STRUCTURAL INVESTIGATION OF ISOPROPANOL AND ALKALINE pH-INDUCED TRYPSIN GEL AND THIN FILM AND ITS BIOTECHNOLOGICAL APPLICATIONS

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Bilge KARAÇİÇEK

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We approve the thesis of **Bilge KARAÇİÇEK**

Assist. Prof. Dr. Çağatay CEYLAN
Supervisor

Prof. Dr. Ahmet YEMENİCİOĞLU
Committe Member

Assoc. Prof. Dr. Oğuz BAYRAKTAR
Committe Member

13 December 2011

Assoc. Prof. Dr. Volga BULMUŞ
Head of the Department of
Biotechnology and Bioengineering

Prof. Dr. R. Tuğrul SENGER
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

STRUCTURAL INVESTIGATION OF ISOPROPANOL AND ALKALINE pH-INDUCED TRYPSIN GEL AND THIN FILM AND ITS BIOTECHNOLOGICAL APPLICATIONS

Trypsin is a biologically and industrially important member of serine protease family. Gelation forms a three dimensional network structure through the interaction of protein molecules among themselves and also with the environment.

The aim of the study was the investigation of structural and the functional properties of bovine pancreatic trypsin after gelation and aggregation processes. The phase behaviour of trypsin was determined for different protein concentration, NaOH concentration and $CaCl_2$ concentrations. In addition, the effect of sucrose addition to gelation time was observed. Increasing protein concentrations caused a decrease in gelation time. Increasing NaOH concentrations resulted in a decrease in gelation time. In low $CaCl_2$ concentrations gelation was observed but in high $CaCl_2$ concentrations aggregation was observed. The gels were resolubilized in water. Trypsin stability studies showed that there was a nearly 50% specific activity loss after the gelation process. According to FTIR studies β–sheet structure in 1637 cm^{-1} band disappeared in trypsin gel and trypsin aggregates. Increases in α–helix structure in 1651 cm^{-1} in trypsin gel with sucrose and aggregate with and without sucrose were observed. Iodoacetamide was shown to delay in gelation indicating the importance of intermolecular disulfides in the gelation process. The QCM studies showed that the film formed after gelation had absorbtion ability to different gases (benzene, carbon monoxide, carbon dioxide, dichloromethane, hydrogen peroxide and propanol) and can be used for gas sensing purposes. GI-XRD studies showed that trypsin thin film did not contain any crystalline structures.

ÖZET

İZOPROPANOL VE ALKALİ pH İLE İNDÜKLENEN TRİPSİN JELİ VE İNCE TABAKASININ YAPISAL İNCELENMESİ VE BİYOTEKNOLOJİK UYGULAMALARI

Tripsin serine proteaz familyasının biyolojik ve endüstriyel açıdan önemli bir üyesidir. Jelleşme, protein moleküllerinin birbirleriyle ve çevreleriyle etkileşimleri sonucu oluşan üç boyutlu ağ yapısıdır.

Bu çalışmanın amacı, sığır pankreatik tripsinin jelleşme ve aggregasyon işlemleri sonucunda yapısal ve fonksiyonel değişikliklerin incelenmesidir. Tripsinin farklı protein, NaOH ve $CaCl_2$, konsantrasyonları altında faz davranışı belirlenmiştir. Ayrıca sukroz eklenmesinin jelleşme zamanı üzerine etkileri incelenmiştir. Artan protein konsantrasyonları jelleşeme zamanında azalmaya yol açmıştır. Artan NaOH konsantrasyonlarının jelleşme zamanında azalmaya yol açtığı gözlemlenmiştir. Düşük $CaCl_2$ konsantrasyonların da jelleşme, yüksek $CaCl_2$ konsantrasyonlarında aggregasyon gözlenmiştir. Tripsin jeller suda tekrar çözünmüştür. Stabilite çalışmaları jelleşme işlemi sonrasında tripsin stabilitesinin yaklaşık % 50 oranında kaybolduğu saptanmıştır. FTIR çalışmalarına göre orijinal tripsinde bulunan 1637 cm^{-1} bandındaki β yapısı, jel ve aggregat yapısında kaybolmuştur. Bunun yanı sıra sukrozlu jel ve sukrozlu ve sukrozsuz aggregat yapısında 1651 cm^{-1} bandındaki α heliks yapısında artış gözlenmiştir. İyodoasetamidin eklenmesi jelleşmeyi geciktirdiği gözlenmiş ve bu da intermoleküler disülfit yapılarının jelleşmedeki önemini açığa çıkarmıştır. Jelleşme işleminde QCM çalışmalarında tripsin ince film tabakasının kullanılan gazlara (benzen, karbon monoksit, karbon dioksit, diklorometan, hidrojen peroksit ve propanol) duyarlı olduğu gözlenmiştir ve bu özelliğinde gazların saptanmasında kullanılabileceğini göstermektedir. GI-XRD çalışmalarında tripsin ince film tabakasında kristal yapısına rastlanmamıştır.

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LIST OF ABBREVIATIONS

Asp : Aspargine
BAEE : N-benzoyl-L-arginine ethyl ester
FTIR : Fourier Transform Spectroscopy
GI-XRD : Grazing Incidence X-Ray Diffraction
IAA : Iodoacetanide
IR : Infrared
KBR : Potassium Bromide
kDa : Kilo Dalton
Lys : Lysine
mg : Milligram
ml : Milliliter
min : Minute
µl : Microliter
nm : Nano Meter
pI : Isoelectric Point
QCM : Quartz Crystal microbalance
SEM : Scanning Electron Microscopy
Ser : Serine
XRD : X-Ray Diffraction

CHAPTER 1

INTRODUCTION

Serine proteases are one of the biggest groups of proteolytic enzymes (Singh et al., 2008) and found in many organisms such as animals (Balti et al., 2009), plants (Singh et al., 2008), and viral genomes (Bazan and Fletterick, 1988). Trypsin is an important member of the serine protease family (Hehemann et al., 2008; Yamashiro et al., 1997). Although the main activity of trypsin is food digestion in the duodenum (Katona et al., 2002), it is also necessary in many physiological functions such as cellular and humoral immunity (Page and Cera, 2010; Mendoza et al., 2005; Cera, 2009; Shi et al., 2009), blood coagulation, fibrinolysis, embryonic development, digestion, apoptosis, hemostatis, homeostasis and fertilization (Cera, 2009). Trypsin function is also implicated in some diseases (Chander et al., 2006). In addition, trypsin also is an industrially enzyme with many uses as a detergent additive, in animal cell cultures, in leather processing, as a dietary supplement, in therapies for the patients with pancreatic disease (Kim et al., 2011).

Gelation is the process of three dimensional network formations of proteins. Gelation of proteins is used in many areas from tissue cultures to desired food texture properties (Zhang et al., 2007). Similar to gelation, protein aggregation occurs among the unfolded proteins interaction (Golub et al., 2007). It is widely observed in several human diseases such as Alzheimer and Parkinson's diseases (Cellmer et al., 2007).

In this research bovine pancreatic trypsin was used as the model protein to investigate the structural and functional changes were observed in the cold gelation. Gelation time was determined in a range of three different variables (trypsin concentration, NaOH concentration and $CaCl_2$ concentration). The effect of sucrose addition to the gelation time was also studied. Resolubilization studies were carried out to observe the trypsin dissolving behaviour in different solutions (starting solution, water, isopropanol and isopropanol-water mixture). Trypsin stability studies were performed to observe the specific activity loss after the gelation process in trypsin gel and trypsin gel with sucrose.

FTIR (Fourier Transform Infrared Spectroscopy) method was used to observe the secondary structural changes after gelation and aggregation applications. Also, Iodoacetamide (IAA) which is a disulfide blocking agent was used to observe its effects on gelation. The adsorption tendency of the protein film formed for several gases after gelation was demonstrated as a potential biotechnological application of the gelation process. For this purpose QCM (Quartz Crystal Microbalance) method was used to characterize a biotechnological application of trypsin thin film as a sensor of gases namely benzene, carbon monoxide, carbon dioxide , dichloromethane, hydrogen peroxide and propanol. SEM studies were carried out for the morphologic changes in protein gelation and aggregation processes. GI-XRD (X-Ray Diffraction) studies were carried out to observe if trypsin thin film contains any crystalline structures on the surface of the gold substrate different from the original trypsin.

CHAPTER 2

LITERATURE REVIEW

2.1. Serine Proteases

Serine proteases are one of the biggest groups of proteolytic enzymes (Singh et al., 2008). They are found in many organisms such as human beings (Chander et al., 2006), animals (Balti et al., 2009), plants (Singh et al., 2008), and viral genomes (Bazan and Fletterick, 1988) and form one third of the proteolytic enzymes (Cera, 2009).

Serine proteases provide hydrolization of the specific peptide bonds in their substrates. This activity is determined by a set of amimo acids in the active site of the enzymes, such as serine (Ser) (Singh et al., 2008). The characterization of serine proteases are provided by the serine residue presence in the active site of the enzyme (Shi et al., 2009). In addition, serine proteases show various kinds of enzymatic activities such as endopeptidase, omegapeptidase, oligopeptidase and exopeptidase (Singh et al., 2008).

They have important roles in the aspects of physiological processes such as embryogenesis, immune response, food digestion, blood clotting (Simonet et al., 2002), regulation of the proteolytic activation of precursor proteins (Perona and Craik, 1995), hormone activation and signal transduction (Shi et al., 2009).

2.2. Trypsin

Trypsin is a monomeric protein which contains 229 amino acid residues, and 12 cystein residues which were linked by 6 disulfide bridges. Trypsin has a molecular mass of 23.8 kDa. Trypsin is an alkali digestive protease and it owns an isoelectric point of 10.5 (Balti et al., 2009; Forbes et al., 2007). It was observed that based on X-ray analysis, trypsin was composed of approximately 55% α-sheet, 13% β-helix (Forbes et al., 2007).

