Effect of Temperature/Ethanol on the Secondary Structure of Bovine Apo Alpha-Lactalbumin Investigated by FTIR/2D IR Correlation Spectroscopy

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ABSTRACT

Whey proteins play a vital role in the manufacture of food products due to their nutritional value and versatile functional properties. α-Lactalbumin (α-LA) is the second most abundant protein in bovine whey and the most abundant protein of human whey. α-LA is a low-molecular-weight (14.2 kDa) and acidic (pI 4-5) protein that is produced in the lactating mammary glands and has a role in lactose biosynthesis. BAMLET/HAMLET (bovine/human alpha-lactalbumin made lethal to tumor cells) are complexes of α-LA and oleic acid that have been shown to have cytotoxic effects on tumor cells but not on healthy cells. In vitro, it has been reported that BAMLET-type complexes can be prepared by heating a solution of bovine apo (calcium-depleted) α-LA in sodium phosphate buffer to which an ethanol solution of oleic acid has been added. However, the possibility that the presence of ethanol may facilitate the complexation of oleic acid with α-LA by affecting the thermal denaturation of the protein has not been investigated In the present study, the combined effects of ethanol and temperature on the secondary structure of bovine apo α-LA were examined by variable-temperature Fourier transform infrared (VT-FTIR) spectroscopy in conjunction with Fourier self-deconvolution (a resolution enhancement technique) and two-dimensional cross correlation spectroscopy (2D CCS). At room temperature, an increase in α-helical and β-structure content at the expense of 3_{10}-helices and turns was observed as a function of increasing the concentration of ethanol (from ~2.5 to 33% w/v). These findings are consistent with the fluorescence and proteolysis studies of α-LA reported in the literature, which showed a similar effect of ethanol on the secondary structure of α-LA. Subjecting bovine apo α-LA solutions to a heating–cooling cycle (heating from 25 to 95^0C and cooling from 95 to 25^0C) in the presence of varying concentrations of ethanol was found to alter the protein's secondary structure. At any concentration of ethanol the α-helix and 3_{10}-helices of the secondary structure of bovine apo α-LA were lost upon heating of the protein. The sequences of the changes in secondary structure during the heating and cooling cycles were elucidated by 2D CCS. The results revealed that the protein refolded during the cooling cycle by reversal of the sequence of unfolding events during the heating cycle only in the presence of 20% or higher concentration of ethanol. Overall, the present study supported the ethanol-induced reversible thermal denaturation of bovine apo-α-LA.

RÉSUMÉ

Étant donné leur valeur nutritive et leurs multiples propriétés fonctionnelles, les protéines du lactosérum jouent un rôle crucial dans la fabrication de denrées alimentaires. L'α-lactalbumine (α-LA) est la plus abondante protéine parmi celles du lactosérum bovin et la seconde plus abondante protéine parmi celles du lactosérum humain. L'α-LA , une protéine acide (pI 4-5) à faible poids moléculaire (14.2 kDa) produite dans les glandes mammaires, joue un rôle important dans la biosynthèse du lactose. Composés des complexes d' α-LA et d'acide oléique, le BAMLET/HAMLET (*b*ovine/*h*uman *a*lpha-lactalbumin *m*ade *le*thal to *t*umor cells) a démontré des effets cytotoxiques sur les cellules tumorales, tout en laissant les cellules saines indemnes. On rapporte que les complexes de type BAMLET peuvent être préparées en chauffant une solution d'apo (décalcifiée) α-LA bovine dans un tampon de phosphate de sodium à laquelle on ajoute une solution d'acide oléique dissoute dans de l'alcool éthylique. Cependant, la possibilité que la présence d'alcool éthylique facilite la complexation de l'acide oleique acid à l'α-LA en influençant la dénaturation thermique de la protéine n'a pas été évaluée. Dans la présente étude, les effets combinés de l'alcool éthylique et de la température sur la structure secondaire de l'apo α-LA bovine furent examinés par spectrométrie infrarouge à transformée de Fourier opérant à température variable (VT-FTIR) en combinaison avec une autodéconvolution de Fourier (technique d'amélioration de la résolution) et de spectroscopie à corrélation croisée en deux dimensions (2D CCS). À la température de la pièce, une augmentation de la concentration en alcool éthylique d'environ 2.5% à 33% w/v donna lieu à une augmentation des structures en hélices alpha et à feuillets bêta, aux dépends d'hélices et coudes 3_{10}. Ces résultats sont conformes à ceux de plusieurs études fluoroscopiques et protéolytiques de l'α-LA préalables, montrant un effet semblable de l'alcool éthylique sur la structure secondaire de l'α-LA. Soumettant l'apo α-LA bovine à un cycle de chauffage-refroidissement entre des extrêmes de 25°C et 95°C, en présence de différentes concentrations d'alcool éthylique, s'avéra modifier la structure secondaire de la protéine. A toutes concentrations d'alcool éthylique, les hélices alpha et 3_{10} de la structure secondaire de l'apo α-LA bovine furent perdues lorsque la protéine fut chauffée. La séquence des altérations de la structure secondaire durant les cycles de chauffage-refroidissement fut suivie par 2D CCS. Les résultats démontrèrent que ce fut seulement en la présence d'une concentration de 20% ou plus d'alcool éthylique que la protéine se replia durant le

refroidissement, en suivant une séquence inverse à celle de son dépliement durant le réchauffement. Globalement, la présente étude confirma la dénaturation thermale réversible de l’apo-α-LA bovine comme étant induite par l'alcool éthylique.

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ABBREVIATIONS

2D - Two-dimensional

2D CCS - Two-dimensional cross correlation spectroscopy

A.U. - Arbitrary units

BAMLET - Bovine alpha-lactalbumin made lethal to tumor cells

BCAAs - Branched-chain amino acids

CD - Circular dichroism

DMPC -Dimyristoylphosphatidylcholine

DSC - Differential scanning calorimetry

DTT - Dithiothreitol

EAI - Emulsion activity index

EDTA - Ethylenediaminetetraacetic acid

EGTA - Ethylene glycol tetraacetic acid

EPC - Endothelial progenitor cell

ES - Emulsion stability

FSD - Fourier self-deconvolved/self-deconvolution

FTIR - Fourier transform infrared spectroscopy

HAMLET - Bovine alpha-lactalbumin made lethal to tumor cells

HFIP - Hexafluoroisopropanol

HP - High pressure

ITC - Isothermal titration calorimetry

kDa - kilodalton

K_m - Michaelis constant

α-LA - α-Lactalbumin

β-LG - β-Lactoglobulin

MAL - Multimeric α-lactalbumin

NMR - Nuclear magnetic resonance

O/W - oil in water

pI - Isoelectric point

PS - Phosphatidylserine

RP-HPLC - Reversed-phase high-performance liquid chromatography

TFE - Trifluoroethanol

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

UV - Ultraviolet

VT-FTIR - Variable-temperature Fourier transform infrared spectroscopy

WPC - Whey protein concentrate

WPI - Whey protein isolate

CHAPTER 1: GENERAL INTRODUCTION

Multiple food groups contribute in maintaining a healthy body. Because of their nutrient rich package dairy/milk derived food products play a vital role in promoting the human health. Protein is a major component of the milk. Most of the nitrogen content in milk is present in the form of proteins. The concentration of the protein in milk is generally reported as 3.1 to 3.6% (Barbano, 1990). The protein fraction of milk is divided into casein and whey protein as 80 and 20% respectively. There are twenty amino acids found in the whey fraction of protein.

Whey proteins possess multi health benefits such as tissue repair and healing, immune enhancement, energy production, fighting infections, and perhaps even retard cancer. In the current study, the effect of ethanol and temperature on the secondary structure of the most abundant protein in human whey and second most abundant protein in bovine whey, α-lactalbumin (α-LA) undertaken. α-LA is responsible for lactose synthesis in mammals. Other than this α-LA is a substantial source of essential amino acids, which may play a role in the muscle recovery/development (Lien, 2003). It is used as an emulsifier in the food industry and as an ingredient in the manufacture of various foods.

α-LA contains two calcium ions which confer considerable thermal stability to the protein. Depletion of attached calcium (termed as apo α-LA) is of great concern and may cause the significant alteration in its secondary structure upon heat treatments or exposure to other physiological conditions such as pH (Griko et al., 1994). Moreover, the interaction of whey proteins especially α-LA with fatty acids has received considerable attention in the last decade (Pettersson et al., 2006). With regard to the latter apo α-LA-oleic acid complexes were found to be cytotoxic against various human and mouse cancer cell lines (Barbana et al., 2006). The complex is known as human/bovine alpha-lactalbumin made lethal to tumor cells (HAMLET/BAMLET). These complexes are made in the presence of ethanol (which helps to solubilize the fatty acid prior to mixing with the protein solution). It was also noted that the effect of temperature during complex formation can have a significant effect at the cytotoxic concentration of the complex.

Accordingly, detailed study of the effects of alcohols on the structure of proteins is of interest. The three major effects of ethanol on the protein structure can be (1) dissolution of peptides or its

aggregates, (2) increase of α-helical structure and most importantly (3) loss of rigid native structure (Hirota, 1998). Alcohols have also been reported to stabilize β-sheet harpins in α-LA by altering localized hydrogen bond interactions (Blanco et al., 1994) as well as binding to the specific hydrophobic binding sites there by displacing the endogenous ligands or by replacing hydrogen bounded water molecule (Avdulov et al., 1996). It has been reported that at higher concentration of ethanol, α-LA may undergo conformational changes followed by the changes in its thermal stability (Grinberg et al., 1998) and spectral properties (Polverrino de Laureto et al., 2002).

One of the most versatile technique in the study of the effect of varying physiochemical conditions on protein secondary structure is the infrared spectroscopy. Infrared spectra of proteins are characterised by a set of absorption regions known as amide modes. Due to the advancements in Fourier transform infrared (FTIR) spectroscopy, high quality spectra of proteins solutions dissolved in D_2O can be obtained (Haris et al., 1992). Moreover the various resolution enhancement techniques such as Fourier self deconvolution, second derivative and difference spectroscopy can be coupled with the FTIR technique (Dong, 2002). These methods can be employed to mathematically resolve the overlapping band components within the broad amide bands. Conformational changes in the proteins can be more clearly analysed with the help of difference spectroscopy. The main advantages of the FTIR spectroscopy includes: inexpensive compared to other spectroscopic techniques such as X-ray diffraction, NMR and CD spectroscopy; no light scattering or fluorescence effects; amount of protein sample required is comparatively small (10 μg); can perform quantitative as well as qualitative analysis.

Two-dimensional infrared (2D IR) spectroscopy is a novel technique which is based on the time resolved spectroscopy. In 2D IR, a system is excited by an external perturbation (temperature, pressure or any chemical), which induces a dynamic fluctuation of the IR spectrum. In the 2D correlation spectroscopy, the spectrum is obtained as a function of two independent wavenumber axes. The peaks located on the spectral plane are used to study intra- and intermolecular interactions among the functional groups. The basic concept or theory of 2D correlation analysis is somewhat comparable in both 2D IR and 2D NMR. In the 2D correlation analysis, by spreading IR peaks over the two dimensional base, a complex spectrum consisting of overlapped peaks can be simplified and the spectral resolution can be enhanced. It is a powerful technique in

terms of revealing the sequence of changes in the secondary structure that occur when a protein is exposed to an incremental change in a specific physicochemical parameter. The spectral information obtained from the 2D contour maps (synchronous and asynchronous) can be simplified by following the rules proposed by Noda (Noda, 1993).

By considering the importance of BAMLET complexes, we deliberately selected the objective of the research to study the conformational changes in the secondary structure of bovine α-LA in the absence and presence of varying concentrations of ethanol by FTIR spectroscopy and 2D correlation technique. The two specific objectives of this study were-

1. Study of the combined effect of ethanol and temperature on the secondary structure of bovine apo α-LA by variable temperature FTIR spectroscopy

2. 2D Correlation analysis of the changes in the secondary structure of bovine apo α-lactalbumin subjected to thermal treatment in the presence and absence of ethanol

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction to whey proteins

Whey protein is a by-product of the cheese and curd manufacturing. It contributes about 90% to the total milk protein volume and accumulates between 6.0 and 6.4% of dry matter (Tunick, 2008). It is one of the two primary sources of milk protein. The other important form of milk proteins is casein. Casein is actually a mixture of milk proteins, which precipitates at pH 4.3 for human and 4.6 for bovine. It is composed of 80% of total milk proteins for bovine and 35% of total human milk proteins. After processing, caseins are the proteins responsible for curd manufacturing, while whey remains in an aqueous state. Whey protein was once considered as waste product of cheese and curd manufacturing. But after recognizing whey as a functional food with its great nutritional values and applications, it is being considered as a co-product in the manufacturing of cheese.

As compare to the vegetable protein sources, whey proteins contain all the essential amino acids in higher concentrations. In whey, the amino acids are efficiently absorbed and utilized, relative to free amino acid solutions. It contains a high concentration of branched-chain amino acids (BCAAs) – leucine, isoleucine, and valine. The most important amino acid in protein metabolism is leucine. Whey proteins are rich in the sulphur containing amino acids such as cysteine and methionine, which enhance immune function through intracellular conversion to glutathione. The primary components of whey protein and their benefits are listed in Table 2.1.

Whey Component	% of whey protein	Benefits
Beta-Lactoglobulin	50-55%	-Excellent sources of essential and branched chain amino acids-spares muscle and glycogen during exercise -Binds fat soluble vitamins, increasing bioavailability
Alpha-Lactalbumin	20-15%	-Primary protein found in human breast milk -Excellent source of essential and branched chain amino acids -High in the essential amino acid tryptophan, which helps regulate sleep, mood, stress

Immunoglobulins	10-15%	-IgA, IgD, IgE, IgG, IgM – Primarily IgG -Primary protein found in colostrums -Immune enhancing benefits to all ages, particularly infants
Lactoferrin	1-2%	-Antioxidant found in the breast milk, tears, saliva, blood -Antiviral, antibacterial, antifungal -Promotes growth of beneficial bacteria -Regulates iron absorption and bioavailability
Lactoperoxidase	0.5%	-Inhibits growth of bacteria
Bovine Serum Albumin	5-10%	-Large-sized protein with good profile of essential amino acids -Fat-binding properties
Glycomacropeptide	10-15%	-Does not contain amino acid phenylalanine, so is often used in infant formulas for infants with phenylketonuria -Inhibits formation of dental plaque and cavities

Table 2.1: Primary Components of whey Protein **(**Alternative Medicine Review Volume 13, Number 4, 2008)

Because of the high nutritional value of whey proteins and after considering their functional properties, food industry these days is showing a great interest for their recovery after the processing of milk. Nowadays, other techniques for recovery of whey proteins such as ultrafiltration and ion exchange chromatography are coming into play for adding value to whey through the production of whey protein concentrates (WPC) and isolates (WPI) (Morr, 1992; Holt et al., 1999).

The biological properties of whey proteins, such as the defence against bacterial or viral infections (Bellamy et al., 1992; Arnold et al., 2002; Clare et al.,2003), improvement of nutrient absorption (P´erez and Calvo, 1995), regulation of cell growth (Aranda et al., 1996; Pocov´I et al., 2009), transport of vitamins (Puyol et al., 1991; Wang et al., 1997) and oligo-elements (S´anchez et al., 1992) of whey proteins are the main reasons for special attention towards it by

researchers. Some of these biological activities depend on the interaction of proteins with specific ligands of low molecular weight.

After spray-drying, whey protein consists of lactose (73-74%), minerals (12%) and a protein component (11-12%), which in turn is comprised of β-lactoglobulin (~47%), α-lactalbumin (~13%), serumalbumin (~4.6%), and various immunoglobulins (~12.7%). Whey protein isolates may contain different protein ratios with the most common being: β-lactoglobulin (55%), α-lactalbumin (24%), serum albumin (5%), and immunoglobulins (15%) (Swaisgood, 1996). Other proteins may also be present in limited quantity such as partially hydrolyzed caseins, lactoperoxidase, lysozyme, lactoferrin and lactallin (Bottomley et al., 1990). β-LG and α-LA are the most potent proteins among bovine whey and are greatly responsible for the physicochemical characteristics of whey protein product and also contributing to its solubility, gelation, foaming, emulsification, and binding properties (Morr and Ha, 1993; Puyol et al., 1999).

As whey is a by-product of cheese formation and may be used for the isolation of bovine *α*-LA to form the bioactive form of the protein. The cytotoxic activity of bovine α-LA against tumor or cancer cells has opened new ways for its use as a potential ingredient to be added in functional health promoting foods (Svensson et al., 2000; Pettersson et al., 2006). In this review, mainly the structural and physico-chemical properties of α-LA are focused. The interaction between α-LA and fatty acids such oleic acid is of great interest. So, the biological implications and applications of the complex formed between *α*-LA and fatty acids are also highlighted.

2.2. α-Lactalbumin

α-LA is a small (14.2kDa) and acidic (pI 4–5) globular whey protein. α-LA is the main protein of human whey and the second most abundant protein of bovine whey, after β-lactoglobulin. α-LA is the most renowned protein of whey by characteristic point of view. It is present in the milk of all mammalian species in relatively high concentration, around 1.1–1.5 g/L, except in that of the seal and sea lion, because they produce milk with very low concentrations of *α*-LA and lactose (P´erez et al., 1990; L¨onnerdal and Lien, 2003). Four disulfide bonds are responsible for the structural stabilization of *α*-LA. Besides, the binding of calcium to the high affinity Ca^{2+} binding site in α-LA greatly responsible for its structure stability. The ability of α-LA to interact with

fatty acid is of great concern as it is very unique property of this protein. However, this interaction depends on the conformational state of the protein. It is believed that the unfolding of protein by treating it with chelating agents or by heat treatment is necessary for its interaction with fatty acids. Reason being the native form of this protein seemed to be unable to bind fatty acids (Svensson et al., 2000; Barbana et al., 2006; Kamijima et al., 2008).

In the lactating mammary glands, the main function of α-LA is to participate in lactose biosynthesis and it works as the regulatory component of the lactose synthase complex (Brew, 2003). New approaches have been suggested to elucidate the biological function of α-LA in the last decade. It has been experienced that α-LA and some fragments obtained by proteolysis have an interesting antimicrobial effect on some bacterial cell. The another subject came into notice is that a particular form of human α-LA induces apoptosis in tumor and immature cells but does not affect significantly to healthy differentiated cells. The conformational change of the protein to a molten globule state and the binding of oleic acid as a stabilizing cofactor are the most basic conditions to achieve the biological functionality of α-LA (Hakansson et al., 1995; 1999; Svensson et al., 2000).

2.2.1. Structure of α-LA

The structure of α-LA is stabilized by four disulfide bonds and about 10% of bovine α-LA is glycosylated (Brew et al., 1992). α-LA is a small acidic protein composed of 123 amino acid residues (Brew,2003). X-ray crystallography has shown that the protein is structurally very similar to lysozyme (Bottomley et al., 1990). The secondary structure of α-LA (figure 1) contains four α-helices, several regions of 3_{10}-helices, and an antiparallel β-sheet separated by irregular β-turns (Brew et al., 1992; Chrysina et al., 2000). Circular dichroism studies have shown that α-LA is 26% α-helix, 14% β-structure and 60% unordered structure (Bottomley et al., 1990). α-LA has a strong binding site called “elbow”, which is particularly binding site for calcium, formed from a helix-turn-helix motif separated by an irregular β-turn. Calcium binds to five aspartic acid residues, which are present at this site (Brew et al., 1992). There are some metal binding sites that are not well characterized especially for manganese (Brew et al., 1992). There is also a region that separates the molecule into two halves called cleft. Cleft is responsible for hydrolytic activity in lysozyme but has no known function in α-LA (Brew et al., 1992).

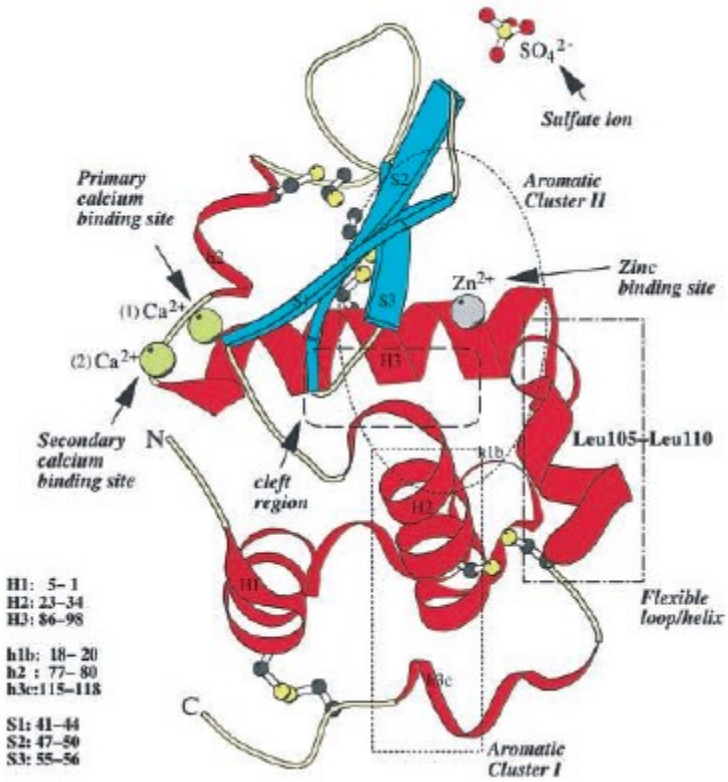


Figure 2.1: The structure of α-LA and the functional regions of the molecule showing the location of metal ions identified in α-LA crystal structure. Secondary structural elements are marked (Reproduced with permission from Chrysina et al., 2000).

Most stable conformation of α-LA lies between pH 5.4 and 9.0. Below pH 4.0 and above pH 9.0, there occur conformational changes in α-LA (Bottomley et al., 1990). Metal binding properties are affected by these conformational changes, but they do not cause any change in secondary structure (Brew et al., 1992). Heating the molecule between 35-40°C may cause changes in its tertiary structure but not in its secondary structure. There is a breakdown of the 3_{10}-helix and an increase in turns (Boye et al., 1996) at the temperature above 65°C along with physiological pH. This action is reversible and upon cooling 80-90% of the protein gets back to its native structure. This factor proves that α-LA is the most heat stable whey protein reason being the removal of calcium ion and again its re-association to protein (Singh et al., 1992). Above pH 9 and below pH 6, partial irreversible aggregation does occur (Boye et al., 1997). α-LA may be stabilized by increasing the salt concentration (Boye et al., 1997) but an increase in denaturation is observed with an increase in concentration of α-LA (Singh et al., 1992).

