien zeigte sich, dass das native β -Lactoglobulin aus 42% β -Faltblatt-, 22% β -Schleife-, 7% α -Helixstrukturen und 28% ungeordneten Strukturen besteht. Dieser wird bei einem 10:1 5h Trypsinverdau folgendermaßen verändert: 24% β -Faltblatt-, 16% β -Schleife-, 5% α -Helixstrukturen und 54% ungeordnete Strukturen. Bei beiden angewandten Spektroskopieverfahren wurde gefunden,

dass die β -Faltblattstrukturanteile wesentlich verringert werden, was mit einem parallelen Anstieg an ungeordneten Strukturen einhergeht und zu einem Zerfall des β -Lactoglobulin in kleine Fragmente führt. Gleichzeitig zeigte sich, dass Enzym-Substrat-Verhältnisse von 10:1 und 50:1 ähnlich effizient sind, wobei das 10:1 Verhältnis eine größere Ausbeute bewirkt, was eventuell mit einer leichteren Zugänglichkeit des Trypsins zu den Peptidbindungen erklärt werden könnte.

Die Temperaturabhängigkeit der β -Caseinstruktur wurde ebenfalls mit Hilfe der FTIR-Spektroskopie untersucht. Dabei wurde gefunden, dass es strukturelle Umlagerungen gibt, die jedoch nicht zur Entfaltung des Proteins führen, die bis zu 90°C reversibel sind. Die Bande bei 1640 cm^{-1} Bande (ungeordnete Strukturen) verschiebt sich sogar bis 1646 cm^{-1} bei 90°C. Dies ist ein Hinweis darauf, dass sich die Polyprolin II Struktur von einer wässrigen in eine hydrophobe Umgebung bewegt.

Der Verdau von β -Casein mit Trypsin bei 37°C in D_2O Puffer wurde mittels FTIR- und CD-Spektroskopie verfolgt. Nach den Ergebnissen der FTIR Studien besteht natives β -Casein aus 20% β -Faltblatt-, 28% β -Schleife-, 16% α -Helixstrukturen und 36% ungeordneten Strukturen. Das 10:1 4h enzymatisch (Trypsin) abgebaute Protein weist 20% β -Faltblatt-, 27% β -Schleife-, 14% α -Helixstrukturen und 39% ungeordnet Strukturen auf. Erstaunlicherweise beträgt der Anteil an α -Helixstrukturen des verdauten Proteins lediglich 8% bei $t=5\text{min}$. Bei den CD Studien wurden für die Struktur des nativen β -Caseins 23% β -Faltblatt-, 15% β -Schleife-, 12% α -Helixstrukturen und 50% ungeordnete Strukturen gefunden. Das 10:1 4h mit Trypsin-hydrolysierte Protein besteht dagegen aus 23% β -Faltblatt-, 18% β -Schleife-, 16% α -Helixstrukturen und 43% ungeordneten Strukturen. Der Verlust von ungeordneten Strukturen geht dabei mit einer Erhöhung des Anteils an α -Helices einher. Die FTIR Ergebnisse zeigten außerdem für die 10:1 und 50:1 Enzymkonzentrationen eine Verstärkung der 1645-1649 cm^{-1} Bande während der Proteolyse. Zusammengenommen deuten die Daten darauf hin, dass die Zunahme an α -Helixstrukturen und die Abnahme an ungeordneten Aufwicklungen durch das Polyprolin II hervorgerufen werden oder aber es bilden sich dicht gepackte Aggregate während der Hydrolyse.

Zusammenfassend konnten wir mit Hilfe der FTIR- und CD-Spektroskopie unbedeutende, aber auch signifikante, strukturelle Umlagerungen während des enzymatischen Verdaus von Proteinen detektieren. Dabei fanden wir heraus, dass bei den untersuchten Substraten (BSA, β -Lactoglobulin und β -Casein) Sekundärstrukturen abgebaut werden und zu freien

Peptidfragmenten konvertiert werden, die nicht aggregieren. Dadurch wird das Proteinrückgrat des nativen Proteins verändert und es kommt zur Degradation. Befinden sich die zu hydrolisierenden Peptidbindungen in einer hydrophoben Region im Innern des Proteins, so sind sie schwerer zugänglich und das Protein ist resistenter gegenüber einer Protease. Letztlich folgern wir, dass die Effizienz der Proteolyse maßgeblich von der strukturellen Stabilität und der Hydrophobizität des Substrates abhängt, sowie von der Enzymspezifität.



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