Trypsin is a member of serine protease family (Hehemann et al., 2008; Yamashiro et al., 1997; Sainz et al., 2004; Chander et al., 2006; Katona et al., 2002; Kishimura and Hayashi, 2002; Garcia et al., 2009; Kim et al., 2011) and was first isolated from pancreatic juice of animals, but later it was observed that many different tissues and organisms owned trypsin (Katona et al., 2002). Trypsin is also isolated from humans, bovine, ovine, porcine, canine, rabbits, rats, mice and from non-mammalian such as turkey, ostrich, rainbow trout, cod, dog-fish, shrimp, crayfish and lobster (Steiner et al., 1997) and also fish and shellfish (Klomklao et al., 2006). The main activity of the trypsin is the food digestion in the duodenum (Katona et al., 2002). Trypsin is needed in food digestion because it functions as a mediator between the taken food and nutrient degradation (Sainz et al., 2004). Trypsin exhibits an important function in the activation of pancreatic enzymes which is needed in digestion (Kim et al., 2011).

Trypsinogen (inactive form of the trypsin) which is found in the intestinal mucosa, activated by enterokinase and the activated trypsin has a function in the activation of the rest zymogens (Yamashiro et al., 1997).

Trypsin is released from pancreatic acinar cells (Kim et al., 2011; Kishimura and Hayashi, 2002) which stored in the zymogen granules. Enterokinase distinguishes the (Asp)4-Lys sequence from the propeptide and provides the cleavage after lysine residue to cleave the active trypsin. The propeptide removal causes conformational alterations which give rise to structural formation of an active site. Trypsin provides the cleavege of the peptide bonds at the carboxyl-terminal end of lysine and arginine residues (Kim et al., 2011; Balti et al., 2009; Mendoza et al., 2005; Shi et al., 2009; Fernández et al., 2004; Yamashiro et al., 1997; Klomklao et al., 2006). In addition, it can activate its own zymogen. When trypsin is activated it also activates the other digestive proteases which were previously synthesized as zymogens (Kim et al., 2011).

2.2.1. Biological Importance of Trypsin

Trypsin activity is also significant in physiological functions like cellular and humoral immunity (Page and Cera, 2010; Mendoza et al., 2005), blood coagulation (Mendoza et al., 2005; Cera, 2009) fibrinolysis (Mendoza et al., 2005; Page and Cera, 2010; Shi et al., 2009; Cera 2009) embryonic development (Mendoza et al., 2005; Page and Cera, 2010; Cera, 2009), digestion (Mendoza et al., 2005; Shi et al., 2009; Cera, 2009), apoptosis (Page and Cera, 2010; Cera, 2009), hemostatis, homeostasis (Page and Cera, 2010) and fertilization (Cera, 2009). Besides extracellular functions trypsin has intracellular functions such as the digestion of bacteria in neutrophils in mammals (Shi et al., 2009) and also it provides the activation of cell surface receptors (PAR family), polypeptide hormon precursor processing and digestion of the proteins of extracellular matrix which provides the migration of cells (Katona et al., 2002).

Also, it was observed that trypsin takes place in some diseases. Alzheimer is a neurodegenerative disease and related to the proteolytic activity in brain. It was observed that overexpression of human trypsinogen IV, in the brain, gave rise to improved expression of glial fibrillar acidic protein and an increase in the amyloid fragment number (Chander et al., 2006). Some neurodegradative brain illnesses like Alzheimer disease mainly related with the proteolytic activity in the brain. The proteinases which is expressed in the brain like human trypsin IV is needed in the pathogenic mechanism of Alzheimer disease (Katona et al., 2002).

2.2.2. Industrial Uses of Trypsin

Trypsin is used for many fields such as detergent industry, animal cell cultures, and leather processing, in the food industry as a dietary supplement, in therapies for the patients with pancreatic disease and also trypsin is demanded by biopharmaceutical manufacturers (Kim et al., 2011).

Trypsin is being used as a pathogenic agent remover in bovine embryos (for bovine herpesvirus-1 and -4, rhinotracheitisvirus). Trypsin, different from other serine proteases, provides breaking the bonds related to the adhesion to sperm surfaces for some viruses such as *Bovine herpesvirus 1 (BHV-1)* (Mattson et al., 2008).

Trypsin is being used as a biocatalyst in dipeptide synthesis either using arginine or lysine esters as acyl donor and kinds of amino acids as nucleophilic agents (Fernández et al., 2004).

Bovine pancreatic trypsin (EC 3.4.21.4), which is a serine protease, is extensively used in food industry, biotechnology industry and biomedicine and in protein analysis. Also, it is used as a model in the improvement of different kinds of methods for stabilizing enzymes (Garcia et al., 2009).

Bovine trypsin is the most commonly used enzyme for the commercial processes to digest or process other proteins which also comprises some therapeutic proteins (Tubìo et al., 2007).

It is used as a dietary supplement in food industry and as a substitute in therapies for the patients who have pancreatic diseases (Tubìo et al., 2009).

2.3. Protein Structure and Stability

2.3.1. Primary Structure

The primary structure of protein is provided by linear alignment of amino acid sequences (Voet et al., 1999). The primary structure contains covalent bonds between amino acids and also it contains disulfide bonds location in the structure (Lehninger et al., 1993).

2.3.2. Secondary Structure

Secondary structure is commonly observed in proteins. The most known secondary structures are α-helix and β-sheet conformations. Secondary structure is mainly stabilized by hydrogen bonding (Lehninger et al., 1993).

2.3.2.1. α-helices

The simplest arrangement of polypeptide chain is α-helix structure. In α-helix structure, the polypeptide backbone is tightly wound around the long axis of the molecule and the R groups of the amino acid residues which forms the helical backbone. α-helix is one of the commonly observed secondary structures in α-keratins. Globular proteins nearly one-fourth of all amino acid residues found in α-helices.
α-helix structure contains internal hydrogen bonds (Lehninger et al., 1993).

2.3.2.2. β-sheets

One of the most seen secondary structures is β sheet. It is a more extended conformation of polypeptide chains (e.g. silk protein fibroin). Also, β-sheet structure is as common as α-helix structure, in β-sheet, the backbone of the polypeptide chain extended into zigzag form. In fibroin the zigzag polypeptide chains are arranged side by side to form a structure which looks like a series of pleats and this structure named as β pleated sheet. The hydrogen bonds may be found as intrachain or interchain between the peptide linkages of adjacent polypeptide side chains (Lehninger et al., 1993).

2.3.3. Tertiary Structure

Tertiary structure is the entire three-dimensional structure of the polypeptide (Voet et al., 1999; Lehninger et al., 1993). The tertiary structure is the relationship between all amino acids in the polypeptide (Lehninger et al., 1993). In other words the folding of the secondary structural elements forms tertiary structure (Voet et al., 1999).

2.3.4. Quarternary Structure

Quarternary structure consists of two or more polypeptide chains which are known as subunits (Voet et al., 1999; Lehninger et al., 1993). The proteins whose molecular masses are more than 100 kD and composed of two or more subunits form quarternary structure (Lehninger et al., 1993).

Protein structure stabilization is provided by balances of the noncovalent and covalent forces. The noncovalent interactions (e.g. hydrophobic effects, electrostatic interactions and hydrogen bonding) and covalent interactions (e.g. disulfide bonding) have important role in the stabilizing the protein structure. In brief, the forces which affect proteins are necessary to maintain the protein structure to make protein functional (Voet et al., 1999). All protein structures were shown in the Figure 2.1.

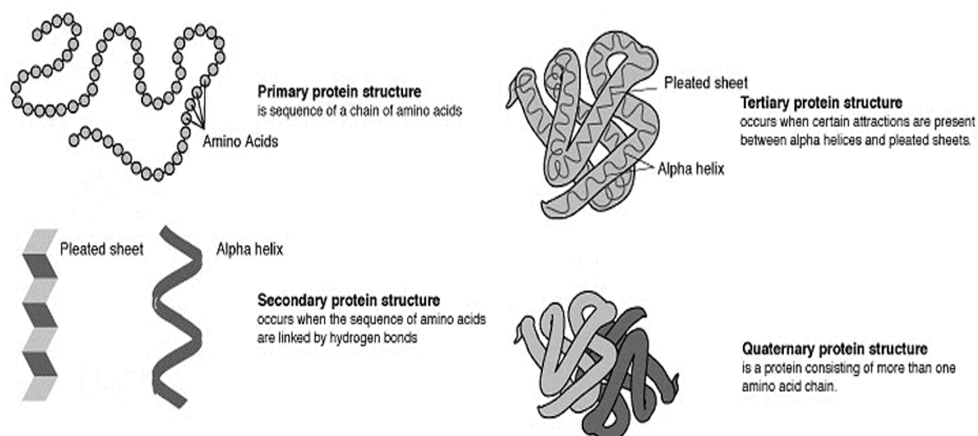


Figure 2.1. Protein structure
(Source: Umass, 2011)

2.4. The Bonds Which Stabilizes Folded Proteins

The stability of the folded proteins is provided by the interactions of;

2.4.1. Hydrophobic Interactions

Hydrophobic interactions are highly attractive forces among the non-polar groups which are divided by water. They are formed due to the water molecules ability to form hydrogen bonds although non-polar groups form Van der Waals interactions by the nearer molecules to themselves. Hydrophobic interactions highly depend on the temperature. As the temperature increases, the interaction becomes stronger (Braynt and McClements, 1998).