2.2.2. Biological Properties of α-LA

The main biological function of α-LA is its functionality in lactose biosynthesis. α-LA works as a regulatory component for lactose synthase complex which helps in catalyses of lactose biosynthesis during lactation in the mammary gland. Galactosyltransferase is the catalytic component of lactose synthase, which catalyzes the transfer of galactose from UDP-galactose to glucose. However, because of its very low affinity for glucose, the efficiency of this enzyme for catalyzing lactose synthesis is not very much (reflected in a Km of about 2 M). α-LA plays the main function to enhance the binding of glucose to galactosyltransferase through a 1:1 protein interaction which reduces the Km for glucose by about 1000-fold, so that lactose synthesis could be catalyzed effectively at the physiological concentrations of glucose present in mammary epithelial cells (Brew, 2003).

In addition, there are several other biological functions which have been attributed to α-LA. Hakansson et al. (2000) noticed a folding variant of human α-LA with strong bactericidal activity against antibiotic resistant and susceptible strains of Streptococcuspneumonia whereas it was ineffective against Staphylococci or Enterococci and most Gram-negative bacteria. The characterization of α-LA by spectroscopic analysis indicated that the protein and its native form has an identical secondary structure but with a fluctuating tertiary structure.

Besides, Pelligrini (2003) observed that polypeptides with bactericidal properties were yielded due to the digestion of bovine α-LA by trypsin and chymotrypsin, being mostly active against Gram-positive bacteria. These findings suggest that α-LA plays a biological function as an antimicrobial protein. It has been shown that the growth of mammary epithelial cells, rat kidney cells, and bovine epithelial cells was inhibited by native α-LA derived from human milk in a dose and time dependent manner, whereas cell division of fibroblasts was unresponsive (Rejman et al., 1992; Thompson et al.,1992; Alston et al., 1998). Likewise, native α-LA preparations from several mammalian species, including goat, cow, and camel were all found to have a growth inhibitory activity on cultured mammary epithelial cells (Thompson et al., 1992). These results demonstrated the possibility that during lactation α-LA, a product of mammary cell differentiation could be a physiologically relevant feed-back inhibitor of mammary cell growth and perhaps of other cell types as well.

In addition, it has been noticed that human and bovine α-LA has anti-proliferative effects on a human breast cancer cell line (MCF-7) (Thompson et al., 1992) and human colon adenocarcinoma cell line (Caco-2) (Sternhagen and Allen, 2001). Recently, Lin et al. (2008) showed that at the concentration of 100 μg/mL, the addition of α-LA to a mouse macrophage cell line caused a decreased cell growth in a time and dose-dependent manner and induced morphological changes, DNA fragmentation and apoptosis. On the basis of these results, it can be believed that α-LA could participate in the inhibition of growth of potential cancer cells.

Hakansson et al. (1995) found that apoptosis of tumor and immature cells was induced by a multimeric form of human *α*-LA, called MAL (Multimeric Alpha-Lactalbumin), but spared all the matured cells. MAL, an active form of α-LA was obtained after precipitating the casein fraction of milk at low pH. Unlike the casein components, α-LA was retained on an anion-exchange matrix and eluted only with 1M NaCl. It was revealed by Chromatographic separation of the eluted α-LA peak on a size-exclusion column that α-LA fraction contained dimers, trimers and higher order oligomers of the protein. Circular dichroism and fluorescence spectroscopic studies have been showed that the active form of α-LA had retained secondary structure but had a more loosely organized tertiary structure as compare to its native state, similar to that exhibited in the molten globule state of the protein (Svensson et al., 1999. This active form of α-LA differed from the molten globule state in terms of stability at neutral pH in an oxidizing environment and in the presence of Ca^{2+}.

2.3. Native and apo conformers of α-LA

The folded or native structure of a protein is a three-dimensional network, which results from a balance of various attractive and repulsive forces within the polypeptide itself and also between the polypeptide and the molecules of solvent (Privalov, P. L. and Khechinashvili, N. N., 1974). It is believed that the regular repeated patterns of folding of the polypeptide backbone to form a three-dimensional network results into the native state of globular proteins. It is trusted that this structure is stabilized by non-covalent linkages. On the other side, it is thought that denatured state of a protein is obtained from the breaking up of labile linkages leaving a disorganized structure with an altered surface and more of its interior exposed (Dill, K. A., 1990). The term

denatured is meant to an alteration in the original native structure without hydrolysis of primary covalent bonds (Colvin & J. R., 1964). The denatured arrangement of a molecule is defined as its conversion from the native conformation to other spatial arrangements of the macromolecule which may be induced by heat, pressure, pH changes, chemical agents (heavy metals, guanidine hydrochloride and urea) and other factors like light.

Acharya (Acharya et al., 1989) reported the high-resolution structure of α-LA (at 1.7 **A**), which demonstrated that the tertiary structure of α-LA contains several regions of regular secondary structure: four α-helices (30%), five 3_{10}-helices (20%), two β-structures (6%) and, an appreciable proportion of random coil (44%). Seven Ca^{++}coordinating oxygen atoms are involved in the Ca^{++}binding site in α-LA that are provided by the carboxylate groups of Asp 82, 87, and 88, by the carbonyl oxygen atoms from Lys 79 and Asp 84, and by two H_2O molecules.

In their recent reviews Kronman, M. J. (1989) and Brew, K.,et al. (1992) particularly emphasize the following points:

- The apo form (acid conformer) has less conformational stability than the native form.
- Chelation of calcium induces the transition of N-form (holo form) to A-form (apo form), where the A-form (apo) is a conformer with less conformational stability on heat treatment than the N-conformer.
- The tertiary structure of the N-form is stabilized by binding of several metal cations at several putative binding sites with respect to thermal denaturation.

Therefore, N-conformer of α-LA when subjected to various physicochemical conditions such as pH, heat treatment above 50°C and binding of ions may undergo various conformational transitions to forms with different physical properties.

On the basis of sedimentation experiments (Kronman, M. J.,et al, 1964), it has been proposed that at a pH less than 4, the apo-form exists in an intermediate state. However, laterally it has been shown (Dolgikh, D. A., 1981:1985) that α-LA in the apo form is partially unfolded (it retains the pronounced secondary structure of the native state). In solution the apo form has

intrinsic viscosity similar to that of the denatured form. However, it has no cooperative melting temperature. This conformation is named as "molten-globule" state. The apparent radius of gyration (14.5 to 19.9 A) has been determined by the sedimentation velocity (Kronman, M. J. et al., 1964), X-ray diffraction methods (Izumi, Y. et al., 1983) and by quasi elastic light scattering (Gast, K. et al., 1986). The intrinsic viscosity (Dolgikh, D. A. et al., 1981) has been determined for both N- and A-conformers. The values demonstrated that the native- and apo- forms have similar molecular dimensions. In the far-UVwavelength range, the CD spectra of N- and A-conformers (Figure 2.2) were rather similar (Dolgikh, D. A. et al., 1981). The changes in the secondary structure from N to the chemically denatured state (6 M guanidium-HCl) were more as compare to from N- (pH 7) to the A- (pH 2) state. It has been realized that the proportion of β-structure (calculated at 208 nm) was lower for the apo form than for the native form (Kronman, M. J., 1989). The observed helical content in the apo form was higher than for the native state.

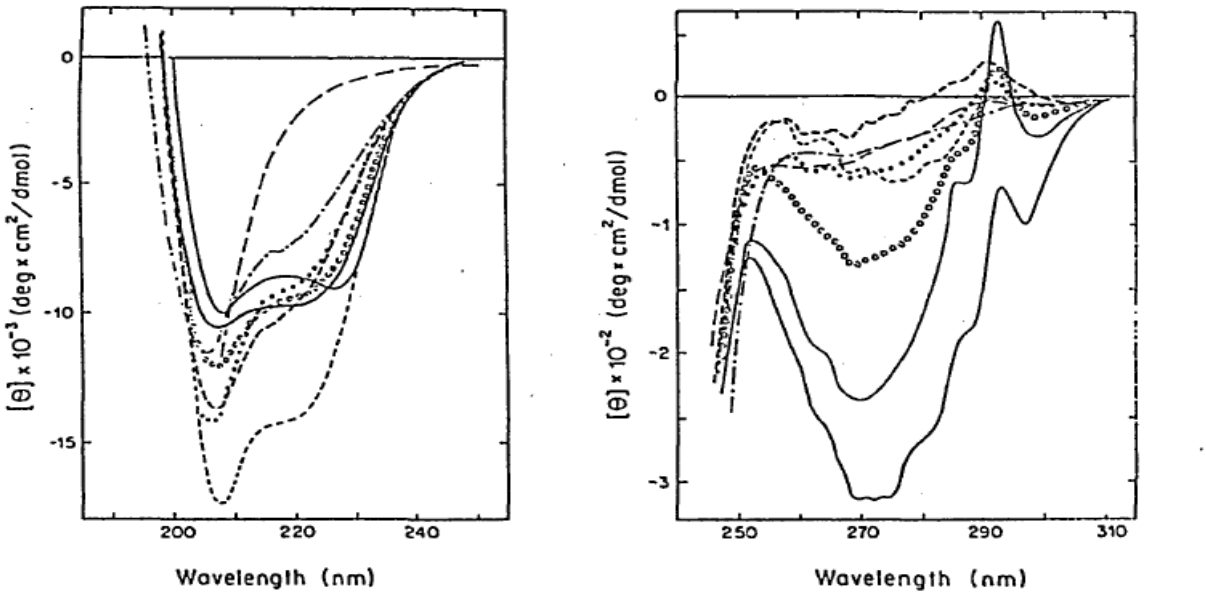


Figure 2.2: CD spectra in far-UV and near-UV regions: Bovine α-LA: N-form; A-form; T-form. Human α-LA: N-form; A-form; P-form; apo form; U-form. (Dolgikh, D. A., 1981)

Moreover, it has been observed the disulfide bond between Cys73 and Cys91 is highly Ca^{++} dependent. The presence of Ca^{++} favours the disulfide pairing observed in the native form and induces a suitable arrangement of the Ca^{++} ligands. When α-LA is re-oxidized in the presence of EGTA, it refolds with randomly arranged disulfide bonds as indicated by the peptide mapping

studies. If the C^{++} is present in a stoichiometric ratio of 1:1, it refolds into the native form with appropriate disulfide pairing (Brew, K. and Gobler, J. A., 1992). It was concluded that refolding of reduced protein into the native form requires Ca^{+}.

2.4. Influence of calcium on the stability of α-LA

Some specific interactions of α-LA with Ca^{++} significantly alter its physical characteristics and folding properties. The thermal stability of α-LA is greatly decreased by removing the bounded calcium, but the protein retains essentially the same folded conformation (W. Pfeil et al., 1985; K. Kuwajima, 1986). On the other hand, according to the number of authors (D.A. Dolgikh et al., 1981 & A. Okazaki, 1994), α-LA gets converted into the molten globule state by removing the Ca^{2+} at low ionic strength and neutral pH. Yutani (K. Yutani et al., 1992) did calorimetrical study of the calcium free apo α-LA, which was supposed to be in the molten globule state in low ionic strength solutions. They did not notice any excess heating effect upon heating. So, it was believed that this state of α-LA is close to the unfolded state (Okazaki et al., 1994).

However, Relkin (P. Relkin et al., 1992) by using a Perkin-Elmer scanning calorimeter studied α-LA at low concentration of calcium. Two heat absorption peaks were found upon heating the solution. The first peak was believed to be belonged to the thermal denaturation of the apo form and the second one was attributed to the holo form. This observation was unable to get significant consideration, reason became the insensitivity of the instrument used in calorimetric experiments [conducted using high concentrations of protein (3 mM) and very fast heating rates (more than $10Kmin^{-1}$)],which was not suitable for the quantitative thermodynamic analysis.

In addition, after period calorimetric studies showed that if the ionic strength of the solution is low, apo α-LA is in the partly unfolded state at room temperature and neutral pH (Y.V. Griko et al., 1994). The reason for occurrence of this partly unfolded state was the removal of Ca^{2+}, which resulted in the appearance of strong repulsive forces between uncompensated negative charges at the calcium binding site. Apo α-LA has a native-like structure in the presence of monovalent salts that unfolds cooperatively upon heating with significant heat absorption, although at much lower temperatures than the holo α-LA (Pfeil, et al., 1985; Griko, et al., 1994).

α-LA solutions having lower concentrations of calcium as compare to that of protein contain a mixture of the apo and holo forms do not interconvert readily (T. Hendrix et al., 2000). This inter-conversion gives a hint that the binding constant of Ca^{2+} to α-LA is very high, and correspond rate of calcium release by the holo form (as well as by EDTA) is much lower than the rate of calcium binding by the apo form (E.A. Permyakov et al., 1987). If it is to be assumed that two forms differ drastically in stability and the denatured protein does not specifically bind calcium, it could be explain by the following scheme:

$$\begin{array}{ccc} P + L & \leftrightarrow & PL \\ \downarrow & & \uparrow \\ D & & \end{array}$$

Where P is the apo α-LA, D is the denatured α-LA, PL is the holo α-LA with bounded ligand and L is the ligand, i.e. calcium ion. Brandts et al. (J.F. Brandts, L.N. Lin, 1990) and Shrake et al. (A. Shrake, P.D. Ross, 1990) analyzed this process in detail. Using the formalism suggested by these authors, the calorimetrically determined heat capacity functions of α-LA in the presence of various concentration of calcium were simulated (T. Hendrix et al., 2000). In this simulation it was assumed that the enthalpy and heat capacity effect of calcium binding by α-LA are zero. It was found that the binding constant decreases with the increase of the calcium concentration and average calcium binding constant K was calculated as $2.9*10^8\ M^{-1}$ (T. Hendrix, et al., 2000).

Many authors using various modifications of the Hummel-Dryer procedure determined the binding constant of Ca^{2+} to α-LA and reported values between 10^6 and $10^9\ M^{-1}$ spread across three orders of magnitude (M.J. Kronman, 1989). According to Kronman, the most reliable values were obtained by his group and lied between $1.5*10^6\ M^{-1}$ (M.J. Kronman et al., 1981) and $2.5*10^6\ M^{-1}$ (S.C. Bratcher et al., 1984), however, all their measurements were carried out at room temperature (20-25^{0}C) at neutral pH. As shown in Fig 2.3. (T. Hendrix et al, 2000), the denaturation of apo α-LA in solutions with pH 8.0 began below 10^{0}C and at 20^{0}C a significant portion of apo-LA were denatured. Therefore, the values of the binding constant offered by Kronman have all been underestimated (T. Hendrix et al., 2000). The lower values of the apparent binding constant for calcium can be explained by this situation. The value of binding

constant that was obtained by Hendrix is $2.9*10^8$ M^{-1}, which is close to the value $K_a = 5*10^7$ M^{-1} obtained from a direct titration isotherm at 5^0C (Yu. V. Griko. et al., 1999).

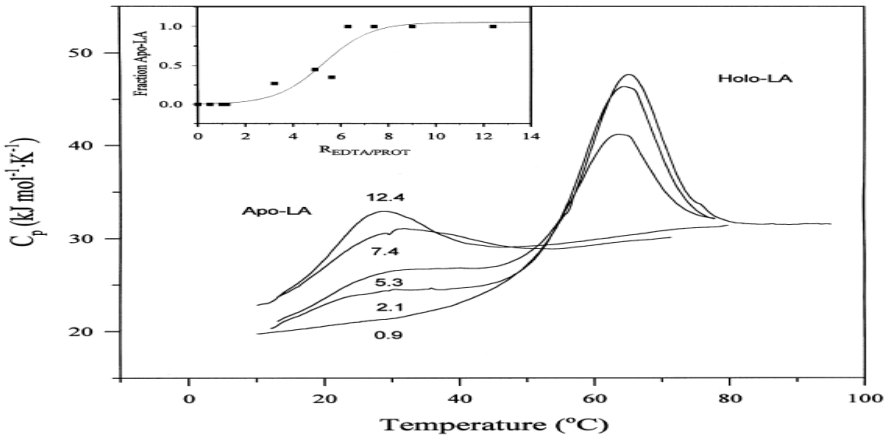


Figure 2.3: The calorimetrically measured heat capacity profiles of α-LA in 10 mM Tris (pH 8) and various amounts of EDTA expressed as *R*=[EDTA]/[protein], and indicated by the curves. The inset shows the calculated fraction of apo α-LA vs. *R*, the fractional amount of EDTA. (T. Hendrix et al, 2000)

With an increase in calcium concentration, the decrease of binding constant of calcium might be caused by an increasing nonspecific influence of the ionic strength resulting in decreased electrostatic interactions. The stabilization of holo α-LA at high concentration of calcium may be caused by the presence of excess free calcium. This effect seems to be entropic resulting from the entropy of mixing of the calcium released upon α-LA denaturation with the calcium of the bulk solution. With increasing concentrations of Ca^{2+} in the bulk solution, the positive entropy of mixing decreases, thus increasing the transition temperature. This situation is very similar to that of DNA, the stability of which depends on the ionic strength and this dependence is entropic in nature caused by entropy of mixing of the released counter ions (P.L. Privalov et al., 1969 & G.S. Manning, 1972). The suggested effect of excess free calcium on the stability and polyelectrolyte properties of α-LA is not contradictory with the possibility of its direct interaction with the protein at additional low-affinity sites (N. Chandra et al., 1998 & C. Ebel, 1999).

2.5. Denaturation of α-LA

The stability of proteins is a major factor determining their functionality in food systems, because this particular functional property is often governed by a specific conformational state of

a protein and its functionality is affected by any alteration of that state. The term denaturation, which is associated with the structural stability of the native protein is prominent and affects the preparation, processing, nutritional value, quality and safety of food proteins. Brandt (1964:1967:1969), Tanford (1968:1970) and Privalov (1971) reviewed the protein denaturation and protein stability.

The term “Denaturation” means to get away from the native state. Such a definition is open to many interpretations and the lack of precision concerning the definition of the native state itself and what constitutes a change from that state has led to many difficulties and controversies. In fact, an author (Colvin, 1964) wrote a requiem for the term "denaturation". Denaturation can now be defined more precisely as "any major alteration in the original native structure without hydrolysis of primary covalent bonds” i.e. changes are restricted to those occurring in secondary or higher structure.

Modern theories of protein structure proposed that the sequence of amino acids and their environment dictates the final conformation of a protein, i.e. the unique three dimensional structure of a protein is a resultant of various attractive and repulsive interactions of the protein chain within itself and with the surrounding solvent. Sometimes, globular proteins can exist in a number of low entropy but energetically equivalent states. These may be seen as "quasi-native" states and transitions from one to another are not considered as major changes and protein is not considered as denatured. The nature of the forces maintaining and stabilizing the native configuration decides the type of alteration which protein will undergo. These forces may include hydrogen bonding, hydrophobic and electrostatic interactions which lead to the formation of rather labile structures. The stability of the native state is marginal because the free energy changes from the above mentioned stabilizing interactions and just counterbalances the large conformational entropy of the "disordered" random state. Therefore, the net free energy difference is very small, which stabilize the native conformation against transitions to other forms. This free energy difference may be overcome by marginal alterations in the surrounding medium by mild heat treatments or by linking with low molecular weight additives.

2.5.1. Significance of denaturation

The thermal behaviour of the whey proteins is very important in the thermal processing of dairy products. The reduction in protein solubility could be a major consequence of denaturation. The early workers exploited the heat labilities of whey protein to monitor the heat processing history of milk and milk products. Quantification of whey protein denaturation may be used as an index of the heat treatment of milk products by measuring the loss of solubility in isoelectric pH range or on saturation with NaCl (Harland et al., 1945; Kuramoto et al, 1959; Leighton, 1962; McGann et al., 1972).

The thermo-sensitivity of whey proteins has a great influence on whey utilization. The various functional properties of protein such as solubility, emulsifying, foaming and thermosetting properties are affected by denaturation and it limits the range of food uses i.e. denatured whey protein products (Evans and Gordon, 1980; Morr, 1982). For many years, heat-coagulation properties of the whey proteins have been exploited to produce a denatured protein concentrate (Robinson et al, 1976; Jelen, 1977), which could be used as an extender in meat products, in baked goods or biscuits, in roller-dried baby foods and to fortify cereals such as wheat or cornflakes. i.e, where protein fortification is extremely required but general functionality is not important.

Another manifestation of whey protein denaturation is increased side group reactivity, which is thought to be responsible for reduced oxidation-reduction potential, cooked flavour development, and anti-oxidant properties. Some protein-protein interactions may be caused by the increased side group activity upon heating, e.g. the disulphide linkage of α-La. This interaction plays a major role in determining the heat stability of milk (Sawyer, 1969; Fox and Morrissey, 1977; Fox, 1982) and also impairs the rennet clotting properties of milk.

2.5.2. Heat induced denaturation

The change in the conformational structure of a protein depends upon the temperature and duration of the heat treatment to which it is subjected. Before being denatured, globular proteins with quaternary structure dissociate first (Georges, C. et al., 1960). In addition to the disruption of non-covalent bonds, there is more exposure of the thiol (Larson, B. L. et al., 1952 & Shimada,

K. et al., 1989) and hydrophobic groups (Sheraga, H. A. et al., 1962), which were previously buried to the aqueous medium. Tryptophan emission spectra may be helpful for monitoring this consequential exposure of the hydrophobic core of the molecule (Mills, O. E., 1976 & Mills, O. E. et al, 1975). The intensity of the tryptophan fluorescence depends upon the heat treatment and the peak wavelength is shifted toward a longer wavelength. It shows that additional exposure of the non-polar chromophore to an aqueous environment has accompanied molecular unfolding (Mills, O. E., 1976).

The modification of the surface hydrophobicity monitored by means of hydrophobic fluorescence probes has been used to quantify the degree of modification of the tertiary structure of globular proteins (Permyakov, E. A. et al., 1984 & Bonomi, F. et al., 1988). The affinity of ANS is because of the absence of rigid packing of hydrophobic clusters in the stable denatured forms of the protein that means to accessibility of molecules of the solvent to protein hydrophobic core (Semisotnov, G. V. et al, 1991).