2.4.2. Electrostatic Interactions

Electrostatic interactions occur between charged molecules due to the attraction of opposite charged molecules (Petsko and Ringe, 2004). If the atoms have the same charges, the formed interaction is repulsive, whereas the atoms have different charges like positive and negative or the opposite, attractive interaction occurs. pH of the solution effects the magnitude, sign and the distribution of the charge on protein molecule. Proteins are negatively charged above their own pI (isoelectric point). Ionic strength has an effect on electrostatic interactions (Braynt and McClements, 1998). Charged groups are generally found on the surface of proteins. Between these charged groups, electrostatic interactions may occur. Ionizable side chains and the charges at the carboxy- and amino-termini can be given as an example for these charges. In brief, it can be said that electrostatic interactions are important to both destabilizing the native state and extension degree of unfolded state (Shirley, 1995).

2.4.3. Van der Waals Interactions

Due to the distribution of electrons, weak and attractive forces occur between atoms or molecules which are called as *Van der Waals interactions.* These forces occur between electronegative forces, which are found between hydrophobic groups (Petsko and Ringe, 2004). They occur due to the high repulsive interactions among molecules and atoms (Braynt and McClements, 1998). Van der Waals interactions strongly depend on the distance (Shirley, 1995).

2.4.4. Hydrogen Bonds

Hydrogen bonds are one of the non covalent interactions which occur due to the hydrogen atom sharing through the hydrogen bond donor (hydroxyl-OH or amino-NH) and a hydrogen bond acceptor atom (oxygen or nitrogen) (Shirley,1995) or in other words hydrogen bonds occur through the donor atom (positively charged) and the acceptor atom (negatively charged) (Petsko and Ringe, 2004). Many acceptor and donor groups are found in the peptide backbone of the proteins (Shirley, 1995).

2.4.5. Salt Bridges

The donor and the acceptor molecules are charged in salt bridges which are a kind of hydrogen bond (Petsko and Ringe, 2004). The opposite charged to ionic protein groups association is known as an ion pair or salt bridge. Nearly 75% of the charged residues in proteins are members of ion pairs which are generally located on the protein surface. Although the strong electrostatic attraction between the oppositely charged members of a salt bridge, these interactions contribute little to the stability of native proteins. The reason for this, the free energy of an ion pair's charge-charge interactions generally fails to compensate for the loss of entropy of the side chains and the loss of solution free energy where the charged groups form a salt bridge (Voet et al., 1999).

2.4.6. Hydration Interactions

Hydration interactions occur when the two hydrated molecules, in short ranges, become nearer to each other. They are formed through the disruption of the hydrogen bonds in molecules and water molecules. The strength of this type of the interaction is determined by the hydration degree of the molecule. If the hydration degree is higher, the distance between the interacted molecules is long and the attraction is strong. If the protein molecule aggregation is an undesired case, in high salt concentrations, this type of interaction counteracts the aggregation. This stems from the binding of hydrated ionic molecules to protein surface and the protein molecules no more interact for aggregation (Braynt and McClements, 1998).

2.4.7. Disulfide Bridges / Disulfide Bonds

The disulfide bridges take place inside the protein structure and they are formed between neighbouring cystein residues. Disulfide bridges form inner part of protein structure due to the reducing environment (Petsko and Ringe, 2004). Proteins have the ability to form intermolecular and intramolecular disulfide bonds under suitable conditions (Braynt and McClements, 1998). Table 2.1 shows the interactions of two similar protein molecules in aqueous solutions.

Table 2.1. General characteristics of molecular interactions between two similar protein molecules in aqueous solution. (Source: Braynt and McClements, 1998)

General characteristics of molecular interactions between two similar protein molecules in aqueous solution						
Type	**Sign**	**Strength**	**Range**	**pH**	**I.S.**	**Temperature**
Hydrophobic	Attractive	Strong	Long	No	No	Increases
Electrostatic	Repulsive	Weak→Strong[a]	Short→Long[a]	Yes	Decreases	Increases
Hydrogen bonding	Attractive	Weak	Short	No	No	Decreases
Hydration	Repulsive	Strong	Short	No[b]	No[b]	Decreases
Van der Waals	Attractive	Weak	Short	No	No	–
Disulfide bonds	Attractive	Very	Strong	Short	Yes	No
[a]Depends on pH and ionic strength (I.S.)						
[b]Indirectly depends on pH and I.S. because these factors influence the degree of protein hydration						

2.5. Carbonhydrate-Protein Interactions

Proteins are in an interaction with aqueous media in general. In aqueous media many cosolvents are found (e.g. sugars, minerals, alcohols, surfactants) and they affect proteins in different physiochemical mechanisms such as electrostatic screening, changes in water binding and inter-intramolecular binding (Guzey et al., 2003).

Sugars affect protein functionality in two ways. The first way is through physical interactions. The second way is the covalent bonding of sugars with proteins by Maillard reaction (Semenova et al., 2002).

Similarly, sugars affect protein gelation in two aspects. One of these aspects is the stabilization of the proteins against heat denaturation. This affects gel strength by an increase in temperature of heat denaturation and changing bond formation in gelation. Another one is the Maillard reaction. During heating, sugars are in an interaction with the amines and covalent cross-links were formed in the protein network. It is possible to observe rheological property changes due to crosslinking (Rich and Foegeding, 2000).

In the presence of sugars, globular proteins (in heat set galation) are protected from unfolding by some factors. Firstly, in the presence of sugars the direct contact between protein and water become thermodynamically undesirable which was proven by the enhancement of hydrophobic interactions. Secondly, a decrease in water activity was observed in the presence of sugars due to making water-protein interactions less effective. Thirdly, sugar-protein interactions by the help of hydrogen bonding may cause alterations in protein surface hydrophilicity (Dickinson and Merino, 2002).

It was proven by many researches that sugar leads to an increase in the thermal stability of the proteins or it prevents protein denaturation by interacting other agents in the medium. The stabilizing effect of the sugars comes from its hydroxyl group number and position. Also the stabilization effect may be a result of preferential hydration of proteins which is the result of the increase in the surface free energy of water (Castronuovo et al., 2002).

In heat induced globular protein gelation sugars may change the gelling mechanisms of globular proteins in some aspects such as i) causing protein gelling temperature increase (in the presence of sucrose, higher temperatures needed for the first stage of the heat set geling method for the protein molecules unfolding) ii) when sugar is found in the medium, it gives rise to slower gelation rate iii) The presence of sugars also gives rise to rigidity increase in the protein gels due to heating for long times above the temperature of protein denaturation which results in irreversible protein denaturation (Christ et al., 2005).

Protein and sucrose interactions are important to their industrial role (Semenova et al., 2002) due to their common use in foods (e.g. yoghurt-style desserts and ice-cream) (Belyakova et al., 2003). In foods, globular proteins are enclosed by a complex mixture of molecules such as surfactants, water, sugars, lipids, polysaccharides, and salts. These molecules can indirectly or directly affect the physico chemical properties of proteins to change their functional properties such as thermostability, surface activity, structure formation, and catalytic activity (Baier and McClements, 2003).

Foods and pharmaceutical products commonly contain proteins and low-molecular-weight sugars (Belyakova et al., 2003; Raikos et al., 2007). As additives and preservatives low-molecular-weight sugars are being widely used (Semenova et al., 2002) and sucrose is commonly used for this purpose.

The functional properties such as foams, gel formation and emulsion stabilisation are the results of the interactions of protein molecules with different ones such as water, high and low molecular weight molecules. The content and the type of the added sugar are important to the quality of food products (Semenova et al., 2002). The interaction of these molecules are plays a significant role in the aspects of stability, functionality of protein, general structure and product rheology (Belyakova et al., 2003; Semenova et al., 2002; Ebel et al., 2000).

2.6. Gelation of Proteins

2.6.1. What is a Gel?

A gel can be explained as elastic, deformable, soft solids which are composed of combined networks such as large molecules or small particles. Gels can be opaque or translucent due to the size and adjustment of the molecules which form them. Nowadays, globular protein gelation is being provided at low temperatures. This method is known as cold gelation which was described by Foegeding and Barbut (Hongsprabhas, Barbut and Marangoni, 1999). According to this procedure, gelation of the globular proteins is provided by unfolding of the proteins through preheating, cooling and salt addition. In this kind of gelation process, much more elastic and transparent gels are obtained. In contrast to heat induced gelation, the gels are not transparent and elastic (Hongsprabhas, Barbut and Marangoni, 1999).

2.6.2. What is a Protein Gel?

Protein gels can be explained as a three-dimensional network which is formed by the combination of polypeptides and the entrapment of the water molecules. The characterization of the gels is defined by their elasticity, viscosity and plasticity properties/abilities. When proteins (e.g. whey proteins) form gels, they have the ability to hold flavor, water, sugar, food ingredients in its structure which provides unique properties for food applications.

The proteins gelation or gelling ability is important to meat products such as sausage and various kinds of oriental textured foods such as tofu (Zhang et al., 2007).

Gel formation is considerable function of texture formation in some foods (Sun and Arntfield, 2010; Sun and Arntfield, 2011). Textural properties of food products (e.g. sausages, cheeses, yoghurt, etc.) are determined by the type of the gel which is related to its forming process (Hongsprabhas and Barbutt, 1997). In addition gelling of proteins is also important in the aspects of enhancing water holding capacity and appearance in food processing (Rosa et al., 2006).

Protein gels are formed due the conformational alterations in the protein structure (Ikeda et al., 2001). Gelation process is composed of two stages such as i) the protein molecules partial denaturation, ii) the aggregation or combination of denatured (unfolded) proteins (Hongsprabhas and Barbutt, 1997). High temperatures and other adverse conditions denature protein structure. Similarly, they also affect gelling property of proteins. By changing these factors, the desired properties related to the gel formation can be provided (Sun and Arntfield, 2010; Sun and Arntfield, 2011; Donato et al., 2005).

2.7. The Difference between the Heat-Set and Cold-Set Gelation of Proteins

Gelation of proteins can be provided by many ways. The most common method is heat-induced gelation. In addition to heat-induced gelation, cold gelation of globular proteins is being recently used (Rosa et al., 2006).

In the heat set gelation process, the gel is obtained in two stages such as denaturation and cross-linking (Baier and McClements, 2005) and the proteins are initially denatured (unfolded), associated and simultaneously aggregated due to the hydrophobic interactions and inter/intra-molecular disulfide bonds (Hongsprabhas and Barbutt, 1997).

In cold set gelation, two steps are needed. The protein is heated and denatured protein particles are formed. For gelation or in other words network formation, the denatured particles interaction is needed. This interaction is provided by decreasing the pH far away from the pI (Rosa et al., 2006; Vilela et al., 2011).

Cold gelation of the protein gels serves to enhance and increase the functional properties of the proteins. Nowadays, the cold set gelation process in food applications is very important in food industry and it has been used greatly in food industry such as yoghurt and processed fish (Cramp et al., 2008).

Whey proteins due to their gelling ability and nutritional values are excessively used in food applications (Bryant and McClements, 2000; Fitzsimons et al., 2007; Remondetto et al., 2002; Donato et al., 2010). In whey protein gelation, cold set gelation is commonly used (Braynt and McClements, 2000; Remondetto et al., 2002; Donato et al., 2010). Also, cold-set gelation is being applied to whey protein isolate, soy protein, whey protein concentrate, and α-lactoglobulin (Donato et al., 2010).

Nowadays, the gelling abilities of whey proteins are widely used in food products such as ice cream, infant foods, beverages and dips, processed meats, pasta, etc. (Fitzsimons et al., 2007). The desired gel structure formation depends on many factors such as heating conditions (temperature and heating rate), pH, protein concentration and the existence of salts in the medium. In whey protein gelation, if all conditions are provided related to desired gel property, the desired gel will be obtained (Barbut and Drake, 1997; Remondetto et al., 2002).

Because of their functional features and nutritional values soy proteins are highly used in food industry. In addition to these properties, owning gelation ability makes soy protein one of the unique proteins. The gelation of soy proteins (at the same time globular proteins) occurs either through heat-set gelation or cold set gelation (Cramp et al., 2008).

2.8. Molecular Aspects of Protein Gelation

Several procedures are used for protein gelation, including heating. In the formation of network structure, the attractive and the repulsive forces balance is provided by the chemical and the physical properties of the protein. Due to the exposion of the hydrophobic groups interactions occur and the crosslinking of the molecules allow for the gel formation.

In addition to hydrophobic interactions, disulfide-sulfhydryl interchange reactions occur due to the hidden sulfhydryl group exposion due to the unfolding of proteins which is also called disulphide crosslinking (Boye et al., 1996).

2.8.1. Disulfide Crosslinking

Since foods contain proteins, environmental conditions such as pH and temperature affect the protein structure and also disulfide bonding. Disulfide bonds may undergo inter/intramolecular bonding. Generally, sulfhydryl groups go to inter or intramolecular bonding by applications (e.g temperature, pH, solvents and composition) and they become in a relation with their environment and as a result, novel intermolecular and intramolecular cross-links were formed between molecules (Swaisgood, 2005). This is also termed as disulfide reshuffling (Wedemeyer et al., 2000).

Disulfide reshuffling is found in β-Lactoglobulin, whey protein and in many proteins which contains cystein residues (Floris et al., 2008; Muhammad et al., 2009; Foegeding et al., 2002).

β-Lactoglobulin contains free sulfhydryl groups inside structure. The interactions of the sulfhydryl groups with the environment (solvents, compound) lead to the sulfhydryl group to be reactive. Also β-Lactoglobulin undergoes some structural changes due to the denaturation, and the hidden and unreactive sulfhydryl groups become reactive due to the environmental interactions. The hidden sulfhydryl groups become exposed and appropriate for (Figure 2.2) sulfhydryl/disulfide interchange and oxidation (Floris et al., 2008; Muhammad et al., 2009).

In whey protein polymers, the driving force in polymer formation mainly depends on the disulfide bonding and non-covalent interactions. The disulfide formation provides a strong network in whey proteins (Foegeding et al., 2002).

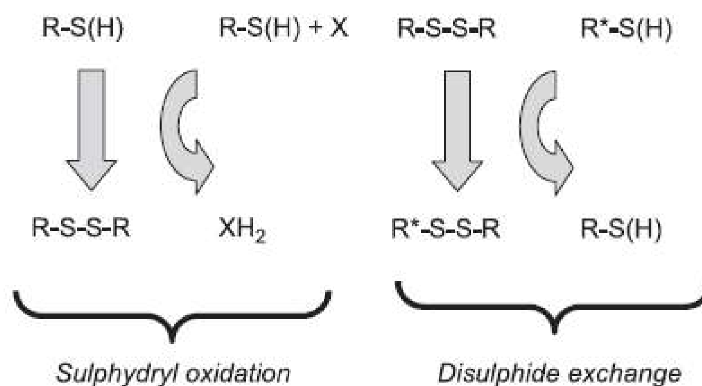


Figure 2.2. Disulfide crosslink formation.
(Source: Visschersa and Jongh, 2005)

2.8.2. Inter/Intramolecular β-Sheet Interactions

In proteins, β-sheet conformations are found as the lateral combination paralel or antiparallel β-sheet structures. β-sheet structure stabilization is provided by hydrophobic interactions and hydrogen bonding. The forces which provide the stability and interaction of β-sheets are important to protein function. Intramolecular β-sheet interactions are related to protein folding and necessary in protein secondary and tertiary structure (Khakshoor and Nowick, 2008; Khakshoor et al., 2007) whereas intermolecular β-sheet interactions are related with protein-protein interactions, quarternary structure, protein aggregation and molecular recognition (Khakshoor and Nowick, 2008; Khakshoor et al., 2007).

Besides their biological activities, β-sheets take place in many diseases such as cancer, HIV and neurodegenerative diseases like Huntington’s disease, Alzheimer’s disease (Khakshoor et al., 2007; Khakshoor and Nowick, 2008; Banerjee et al., 2003; Masuda et al., 2008). Intermolecular β-sheet interactions may be either paralel or antiparallel (Figure 2.3) due to the primary structure of the peptide (Masuda et al., 2008). In amyloid diseases such as Alzheimer’s disease and Parkinson’s disease insoluable, generally fibrous and aggregates are formed which are rich in β-sheet conformation (Bauer et al., 2011). β-sheet forming proteins are important to fibril and gel formation and in tissue engineering applications (Banerjee et al., 2003; Park et al., 2006).

Hydrogels composed of β-sheet forming peptides provides an appropriate three-dimensional cell culture matrix or scaffold (e.g. neural progenitor cells, chondrocytes, and endothelial cells) for tissue engineering applications (Park et al., 2006).

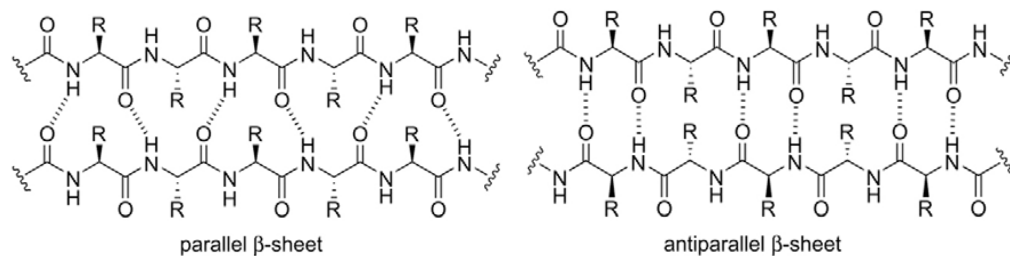


Figure 2.3. Parallel β-sheet and antiparallel β-sheet.
(Source: Khakshoor and Nowick, 2008)

2.9. Protein Aggregation

Protein aggregate formation is a consequence of unfolded proteins interaction which causes agglomerate formation with different kinds of molecular alterations (Golub et al., 2007). Aggregated proteins generally own reduced activity or no biological activity (Wang, 2005).

Protein aggregation can be divided into two categories such as physical aggregation and chemical aggregation. In physical aggregation, protein molecules have the ability to aggregate with each other without any alterations in primary structure. If protein aggregation occurs by new covalent bond formation, it is called as chemical aggregation. The bond formation may occur directly by crosslinking or indirectly by changing the aggregation affinity of the original protein. The formed aggregates may be soluble or insoluble (Wang, 2005). Protein aggregation is being affected by internal (structural) and external (environmental) factors. Internal factors include primary and secondary structure. Protein aggregation primarily depends on the primary structure sequence of the protein. The nature of the amino acids in the sequence like hydrophilic, hydrophobic, polar, non-polar determines the aggregation affinity of the protein (Wang, 2005).

Protein aggregation related with the secondary structure rises from α-helix and β-sheet structures. It was observed that β-sheet structures are much more needed than α-helix structures in protein aggregation. α-helix structure was less observed in aggregated proteins. This happens because α-helices own strong dipole moment than β-sheets (Wang, 2005).

Aggregation of proteins are observed in some human diseases such as Mad Cow, Alzheimer's, Huntington's, Parkinson's (Cellmer et al., 2007) and cataract formation (Grigsby et al., 2002). Diseases which are related to protein aggregation are divided into two groups. The first groups are composed of aggregated proteins which lost their biological function (e.g. cystic fibrosis, Marfan syndrome). The second group is composed of protein aggregates which own cytotoxic effect (e.g. Parkinson's, Huntington's and Alzheimer's diseases) (Cellmer et al., 2007).

2.10. Thin Films in Biotechnology

Thin films can be explained as two dimensional material, the thickness, the third dimension, is so small (from 1 μ to 1 nm) and there is a significant increase in the surface-to-volume ratio (up to 10^6) (Tvarozek et al., 1998).

Nowadays, related to their chemical and physical properties, thin films of bio-polymers, proteins and DNA are interesting. Also, in food packaging edible thin films are used for food preservation purposes (Tharanathan, 2003). Soy milk and cereal proteins are used in protein films in various applications (Nakata et al., 2007).