The changes of peptide backbone and side-chain chromophores have been shown by circular dichroism. Two dominant negative peaks at about 208 and 222 nm and a positive peak in the vicinity of 195 nm has been showed by CD spectrum of globular proteins in their native states. The negative peaks are due to the presence of a highly ordered structure (α+β, α/β, and α-helical regions). A loss of α-ordered characteristics in favour of β-structure has been showed by the decrease in the far-UV bands observed in heat-treated protein solutions (Townend, R. et al., 1967). Aromatic side-chain contributions to the CD spectra of heated solutions could arise from clustering of aromatic amino groups. As shown by the Infrared spectroscopy of β-LG solution, during first step of denaturation, the helical structure was destroyed, whereas a residual secondary structure was still present after a heat treatment of 90°C (Casal, H. L. et al., 1988).

A long time ago, heat-induced conformational change in small single-domain globular proteins was suggested to be a reversible two-step process leading from the compact native state to the denatured one (Brandts, J. F., 1969 & Privalov, P. L., 1980) . There was a loss of the intra-molecular bonds that stabilize the tertiary and secondary structures. So that only the spatial arrangement of the protein backbone was altered in favour of a more disordered form, is a highly

cooperative transition. Recently, Kuwajima (Kuwajima, K., 1989) reviewed this classical view of unfolding of globular proteins by considering the concept of the so-called "molten-globule", intermediate state between the "native" and "denatured" ones.

Calorimetric measurements provide valid information on heat-induced transitions. According to them these molten-globule states, characterized by residual structure might be present in the unfolding reactions. Kuwajima (Kuwajima, K., 1989) proposed that the "molten globule" and final "denatured" states are although structurally different but they are "calorimetrically" indistinguishable.

2.5.3. High pressure induced denaturation

In addition to the denaturation caused by temperature, high pressure (HP) treatment also affects protein conformation in a manner depending on the protein itself, its environment, and the applied pressure, temperature and duration of treatment.

The primary structure of protein remains intact on HP treatment (Gross & Jaenicke, 1994) (Mozhaev et al., 1994). Hydrogen bonds, which are responsible for the stability of secondary structure are enhanced at low pressures and ruptured only at very high pressures (Hendrickx et al., 1998). Significant changes in the tertiary structure of proteins, which is maintained by hydrophobic and ionic interactions are observed >200 MPa (Hendrickx et al., 1998). Pressure strongly destabilised the Ionic bonds in aqueous solution (Gross & Jaenicke, 1994), as there are hydrophobic interactions between aliphatic groups (Heremans, 1982; Mozhaev et al., 1994). It is believed that high pressure treatment at moderate temperatures disrupts only relatively weak bonds such as hydrogen, hydrophobic and ionic bonds (Hendrickx et al., 1998).

HP-induced denaturation of the major whey protein, α-LA is of great importance while processing of milk and other dairy products. Studies on milk have shown that α-LA is more baro-resistant than β-LG (Lo´pez-Fandin˜o et al., 1996; Felipe et al., 1997; Lo´pez-Fandin˜o & Olano, 1998, Garcia-Risco et al., 2000, Scollard et al., 2000; Huppertz et al., 2004). Upon HP treatment of milk denatured β-LG may form small aggregates (Felipe et al., 1997) or interact with casein

micelles (Scollard et al., 2000). Huppertz et al. (2004) showed that the majority of denatured β-LG in HP-treated milk was co-sedimentable with the casein micelles.

HP-induced denaturation of α-LA in aqueous solution and buffers has been studied in considerably more detail as compare to milk. In these kinds of systems, it has been noticed that through sulphydryl-disulphide interchange reactions high pressure promotes unfolding and aggregation of protein (Dumay et al., 1994, Funtenberger et al., 1997, Van Camp et al., 1997). Prior to HP treatment, by adding the sulphydryl-blocking (Tanaka et al., 1996; Funtenberger et al., 1997) or disulphide-reducing agents (Funtenberger et al., 1997) to the solutions, HP induced aggregation of protein can be prevented. In HP-treated whey protein solutions in buffer, mixed aggregates of denatured α-LA has been shown (Jegouic et al., 1997). This section of the review focussed on the temperature & HP-induced denaturation of α-LA in whey, milk and mixtures. The influence of sulphydryl-modifying agents and calcium on the denaturation of α-LA was also targeted to further elucidate the mechanism for heat & high pressure-induced denaturation of α-LA in milk and whey.

2.6. Influence of alcohols on α-LA

Since past few decades, the effects of alcohols on proteins and peptides have been studied extensively (Schrier, E. E. et al., 1965 & Nelson, J. W. et al., 1986). The wide range of applications of the alcohols in many fields makes these studies even more important. The Previous studies mainly focussed on the effect of alcohols on proteins as denaturants. It has been demonstrated that rigid native state of proteins is denatured by various alcohols and subsequently the α-helical structure gets stabilized.

The α-helical structure in denatured proteins and their fragments has been recently stabilized by using alcohols, in particular, 2,2,2-tri-fluoroethanol (TFE) (abbreviations used for alcohols are summarized in Table 2) (Nelson, J. W. et al., 1986 & Yang, J. J. et al., 1995). The secondary structures which have been stabilized in alcohols are suggested to be the initiation site of protein folding (Dyson, J. et al., 1993). With N-labelled hen egg-white lysozyme the structural and dynamic properties of the TFE-induced helical state and its relation to the native state has been showed by the nuclear magnetic resonance studies by Dobson and co-workers (Buck, M. et al.,

1996) (Thomas, P. D. et al., 1993), which is important to analyze the mechanism of protein folding. The aggregations, which some-times occur during peptide synthesis are dissolved by using some alcohols such as 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP). HFIP is also used for same purpose by the Researchers investigating prion diseases (Gasset, M. et al., 1992 & Zhang, H. et al., 1995) and Alzheimer's amyloid peptides (Wood, S. J. et al., 1996). So, it has been demonstrated that as compare to TFE, HFIP has a much stronger potential for denaturing the protein's native structure and also in inducing the helical structure (Hirota, N. et al., 1997).

Alcohol	Abbreviation used	*m* of β-lactoglobulin (± error) (kJ/mol/M)	*m* of melittin (± error) (kJ/mol/M)
Alkanols			
(1) Methanol	MeOH	1.97 (±0.03) 3.75 (±0.19)*	0.78 (±0.02)
(2) Ethanol	EtOH	3.92 (±0.07) 7.45 (±0.51)*	1.57 (±0.02)
(3) 1-Propanol	*n*PrOH	8.45 (±0.13)	3.68 (±0.10)
(4) 2-Propanol	*i*PrOH	6.01 (±0.14)	3.25 (±0.06)
(5) 2-Methyl-2-propanol	*t*BuOH	12.0 (±0.17)	4.75 (±0.12)
(6) 2-Butanol	*s*BuOH	13.0 (±0.18)	6.00 (±0.14)
Diols			
(7) 1,2-Ethanediol	$Et(OH)_2$	2.63 (±0.04)*	0.90 (±0.22)
(8) 1,2-Propanediol	$Pr(OH)_2$	2.75 (±0.07) 5.89 (±0.33)*	1.47 (±0.05)
(9) 1,4-Butanediol	$1,4Bu(OH)_2$	3.73 (±0.13) 6.52 (±0.16)*	1.42 (±0.03)
(10) 2,3-Butanediol	$2,3Bu(OH)_2$	7.63 (±0.18)*	2.86 (±0.13)
(11) 1,5-Pentanediol	$Pen(OH)_2$	5.15 (±0.26) 11.3 (±0.02)*	2.10 (±0.09)
(12) 2-Methyl-2,4-pentanediol	MPD	8.05 (±0.67)	4.44 (±0.28)
Triol			
(13) 1,2,3-Propanetriol	Glycerol	2.18 (±0.4)*	0.95 (±0.02)
Halogenols			
(14) 2-Fluoroethanol	FEtOH	3.16 (±0.14)	1.39 (±0.04)
(15) 2,2,2-Trifluoroethanol	TFE	9.15 (±0.11)	5.05 (±0.12)
(16) 1,1,1,3,3,3-Hexafluoro-2-propanol	HFIP	37.3 (±0.41)	18.2 (±1.19)
(17) 2-Chloroethanol	ClEtOH	8.23 (±0.22)	3.17 (±0.13)
(18) 3-Chloro-1-propanol	ClPrOH	16.7 (±0.26) 22.1 (±2.93)*	6.71 (±0.17)
(19) 2-Bromoethanol	BrEtOH	12.8 (±0.54)	9.04 (±0.16)

*Asterisks indicate data obtained in the presence of 20% ethanol.

Table 2.2: Abbreviations used for various alcohols along with m Values of various alcohols (Reproduced, with permission, from Hirota, N. et al., 1998)

By the decreased polarity of solvent, these effects of alcohols can be explained to some extent (Hirota, N. et al., 1997 & Liu, Y. et al., 1995). Hydrophobic interactions stabilizing the native structure are weakened in low polarity solvents and simultaneously the local hydrogen bonds are strengthened, resulting in denaturation, and stabilization of the extended α-helical structures. However, the exact relation between the alcohol-induced α-helix formation and the alcohol-induced denaturation of the native state of proteins are still unclear. Luo and Baldwin (Luo, P. & Baldwin, R. L., 1997) studied the mechanism of helix-induction by TFE. They analyzed the helix formation of the alanine based short peptides on the basis of the Lifson-Loig helix-coil transition theory (Rohl, C. A. et al., 1996). At the same time they measured the strength of the hydrogen

bond in a model compound, salicylic acid in TFE/water mixtures. They demonstrated that curve of the increase of TFE concentration versus hydrogen bond strength matches both in shape and magnitude of the increase in average helix propensity in TFE/water mixtures. They finally summed up that strengthening the hydrogen bonds is main responsible factor for the TFE effects on the helix formation of short peptides. This conclusion suggested that the mechanism of the alcohol-induced denaturation of proteins is different from the mechanism of alcohol-induced helix formation of peptides.

In the previous study (Hirota, N. et al., 1998) for understanding the mechanism of the alcohol effects on proteins and peptides, the effects of various alcohols including TFE on melittin, a major component of honeybee venoms was investigated. Melittin is made up of 26 amino acid residues of which no one is acidic, 5 are basic and the C-terminal carboxyl group is amidated (Haberman, H., 1972).

Under conditions of low salt at neutral pH, melittin is in a monomeric unfolded state and it transforms into α-helical tetramer under high salt or high acid conditions, at alkaline pH or at high peptide concentrations (Hagihara, Y. et al., 1992). Melittin get converted into a monomeric α-helix by the addition of alcohols (Bazzo et al., 1988). An approach was undertaken in order to obtain good understanding of the contribution of each group like hydrocarbon (CH) group, hydroxyl (OH) group and halogen group to the alcohol effects on melittin. The effectiveness was proportional to the bulkiness of hydrocarbon groups for alkanols, indicating that the hydrocarbon group contributes positively to the alcohol effects. Hydroxyl groups contribute negatively to the alcohol effects, which have been shown by the comparison of alcohols with the same hydrocarbon group but different number of hydroxyl group. Halogen increases the effectiveness in the order of F<Cl<Br indicated by the comparison of several halogenols. On the basis of these results it could be believed that the effects of alcohols can be interpreted by the additive contributions of each of the constituent groups of an alcohol.

2.6.1. Influence of ethanol on the structure of α-LA

Figures 2.5 & 2.6 are representing the results of spectral measurements of ethanol-induced conformational changes of holo and apo α-LA. For native form of α-La, the maximum

tryptophan emission was counted at 330 nm. With the increase in ethanol concentration this maximum shifted to longer wavelengths and its magnitude increased, being most intense in the presence of 40% ethanol (Fig. 2.4a). Red shift of the emission maximum shows that under the influence of ethanol the tryptophanyl residues, which in aqueous solution are sheltered in the hydrophobic core of the protein molecules become more exposed to a polar environment (Stryer, L., 1968). On the other side, for the apo form fluorescence intensity reached its maximum at 350 nm. No changes were observed in the maximum wavelength, whereas by increasing ethanol concentrations the intensity magnitude increased (Fig. 2.4b). This increase in fluorescence intensity corresponds to an increase in solvent polarity and may be due to a decrease of the quenching of some tryptophanyl residues.

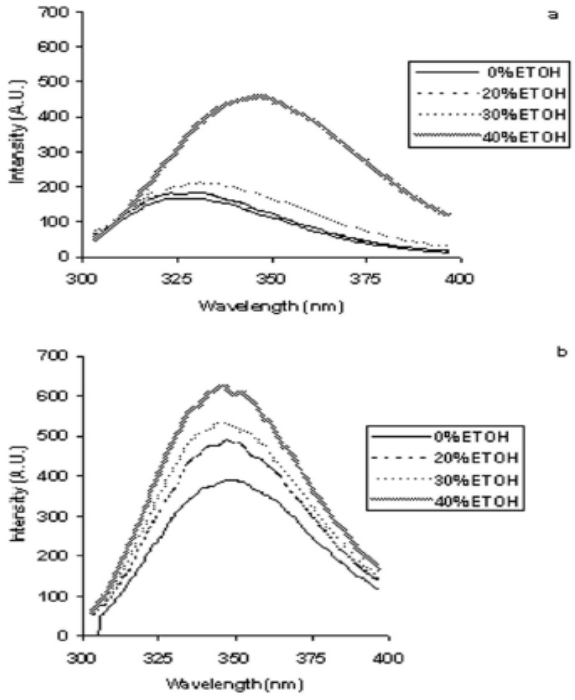


Fig 2.4

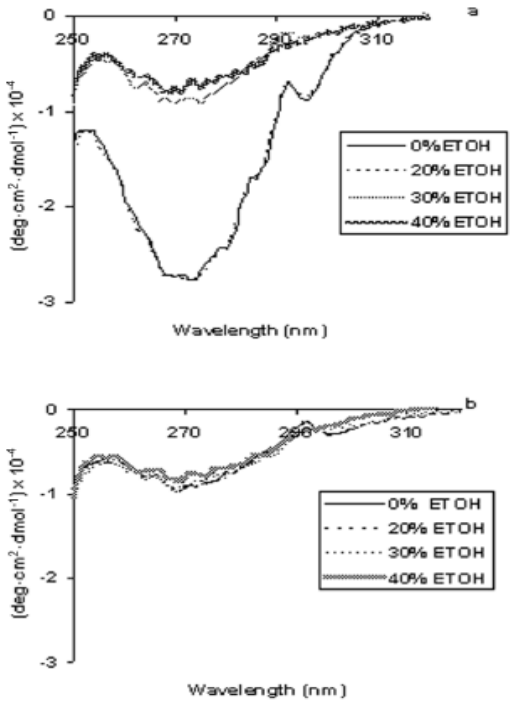


Fig 2.5

Figure 2.4: Fluorescence spectra of holo (a) and apo (b) α-lactalbumin with increase in concentration of ethanol. Protein concentration was 10 µM in 25 mMTris-HCl buffer, pH 8.0. The spectrums were recorded at 25^0C (A.U., arbitrary units) (Reproduced, with permission, from Wehbi, Z., 2006)

Figure 2.5: Near-UV CD spectra of holo (a) and apo (b) α-lactalbumin with increase in concentrations of ethanol. Protein concentration was 140 µM in 25 mMTris-HCl buffer, pH 8.0. The spectrums were recorded at 37^0C (Reproduced, with permission, from Wehbi, Z., 2006)

Fig 2.5 represents the Near-UV CD spectra of holo and apo α-LA. There are two minima at 270 and 298 nm in the spectrum of holo form, arising from spectral contribution of aromatic chromophores (tryptophan, tyrosine and phenylalanine) (Barbana, C. et al., 2006 & Grinberg, V. Y. et al., 1998). The holo protein in the presence of 20% ethanol and in the absence of alcohol showed the similar spectrum (Fig. 2.5a). In contrast, in the presence of 30 and 40% ethanol, the tertiary structure was largely disorganized as shown by the strong reduction in the CD signal in the 250–300nm region. These results are in agreement with previous measurements performed for α-LA in the presence of increasing concentrations of ethanol (Grinberg, V. Y. et al., 1998). In the case of apo α-LA, the near CD spectra showed the absence of signal at 270nm, as compared to that previously reported (Segawa, T. et al., 1983 & Barbana, C. et al., 2006). These results gave a clue of an increase of the rotation freedom of aromatic chromophores (loss of the rigid tertiary structure of the protein).

Furthermore, in the presence of increasing concentrations of ethanol, the near-UV CD spectra of the apo form were hardly distinguishable from the spectra of apo form without ethanol (Fig. 2.5b). With holo α-LA, far-UV CD studies showed that increased concentrations of ethanol increased the amplitudes of spectral bands at 208 and 232 nm, which are characteristics of increased α-helix content (Grinberg, V. Y. et al., 1998).

By near-UV CD spectroscopy, the structural conformation of apo α-LA previously incubated with oleic acid in the presence of increasing ethanol concentrations was studied. But after comparing with the CD spectra of same protein under the same solvent conditions, no significant changes were observed. However, Polverino (Polverino de Laureto, P. et al., 2002) observed a reduction of the CD signal at 270 nm, when oleic acid added to the apo α-LA and dissolved in the aqueous solution at pH 8.3. This strange behaviour of the apo form could be associated to the different temperatures at which measurement was performed or to a decreased signal due to micellization of oleic acid.

2.6.2. Proteolysis of α-LA as a function of ethanol concentration

Holo and apo forms of α-LA were subjected to trypsinolysis concentrations in the presence of increasing ethanol concentration and tryptic peptides were separated by RP-HPLC. Being stable

and active at neutral pH in the presence of aqueous organic solvents is an advantage of this protease (Simon, L. M. et al., 2001). Because of the substrate specificity of tryptic cleavage, it is expected to mainly depend on the stereochemistry and dynamics of the α-LA substrate and not on the specificity of the enzyme.

Fig 2.6 shows the rate of disappearance of holo and apo α-LA. Holo α-La was resistant to hydrolysis by trypsin in the absence of ethanol (more than 95% of the protein remained undigested after 24 hrs of incubation) as expected for a fully folded and rigid structure of the calcium-loaded protein. These results were identical to the results reported previously under similar experimental conditions (Schmidt, D. G. et al., 1991 and Hirai, Y. et al., 1992).

Holo α-LA was susceptible to trypsinolysis in the presence of 20% ethanol. About 57% of the protein remained non-hydrolyzed after 6 hours of hydrolysis. In the presence of 30% ethanol hydrolysis rate increased substantially. The proportion of non-hydrolyzed α-LA decreased from 50% (after 1 hrs) to 10% after 6 hrs of hydrolysis (Fig 2.6a). Structural changes induced by ethanol make the protein more susceptible to hydrolysis as mainly because of exposure of its hidden tryptic cleavage sites as it has been also found with other globular proteins in alcohols (Dalgalarrondo, M. et al., 1991). Also, it is noticed that α-LA was susceptible to tryptic hydrolysis after its thermal denaturation. It was due to a looser conformation that facilitated the accessibility of the enzyme (Bertrand-Harb, C. et al., 2002).

It is quite interesting that spectral measurements did not show any significant secondary or tertiary changes (Grinberg, V. Y. et al., 1998) in the presence of 20% ethanol. There might be transitory conformational changes, indicated by enhanced proteolysis such as an increase in amino acid side chain rotation. Therefore, limited proteolysis might be a more efficient tool as compare to spectroscopic techniques to determine conformational changes of α-LA.

Only a small peak eluting just before α-LA was observed in the presence of 40% ethanol at all incubation times (Wehbi, Z. et al., 2006). After reduction with DTT, this peak disappeared, indicating that the protein aggregates formed would hinder the sites of cleavage. The analysis of the results of limited proteolysis experiments which were performed on globular proteins of

known structure revealed that helical segments are not the site of proteolytic attack (Fontana, A. et al., 1993). Therefore, the high helical conformation state acquired by α-LA dissolved in aqueous buffer containing 40% ethanol (Grinberg, V. Y. et al., 1998) might be the origin of this inhibitory effect as it was also reported in the case of α-LA in the presence of high concentration of TFE (Polverino de Laureto, P., et al., 1995) and in the case of other globular proteins in high ethanol concentration (Grinberg, V. Y. et al., 1998; Dalgalarrondo, M. et al., 1991; Dalgalarrondo, M. et al., 1995).

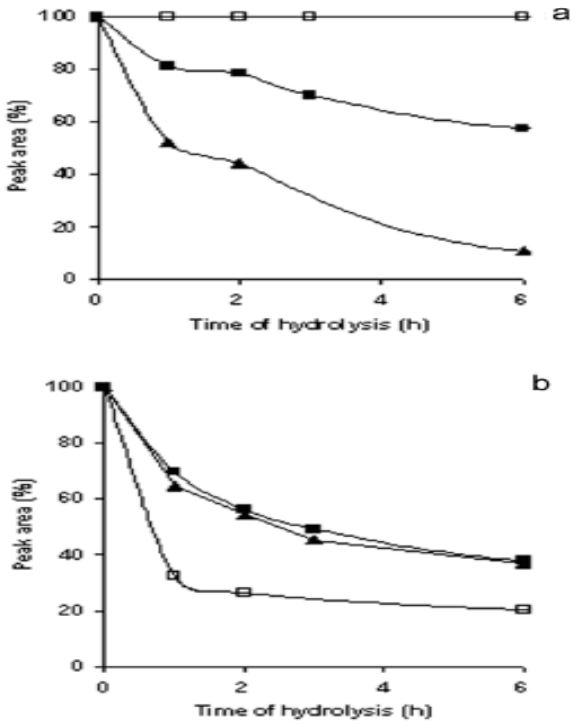


Figure 2.6: Disappearance with time of bovine holo (a) and apo (b) α-LA peak after proteolysis with trypsin as a function of ethanol concentration: 0% (□), 20% (■) and 30% (▲). Peak area corresponds to residual α-LA (%) (Reproduced, with permission, from Wehbi, Z., 2006)

The disappearance of apo α-LA is shown by figure 2.6b. The protein is degraded quickly in the absence of ethanol, leaving only 30% of the intact protein after 1 h of trypsinolysis. These results are in consensus with those reported by Hirai (Hirai, Y. et al., 1992), who determined the lactose synthase activity of α-LA after hydrolysis by trypsin. Calcium loaded protein had 150 times higher halftimes of the tryptic digestion as compare to apo α-LA. It was suggested by these authors that calcium provide α-LA with a very strong resistance to tryptic attack while apo α-LA

was highly susceptible to digestion (Hirai, Y. et al., 1992). Likewise, the native form was resistant to proteolysis, whereas the partially folded states of α-LA exposed to acid solutions at pH 2.0 or at neutral pH upon EDTA-mediated removal of the single protein-bound Ca^{2+} ion were easily digested by chymotrypsin and proteinase K (Polverino de Laureto, P. et al., 1999).

In contrast, an inhibitory effect of proteolysis can be observed when adding 20 and 30% ethanol to the apo form and the overall reaction rates slightly lowered. After 1 and 6 hour of hydrolysis, the proportion of non-degraded apo α-LA decreased from 64 to 36% respectively, at both ethanol concentrations. Identical results were obtained in the presence of increasing concentrations of TFE for the apo form of α-LA (Polverino de Laureto, P. et al., 1995). These authors noticed that proteolysin by thermolysin is greatly accelerated by adding TFE up to 20% though at higher concentrations the proportion of degraded protein decreased considerably. No hydrolysis of the apo α-LA was observed after 24 h of incubation in the presence of 40% ethanol as it was observed in the case of the holo form of the protein (Wehbi, Z. et al., 2006).

The rate of disappearance of the protein was similar when apo α-LA was previously incubated to form a complex with oleic acid, to that obtained in the case of the apo form without bounded fatty acid (results not shown). It was reported that the resistance of apo α-LA to tryptic digestion was increased upon its binding to other low molecular mass apolar substances, such as 4,4'-bis-1-(phenylamino)-8-naphtalene sulfonate (Hirai, Y. et al., 1992). Results have shown that to increase the conformational stability of α-LA in order to resist trypticdegradation, its binding to oleic acid is not sufficient (Wehbi, Z. et al., 2006).These results are in contradiction with those observed in the case of other fatty acid binding proteins such as β-lactoglobulin (Puyol, P. et al., 1993).

2.7. Interaction of α-LA with fatty acids

Various techniques such as fluorescence spectroscopy, NMR spectroscopy, partition equilibrium, CD (circular dichrosim) and many other chromatographic techniques have been used in order to determine the interaction of α-LA with fatty acids. Previous research has been shown that the holo (native form) form of α-LA is unable to bind to the fatty acids.

In addition, the inability of α-LA of bovine or human origin to bind with fatty acids has been demonstrated with the help of size-exclusion chromatography and autoradiography. Only serum albumin from human as well bovine origin and β-lactoglobulin from bovine sources showed radioactivity (P´erez et al., 1989). When analysed by gas-liquid chromatography, α-LA in native form (non-denatured) obtained from human and bovine milk did not showed any traces of fatty acids (Barbana et al., 2006; 2008). Similar results were collected by using other techniques such as fluorescence spectroscopy, partition equilibrium and far-UV circular dichroism, which indicated that the concerned whey protein either from bovine or human origin does not interact with oleic and palmitic acids (Barbana et al., 2006; 2008; Knyazeva et al., 2008). These results proved that holo form of the α-LA does not contain fatty acids bound in vivo and also are unable to bind with them in vitro.

In addition, Cawthern et al. (1997) using fluorescent indicator composed by the acrylodate intestine fatty acid-binding protein could not determine the interaction of the holo bovine α-LA with stearic acid. These results caused the contradiction to those acquired with the help of electron spin resonance and intrinsic protein fluorescence methods by the same author, according to which α-LA has binding capacity to bind stearic acid with a dissociation constant in the range of 10 to 100 μM.

Interestingly, it has been reported that the apo form (calcium depleted form) of α-LA of both human and bovine origin obtained by treating against EDTA have the capability to bind the fatty acids (Cawthern et al., 1997; Barbana et al., 2006; Yang et al., 2006a; Barbana et al., 2008; Knyazeva et al., 2008; Zhang et al., 2009). By using fluorescence spectroscopy, Cawthern et al. (1997) reported that bovine apo α-LA shows one binding site for stearic acid with a dissociation constant of 2.3μM at the pH value of 8.5. However, various binding sites came into notice with a dissociation constant of 35 μM for 5-doxyl stearic acid. Barbana et al. (2006) by using same technique claimed only one binding site in bovine apo α-LA for oleic acid with a association constant of $3.3 \times 106\ M^{-1}$. However, in the same study due to the low fluorescence enhancement, the binding parameters in case of palmitic acid could not be determined, reason being the difficulties in obtaining accurate fit.

Partition equilibrium technique was also used to study the interaction between fatty acids and apo α-LA. It has been reported that apo α-LA possesses one binding site for fatty acids. According to the study performed by Barbana and his group, the association constants for binding of oleic and palmitic acid to bovine α-LA were 4.6×10^6 and 5.4×10^5 M^{-1}, respectively and for human apo α-LA are 1.9×10^6 and 4.2×10^5 M^{-1}, respectively (Barbana et al., 2006; 2008).

Knyazeva and his group (Knyazeva et al., 2008) in their study formed a complex of oleic acid with human apo-α-LA at the pH of 8.3. They found that a temperature of 45^0C, absence of Ca^{2+} and presence of 150 mMNacl are the most favourable conditions for the complex formation. A decrease in oleic acid bound to α-LA was resulted by dropping the incubation temperature from 45 to 17^0C. A higher value of the apparent affinity constant for human apo α-LA to bind oleic acid was noticed at the temperature of 45^0C as compare to 17^0C *(K* = $2\times105M^{-1}$ and *K* =$2\times104M^{-1}$, respectively). As determined by the fluorescence spectroscopy, the temperature also influences the number of binding sites as 2.9 at 17^0C and 9 at 45^0C. The α-helix content of the protein was increased independently of temperature conditions due to binding of oleic acid to *α*-LA. The Ca^{2+} association affinity of the protein did not been effected due to the binding.

As seen in the near-UV region of the CD spectra, the thermal treatment of the complex of oleic acid with human or bovine α-LA at the temperature of 50 or 60^0C for 10 minutes resulted into conformational changes in the protein's structure and directed it towards molten globule state. These results indicated that thermal treatment of protein or protein complex could facilitate the exposure of hydrophobic patches which are normally hidden in the inner surface of protein and could significantly promote the binding of fatty acids to the protein even in the presence of the salt (Kamijima et al., 2008).

It is believed that the pH during incubation influence the binding of fatty acids to apo α-LA. Using UV absorbance spectroscopy and transmission electron microscopy, Zhang et al. (2009) showed that at pH 4.0-4.5, the incubation of oleic acid and linoleic acid with bovine apo *α*-LA induced the formation of intermediates of the protein that form amorphous aggregates in the concentration and time dependent manners. Under the physiological conditions at 37^0C these

aggregates were dissolved and they demonstrated the identical structural characteristics to those shown by the protein during the complex formation between the apo α-LA and oleic acid. The results obtained by using the techniques like fluorescence spectroscopy, electrospray ionization mass spectroscopy, atomic force microscopy and CD (circular dichroism) showed that at the pH between 4.0 and 7.0 the formation of dimeric intermediate of bovine apo α-LA is enhanced by the oleic acid. Whereas, at the pH of 3.0, the molten globule state content is increased in the presence of oleic acid as compared to its absence.

On the other side, it has been suggested that the complex formation of fatty acid and apo α-LA could be performed by other alternative method (chromatographic) rather than by simple blending of fatty acid and protein. The complex is generally formed by passing the calcium depleted (apo conformer) α-LA through an ion exchange matrix which is pre-conditioned with oleic acid. Apparently, the native conformer of α-LA does not retain in the column upon passing through it. Apo α-LA which has ability to be retained on the column, forms a complex with fatty acid and which is eluted as a sharp peak after applying 1M of Nacl (Svensson et al., 2000). This eluted α-LA can be retained in the partially unfolded form even in the presence of salt and at neutral pH, Which is contradict to the literature which claims the reversion of the protein to its native form under such conditions. So it is proposed that fatty acid is the required element or say is cofactor to maintain the molten globule state of the apo conformer of the protein even in the presence of the Ca^{2+}(Svensson et al., 2000; Mok et al., 2007).

To understand the specificity of the binding of fatty acids with apo α-LA, the apo form of protein was subjected to the column matrixes which were pre-conditioned with fatty acids differing in the degree of saturation, conformation (cis/trans) and the carbon chain length (Svensson et al., 2003b). The maximum amount of the protein was retained on the columns, pre-conditioned with unsaturated C18 fatty acids (cis) and formed a complex that was eluted as sharp peak when 1M of NaCl was applied. Therefore, when apo α-LA was applied to the column matrix which was conditioned with oleic acid (C18:1:9 cis) and Vaccenic acid (C18:1:11 cis), about 90% and 70% protein was retained respectively for both fatty acids. However, for other unsaturated fatty acids such as C18:1:6 cis, C18:2:9, 12 cis; C18:3:9, 12, 15 cis and C18:3:6, 9, 12cis, the percentage of protein retention was comparatively lower.

Similarly, the unsaturated fatty acids of cis conformation with shorter (C16:1:9 cis) or longer (C20:1:11 cis and C20:4:5, 8, 11, 15 cis) carbon chains were able to develop the stable complexes, but the protein yield was quite lower as compare to that obtained in the case of oleic acid. On the other side, upon applying the apo α-LA to the column matrix conditioned with the fatty acids of trans conformation such as C18:1:9 trans, C18:1:11 trans, or with the 18:0 saturated fatty acid, it could not result into complex formation. The results of the gas-liquid chromatography have showed that complex formed by passing the protein from a matrix conditioned with oleic acid had 0.6 to 1.3 mole of oleic acid per molecule of the apo α-LA. The given results suggests that upon interaction of fatty acid with apo α-LA, C18 cis fatty acids gives the highest yield and this interaction occurs in the a stereo-specific manner and for best retention of the protein the fatty acids should be of the cis conformation.

Studies of similar nature have been carried out for the apo α-LA from bovine, porcine, equine and other mammals by following the same method of passing apo α-LA through the matrix which is pre-conditioned with oleic acid and the results obtained were analogous to those acquired in the case of protein of human origin (Pettersson et al., 2006). This is true that lower yields were collected for these mammal species. 62% protein was eluted as a sharp peak in the case of human protein, whereas only 24% to 46% of protein was eluted in the case of other mammals. The differences in the amino acid sequence in the fatty acid binding site that results in changes in the affinity of individual α-LA from different species for binding oleic acid (Svensson et al., 2003a; Pettersson et al., 2006) is considered as the main reason behind the difference in protein yield among different mammals.

2.7.1. Effect of pH on the interaction of α-LA with fatty acids

The adoption of the flexible molten globule state by bovine α-LA enables it to insert rapidly into bi-layer at pH around 2, mainly by hydrophobic interactions and by electrostatic bonds between the positive charges of the protein and the negative charged head groups of the phospholipids (Chaudhuri et al., 2004). Besides, hydrophobic interactions can take place between the protein and the apolar lipids of the membrane (Chaudhuri et al., 2004; Chenal et al., 2005; Herreman et al., 1981; Montich& Marsh, 1995).

At pH values (pH=3) corresponding to the adoption of the molten globule state, the insertion rate of bovine α-LA to single bi-layer of EPC was shown optimum. Bañuelos and Muga (1996b) showed that local rearrangements of flexible connecting loops and helical segments of the protein are induced by lowering the pH to values favourable for α-LA/membrane interaction. Montich and Marsh (1995) found that at pH 4, α-LA adopts a molten globule state and associates the lipid membranes affecting their chain mobility and packing.

The interaction of bovine α-LA with the negatively charged membranes of vesicles of PS and EPC was found mainly hydrophobic between pH values of 4 and 4.5 (above the isoelectric point). Overall, the molten globule state was indicated as the optimum conformation of the protein that could associate the lipid bilayers (Agasøster et al., 2003; Chenal et al., 2005; Halskau et al., 2002, 2005; Lala et al., 1995).

2.7.2. Effect of temperature on the interaction of α-LA with fatty acids

By using fluorescence spectroscopy, it was noticed that the accessibility to the tryptophan residues was more significant after protein-lipid association at temperatures below the thermal transition of α-LA, whereas it was observed that some of the tryptophan residues were bound to the apolar phase of the membrane at temperatures above the thermal transition of the protein (Permyakov, 2005).

Bañuelos and Muga (1996a) and Cawthern et al. (1996) showed that bovine α-LA adsorbed onto the lipid surface below 34^0C and that these interactions were dependent on the form of the protein and the temperature. For the α-LA/DMPC complex, the unfolding of the holo α-LA occurred at lower temperatures, whereas for the apo form, the results were inconclusive. Furthermore, high tryptophan fluorescence of the DMPC bound to the thermally apo α-LA was observed, suggesting an insertion of some tryptophan residues into the membrane (Cawthern et al., 1996). Other results have also indicated that the thermally unfolded protein remained strongly bound to the liposomes (Permyakov et al., 1988).

It is realized that at temperature corresponding to the lipid-gel transition (30^0C), the binding of the protein to lipid membranes increased their chain mobility, whereas a limitation of the same

mobility was observed at the temperature that of the lipid fluid phase (20^0C). Moreover, the disruption of the cooperative chain packing of the membranes contributes to the abolition of the cooperative lipid chain-melting transition indicating that the protein may traverse the lipid bilayer (Montich& Marsh, 1995).

2.8. Emulsification properties of α-LA

α-LA has an ability to form and stabilize emulsions (dispersions of two immiscible liquids such as w/o or o/w) (Hill, 1996). Due to their amphiphilic character that enables them to emulsify and improves the stability of formed emulsions, whey proteins are successfully used in several food systems. Whey proteins are able to adsorb to oil droplets. Due to which they reduce the interfacial tension leading to further droplet disruption and to protect oil droplets against coalescence and/or flocculation by forming interfacial continuous protective membranes (McClements, 2008).

The capacity of the proteins to adsorb and spread through a large surface area decides the formation and stability of emulsions. This way they form a protective film around oil droplets (Kinsella, 1984). It also has been analysed that during the adsorption of α-LA to oil droplets, its secondary and tertiary structures also changed that stabilize it even under heat treatment. It suggested a possible transition of the protein structure towards a stable conformation after adsorption to the oil phase (Corredig & Dalgleish, 1995). Using DSC it was noticed that in the adsorbed state at a temperature of 67.6^0C, α-LA displayed a thermal transition, which is comparable to the protein in solution. With the free α-LA, an enthalpy of transition of 22.2 kJ $mol^{-1}$was obtained, which is significantly lower than 123 kJ mol^{-1} (Corredig & Dalgleish, 1995). Compared with the free protein, the conformational change of the adsorbed α-LA to the oil-in-water interface was confirmed recently by Nik, Wright and Corredig (2010). They stated that the protein in solution was totally degraded by the enzyme but adsorbed α-LA was resistant to the pepsin digestion.

Upon adsorption of the protein to the oil droplets, equilibrium between both conformers of α-LA (holo and apo) in solution and in oil droplet interface was observed. The stability of emulsion is greatly influenced by the characteristics of film adsorbed on the interface, the interfacial tension

between phases, the electric charge on the dispersed globules and the viscosity of the dispersion phase. Besides, the stability of emulsion is affected by other factors such proportion of polar lipids or synthetic surfactants in solution and protein concentration etc (Morr& Ha, 1993).

The homogenization process is widely used in the food industry, which enables the reduction of oil droplets size in emulsions and contributes to enhance their stability. In this regard, it has been observed that whey proteins reduce the energy required for the droplets disruption through their dynamic interfacial properties. Consequently, the use of these proteins during homogenization could be beneficial (McClements, 2008; Morr& Ha, 1993).

2.8.1. Capacity of α-LA to stabilize emulsions

Conformation of protein and their ability to increase the surface area are the key factors for the emulsion stabilisation capacity (Sitohy et al., 2001). Kinsella (1984) reported that surface area of 1-3 m^2mL^{-1} in emulsions was obtained with approximately 2 mg m^{-2} of whey proteins. In terms of droplet size, interfacial area and droplet coalescence rate, the addition of 2% (w/v) of whey proteins to peanut oil was found optimal to stabilize the emulsion at pH range of 4 to 7 (Klemaszewski & Kinsella, 1991).

The flexible structure of bovine α-LA enhances the emulsion stability as it allows α-LA to form films and decrease the interfacial tension (Kinsella, 1984). However, bovine α-LA enriched in solutions at pH 7 showed a lower emulsion activity index (EAI) (28.8 ± 1.3 m^2 g^{-1}) as well as a lower emulsion stability (ES) (13 ± 2 min) than that of the enriched fraction of β-LG (EAI of 32.6 ±3 m^2 g^{-1} and ES of 23 ± 5 min) and whey protein concentrate (WPC), which is composed of 17.2% (w/w) of α-LA and 64% (w/w) of β-LG (EAI of 34 ± 1.4 m^2 g^{-1} and ES of 16.5 ±5 min) (Tomasula & Yee, 2001). As compare to other milk proteins such as bovine serum albumin and immunoglobulins, the polymerization after the formation of oil-water emulsions was not observed in case of monomeric α-LA (Monahan, McClements, & Kinsella, 1993). This could be related to the lack of free thiol groups in the structure of α-LA and could explain in part the distinctive emulsifying behaviour of the protein compared with the other milk proteins.

2.8.2. Effect of pH and temperature on emulsifying properties of α-LA

Processing conditions and pH could greatly affect the stability of emulsions. In the presence of 15% (w/v) of sunflower oil, the effect of pH on the emulsifying capacity of bovine whey proteins were evaluated at room temperature through the measurement of the mean diameter of the oil droplet size and distribution by conventional microscopy (Laleye et al., 2008). Through the physical immobilization of oil droplets in the continuous phase, the ability of whey proteins to form a gel upon heating contributes to the emulsion stability. This property of α-LA could be very interesting for thermo-processed products (Kinsella, 1984). It has also been realized that the homogenized o/w emulsions could be stabilized by using heated WPC (Hayes, Stranaghan, &Dunkerley, 1979). However, Tossavainen (Tossavainen et al., 1998) showed that at a fat/protein ratio of 7:5, heat-denatured α-LA decreased emulsion stability.

2.8.3. Effect of salts on emulsifying properties of α-LA

Hunt and Dalgleish (1996) investigated the effect of KCl on the adsorption of WPI in emulsions containing 20% (w/w) soya oil. It has been proved that diameter of droplet emulsions was little bit increased from 0.45 to 0.65mm by adding the KCL up to 2oo mM at pH 7. However at pH 3, KCl concentrations above 50 mmol dm^{-3} increased the droplet size from 0.64 to 3.35 mm, which brought an increase in the emulsion viscosity. It is indicating that electrostatic interactions with added salt cause their aggregation. The effect of KCl on the adsorption competition between major whey proteins was studied. The addition of KCl up to 100 m mol dm^{-3} at pH 7 caused an increase in the amount of α-LA adsorbed to the emulsion interface from 25 to 35%, whereas no effect was observed at pH 3.

There was an observation that the emulsification properties of WPC in oil-in-water emulsions formed by 10% (w/v) sunflower oil and phospholipids were influenced by the concentration of NaCl. The addition of NaCl at concentrations between 0.5 and 1.5% (w/v) is able to promote the increase of droplet size of emulsions, the aggregation of the oil droplets as well as the increase of creaming rate (Sünder et al., 2001). In addition, the heat treatment of 90^0C for 5 min in the absence of NaCl led to the displacement of α-LA by β-LG. However, the addition of NaCl up to 200 mM eliminated the competition during heating. These findings suggest that the aggregation

of α-LA in the aqueous phase is promoted by NaCl and between the adsorbed and non adsorbed forms of the protein, making less easy its displacement by β-LG from the oil droplets (Ye, 2010).

2.8.4. Effect of chelating agents on emulsifying properties of α-LA

Keowmaneechai and McClements (2002) using isothermal titration calorimetry (ITC) investigated the effect of EDTA and citrate on the emulsifying properties of WPI. The o/w system was composed of 6.94% (w/v) soybean oil, 0.35% (w/v) WPI, 0.02% (w/v) sodium azide, 20 mMTris buffer, 10 mM $CaCl_2$, and concentrations between 0 and 40 mM of the chelating agent (EDTA or citrate). The mean particle diameter was lowered by the addition of EDTA or citrate emulsion at chelating agent to calcium ion molar ratio of 0.25, 0.35, 0.5 and 1 and it changed the particle size distribution from a monomodal to a bimodal model. The release of calcium ion from the o/w emulsions inhibited their aggregation and caused the formation of flocs in the system, in fact EDTA showed the greater effect. Since EDTA has a higher binding affinity to calcium in comparison with citrate. The effective concentrations for EDTA and citrate required to prevent emulsion aggregation were higher than 3.5 and 5 mM respectively.

The influence of the chelating agents on rheological properties of the emulsions was also studied. In emulsion systems without $CaCl_2$ and chelating agents, exhibited Newtonian behaviour was characterized by a constant viscosity independent of the shear rate. A change towards a strong shear-thinning behaviour characterized by a higher viscosity was induced by the addition of $CaCl_2$ to the system. This change could be linked to the aggregation of the oil droplets as a result of the presence of Ca^{2+} ions in the system. The injection of chelating agents such as EDTA or citrate to the calcium ion (in the emulsions) with a ratio of 0.35 inhibits the decrease of viscosity and induces a shear-thinning behaviour as result of the disaggregation of the droplets after the release of bounded calcium. However, a change towards behaviour (similar to that observed in control emulsions without chelating agents) was induced by addition of the same chelators at higher ratio levels (0.5-4).

These studies and findings could be of great importance for the formulation of food emulsions, especially which contain whey proteins including α-LA to control the flocculation of the o/w

droplets containing calcium ions, which are commonly used in the food industry (Keowmaneechai and McClements, 2002).