Protein thin films are also commonly used in biomedical materials to enhance the cell adhesion and biocompatibility, and in tissue engineering as a scaffold material (Lubarsky et al., 2007; Uematsu et al., 2004). For growing embryonic stem cells hydrogels are used as a scaffold material (Smith et al., 2010). Thin films are also used in biochips and biosensors (Uematsu et al., 2004). Proteins can form multilayers without losing their functionality. This property provides an important opportunity for biomedical applications. Biocompatible and bioresorbable properties of protein multilayers make them functional for cell adhesion surfaces and drug delivery systems (Su et al., 2009).

Protein-based materials are preferred to other materials because of their biocompatibility, biodegradability and low toxicicty properties. These properties make them popular in the drug delivery systems. Soy protein and gelatin are being commonly used for this purpose. In drug delivery systems, protein cages provide unique properties such as drug loading and releasing systems. They also provide chemical and biological structures for drug delivery. These protein cages protect the drugs from enzymatic degradation during interaction with the environment. These protein based capsules are suitable for the thin film strengthen (Huang et al., 2011).

2.11. FTIR (Fourier Transform Infrared Spectroscopy)

FTIR is a kind of infrared spectroscopy method. IR (infrared) radiation passes through the sample. While IR is passing, some of the IR holded by the sample and some of the IR is transmitted. Final spectrum shows the molecular absorbtion and transmisson. Every molecular structure produces its own infrared spectrum.
By the help of the FTIR:
*Unknown material identification,
*The quality and consistency determination of the sample,
*The amount of components determination in the mixture can be performed.
An infrared spectrum shows the fingerprint of the sample with absorbtion peaks which corresponds to the frequencies of vibrations between the bonds of the atoms making up the material (ThermoNicolet, 2011).

FTIR is a method which is commonly used in the determination of protein secondary structure (Kong and Yu, 2007; Weert et al., 2001). FTIR method is preferred because the scattering is decreased and the method mainly depends on wavelength radiation (Weert et al., 2001). By the FTIR specroscopy method, the intensity and the wavelength of the IR radiation absorbtion of the sample is being measured. Repeating units of the vibrated molecules provides the IR data analysis. Repeating units in protein or polypeptide chains comprise specific IR bands which are formed due to the molecules vibrations. Specific IR bands are composed of nine absorption bands which are A, B, I-VII.

Among nine specific absorption bands, Amide I band has an important role in the determination of the protein secondary structure. Amide I band has the absorbtion wavelength region/range between 1700 cm^{-1} and 1600 cm^{-1} and it mostly depends on C=O stretching. It was observed that the frequencies related to the protein secondary structural elements were meaningful for the Amide I absorption bands. As Amide I band has specific affinity to changes in the hydrogen bonding and molecular geometry, it is important related to considering the analysis of the protein conformation and secondary structural changes. The secondary structure of the proteins like α-helix and β-sheet has spesific C=O stretching frequencies related with their spesific hydrogen bonding and molecular geometry. Amide II band is also used in protein structure studies and depends on NH bending but for the proteins secondary structure Amide I band is more significant than Amide II band (Kong and Yu, 2007).

2.12. QCM (Quartz Crystal Microbalance)

QCM is an appropriate device in transducing chemical and biochemical signal sensing. By coating QCM probe with sensitive coatings, the resonance frequency changes will be determined by QCM after sensing analysis.

The method mainly depends on the frequency shift of the coated quartz crystal related to the sensing (Okur et al., 2010). QCM provides the measurement of the resonance frequency change of the quartz crystal between gold electrodes (Erol et al., 2011). It is being used widely as a sensor of gas and liquid materials (Wyszynski et al., 2007; Nakamura et al., 2000; Smith and Shirazi, 2005; Nakamoto et al., 2002). The QCM sensing principle depends on the resonance frequency change because of the mass change on the QCM probes due to the sensing ability of the sensing film (Nakamura et al., 2000; Atashbar et al., 2005). Also, this method is used for immunoassays and protein sensing (Atashbar et al., 2005). The QCM frequency response is affected by mass, effective stiffness and viscosity, conductivity, dielectric constant or electrode morphology (Rickert et al., 1997).

2.13. XRD (X-Ray Diffraction)

X-rays are electromagnetic radiation with photon energies between the ranges of 100 eV-100 keV. In diffraction applications, short wavelength x-rays are commonly used (1 keV-120keV). The reason is the wavelength of x-rays is comparable to the size of atoms, as they are appropriate for probing the structural arrangements of atoms and molecules in a wide range of materials.

They are produced whether by x-ray tubes or synchotron radiation. X-ray tube is a kind of primary x-ray source which is used in labs x-rays instruments. X-rays are produced when a focused electron beam accelerated across a high voltage field bombards a stationary or rotating solid target. When electrons bumped into atoms which are in the target, ongoing spectrum of x-rays are scattered. The high energy electrons are also emitting inner shell electrons in atoms through the ionization process. As a free electron fills the shell, an x-ray photon with energy characteristics of the target material is scattered (Materials and Research Laboratory-University of California, 2011).

X-ray diffraction is an important characterization method to determine the 3D structure of proteins. XRD provides structural knowledge related to the molecular function. It is used to determine crystalline structures on samples. X-Ray diffraction is also used in sugar-protein mixtures for the particle determination of sugar crystallines (Adhikari et al., 2009).

XRD is commonly used in the thin protein films, and in macromolecular films for the structural determination (Birkholz, 2010). In protein thin films, protein crystallization and for the thin film characterization XRD method is used (Pechkova and Nicolini, 2002; Byrne et al., 2009).

2.14. Thesis Objectives

The aim of this study was to investigation the structural and functional properties of bovine pancreatic trypsin after gelation and aggregation processes. Protein concentration, NaOH concentration and $CaCl_2$ concentration affects on gelation were determined by gelation time studies. Trypsin stability studies were carried out to observe the specific activity lost after gelation process. Resolubility studies were performed to observe trypsin gel resolubilization behaviour in different solutions. Secondary structural changes were studied using the FTIR method. GI-XRD studies were use to investigate any crystal formation in the thin film. SEM was performed for visualizing the morphological changes after gelation and aggregation processes. Iodoacetamide was used to observe the affect of intermolecular disulfide bonding on gelation. The QCM experiments were carried out to observe the trypsin thin film gas absorbtion ability as a potential biotechnological application.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

The reagents and solutions used throughout the experiments are presented in Appendix A. The experimental procedures are presented in Appendix B.

3.2. Methods

3.2.1. Trypsin Gel and Aggregate Preparation

Trypsin gelation and aggregation experiments were performed between the trypsin concentrations of 2 mg/ml and 10 mg/ml. Both in trypsin gelation and aggregation the starting solution and isopropanol were used. For sucrose added trypsin gels, concentrations of 10 mg/ml, 20 mg/ml and 30 mg/ml sucrose was used for the gel preparations. For trypsin gels with different NaOH concentrations, gelation was performed with the NaOH concentrations of 0.001 M, 0.01M, 0.1 M, 0.5 M, 1 M, 2 M, 2.5 M, 4 M and 5 M. For trypsin aggregation experiments concentrations of 2.2 mg/ml, 6.6 mg/ml, 11.1 mg/ml, 20 mg/ml, 22.2 mg/ml, 33.3 mg/ml, 40 mg/ml, 44.4 mg/ml and 55.5 mg/ml $CaCl_2$ were used.

3.2.1.1. Trypsin Gelation Procedure

In the gelation experiments, various amounts of trypsin concentrations from 2 mg/ml to 10 mg/ml were used. Trypsin from bovine pancreas was used. Trypsin concentrations between 2 mg/ml and 10 mg/ml were weighed in ependorff tubes and protein solutions with certain concentrations were prepared in 0.1 ml volume of starting solution and 0.1 ml of isopropanol were added onto trypsin solution.

Finally, 0.05 ml of 0.1 M NaOH was added. Protein gelation takes place in a few minutes. The concentrations of 10 mg/ml, 20 mg/ml and 30 mg/ml sucrose and 2.2 mg/ml, 6.6 mg/ml, 11.1 mg/ml, 20 mg/ml, 22.2 mg/ml, 33.3 mg/ml, 40 mg/ml, 44.4 mg/ml, 55.5 mg/ml $CaCl_2$ and 0.001 M, 0.01 M, 0.1 M, 0.5 M, 1 M, 2 M, 2.5 M, 4 M, 5 M NaOH were used in the gelation experiments (Figure 3.1).

Figure 3.1. Trypsin Gelation Procedure

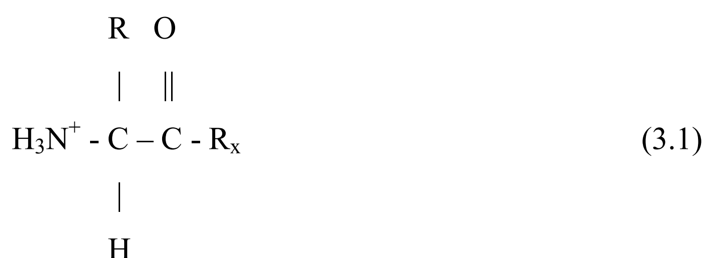
3.2.2. Inverted Tube Method for the Gelation Time Determination

Gelation time determination was carried out according to the inverted tube method. Gelation is determined regarding the gel in the tube which doesn't flow or slip (Lawal et al., 2004; Gupta et al., 2006; Huda et al., 2001).

3.2.3. Enzymatic Assay and Standard Curve Development

3.2.3.1. Enzymatic Assay

The biological activity of the protein is mainly determined by its active site. Structural alterations lead to either loss of biological activity or a decline in it. An enzymatic assay is a unique tool in determining the quality of protein samples. For an accurate measurement of biological activity, reliable and reproducible assay procedure is involved. The assay which was used in measurements was the assay procedure of Schwert and Takenaka (1955) and it was later modified by Fenton (1993).