2.9. HAMLET & BAMLET

Studies showed that bovine α-LA under the same conditions used for the conversion of the human protein, could be converted to an apoptosis inducing form. It requires a folding change in bovine *α*-LA by the removal of $Ca^{2+}$with EDTA. The Ca^{2+}free protein was applied to an oleic acid conditioned ion exchange matrix and elute with high salt was assayed using the mouse lymphoma cell line L1210. In the apoptosis assay, the converted bovine protein induced DNA fragmentation and also reduced cell viability from 98% to 8%. At similar protein concentration, there was no apparent difference in efficiency of apoptosis induction between human and the bovine equivalent. So, the molecular complex of human apo α-LA and oleic acid was named HAMLET (Human Alpha-lactalbumin Made Lethal to Tumor cells) and bovine apo α-LA and oleic acid was named BAMLET (Bovine Alpha-lactalbumin Made Lethal to Tumor cells) (Svensson et al., 2003a).

In a series of experiments, *α*-LA from equine, caprine, and porcine species, was treated with EDTA, subjected to ion exchange chromatography on an oleic acid conditioned column, and complexes eluted with a NaCl gradient. α-LA/oleic acid complexes from all species was effective for causing a reduction in cell viability of L1210 cell line of about 35%, which was similar to that of human species assayed at the same protein concentration. In other words, all the complexes caused detectable DNA fragmentation. These results explained that purified α-LA species variants after partial unfolding and coupling to oleic acid can form complexes with anti-tumor activity (Pettersson et al., 2006). It was explored that how to prepare the α-LA/oleic acid complex with structural and cytotoxic properties similar to those of HAMLET, but under solution conditions. The active complex was formed by titration of human apo α-LA with oleic acid at pH 8.3 at 17°C and 45°C. These complexes possessed physicochemical and structural properties as well as cytotoxic effects on human larynx carcinoma HEp-2 cells, closely resembling those of HAMLET (Knyazeva et al., 2008). Similarly, it was shown that, when incubating Ca^{2+} depleted bovine α-LA with oleic or linoleic acids, cytotoxic aggregates were formed. The structural characteristics of these aggregates were very close to HAMLET. It has

been noticed that aggregates of bovine apo α-LA induced by these fatty acids showed significant dose-dependent cytotoxicity to human lung tumor cells. It is therefore expected that these cytotoxic aggregates of apo α-LA could be potential antitumor agents (Zhang et al., 2009).

The loss of viability of L1210 leukemia cells has been noticed after the heat treatment of a mixture containing the native form of human or bovine α-LA and oleic acid at 50 or 60°C for 10 minutes. The mean values of the viability ratio obtained with the human protein were 3% and 40% for heat-treated samples at 50°C and 60°C, respectively, which were identical to HAMLET. Such kind of apoptotic complexes are also formed by bovine protein under the same conditions, although the activities were lower than those of HAMLET or heat-treated human *α*-LA complexes. In all the cases DNA fragmentation was observed in cells incubated with the heat-treated samples of both species (Kamijima et al., 2008).

Furthermore, the application of HAMLET topically to skin papillomas for three weeks has been claimed to reduce the papilloma volume by 75% in 100% of the patients compared to 15% in the placebo group (Gustafsson et al., 2004). HAMLET also has been proven to be effective against bladder cancer cells in vivo. The intravesical instillation of HAMLET stimulated a rapid increase in the shedding of tumor cells into the urine during the days of instillation. Most of the shed cells were dead and an apoptotic response was detected in 6 of 9 patients, by using the TUNEL assay. By using endoscopic photography, morphological changes in the tumors were documented and a reduction in tumor size or change in tumor characteristics was observed in 8 of 9 patients (Mossberg et al., 2007 & Hallgren et al., 2008).

Several approaches have taken to identify the mechanisms of apoptosis in response to HAMLET and to understand the difference in sensitivity between tumor and healthy cells. HAMLET induced cell death has shown the features of classical apoptosis but many aspects of the cellular response to HAMLET are unconventional, suggesting that it activates programmed cell death in tumor cells through other pathways. The availability of surface receptors is not the primary factor determining the sensitivity of tumor and healthy cells, as both cell types showed rapid surface binding of HAMLET. Likewise, translocation of HAMLET to the cytoplasm was similar

in both cell types, though the massive cytoplasmic accumulation of HAMLET characterizes its activity on tumor cells (Gustafsson et al., 2005).

The redistribution of HAMLET from the cytoplasm to the perinuclear region accompanied by the movement of mitochondria, occurred only in tumor cells and not into healthy cells. Also, HAMLET was found in tumor cell nuclei. These findings indicate that sub-cellular localization of HAMLET reflect the differences in cell susceptibility to this complex.

Histones were identified as the nuclear target molecules for HAMLET, with a preference for H3 and H4 histones (D¨uringer et al., 2003). Because of its high affinity for histones, HAMLET disrupts the association between histones and DNA, and thus induces changes in the global chromatin structure. The perturbation of chromatin structure is an important feature of HAMLET-induced cell death (Ḋuringer et al., 2003; Svanborg et al., 2003; Brest et al., 2007; Mok et al., 2007).

However, Permyakov et al. (2004; 2005) claimed that for the efficient interaction of the protein with histone H3, the binding of oleic acid to α-LA was not necessary. He showed that both holo and apo α-LA were able to bind to the histone and also to basic poly-amino acids (poly-Lys and poly-Arg), that represent simple models of histone proteins and the binding process was driven by electrostatic interactions. These studies explain the antiproliferative effect observed by native α-LA in several tumor cell lines (Thompson et al., 1992; Sternhagen and Allen, 2001).

Moreover, Tolin et al. (2010) have recently shown that peptic fragments of bovine α-LA bound to oleic acid displayed a apoptotic activity on Jurkat tumor cell, analogous to that caused by BAMLET. He believed that to form an apoptotic oleic acid complex, the entire sequence of α-LA is not required. He suggested that for its apoptotic activity, BAMLET does not require specific binding of the protein and mentioned that oleic acid itself is the active component of the cytotoxic protein/peptide complexes. So, all these studies proven that several aspects of the mechanisms, which were proposed to explain cell death caused by HAMLET and BAMLET are still vague.

In contrast, it has been shown that HAMLET interacts with mitochondria in the cytoplasm of tumor cells and triggers membrane depolarization, the release of cytochrome 'c' and activates pro-apoptotic caspases, including caspase 3 and 6. However, HAMLET induced cell death is different from various other classical apoptotic processes, since caspase inhibitors do not rescue cells and therefore the role of caspases remains undefined (Köhler et al., 2002). Recently (2009), it has been explained that HAMLET can induce extensive cytoplasmic vacuolization and forms double-membrane-enclosed vesicles in tumor cells. These findings suggested that HAMLET may cause macro-autophagy in tumor cells, which may become the reason for HAMLET-induced tumor cell death (Aits et al., 2009).

In the next Chapters, a detailed study of the effect of temperature and ethanol concentrations on bovine apo α-LA will be systematically undertaken in support of future studies aimed at the elucidation of the mechanism of BAMLET formation in the presence of varying concentrations of oleic acid.

CHAPTER 3: Study of the combined effect of ethanol and temperature on the secondary structure of bovine apo α-lactalbumin by variable temperature-FTIR spectroscopy & Fourier self deconvolution

3.1. Introduction

α-LA is a small (14.2kDa) & acidic (pI4–5) globular whey protein. α-LA is the main protein of human whey and the second most abundant protein of bovine whey after β-lactoglobulin. It is composed of 123 amino acid residues (Brew, 2003). α-LA is present in the milk of all mammalian species in relatively high concentration, around 1.1–1.5 g/L except in that of the seal and sea lion, because they produce milk with very low concentrations of *α*-LA and lactose (P´erez et al., 1990; L¨onnerdal and Lien, 2003).

The main biological function of *α*-LA is its functionality in lactose biosynthesis. It works as a regulatory component for lactose synthase complex which helps in catalyzes of lactose biosynthesis during lactation in the mammary gland. *α*-LA plays the main function to enhance the binding of glucose to galactosyltransferase through a 1:1 protein interaction which reduces the Km for glucose by about 1000-fold, so that lactose synthesis could be catalyzed effectively at the physiological concentrations of glucose present in mammary epithelial cells (Brew, 2003).

The structure of α-LA is stabilized by four disulfide bonds and about 10% of bovine α-LA is glycosylated (Brew et al., 1992). The secondary structure of α-LA contains four α-helices, several regions of 3_{10}-helices and an antiparallel β-sheet separated by irregular β-turns (Brew et al., 1992). Circular dichroism studies have shown that α-LA is 26% α-helix, 14% β-structure and 60% unordered structure (Bottomley et al., 1990). α-LA has a strong binding site called “elbow”, which is particularly binding site for calcium formed from a helix-turn-helix motif and separated by an irregular β-turn. Calcium binds to five aspartic acid residues, which are present at this site (Brew et al., 1992). The binding of calcium to the high-affinity Ca^{2+} binding site in *α*-LA contribute greatly for its structure stability. The calcium depleted conformer of α-LA is called as apo α-LA.

Most stable conformation of α-LA lies between pH 5.4 and 9.0. Below pH 4.0 and above pH 9.0 conformational changes occurs (Bottomley et al., 1990). Metal binding properties are affected by these conformational changes but secondary structure mostly been unaffected (Brew et al., 1992). Heating of the protein between 35-40°C may cause changes in its tertiary structure but secondary structure remains unchanged. There occurs a breakdown of the 3_{10}-helix and increase in turns (Boye et al., 1996) at the temperature above 65°C along with physiological pH. This action is reversible and upon cooling 80-90% of the protein may return to its native form. This factor proves that α-LA is the most heat stable whey protein (Singh et al., 1992). However, above pH 9 and below pH 6 partial irreversible aggregations does occur but α-LA may be stabilized by increasing the salt concentration (Boye et al., 1997).

By the combination of spectral and proteolysis techniques, the group of Wehbi (Z.Wehbi et al., 2006) studied the conformational properties of partly unfolded forms of α-LA induced by ethanol. By using different concentration of ethanol they analysed the structural changes and proteolytic susceptibilities of holo and apo α-LA, the latter with and without oleic acid at pH 8.0. Fluorescence spectroscopy showed that with the increase in concentration of ethanol the tryptophanyl residues become more accessible to the solvent. The tertiary structure of holo α-LA was maintained in 20% ethanol whereas addition of 30 and 40% of ethanol altered its tertiary structure as shown by near circular dichroism spectra. On the other side for apo α-LA, spectra were identical for all percentages of treated ethanol (Wehbi, Z. et al., 2006).

In the current study, the combined effect of temperature and ethanol on the secondary structure of bovine apo α-LA is determined by employing the variable temperature-Fourier transform infrared spectroscopy (VT-FTIR). The Amide I' spectral region (1600-1700 cm^{-1}) was examined in order to ascertain the changes in the secondary structure of protein. Infrared spectroscopy constitutes one of the oldest methods for studying the secondary structure of polypeptides and proteins. Since H_2O absorbs strongly in the most important spectral region around 1640 cm^{-1}, studies in aqueous solution are difficult unless deuterium oxide is used as a solvent. Since FTIR spectra are composed of many overlapping bands that have inherently large half-bandwidths, resolution enhancement methods such as curve fitting, second derivative analysis, difference spectroscopy and most importantly Fourier self deconvolution (FSD) are usually required to

detect the spectral changes. In this study the VT-FTIR spectra of bovine apo α-LA without and with varying amount of ethanol was enhanced by employing Fourier self deconvolution technique as the means of resolution enhancement.

3.2. Material and methods

Materials: Bovine apo α-LA (calcium depleted) was obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Deuterium oxide was bought from Cambridge Isotope Laboratories, Inc. (Montreal, QC). Ethanol was from Greenfield Ethanol.Inc. All reagents and chemicals used were of analytical grade.

Sample Preparation: Five 10% (w/v) solutions of bovine apo α-LA were prepared in deuterium oxide (solvent). The prepared 10% (w/v) samples (40µl each) were spiked with the different volumes of ethanol as 0, 1, 10, 15 and 20µl and resulted into final samples with the ethanol concentrations of 0, 2.4, 20, 27.3 and 33.3% (w/v) respectively. Samples were stored at 4^0C for 24 hours prior to use.

Fourier Transform Infrared (FTIR) Spectroscopy: Infrared spectra of α-LA solutions (10% W/V) prepared in D_2O in the absence and presence of varying concentrations of ethanol were recorded with an Excalibur FTIR spectrometer (Agilent Technologies) equipped with a deuterated triglycine sulfate detector. A total of 256 scans were coded and averaged at $4cm^{-1}$resolution. Wavenumber accuracy was within $\pm 0.01 cm^{-1}$. The spectrometer was purged with dry air from a Balston dryer (Balston; Haverhill, MA). Single-beam background spectra in the beginning of each experiment were recorded at 25^0C with one CaF_2 window. The samples were held in an IR cell with 25µm Teflon spacer and two CaF_2 windows. The temperature of the samples was regulated by placing the cell in a thermostat holder employing an Omega temperature controller (Omega Engineering, Laval, QC). The temperature was increased& decreased in 5°C increments [while heating (from 25 to 95^oC) & cooling (from 95 to 25^0C) respectively]and the cell was allowed to equilibrate for 10 minutes prior to data acquisition. Fourier self deconvolution of the obtained spectra was performed by using OMNIC 8.2.0.387 software from Thermo Fisher Scientific Inc. Fourier self deconvolution of the infrared spectra was undertaken as described by Kauppinen et al. (1981). The signal-to-noise ratio was >10 000:1

and the bandwidth used for deconvolution was 27 cm^{-1} with a narrowing factor of 2.7 (Prestrelski et al., 1991 a & b).

3.3. Results and Discussion

The FSD of the amide I' band (ranged from 1600 to 1700 cm^{-1}) corresponding to the spectrum of bovine apo α-LA (without ethanol) at 25°C is shown in figure 3.1. It has been previously reported that the structure of apo α-LA has a very high content of helical structure (Acharya et al., 1989) which is a mixture of α-helix (31% of the amino acids) and 3_{10}-helix (about 21% of the amino acids). It is therefore expected that FTIR spectrum of α-LA would show strong bands reflecting the significant contribution from these structures. The β-sheet forms up only about 6% of the structure, and therefore was expected to contribute only a small proportion to the spectrum. The spectrum of bovine apo α-LA in Figure 3.1 showed four main bands of interest at wavenumbers of 1628, 1639, 1652 & 1677cm^{-1}. This observation is consistent with the previous studies (Boye et al., 1997), except the band at 1662cm^{-1} which was not noticed in the given spectrum. The band at 1628cm^{-1} has been assigned to extended antiparallel β-sheet (Yang et al., 1985) and the band at 1639 cm^{-1} to 3_{10}-helices (Holloway &Mantsch, 1989). The band at the wavenumber 1652 cm^{-1} is attributed to α-helical structure (Susi & Byler, 1988). The band at 1677 cm^{-1} has been assigned to β-turns or to the high frequency component of the antiparallel β-sheet (Casal et al., 1988).

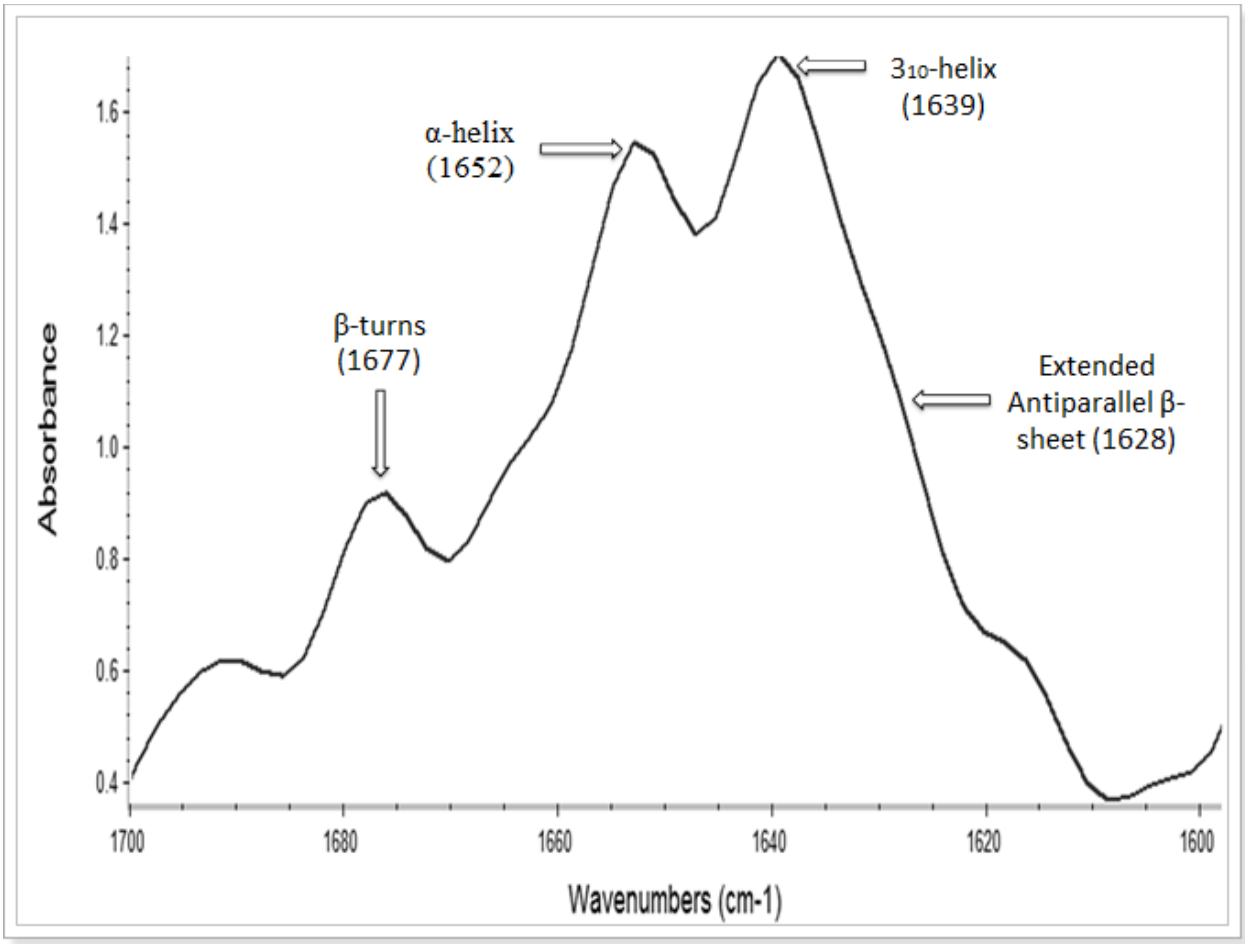


Figure 3.1: Peaks assignment of Fourier self deconvolved (FSD) FTIR spectrum of amide I' band (1600-1700 cm^{-1}) of bovine apo (calcium depleted) α-LA acquired at 25°C.

In this study, amide I' band of the spectra of α-LA in solution with different concentrations of ethanol was analysed to determine the effect of ethanol on the secondary structure of protein. Figure3.2 depicts the FSD spectra of bovine apo α-LA without and with varying concentrations of ethanol as 2.4, 20, 27.3 and 33.3% (w/v) ethanol at 25^0C (before thermal treatment). Interestingly, ethanol (without thermal treatment) has potential to induce the conformational changes in the secondary structure of α-LA. It has been observed that by treating the α-LA with even 2.4% (w/v) ethanol caused a decrease in the intensity of band at 1639 cm^{-1} relative to the band at 1652 cm^{-1} (band assignment is already discussed above). This indicates that loss of 3_{10}-helix content is compensated by the increase in α-helix. Furthermore, the given change was enhanced as the ethanol content increased at 20, 27.3 & 33.3%. At the ethanol concentration of 20% the band at 1639 cm^{-1} completely disappeared. The 1628 cm^{-1} band became more prominent with the increase in ethanol content while the band at 1676 cm^{-1} decreased its intensity in the

favour of a new band at 1684 cm^{-1}, which is attributed to β-structure (Dzwolak et al., 2001). These results are in agreement with the previous measurements performed by Circular dichroism (CD) spectroscopy (Wehbi et al., 2006).

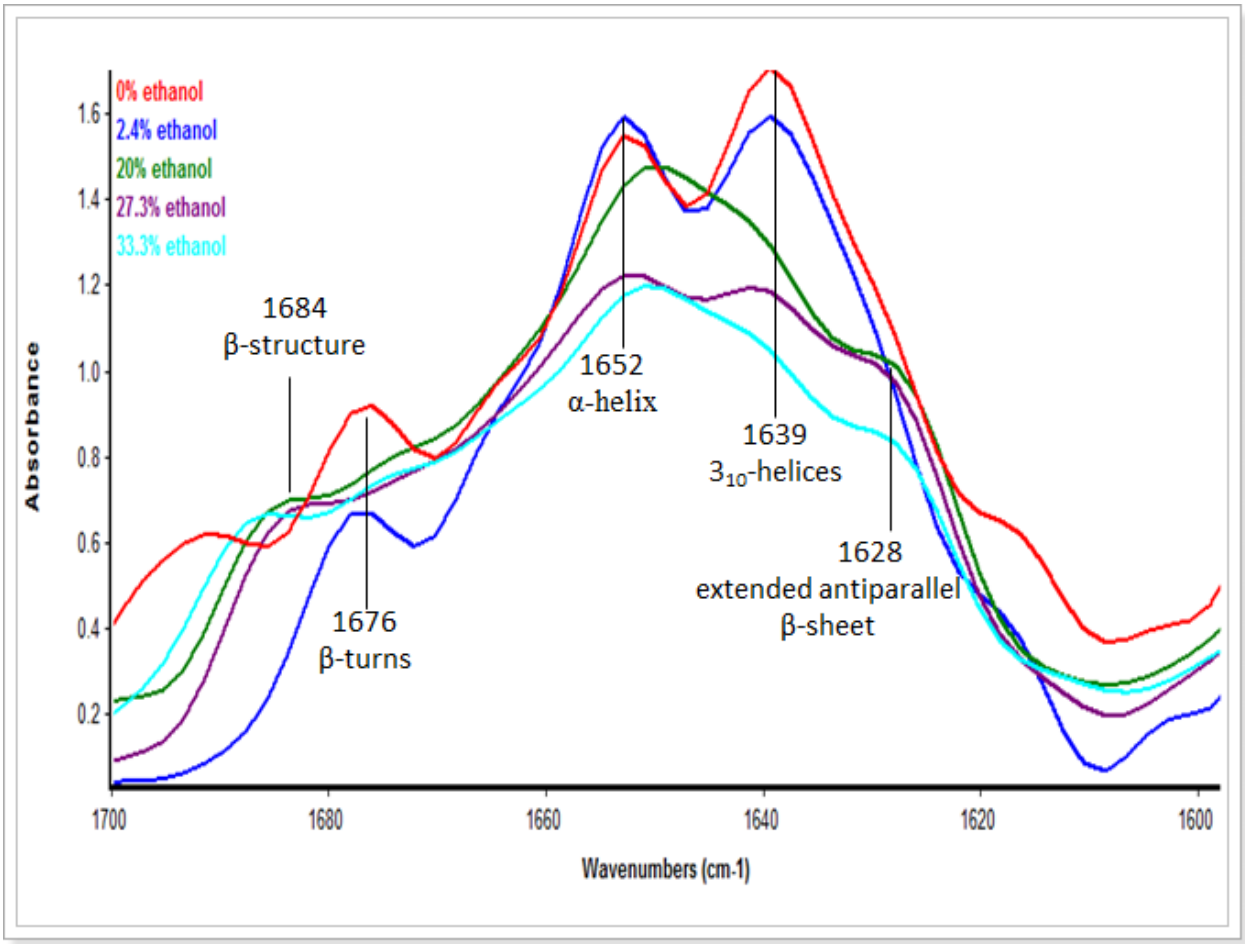


Figure 3.2: Fourier self deconvolved (FSD) spectra of bovine apo α-LA without and with on different concentrations of ethanol at 25^0C before heating

Furthermore, the amide I' band of the deconvolved spectra of bovine apo α-LA in solution at 2.4, 20, 27.3 & 33.3% (w/v) ethanol concentrations, heated to 95°C was analysed (Figure 3.3). In the spectrum of α-LA with 2.4 (w/v) ethanol, the amide I' band was basically a composite of two major peaks situated at 1651 & 1672 cm^{-1}, which were assigned to α-helix and β-turns respectively (Susi & Byler, 1988). The intensity of the band at 1651cm^{-1} decreased as the ethanol concentration increased, which may be attributed to the dilution of protein sample due to the addition of ethanol as the band intensity in FTIR spectrum is directly proportional to the concentration of sample. Moreover, the band at 1672 cm^{-1} decreased in intensity relative to the α-

helix by increasing ethanol concentration and eventually disappeared at the ethanol content of 33.3% as a function of heating.

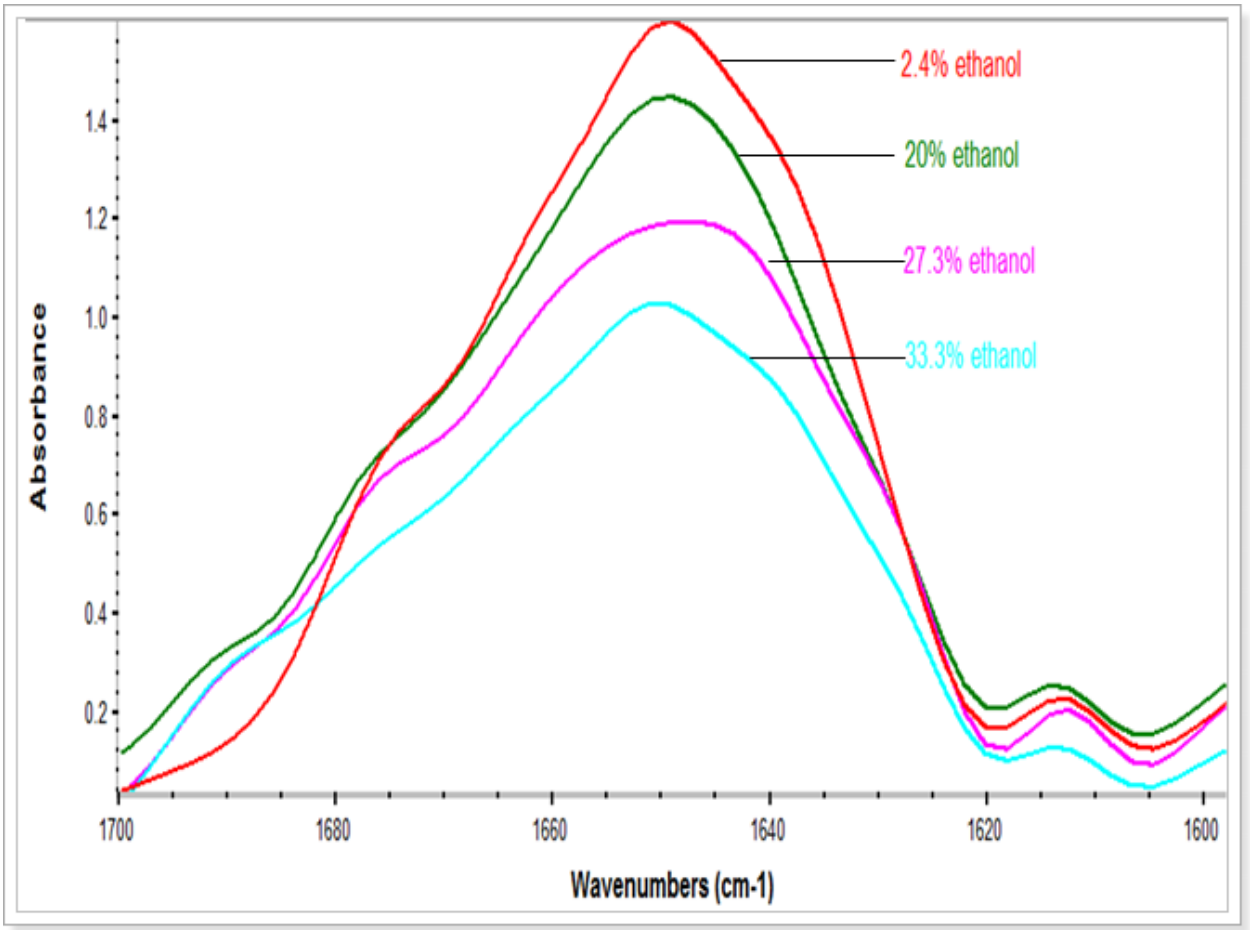


Figure 3.3: Fourier self deconvolved (FSD) spectra of bovine apo α-LA with 2.4, 20, 27.3 & 33.3% (w/v) of ethanol acquired at the end of heating cycle (at 95°C)

The figure 3.4 (A, B & C) represents the extent of mutual reversibility between the unfolding and refolding [during heating (from 25 to 95^0C) & cooling (from 95 to 25^0C respectively] of the secondary structure of bovine apo α-LA induced by the ethanol. It had been reported that the increase in the ethanol concentration results in irreversible changes in the conformation of bovine apo α-LA. But on the other side, it showed an excellent reversibility effect on the thermal denaturation of protein. The spectral pairs illustrated in the figure 3.4 (A, B & C) were acquired prior to the heating cycle (from 25 to 95^0C) (upper trace) and subsequent to the cooling cycle (from 95 to 25^0C) (lower trace). This study revealed that in the absence of ethanol the conformational changes occurred in the bovine apo α-LA due to the temperature increase were

irreversible upon cooling (figure 3.4A). Whereas, upon cooling, the spectra acquired of bovine apo α-LA spiked with even the minimal concentration of ethanol (2.4% w/v) showed a dramatic reversibility of the changes which occurred due to heat treatment from 25 to 95°C (figure 3.4B). It is observed that the band at 1652 cm^{-1} (α-helix) rebounded to its characteristic band shape, which was absent in the spectra of bovine apo α-LA without ethanol.

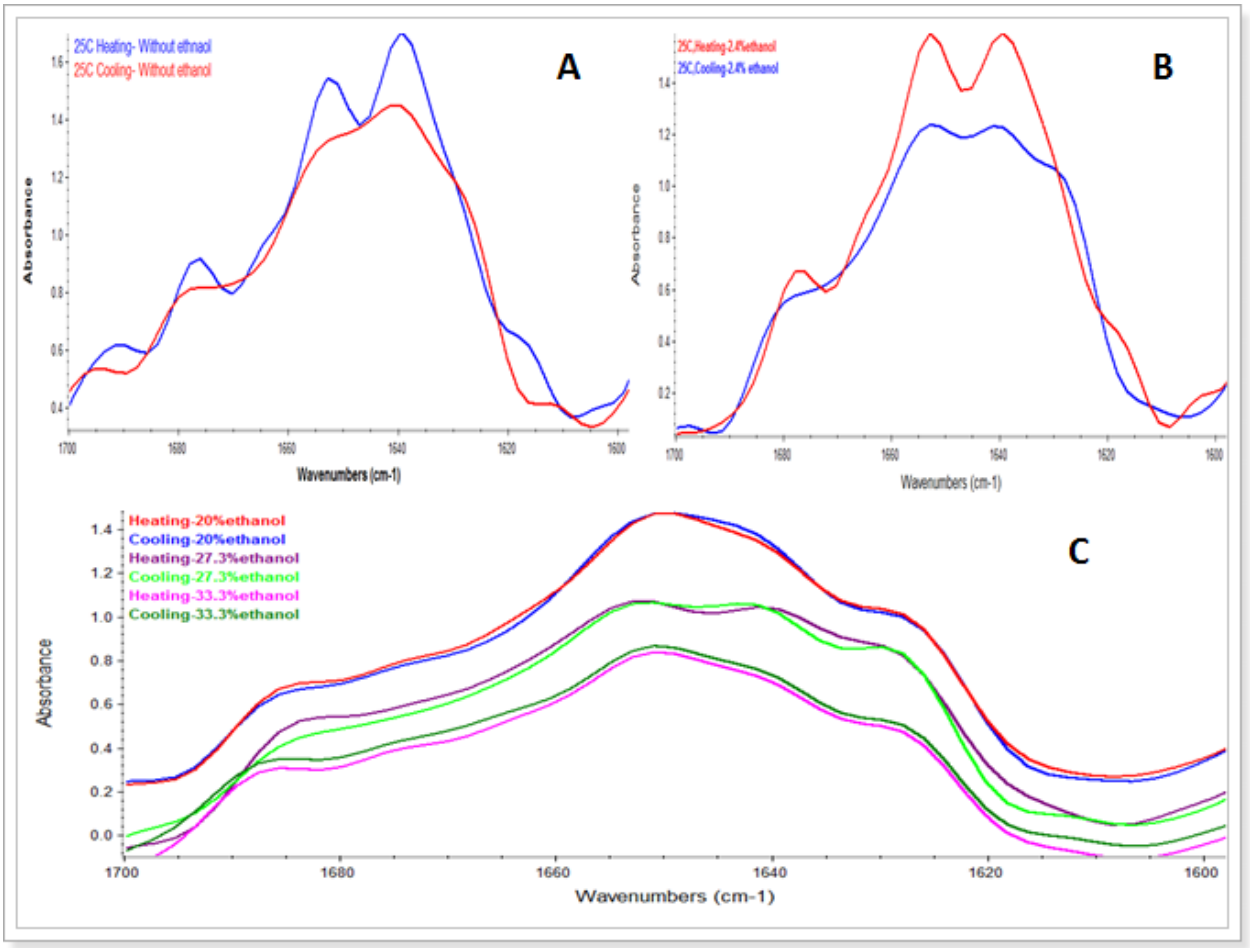


Figure 3.4: Demonstration of the extent of reversibility of the thermal denaturation (upon cooling) of bovine apo α-LA as a function of ethanol concentration

In addition, figure 3.4.C indicates that upon increasing the ethanol concentration as 20, 27.3 & 33.3% (w/v), the thermal denaturation of ethanol modified-protein was completely reversible and upon cooling (25^0C of the Cooling phase) it achieved the same conformation which it showed in the beginning of the heating cycle (25^0C of the heating phase). This enhancement in the reversibility of the thermal denaturation of bovine apo α-LA due to ethanol [20, 27.3 & 33.3%

(w/v)] can be estimated by superimposing the spectra obtained in the beginning of heating phase and at the end of the cooling phase (figure 3.4.c) as they overlapped completely.

The mutual reversibility between the heating & cooling cycles of bovine apo α-LA induced by ethanol and then enhanced by increasing the concentration of ethanol is supported by the normalised graphs plotted between the height of different bands found in the spectra of bovine apo α-LA in conjunction with 2.4, 20, 27.3 & 33.3% (w/v) ethanol and heating/cooling temperature. Figure 3.5 depicts the changes occurred in the height of different bands observed in the amide I' band of bovine apo α-LA solution with 2.4% (w/v) ethanol. The plot revealed that the bands located at wavenumbers 1670 and 1647 cm^{-1}, which were are assigned to the turns (Boye et al., 1997) and random coil (Byler et al., 1986) respectively gradually increased in height with increasing temperature. On the other hand, the band at 1625 cm^{-1}, attributed to extended antiparallel β-sheet (Yang et al., 1985) showed the opposite trend and decreased in height by increasing the temperature of α-LA-ethanol sample. Interestingly, during the cooling cycle both three bands followed a reversible path to the heating cycle.

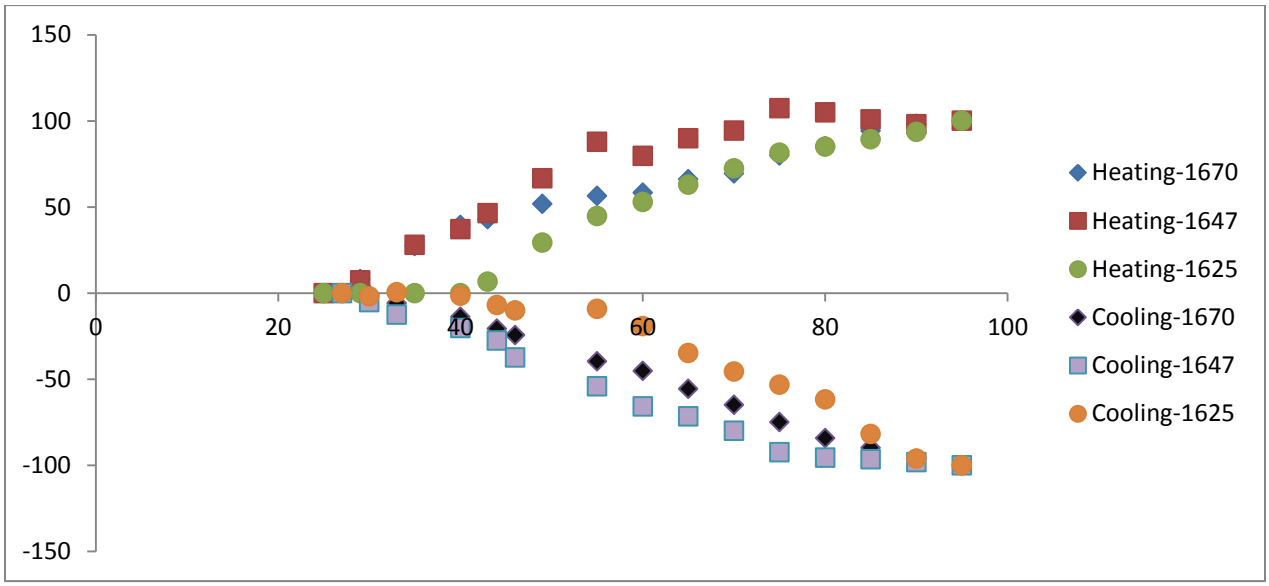


Figure 3.5: Plot demonstrating the changes in heights of different bands in amide I' band of bovine apo α-LA in solution with 2.4% (w/v) ethanol as a function of temperature

In addition, the data obtained from bovine apo α-LA treated with 20% (w/v) of ethanol was further examined (Figure 3.6). The amide I' band of bovine apo α-LA in solution with 20% (w/v)

ethanol indicated the three main bands of interest as 1685, 1662 & 1625 cm^{-1}. The plot in figure 3.6 reveals that the height of bands at wavenumbers 1685 and 1662 cm^{-1}, corresponding to β structure (Clark et al., 1981) and turns (Susi & Byler, 1985) respectively, increased as a result of increasing temperature (from 25 to 95^{0}C) of protein-ethanol (20%) solution, while the remaining of the amide I' band (specifically band at 1625cm^{-1}) decreased its intensity with increase in temperature. The band at 1685cm^{-1} attained its maximum change around the temperature of 65^{0}C, one at 1625cm^{-1} around 90^{0}C and band at 1662cm^{-1} showed maximum change around the final temperature of the heating cycle (95^{0}C). Furthermore, during heating cycle, the bands at 1685 and 1625 cm^{-1} showed a greater rate of change as compared to band at 1662cm^{-1}. The bands at 1685 and 1625 cm^{-1} showed little change above 75^{0}C. According to the plots in figure 3.6, the cooling cycle depicts the reverse trend to heating cycle, which is in congruence with the data plotted for bovine apo α-LA with 2.4% (w/v) ethanol (figure 3.5).

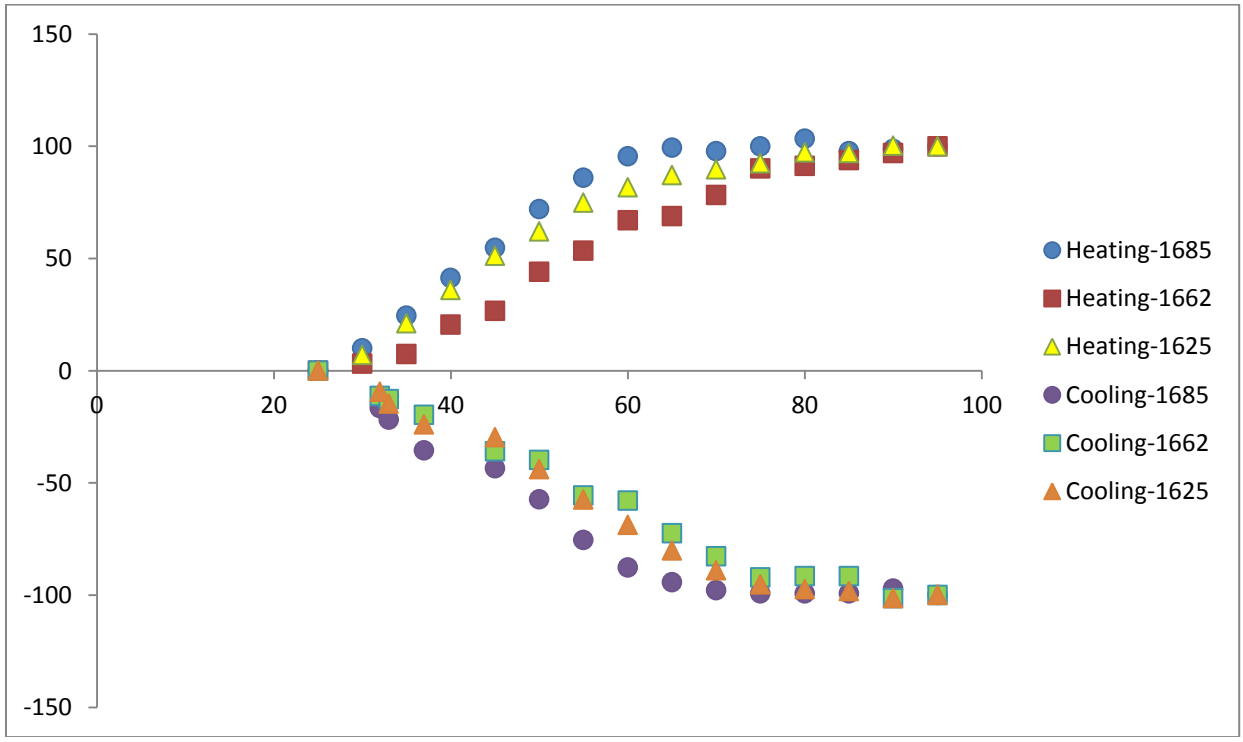


Figure 3.6: Plot demonstrating the changes in heights of different bands in amide I' band of bovine apo α-LA in solution with 20% (w/v) ethanol as a function of temperature

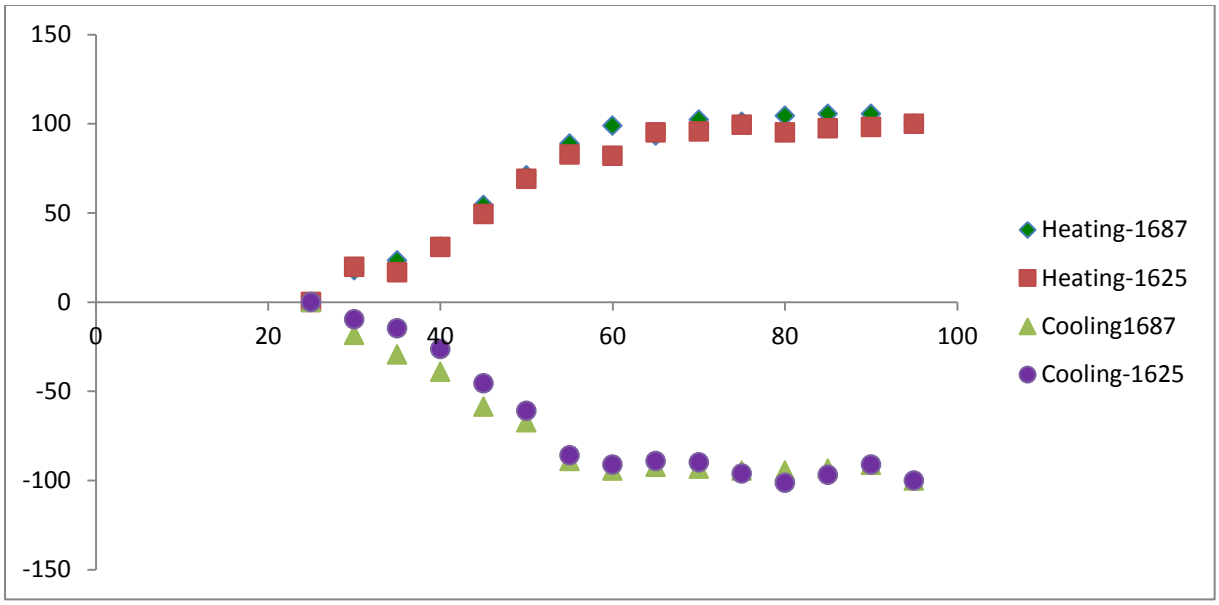


Figure 3.7: Plot demonstrating the changes in heights of different bands in amide I' band of bovine apo α-LA in solution with 33.3% (w/v) ethanol as a function of temperature

Furthermore, bovine apo α-LA treated with 27% (w/v) ethanol (data not shown) demonstrated the four main bands of interest located at the wavenumber positions of 1685, 1662, 1647 & 1625cm^{-1}(band assignment has already been discussed). It is discerned that all the changes in the amide I' band as a function of heating were reversible upon cooling. Similarly, the spectra (amide I' band) of bovine apo α-LA heated from 25 to 95^{0}C in a solution containing 33.3% (w/v) ethanol exhibited two major bands of concern around 1687 and 1625cm^{-1}. Both the stated bands expressed a transition in both heating and cooling cycles around the temperature of 50^{0}C. Apparently, the changes occurred in both the bands showed reversible trends between heating and cooling cycles, which is in consensus with the data obtained from other samples of bovine apo α-LA in solution with different concentrations of ethanol.