(3.1)

In the depict formula R_X may be an amino, peptide, or ester linkage. As trypsin owns strong esterolytic reactivity, it provides a useful property in biological activity determination of the sample. According to Fenton (1993) assay method, which was used in this study, trypsin catalyzes the hydrolysis of N-benzoyl-L-arginine ethyl ester (BAEE) to N-benzoyl-L-arginine (BA) and ethanol.

$$BAEE \xrightarrow{Trypsin} BA + Ethanol \quad (3.2)$$

This reaction is known to follow Michaelis-Menten kinetics.
With the help of the spectrophotometry, the trypsin-BAEE assay is performed in simple and straight forward manner. Spectrophotometry provides a linear relation between the fractional decrease and the concentration of an absorbing substance. This relation is also known as Beer-Lambert Law:

$$\mathbf{A=\varepsilon b C_n} \quad (3.3)$$

In the formula **A** denotes absorbance at a specific wavelength, **ε** denotes molar extinction coefficient of substance **n** at that wavelength (L/mmol.cm), and $\mathbf{C_n}$ denotes the molar concentration (mmol/L), and **b** denotes the light path length (cm).

The enzymatic assay provides the monitoring of rate conversion of the substrate, BAEE, to BA by trypsin over time. At 253 nm absorbance, the maximum differential absorbance between BAEE and BA occurs. By using spectrophotometric UV absorbance at 253 nm, it is possible to monitor the amount of BA present over time. As several species (e.g. BAEE and BA) are found in solution at a given time, it is possible to monitor the absorbance values. Due to the presence of multiple species in solution Beer-Lambert Law changes to:

$$A=b(\varepsilon_{BAEE}C_{BAEE} + \varepsilon_{BA}C_{BA}) \quad (3.4)$$

The change in absorbance over time, t, for the solution will be:

$$\Delta A = A_{t = t} - A_{t = 0} \quad (3.5)$$

The previous equations changes to below equation after algebraic manipulations,

$$\Delta A = b(\varepsilon_{BA} - \varepsilon_{BAEE})(C_{BAEE\ t=0} - C_{BAEE}) \quad (3.6)$$

$$\mathbf{\Delta A = b(\varepsilon_{BA} - \varepsilon_{BAEE})C_{BA}} \quad (3.7)$$

In the formula, **ΔA** denotes the change in absorbance at 253 nm, **b** denotes the light path of 1 cm, $\mathbf{C_{BA}}$ denotes the molar concentrations of BAEE and BA present in the solution (mmol/L), and $\mathbf{(\varepsilon_{BA} - \varepsilon_{BAEE})}$ denotes the difference in molar extinction coefficients (L/mmol.cm) of BA and BAEE. One of the important assay conditions, which was the difference. As a conclusion $\mathbf{(\varepsilon_{BA} - \varepsilon_{BAEE}) = 1}$ L/mmol.cm and **b** = 1 cm,

$$\Delta A = C_{BA} \quad (3.8)$$

The slope of the absorbance vs. time graph will be the production rate of BA (e.g. amount BA produced/unit time). Michaelis-Menton constant, which is relatively small, K_m, of 4.3 X 10^{-3} mM for the hydrolysis of BAEE by trypsin is the presentation of the high specificity for BAEE. The Michaelis-Menten kinetic equation degraded to $V = k[E]$, where V denotes the maximum rate of reaction, k denotes for forward rate constant and [E] denotes the total enzyme concentration.

An optimum condition for the trypsin-BAEE assay protocol is alkaline conditions. However, it is known that trypsin owns autocatalytic activity above pH 5.0. Due to this Fenton needed to add $CaCl_2$ that which is necessary to add 20 mM $CaCl_2$ to assay buffer to stabilize trypsin. It is regarded that calcium ions provide a stable complex formation with trypsin and as a conclusion prevents trypsin from autolysis (Carter, 1990).

3.2.3.2. Standard Curve Determination Protocol

7.2 mg trypsin was dissolved in 7.2 ml starting solution for the enzyme solution. The data obtained here are used for standard curve determination. The measurements were performed at 278.0 nm wavelength 3.2 ml quartz cuvettes were used for the measurements. In the cuvette, tris buffer and enzyme solution were mixed by the inversion of the tube and the absorbance was taken. Later, the absorbance (278.0 nm) vs trypsin concentration (mg/ml) graph was performed due to measurements. The extinction coefficient for trypsin was determined from slope of the graph.

Table 3.1. Standard curve for trypsin concentration determination

Enzyme solution (ml)	Tris Buffer (ml)	Trypsin concent.(mg/ml)	Abs. (278 nm)
0,1	0,9	0,1	0,1389
0,2	0,8	0,2	0,2836
0,4	0,6	0,4	0,5541
0,5	0,5	0,5	0,6920
0,6	0,4	0,6	0,8191

By using this table, an equation was performed via the slope of the points. The graph was performed on five points which were on the line.

Table 3.2. The Standard Curve Determination Absorbance Data at 278 nm

Sample ID	Concentration	WL278,0
1	0,1	0,1389
2	0,2	0,2836
3	0,4	0,5541
4	0,5	0,692
5	0,6	0,8191

By using the datas on the above (Table 3.2) standard curve determination graph (Figure 3.2) was performed.

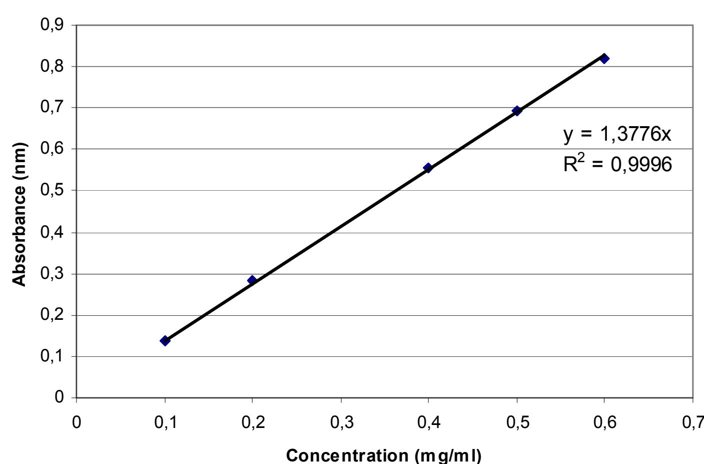


Figure 3.2. Standard curve determination graph (on five points).

According to this graph, the equation of **y = 1,3776x** was obtained and used in $C_{trypsin}$ calculations. y denotes absorbance and x denotes concentration. By putting the absorbance at 253 nm to y place we will able to calculate the protein concentration (x) at that absorbance. All the absorbance measurements were carried out with UV-2450 Shimadzu UV-VIS Spectrophotometer.

3.2.3.3. Protein Concentration Determination

The absorbance at 278 nm is needed to calculate ΔA_{min} value for the specific activity calculation formula. At 278 nm, ΔA_{min} gives the maximum absorbance change in minute.

3050 µl tris buffer and 150 µl substrate solution is needed for blank readings. They are added into 4 ml quartz cuvette. Later, 2850 µl tris buffer, 150 µl substrate solution and 200 µl of our diluted samples of 1/100 original trypsin, 1/10 trypsin gel and 1/10 sucrose added trypsin gel were added and mixed by the inversion of the quartz cuvette and later the absorbance at 278.0 nm was read. This was repeated three times for every sample. Here, absorbance vs time graph is formed due to readings and the slope of the two points on the line will give $\Delta A_{min.}$

3.2.3.4. Trypsin Specific Activity Measurements

This absorbance is needed to calculate the concentration of trypsin or the sample in the 253.0 nm absorbance. The equation of y = 1,3776x was used for the sample concentration calculation. y denotes the absorbance in the 253.0 nm absorbance and x denotes the concentration of the sample in the 253.0 nm absorbance. Here the equation will be used in calculation of the $C_{trypsin}$ value.

1300 µl tris buffer was added into 3.2 ml of quartz cuvette for blank reading. Later our diluted samples 200 µl was added into quartz cuvette by order of 1/100 original trypsin, 1/10 trypsin gel, and 1/10 sucrose added trypsin gel onto the 1300 µl tris buffer and mixed by the cuvette inversion and later the absorbance was read at 253.0 nm. It was repeated 3 times for every sample.

The specific activity formula was given below;

$$\text{Activity ("Sigma" units)} = \frac{(\Delta A_{min}) / (df)}{(0.001)(0.2)(C_{trypsin})} \qquad (3.9)$$

ΔA_{min} = maximum absorbance change/minute

df = dilution factor used

$C_{trypsin}$ = concentration of enzyme solution (mg/ml)

0.2 = volume of enzyme solution used (ml)

0.01 = change in A_{253}/min per unit trypsin at pH 7.6, T=25 C
in 3.2 ml reaction volume

The specific activity of the original trypsin, trypsin gel, trypsin gel with sucrose and trypsin gels with different NaOH concentrations were measured.

3.2.4. The Resolubilization of Trypsin Gel Studies

Resolubilization of gels was carried out by using deionized water, isopropanol, water-isopropanol mixture and starting solution. 5 mg/ml trypsin and 30 mg/ml sucrose were used for experiments. Gelation was performed according to trypsin gel formation procedure. After gel formation 0.4 ml of deionized water, alcohol, water alcohol mixture, and starting solution were added onto gels for dissolution.

3.2.5. Fourier Transform Infrared Spectroscopy (FTIR) Studies

FTIR analyses were carried out for trypsin, trypsin gel and sucrose added trypsin gel to observe the secondary structural changes. The analysis was performed on Perkin-Elmer Spectrum 100 FTIR spectrometer (Perkin-Elmer Inc., Norwalk, CT, USA) was used.