3.4. Conclusion:

In current study, the combined effect of temperature and ethanol on the secondary structure of bovine apo α-LA was determined by analysing the amide I' region of Fourier self deconvolved spectra of protein in the absence and presence of varying concentration of ethanol. It is observed that the ethanol independent of temperature can alter the secondary structure of α-LA. The 3_{-10} helix and β-turns were found to be the most susceptible to the ethanol. Interestingly, after heating the protein in solution containing different concentrations of ethanol from 25 to 95 °C, the α-

helical and antiparallel β-sheet contents also observed to decrease as a function of increasing ethanol concentration. More importantly, the ethanol-modified secondary structure of bovine apo α-LA showed a greater reversible effect between the heating and cooling cycles as a function of ethanol content, which was actually absent in the case of α-LA without ethanol. The changes in the different components (represented by different bands found in amide I' band) of the secondary structure of bovine apo α-LA in solution with different concentrations of ethanol were also monitored by plotting the peak heights of amide I' bands as a function of increasing and decreasing temperatures during the heating and cooling cycles respectively. Finally, it is believed that this work will facilitate further studies to determine the role of ethanol as a solvent in the formation of protein-lipid complexes.

Chapter 4: 2D Correlation analysis of the changes in the secondary structure of bovine apo α-lactalbumin subjected to thermal treatment in the presence and absence of ethanol

4.1. Introduction

In recent years, the development of generalized two-dimensional (2D) correlation spectroscopy as a technique for studying the effects of external perturbation (chemical, pressure or temperature etc) on the proteins and peptides has gained interest. In 2D correlation analysis correlation peaks appearing in the synchronous and asynchronous contour maps respectively represent in-phase and out-of-phase variation tendencies of corresponding band intensities.

The 2D correlation analysis provides the means of elucidating the steps of unfolding and refolding of the secondary structure of bovine apo α-LA. In order to simplify the outcomes of 2D correlation analysis, we transformed the results into tabular form. These tables depicts the sequence of events which occur during the unfolding and refolding of the secondary structure of bovine apo α-LA as suggested by the set of rules proposed by Noda (Noda, I., 1993) and described by Ismoyo (Ismoyo, et al., 2000), Filosa (Filosa et al.,1999) and Gomma (2012).In present work, 2D correlation analysis of variable-temperature FTIR spectra of bovine apo α-LA with and without ethanol presented in the previous chapter will be undertaken.

4.2. Methodology

Second Derivative Difference Spectra: Second derivative spectra of the FTIR spectra acquired in previous chapter were calculated using OMNIC6.0 from Thermo Nicolet Corporation. All second derivative spectra were multiplied by a factor of negative 1000. In both heating and cooling cycles all the spectra (at different temperature) were subtracted from the initial spectrum. For example, in the heating cycle, spectrum at 25^0C was subtracted from all the spectra starting from 30^0C and onwards. Similarly in cooling cycle, the spectrum acquired at 95^0C was subtracted from all the spectra starting from 90^0C and onwards. The subtraction factor in both

thermal cycles was selected as 1. Finally all the spectra were smoothed with a smooth point of seven (13.5 cm^{-1}).

2D IR Correlation Analysis: Generalized 2D IR Correlation analysis of the amide I' region (1600-1700 cm^{-1}) was carried out as described previously (Filosa et al., 1999) using KG2D software written by Wang et al (1998). A 6% correlation intensity cut off was used to generate 2D synchronous and asynchronous contour maps. The KG2D software is able to process any set of spectra (e.g., Raman scattering, infrared. or ultraviolet/visible absorption) that can be imported by GRAMS/32 Spectral Notebase software (Thermo Galactic). Throughout this work, solid and dashed lines in the 2D plots represented positive and negative correlation peaks respectively. According to the rules proposed by Noda (Noda, 1993), the sign of a cross peak (positive or negative) in the asynchronous maps in combination with the sign of the cross peak in the synchronous maps indicated the sequential relationship between two bands. The information reflected by the 2D IR correlation maps is then summarised in the form of tables. These tables facilitate the determination of sequence of events those occur during the unfolding or refolding of the secondary structure of protein due to combined effect of temperature with or without ethanol. It should be noted that the use of 2^{nd} derivative spectra in place of FSD spectra of the protein resulted in superior quality 2D maps in this study.

4.3. Results and Discussion

One-dimensional spectral analysis of bovine apo α-LA (chapter 3) provided a measure of the thermal stability of protein. In this Chapter, the unfolding and refolding pathways of bovine apo α-LA are elucidated by two dimensional correlation analyses of the variable temperature spectra of protein in the absence & presence of varying concentration of ethanol as a denaturant.

Unfolding pathway of bovine apo α-LA during the heating cycle

Figure 4.1a depicts the overlaid second derivative difference spectra (multiplied by -1000 to inverse the scale) of the amide I' region of bovine apo α-LA (without ethanol) as a function of increasing temperature from 25 to 95°C.

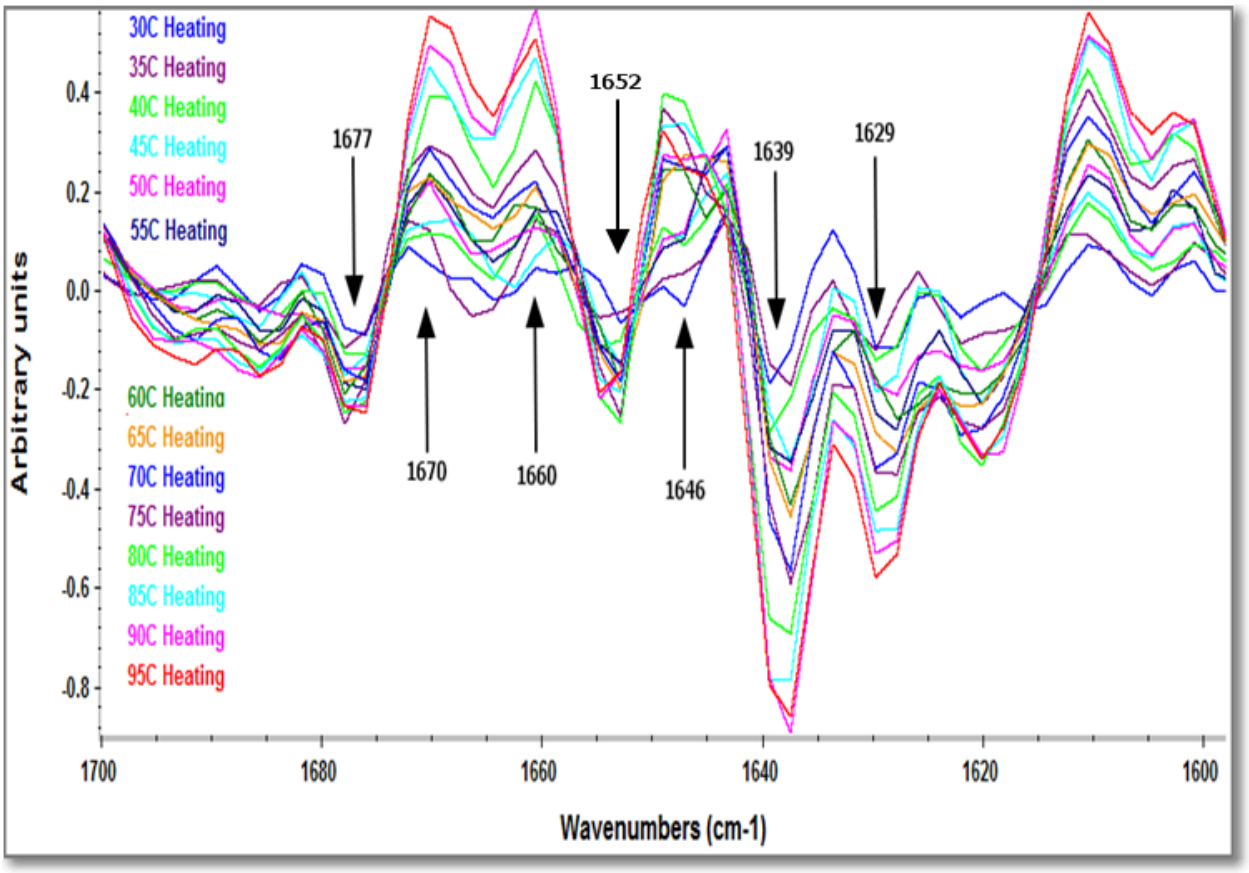


Figure 4.1a: Overlaid second derivative difference FTIR spectra of amide I' region of the bovine apo α-LA as a function of increasing temperature from 30 to 95^{0}C

The intensity of seven major bands was observed to be changed as a function of increasing temperature. The assignment of these bands is as follows: 1670 cm^{-1} was attributed to turns (Boye et. al. 1997); 1629 cm^{-1} was assigned to extended antiparallel β-sheet (Susi et al., 1985); 1652 cm^{-1} was attributed to α-helical structure (Susi & Byler, 1988); 1639 cm^{-1} to 3_{10}-helices (Holloway &Mantsch, 1989); the bands at 1646 & 1660 cm^{-1} were related to random coil and turns (Susi & Byler, 1986 & 1988) respectively; band at 1677 cm^{-1} to β-turns or was assigned to the high frequency component of the antiparallel β-sheet (Casal et al., 1988) and an additional band around 1682 cm^{-1} was attributed to the β-turns by shan-Yang (Shan-Yang lin et al., 2004).

Figure 4.1a demonstrated that increase in temperature from 30 to 95^{0}C caused an increase in the turns and unordered (random) structure at the expense of α-helix, 3_{10}-helixes and β-sheets. In order to reveal the unfolding pathway and to discern the subtle changes that occurred during the

heating cycle, we performed 2D correlation analysis (Ismoyo, et al., 2000) of amide I' band of the bovine apo α-LA, heated over the temperature range of 25-95^{0}C.

Figures 4.1b and c depict the 2D synchronous and asynchronous contour maps respectively of bovine apo α-LA heated from 25 to 95^{o}C. The synchronous map showed seven clear autopeaks at 1629, 1639, 1646, 1652, 1660, 1670 and 1677 cm^{-1} (peak assignment already has been discussed above). In the synchronous contour map, the positive correlation peaks denoted by solid lines represent the intensity variations in the same direction of the corresponding peaks. Whereas the negative correlation peaks depicted by the dashed lines shows the variation of intensity in the opposite direction. For example, the cross correlation peak between the bands at 1629 and 1670 cm^{-1}is negative because the intensity at 1629 cm^{-1} decreased but intensity at 1670cm^{-1} increased due to temperature increase (the direction of change is established by examination of the peaks in difference spectra in figure 4.1a). Similarly the cross correlation peaks corresponding to bands at 1629 & 1646 cm^{-1}; 1629 & 1660 cm^{-1}; 1639 & 1660; 1639 & 1670; 1646 & 1677 cm^{-1}; 1646 & 1652 cm^{-1}; 1652 & 1670 cm^{-1}; 1652 & 1660 cm^{-1}; 1660 & 1677 cm^{-1} and between 1670 & 1677 cm^{-1} are also negative. The cross correlation peak between 1629 & 1677 cm^{-1} is positive because both bands decreased their intensity with the increase in temperature. Similarly, the cross-correlation peaks corresponding to the cross peaks at 1629 & 1652, 1629 & 1639; 1639 & 1677; 1639 & 1652 cm^{-1}; 1646 & 1670; 1646 & 1660; 1652 & 1677 cm^{-1} and 1660 & 1670 cm^{-1} are positive because all these band pairs increased or decreased their intensity together with increasing temperature of the protein solution.

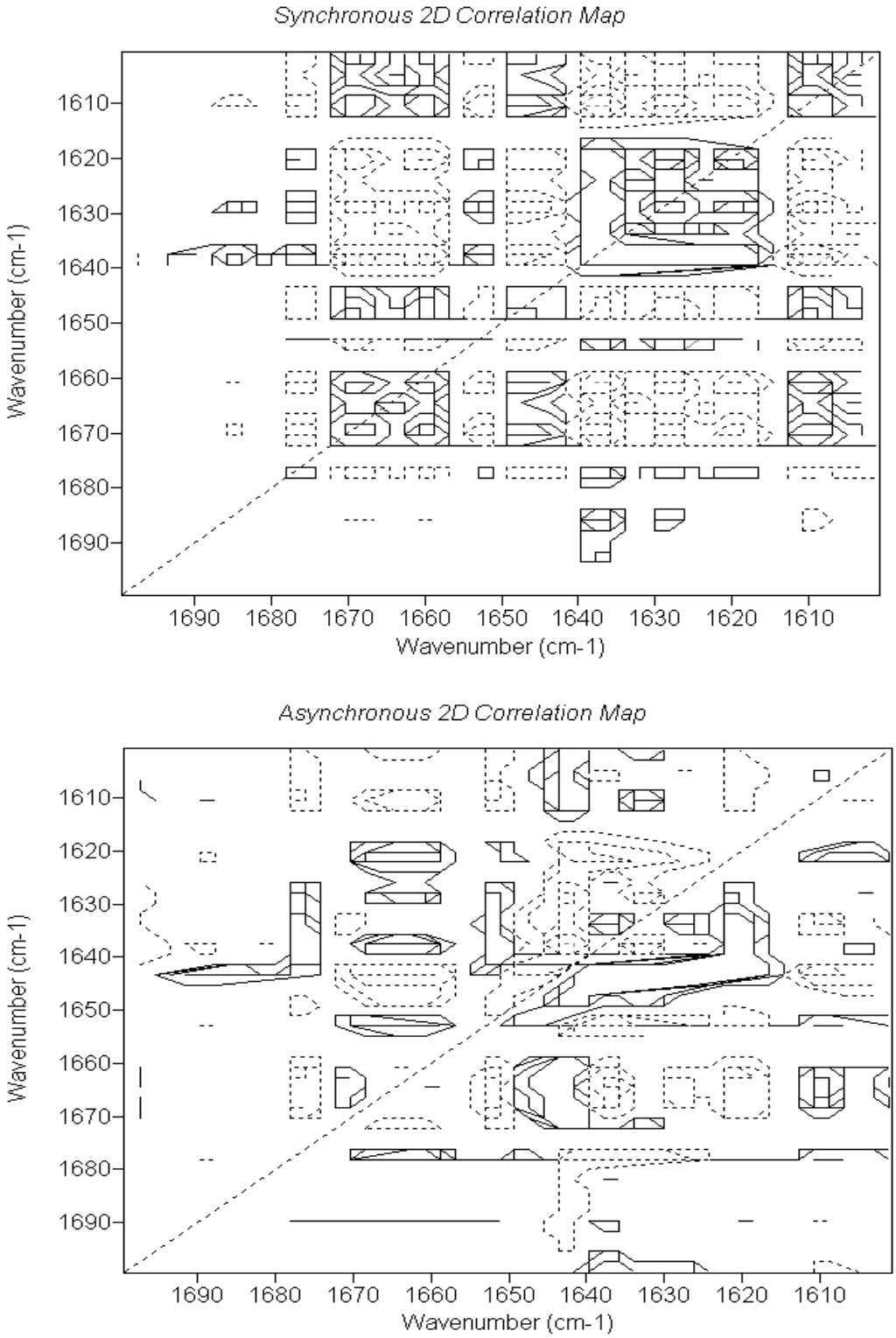


Figure 4.1 b & c: 2D Synchronous (b-top) and asynchronous (c-bottom) contour maps of bovine apo α-LA generated from second derivative difference spectra in figure 4.1a. The solid and dashed lines represent positive and negative peaks respectively.

The asynchronous contour map (figure 4.1c) plays a vital role in understanding the sequence of events. It is asymmetrical with respect to the diagonal line. The peaks in the asynchronous contour map represented by dashed lines are taken as negative and those represented by solid lines are taken as positive peaks. The results obtained from the difference spectra (figure 4.1a) and the 2D maps (figure 4.1a & b) can be represented in tabular form to facilitate interpretation (Filosa et al., 1999). Table 4.1 contains all the main bands of interest with arrows, which indicates the increase or decrease in intensity derived from the overlaid second derivative difference FTIR spectra (figure 4.1a). The procedure to determine the sequence of events for the 2D maps has been described previously by Noda (1993) and Filosa (Filosa et al., 1999). Briefly the first sign in any row or column in the table is taken from the synchronous map and second sign is from the asynchronous map. When the two signs in any row or column are identical (both are positive or negative together), it indicates that changes in the peak placed on the X axis taken place before the changes on the peak at Y axis. On the other hand, when both signs in any row or column are incongruent to each other (One is negative and other is positive), indicates that the changes in the peak situated on Y axis occurred before they occurred in the peak located at X axis. The arrow heading towards right in any row or column shows that peaks on the Y axis changed before the one at X axis. On the other side, an arrow directed towards left represents that the peak on the X axis changed before the one at Y axis. The double-sided arrow indicates that the changes at both bands (wavenumbers) are occurring at the same time.

By considering the directions of these arrows, one can deduce the sequence of unfolding (Filosa, 1999, Gomaa, 2012). The bottom part of the table summarizes the entire sequence of unfolding events obtained from the upper part of the table. For example, the cross correlation peak in the asynchronous map (figure 4.1c) correspond to bands at 1629 and 1677 cm^{-1} is positive, and since the corresponding peak in the synchronous map is also positive, the band at 1677 cm^{-1} changes its intensity before the band at 1629 cm^{-1}. Similarly the peak between the bands 1646 and1677 cm^{-1} is negative in both synchronous as well as asynchronous so the changes in band 1646 cm^{-1} are followed by the changes in the band 1677 cm^{-1}. In same manner, the band at 1646 cm^{-1} changes its intensity before the band at 1629 cm^{-1}. On the other hand, the peak between the bands 1646 and 1670 cm^{-1} is positive in synchronous but negative in asynchronous, which reveals that the changes occurred at the band 1646 cm^{-1} prior to the band 1670 cm^{-1} as in the case

of 1646 vs 1660cm^{-1}. Based on this information the sequence of the unfolding events of bovine apo α-LA during heating is as follows: in the beginning the, α-helix lost and at the same time β-sheet unfolded, which was followed by the formation of random coils. The next event occurred with loss of antiparallel β-sheet, followed by the loss of 3_{10}-helix. Finally, the unfolding of bovine apo α-LA due to heating was accomplished by the formation of turns, indicated by increased intensity of the band at 1670 cm^{-1} followed by the increased intensity of band at 1660 cm^{-1}.

cm^{-1}	1677↓	1670↑	1660↑	1652↓	1646↑	1639↓
1629↓	+, + ⇦	-, + ⇨	-, + ⇨	+, + ⇦	-, - ⇦	+, - ⇨
1639↓	+, + ⇦	-, + ⇨	-, + ⇨	+, + ⇦	-, - ⇦	
1646↑	-, - ⇦	+, - ⇨	+, - ⇨	-, - ⇦		
1652↓	+, 0 ⇔	-, + ⇨	-, + ⇨			
1660↑	-, - ⇦	+, + ⇦				
1670↑	-, - ⇦					
Sequence of events						
⇩	Loss of α-helix (1652 cm^{-1}) and Unfolding of β-sheet (1677 cm^{-1}) Formation of random coil (1646 cm^{-1}) Loss of anti-parallel β-sheet (1629 cm^{-1}) Loss of 3_{10}-helix (1639 cm^{-1}) Turns formation (1670 cm^{-1}) Completion of turns formation (1660 cm^{-1})					

Table 4.1: Sequence of the unfolding events of bovine apo α-LA during the heat treatment from 25 to 95^{0}C

Refolding pathway of bovine apo α-LA during the cooling cycle

The sequence of refolding of bovine apo α-LA during cooling cycle (95-25^{o}C) was compared to the sequence of unfolding during heating cycle to ascertain if the pathways of unfolding & refolding were different. Figure 4.2a depicts the overlaid second derivative difference spectra (multiplied by -1000 to reverse the scale orientation) of bovine apo α-LA acquired during the cooling cycle from 90^{0}C to 25^{0}C. Since Figure 4.2a is a set of difference spectra, the first

spectrum acquired at the initial temperature of cooling cycle (95^0C) is subtracted from all the other spectra taken at lower temperatures during cooling cycle.