For FTIR experiments, concentrations of 6 mg/ml trypsin and 20 mg/ml sucrose were used. The gel was lyophilized by Labconco FreeZone lyophilizator for one night. 225 mg KBr was mixed with 0.5 mg trypsin, lyophilized trypsin gel and sucrose added trypsin gel and the mixture was then exposed to a pressure of 1100 kg/cm^2 in an evacuated to produce a KBr pellet for use in FT-IR spectrometer. The spectra were recorded in the 4000–450 cm^{-1} region at room temperature. A total of 20 scans were taken for each interferrogram at 4 cm^{-1} resolution.

Spectrum 100 software (Perkin-Elmer) was used for all of the data manipulations. From trypsin powder, gel and aggregate (n=3) multiple scans, which gave identical spectra, were performed. These replicates were averaged and the averaged spectra for each sample were then used for further data manipulation and statistical analysis. Then, the spectra were interactively baselined from two arbitrarily selected points. Finally, the spectra were normalized in specific regions for visual comparison of the trypsin samples.

The gel and aggregate formations cause secondary structural variations in the Amide I band (1700-1600 cm^{-1}) intensity calculations provided by second derivative spectra analysis. The spectrum of second derivation was performed by a Savitzky–Golay algorithm with five smoothing points. The second derivative signals of the minima peak were used as the peak positions of original absorption spectra correlation (Ozek et al., 2010; Toyran et al., 2006).

3.2.6. Iodoacetamide (IAA) Studies

Iodoacetamide (IAA) was used as the disulfide blocking agent. 1 M and 2 M IAA were used for 5 mg/ml trypsin and 30 mg/ml sucrose concentrations to observe the gelation time changes in trypsin gel and trypsin gel with sucrose.

3.2.7. Scanning Electron Microscopy (SEM) Studies

Scanning electron microscope (SEM) images were performed in FEI Quanta250 FEG. ESEM mode was used due to its ability to operate under humid conditions. Concentrations of 6 mg/ml trypsin, 30 mg/ml sucrose and 40 mg/ml $CaCl_2$ were used for the SEM experiments. SEM images of trypsin, trypsin gel, trypsin aggregate, trypsin gel with sucrose were taken at 20 μm, 50 μm and 200 μm magnifications. All the samples were left drying for 2 days to get rid of the gel humidity. Later, the dried samples of trypsin gel, trypsin aggregate, trypsin gel with sucrose and trypsin were firstly exposed to N_2 gas (for coating) and later analyzed under the SEM device.

3.2.8. Quartz Crystal Microbalance (QCM) Studies

QCM experiments were carried out with the concentration of 5 mg/ml trypsin. Trypsin gel was performed according to trypsin gel formation procedure and the mixture was added onto QCM plate and waited for the gelation.

Later the gel onto QCM plate placed into ethanol and ultrasonically cleaned, then rinsed by de-ionized water to get rid off the gel bulk. The gel was washed until a thin film formation took place. Later, the thin film on the plate was dried using dry N_2. Finally, the QCM plate with thin film put into the teflon housing and the reference frequency was recorded according to the trypsin thin film's sensing ability for different gases (with the gases of benzene (C_6H_6), carbon monoxide (CO), carbondioxide (CO_2), dichloromethane (CH_2Cl_2), hydrogen peroxide (H_2O_2) and propanol (CH_3CH_2OH)).

A time-resolved electrochemical quartz crystal microbalance (EQCM) with the model of CHI400A Series from CH Instruments (Austin, USA) was used to measure the changes in the resonance frequency of quartz crystals between gold electrodes via both serial and USB interface connected to a computer. The QCM works with oscillation frequencies between 7.995MHz and 7.950 MHz. The density (ρ) of the crystal is 2.684 g/cm^3, and the shear modulus (μ) of quartz is 2.947×1011 $g/cm\ s^2$. Around oscillation frequency of 7.995 MHz, a net change of 1 Hz corresponds to 1.34 ng of materials adsorbed or desorbed onto the crystal surface of an area of $0.196 cm^2$.

3.2.9. X-Ray Diffraction (XRD) Studies

XRD experiments were performed for original trypsin and trypsin thin film. For original trypsin and trypsin thin film GI-XRD studies were performed by using Phillips X́`pert Pro.

CHAPTER 4

RESULTS AND DISCUSSION

Within this chapter, the results of this investigation are given in detail. Section 4.1 gives the details related to variations of gelation time with protein, NaOH and $CaCl_2$ concentrations. Section 4.2 deals with the phase behaviour of trypsin under varying trypsin, NaOH and $CaCl_2$ concentrations. In section 4.3 the investigations related to the effect of sucrose on trypsin gelation under various conditions were presented. In section 4.4 the resolubilization studies of trypsin gel with and without sucrose are presented. In section 4.5 trypsin stability studies following gelation and resolubilization are presented. In section 4.6 the studies carried out to delineate the protein structure using the FTIR method are presented. In section 4.7 Iodoacetamide studies regarding the nature of the intermolecular interactions governing the gelation are presented. In section 4.8 the SEM studies on the dried gels are presented. In section 4.9 the results of the QCM studies are presented. Finally, the results of the XRD studies regarding the investigation of any crystal formation are presented.

4.1. Gelation Time Studies

Gelation time is necessary regarding the behaviour of the protein gelation under different circumstances. Different physical and chemical conditions such as pH, acidity and composition can change gelation time significantly. Therefore, experiments were designed to investigate the gelation process in terms of gelation time under different physical and chemical conditions.

4.1.1. The Effect of Protein Concentration on Gelation Time

In protein gelation, protein concentration is important. The experiments were designed to investigate the effect of protein concentration on gelation time.

Figure 4.1 presents gelation time with different trypsin concentrations. The selected protein concentrations were 10 mg/ml, 7.5 mg/ml, 5 mg/ml, 4 mg/ml and 3 mg/ml. The gelation experiments were carried out in 0.1 M NaOH and in the absence of $CaCl_2$ and in the room temperature.

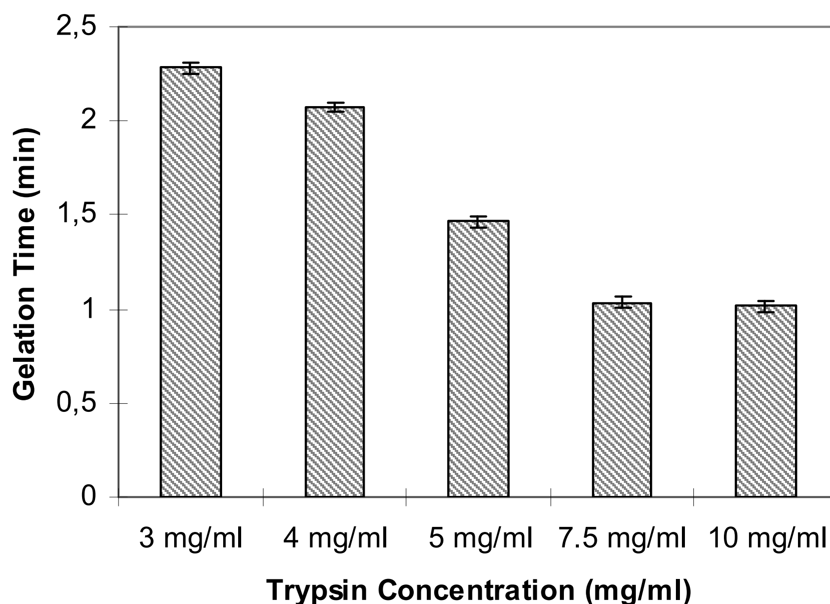


Figure 4.1. The gelation time under different trypsin concentrations (0.1 M NaOH and 3 mg/ml, 4 mg/ml, 5 mg/ml, 7.5 mg/ml, 10 mg/ml trypsin were used in the absence of $CaCl_2$ at room temperature).

Figure 4.1 shows the gelation time behaviour under selected trypsin concentrations. 0.1 M NaOH was used for all the trypsin concentrations in the absence of $CaCl_2$. Here, it is observed that as trypsin concentration increases, the gelation time decreases. It can be seen in Figure 4.1 that as trypsin concentration reaches 10 mg/ml the gelation time reaches a minimum level asymptotically. This behaviour is related to the trypsin molecules interactions among themselves. More trypsin means more interaction of trypsin molecules which leads the gelation time to reaching a point. As trypsin concentration increases, the interaction with the other trypsin molecules will be more probable and gelation happens in a much shorter time than lower trypsin concentrations.

4.1.2. The Effect of NaOH Concentration on Gelation Time

In this part, the behaviour of the trypsin gelation process under different NaOH concentrations was studied. 5 mg/ml was used for all the NaOH concentrations (5 M, 2.5 M, 1 M, 0.5 M, 0.01 M and 0.001 M) in the absence of $CaCl_2$ at room temperature.

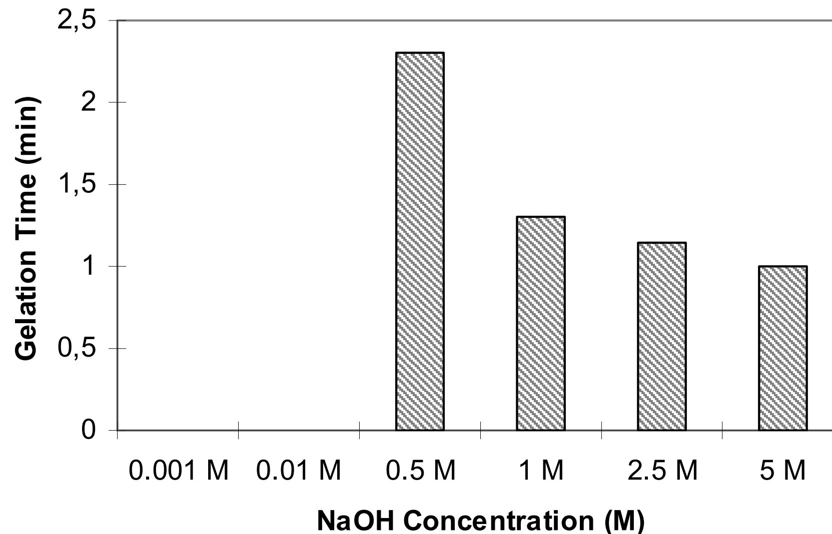


Figure 4.2 The gelation time at different NaOH concentration (5 mg/ml trypsin 0,0001 M, 001 M, 0.5 M, 1 M, 2.5 M, 5 M NaOH were used in the absence of $CaCl_2$ at room temperature).