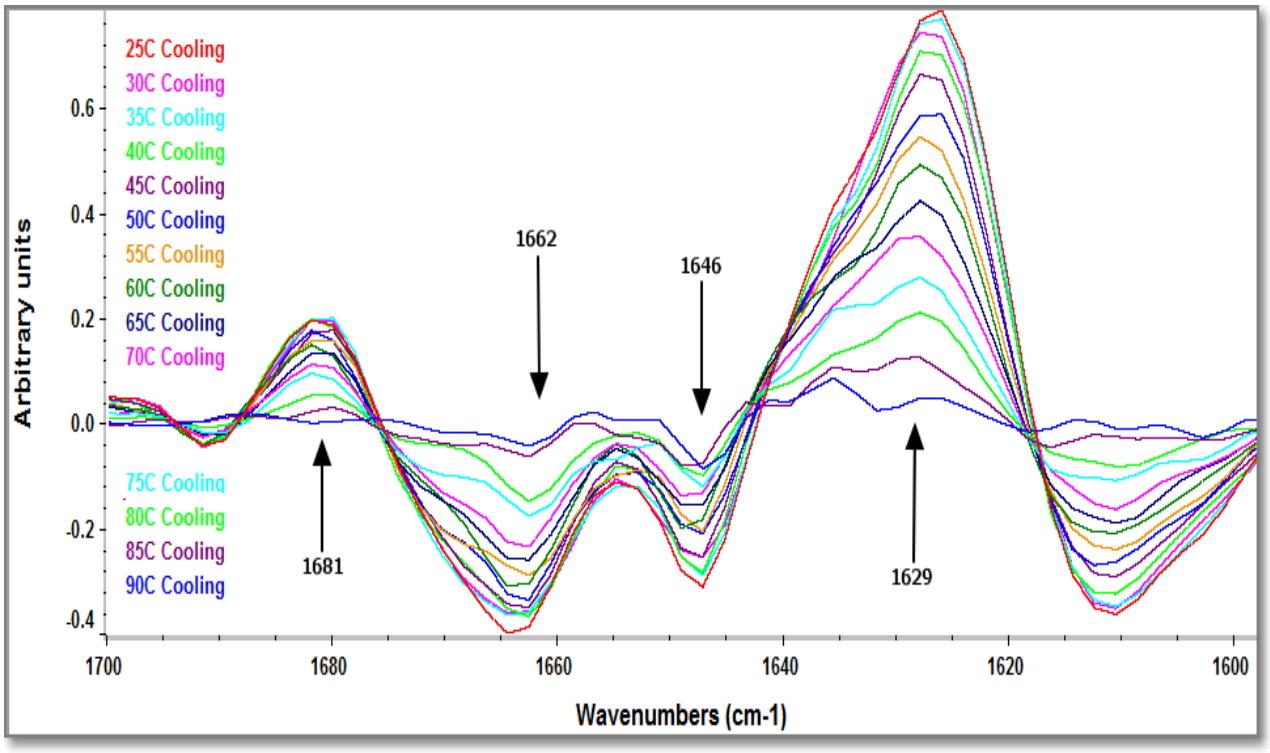
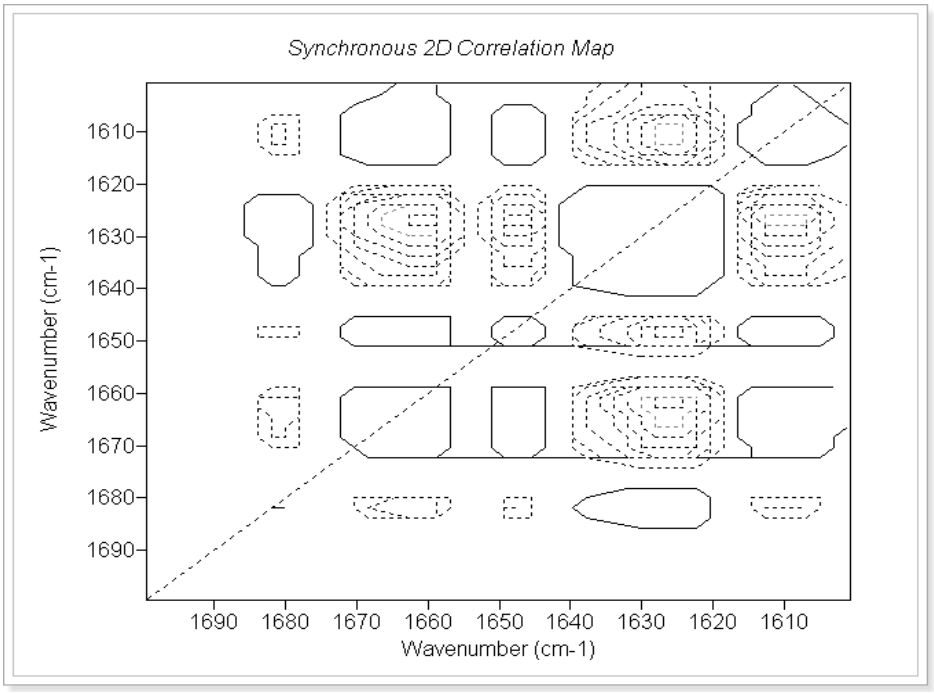


Figure 4.2a: Overlaid second derivative difference FTIR spectra of amide I' region of the bovine apo α-LA as a function of decreasing temperature from 90 to 25^0C

Figure 4.2a, show the overlaid second derivative difference spectra in the amide I' absorption region of the cooling cycle. The band at 1662 cm^{-1} decreased during the cooling cycle. Since the increase in both bands 1660 & 1670 cm^{-1} during heating cycle represented the formation of turns, so the decrease in band 1662 cm^{-1} during cooling cycle indicates the loss of those structures. The rise of the band at 1681 cm^{-1} represent the increase in β-structure which was decreased during the heating cycle, indicated by the decreased intensity of 1677 cm^{-1} band in heating phase. The random coils (1646 cm^{-1}) also lost upon cooling which were formed as one of the consequences of thermal denaturation of protein during heating. According to the figure 4.2a, cooling of protein also resulted in the increase of its β-sheet (1629 cm^{-1}).



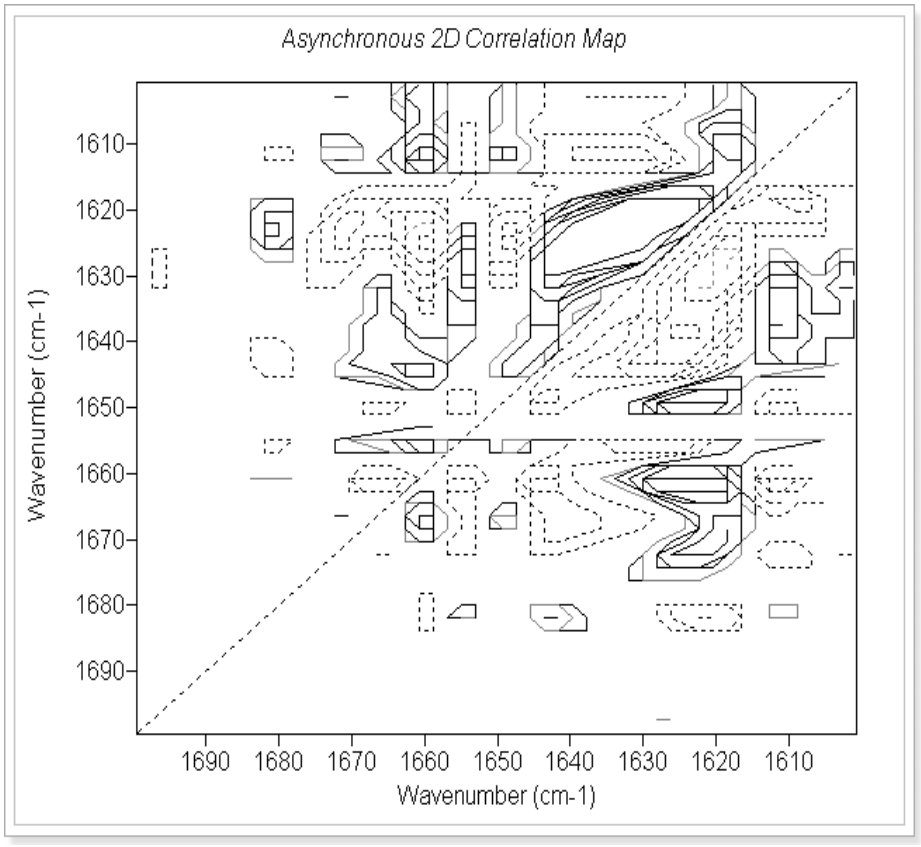


Figure 4.2 b & c: 2D synchronous (b-top) and asynchronous (c-bottom) contour maps of bovine apo α-LA in cooling cycle generated from second derivative difference spectra in figure 4.2a. Solid and dashed lines represent positive and negative peaks respectively.

Figures 4.2b & c represents the synchronous and asynchronous 2D correlation maps of bovine apo α-LA generated from the FTIR spectra shown in figure 4.2a of the cooling cycle. The synchronous contour map (figure 4.2b) showed the same four main peaks of concern which were indicated by arrows in the overlaid second derivative spectra in figure 4.2a. Table 4.2 summarizes the sequence of events of the protein when it refolded during the cooling cycle. The sequence of refolding events of bovine apo α-LA during cooling was different from the unfolding pathway observed during the heating cycle. In other words, the protein did not follow the same route during the cooling phase. In cooling cycle, the refolding pathway commenced by simultaneous refolding of the unfolded β-structure & loss of formed turns, which is signalled by increase & decrease in the height of bands at 1681 & 1662 cm^{-1} respectively. These events were followed by the loss of random coils, as shown by the decrease in the intensity of band at 1646 cm^{-1}. The refolding of the secondary structure of protein was accomplished by the formation (and increase) of β-sheet, demonstrated by the increase in the height of band at 1629 cm^{-1}. These sequences of unfolding and refolding of bovine apo α-LA reveal that the β-sheet is more stable to temperature compared to the helical conformations.

cm^{-1}	1681 ↑	1662 ↓	1646 ↓
1629 ↑	+, + ⇦	-, - ⇦	-, - ⇦
1646 ↓	-, - ⇦	+, + ⇦	
1662 ↓	-, 0 ⇔		
Sequence of events			
⇩	Refolding of β-sheet (1681 cm^{-1}) & loss of turns (1662 cm^{-1}) Loss of random coil (1646 cm^{-1}) Reformation of anti-parallel β-sheet (1629 cm^{-1})		

Table 4.2: Refolding sequence of bovine apo α-LA during cooling cycle from 95 to 25^0C

Unfolding & refolding pathways of bovine apo α-LA solution with 2.4 % (w/v) ethanol

The unfolding and refolding pathway of bovine apo α-LA treated with 2.4 % (w/v) of ethanol was examined by 2D correlation analysis. The overlaid second derivative difference spectra were

generated from the variable temperature FTIR spectra of bovine apo α-LA spiked with 2.4 % ethanol obtained during the heating & cooling cycles.

The sequences of events of unfolding and refolding of the secondary structure during the heating and cooling treatments of bovine apo α-LA solution containing 2.4% ethanol are summarized in the tables 4.3 & 4.4 respectively. In the heating cycle, the 2D synchronous map of protein-ethanol solution showed five main autopeaks at 1683, 1670, 1660, 1647 & 1627 cm^{-1}. We observed that the peaks associated to α-helix (1652) and 3_{10}-helix (1639) were absent in the second derivative difference spectra and 2D maps of apo α-LA with 2.4% (w/v) ethanol. It indicates that due to the action of even minimal concentration of ethanol (2.4%), the secondary structure of the protein was disorganised especially the helical content, which eventually resulted in the formation of turns (1660 & 1670 cm^{-1}) and random coils (1647 cm^{-1}). According to the table 4.3, the first change effected the conformation of protein (in solution with 2.4% ethanol) during heating cycle was the unfolding of β-sheet, which was followed by the formation of random coils. Next event in the unfolding of protein`s secondary structure was formation of turns, followed by the loss of extended antiparallel β-sheet as the final step in the unfolding pathway.

cm^{-1}	1683↓	1670↑	1660↑	1647↑
1627↓	+, + ⇦	-, - ⇦	-, - ⇦	-, - ⇦
1647↑	-, - ⇦	+, - ⇨	+, - ⇨	
1660↑	-, - ⇦	+, 0 ⇔		
1670↑	-, - ⇦			
Sequence of events				
⇩	Unfolding of β-sheet (1683 cm^{-1}) Formation of random coil (1647 cm^{-1}) Formation of turns (1660 & 1670 cm^{-1}) Loss of extended anti-parallel β-sheet (1627 cm^{-1})			

Table 4.3: Sequence of the unfolding events of bovine apo α-LA with 2.4% (w/v) ethanol during heating cycle from 25 to 95^0C

The refolding of the secondary structure of bovine apo α-LA spiked with 2.4% ethanol followed the pathway which was reversible up to some extent to the unfolding pathway (during heating

cycle). The cooling cycle showed three main peaks of concern: 1683, 1647 and 1627 cm^{-1}. The refolding of the protein's secondary structure in the presence of 2.4% ethanol commenced with the reformation of extended antiparallel β-sheet, which was the last event during heating cycle. It was then followed by the loss of random coils along with the simultaneous refolding of β-sheet, which was observed as first event during the heating cycle. Interestingly, the presence of even a minimal amount of ethanol (2.4%) was able to enhance the reversibility between the unfolding and refolding pathways followed by bovine apo α-LA.

cm^{-1}	1683 ↑	1647 ↓
1627 ↑	+, - ⇨	-, + ⇨
1647 ↓	-, 0 ⇔	
Sequence of events		
⇩	Reformation of anti-parallel β-sheet (1627 cm^{-1}) Loss of random coil (1647 cm^{-1}) & refolding of β-sheet (1683 cm^{-1})	

Table 4.4: Refolding sequence of bovine apo α-LA with 2.4% (w/v) ethanol during cooling cycle from 95 to 25^{0}C

Unfolding & refolding pathways of bovine apo α-LA solutions containing 20, 27.3 & 33.3 % (w/v) ethanol

Further 2D correlation analysis of variable temperature (heating and cooling cycles) second derivative difference spectra of bovine apo α-LA in the presence of 20, 27.3 & 33.3% (w/v) ethanol was undertaken. The individual tables depicting the sequence of events were generated for heating and cooling cycles of samples of apo α-LA in solutions with different ethanol content. Table 4.5 & 4.6 summarize the unfolding and refolding pathway respectively of apo α-LA, in the presence of 20% ethanol. Whereas, tables 4.7 & 4.8 summarizes the unfolding and refolding pathway respectively of bovine apo α-LA, in the presence of 27.3% ethanol.

The overlaid second derivative difference spectra as well as synchronous & asynchronous contour maps corresponding to bovine apo α-LA combined with 20, 27.3 & 33.3% ethanol showed a new band around the 1685cm^{-1}. The band 1685 cm^{-1} in the amide I' region has been

previously assigned to β-structure (Dzwolak et al., 2001). It is to be noticed that similarly to the case of protein with 2.4% ethanol, in the second derivative difference spectra of apo α-LA with 20, 27.3 & 33.3% ethanol, the bands at 1652 cm^{-1} and 1639 cm^{-1} assigned to α-helix and 3_{10}-helix respectively were absent. Similar results were reported by Boye et al. (1999), when the 3_{10}-helix and α-helix were lost by heating the α-LA in D_2O to 95°C and which also resulted into the appearance of a new band at 1647 cm^{-1} (assigned to random coil). It confirms that ethanol causes the loss of helical content (α-helix & 3_{10}-helix) of the secondary structure of apo α-LA, which is compensated by the formation of turns and random coils.

Table 4.5 indicates that during the heating cycle, bovine apo α-LA in the presence of 20% ethanol began unfolding by the formation of random coils (1646 cm^{-1}). The next event occurred was unfolding of β-structure (1685 cm^{-1}), which was further followed by the loss of antiparallel β-sheet (1626 cm^{-1}), which was then followed by the formation of turns (1660 cm^{-1}). The pattern of the refolding of secondary structure of protein during cooling was exactly opposite to the unfolding pathway followed during heating. The first event in the cooling cycle (table 4.6) was a loss in turns (1660 cm^{-1}). The next event was increase in the intensity of band at 1626 cm^{-1} (antiparallel β-sheet), which was followed by the increase in β-structure (1685 cm^{-1}) and finally the refolding of protein's secondary structure was completed by the loss in random coils (1646 cm^{-1}), which were formed during the heating cycle.

cm^{-1}	1685↓	1660↑	1646↑
1626↓	+, + ⇦	-, + ⇨	-, - ⇦
1646↑	-, + ⇨	+, - ⇨	
1660↑	-, - ⇦		
Sequence of events			
⇩	Formation of random coil (1646 cm^{-1}) Unfolding of β-structure (1685 cm^{-1}) Loss anti-parallel β-sheet (1626 cm^{-1}) Formation of turns (1660 cm^{-1})		

Table 4.5: Sequence of events in the unfolding of bovine apo α-LA in the presence of 20% (w/v) ethanol during heating cycle from 25 to 95°C

cm^{-1}	1685 ↑	1660 ↓	1646 ↓
1626 ↑	+, - ⇨	-, - ⇦	-, + ⇨
1646 ↓	-, - ⇦	+, + ⇦	
1660 ↓	-, + ⇨		
Sequence of events			
⇩	Loss of turns (1660 cm^{-1}) Reformation of anti-parallel β-sheet (1626 cm^{-1}) Increase in β-structure (1685 cm^{-1}) Loss of random coil (1646 cm^{-1})		

Table 4.6: Sequence of events in the refolding of bovine apo α-LA in the presence of 20% (w/v) ethanol during cooling cycle from 95 to 25°C

The unfolding & refolding pathway of bovine apo α-LA spiked with 27.3% (w/v) ethanol was slightly different as compared to the protein sample with 20% (w/v) ethanol. According to the sequence of events represented by table 4.7, the protein with 27.3% (w/v) added ethanol started unfolding by decreasing the intensity of band at 1685 cm^{-1} (loss of β-structure) followed by the loss of antiparallel β-sheet (1626 cm^{-1}), which was further followed by the formation of random coils (1646 cm^{-1}). The final event noticed in the sequence was formation of turns, represented by the increased intensity of the band at 1660 cm^{-1}.

cm^{-1}	1685 ↓	1660 ↑	1646 ↑
1626 ↓	+, + ⇦	-, + ⇨	-, + ⇨
1646 ↑	-, - ⇦	+, - ⇨	
1660 ↑	-, - ⇦		
Sequence of events			
⇩	Unfolding of β-structure (1685 cm^{-1}) Loss of anti-parallel β-sheet (1626 cm^{-1}) Formation of random coil (1646 cm^{-1}) Formation of turns (1660 cm^{-1})		

Table 4.7: Sequence of events in the unfolding of bovine apo α-LA in the presence of 27.3% (w/v) ethanol during heating cycle (25 to 95°C)

cm^{-1}	1685 ↑	1660 ↓	1646 ↓
1626 ↑	+, - ⇨	-, - ⇦	-, - ⇦
1646 ↓	-, + ⇨	+, + ⇦	
1660 ↓	-, + ⇨		
Sequence of events			
⇩	Loss of turns (1660 cm^{-1}) Loss of random coil (1646 cm^{-1}) Reformation of anti-parallel β-sheet (1626 cm^{-1}) Formation of β-structure (1685 cm^{-1})		

Table 4.8: Sequence of events in the refolding of bovine apo α-LA in the presence of 27.3% (w/v) ethanol during cooling cycle (95 to 25°C)

During the cooling cycle, the first change noticed was the loss in turns (1660 cm^{-1}), followed by the loss of random coils (1646 cm^{-1}). This was subsequently followed by reformation of the antiparallel β-sheet (1626 cm^{-1}) which previously lost as a result of heating process. The final event was increase in the β-structure (1685 cm^{-1}), which lost as first event during the heating cycle. The 2D correlation analysis of bovine apo α-LA in 33.3% ethanol (w/v) solution (data is not presented) also showed that events during the unfolding and refolding of secondary structure of bovine apo α-LA followed the exactly same sequence as followed by the protein in the presence of 27.3% ethanol and hence showed a great reversibility between the unfolding and refolding pathways induced by ethanol.

4.4. Conclusion:

VT-FTIR spectroscopy in conjunction with 2D correlation analysis provided a means to study the unfolding and refolding of bovine apo α-LA in the presence (varying amount) and absence of ethanol. It was observed that the unfolding and refolding pathways followed by α-LA during the heating and cooling cycles respectively were neither identical nor reversible to each other. According to this study, ethanol which acted as an external perturbation has great potential to enhance the mutual reversibility between the unfolding and refolding pathways followed by bovine apo α-LA during heating and cooling respectively. However, the unfolding/refolding pathways followed by bovine apo α-LA were different from sample to sample (samples differed

on the basis of ethanol content), but the reversibility between the unfolding and refolding pathways of individual protein samples was greatly enhanced at the higher level of ethanol content (20, 27.3 & 33.3 %).The current study also supports the utility of resolution enhanced 2D IR correlation analysis using second derivative spectra and as a tool for the elucidation of the mechanism of protein unfolding.

CHAPTER 5: SUMMARY

Bovine whey proteins are of great importance in food products due to their versatile functional properties and nutritional value. About 70,000 tonnes of whey proteins are manufactured as valuable food ingredients (De Wit. JN., 1988). α-LA is the second most dominant protein in the whey fraction of bovine milk. It is used in the development of various high protein foods and nutraceutical products. With regards to the latter, oleic acid-apo α-LA complexes (termed BAMLET) have been found to possess tumoricidal properties. These complexes are formed by heating (40-85^{o}C) a mixture of oleic acid in ethanol and apo α-LA were found to have varying degrees of tumoricidal and varying oleic acid to apo α-LA ratios.

This research focused on the specific effect of varying concentrations of ethanol on the structure of apo α-LA as part of an overall strategy to determine the mechanism of formation of BAMLET complexes and correlate their structure to tumoricidal activities. Two dimensional (2D) correlation analyses were performed in order to reveal the sequence of events leading bovine apo α-LA unfolding and refolding with increasing/decreasing temperature cycles in the presence of varying concentrations of ethanol.

In the chapter 3, the effect of various concentrations of ethanol [0, 2.4, 20, 27.3, 33.3% (w/v)] and varying temperature range (25 to 95°C in heating cycle and 95 to 25°C in cooling cycle) on the secondary structure of apo α-LA was determined by analysing the amide I' region of Fourier self deconvolved (FSD) spectra. The analysis revealed that (1) independent of temperature, ethanol has the potential to alter the secondary structure of apo α-LA; (2) upon heating the protein from 25 to 95°C, the α-helical and antiparallel β-sheet contents decreased as a function of ethanol concentration and (3) the ethanol-modified secondary structure of bovine apo α-LA showed almost complete reversibility above an ethanol concentration of 20% (w/v), which was not the case for apo α-LA alone.

In the chapter 4, the 2D correlation analyses was carried out to exhibit the unfolding and refolding pathways followed by bovine apo α-LA in the absence and presence of varying concentration of ethanol. The study performed in the chapter 4 of this thesis demonstrates that (1) unfolding and refolding pathways during heating and cooling respectively followed by apo α-

LA were neither identical nor reversible to each other, (2) ethanol resulted in reversibility between the unfolding /refolding pathways upon heating and cooling respectively of protein and which were identical above an ethanol concentration of 20%, (3) the predominate helical content of apo α-LA decreased in favour of β-sheet conformations in the presence of ethanol. This work will lay the foundation for future studies aimed at studying the combined effect of free fatty acid/ethanol mixtures on the formation of BAMLET type complexes of apo α-LA.

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