At 5 M, 2.5 M, 1 M and 0.5 M NaOH concentrations gelation was observed. In contrast to at 0.01 M and 0.001 M NaOH concentrations no gelation was observed and the samples remained in solution form (Figure 4.2). Here, it can be claimed that a particular NaOH concentration is needed for gelation to take place and as the NaOH concentration increased the gelation time decreased.

In protein gels, protein solubility is highly influenced by pH (Choi et al., 2000). Setting out from this point according to Figure 4.2 it can be concluded that the increasing NaOH concentration leads to a decrease in trypsin solubility and gelation takes place. As the solubility of trypsin molecules decreased, their interaction with each other and environment increased and as a result, a decrease in gelation time is observed. This is apparent when the basic nature of trypsin is considered (pI = 10.5).

4.1.3. The Effect of $CaCl_2$ Concentration on Gelation Time

In this part, the effect of $CaCl_2$ addition on trypsin gelation was studied. For all the $CaCl_2$ concentrations studied, 5 mg/ml trypsin and 0.1 M NaOH were used.

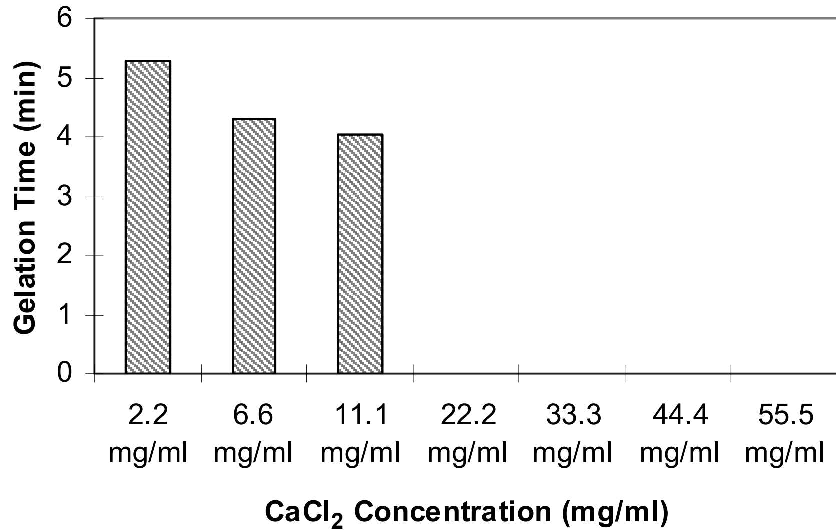


Figure 4.3. The gelation time determination with the presence of $CaCl_2$ (5 mg/ml trypsin and 0.1 M NaOH were used for the concentrations of 2.2 mg/ml, 6.6 mg/ml, 11.1 mg/ml, 22.2 mg/ml, 33.3 mg/ml, 44.4 mg/ml, 55.5 mg/ml $CaCl_2$ at room temperature).

In 2.2 mg/ml, 6.6 mg/ml and 11.1 mg/ml concentrations of $CaCl_2$, gelation was observed whereas in 22.2 mg/ml, 33.3 mg/ml, 44.4 mg/ml and 55.5 mg/ml concentrations of $CaCl_2$ aggregation was observed.

In cold-set gelation of whey proteins $CaCl_2$ and many different salts (e.g. NaCl, KCl) are commonly used (Boulet et al., 2000; Hongsprabhas and Barbut, 1998; Hongsprabhas et al., 1999). $CaCl_2$ addition to medium causes neuralisation of electrostatic interactions at whey protein gel formation (Maltais et al., 2005). In another study (related with whey proteins), it was observed that at low $CaCl_2$ concentrations (10 mM or lower than 10 mM), gelation was slow and ordered, fine stranded gels were obtained. At high $CaCl_2$ concentrations (higher than 10 mM), gelation was fast and aggregates were obtained (Kuhn et al., 2010).

At high salt concentrations electrostatic repulsion between protein molecules screened sufficiently so that protein molecules can come to close distances to form aggregates (Chantrapanchai and McClements, 2002).

According to Figure 4.3 between the $CaCl_2$ concentrations of 2.2 mg/ml and 11.1 mg/ml gelation was observed. Gelation becomes slower as $CaCl_2$ concentration reaches lower/lowest $CaCl_2$ concentrations as in Kuhn's work. Between the $CaCl_2$ concentrations of 22.2 mg/ml and 55.5 mg/ml aggregation was observed instead. Also, Kuhn observed aggregates at high $CaCl_2$ concentrations. In this study, under some concentrations of $CaCl_2$ gelation, aggregation was observed. It can be said that at lower $CaCl_2$ concentrations (2.2 mg/ml, 6.6 mg/ml and 11.1 mg/ml) $CaCl_2$ causes neutralisation of electrostatic interactions and three dimensional network formations which resulted in gelation. At high $CaCl_2$ concentrations (2.2 mg/ml, 33.3 mg/ml, 44.4 mg/ml and 55.5 mg/ml) as $CaCl_2$ concentration is found more than needed for neutralisation, the same charged ions in trypsin molecules will start to repel each other and rather than gelation aggregation will be observed. In addition, $CaCl_2$ binds to water (in food industry $CaCl_2$ is used as a firming agent and recommended by U.S. Food and Drug Administration because of being regarded safe) and as the $CaCl_2$ concentration increases, it holds more water molecules decreasing the water concentration in protein solution and leads to increased protein concentrations thus increasing the protein-protein interactions.

4.2. Phase Behavior of Trypsin under Varying Trypsin, NaOH and $CaCl_2$ Concentrations

Throughout the studies three different phases were observed due to different environmental factors namely protein concentration, $CaCl_2$ concentration and NaOH concentrations. Among these enviromental factors, NaOH concentration determines pH of the solution and $CaCl_2$ concentration determines the ionic strength of the solution. The phase change of trypsin is represented in Figure 4.4 upon addition of NaOH solution.

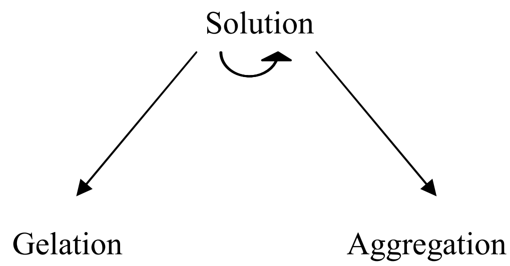


Figure 4.4. The proposed phase change model for trypsin

In Table 4.1, the phase behavior is presented under certain experimental conditions namely trypsin concentration, $CaCl_2$ concentration and NaOH concentration. 2 mg/ml, 6 mg/ml and 10 mg/ml trypsin, 0 mg/ml, 20 mg/ml and 40 mg/ml $CaCl_2$ and 0 M, 2 M and 4 M NaOH were used for the experiments.

Table 4.1. Phase behaviour of at different trypsin, NaOH and $CaCl_2$ concentrations.

Trypsin Concentration (mg/ml)	NaOH Concentration (M)	$CaCl_2$ Concentration (mg/ml)	Observed Phase
2	0	0	Solution
2	0	20	Gelation
2	0	40	Gelation
2	2	0	Solution
2	2	20	Aggregation
2	2	40	Aggregation
2	4	0	Solution
2	4	20	Aggregation
2	4	40	Aggregation
6	0	0	Solution
6	0	20	Gelation
6	0	40	Gelation
6	2	0	Gelation
6	2	20	Aggregation
6	2	40	Aggregation
6	4	0	Gelation
6	4	20	Aggregation
6	4	40	Aggregation
10	0	0	Solution
10	0	20	Gelation
10	0	40	Gelation
10	2	0	Gelation
10	2	20	Aggregation
10	2	40	Aggregation
10	4	0	Gelation
10	4	20	Aggregation
10	4	40	Aggregation

In Figure 4.5, the phase behavior is shown in three dimensional spaces for better demonstration purposes. Each phase is shown with a different color. Since the phase diagram includes three different phases for three different environmental conditions, the conclusions drawn from the figure present a complex array of data. Therefore, the data were analyzed according to varying protein concentration levels for two other environmental conditions.

Independent from trypsin concentration, solution phase was observed in the absence of NaOH and $CaCl_2$. In all the trypsin concentrations studied, gelation was observed in the presence of $CaCl_2$ and NaOH (even in the lowest trypsin concentration). In the lowest trypsin concentration, gelation was observed because $CaCl_2$ holds water and trypsin molecules distances become shorter and through the intermolecular interactions with each other, gelation was observed. At the lowest trypsin concentration (2 mg/ml) in the presence of NaOH and in the absence of $CaCl_2$ gelation was not observed because trypsin concentration was not sufficient to form three dimensional network and trypsin molecules were not in close distances. At higher trypsin concentrations (6 mg/ml and 10 mg/ml) gelation was observed because sufficient trypsin concentration for gelation is provided (in the absence of $CaCl_2$).

At 6 mg/ml trypsin concentration gelation was observed in the presence of either NaOH or $CaCl_2$. In contrast, aggregation was observed in the prensence of both NaOH and $CaCl_2$. In other words, when $CaCl_2$ and NaOH were both present in the medium at the same time, aggregation was observed. Similarly, 10 mg/ml trypsin concentration also showed the same behaviour with 6 mg/ml trypsin concentration.

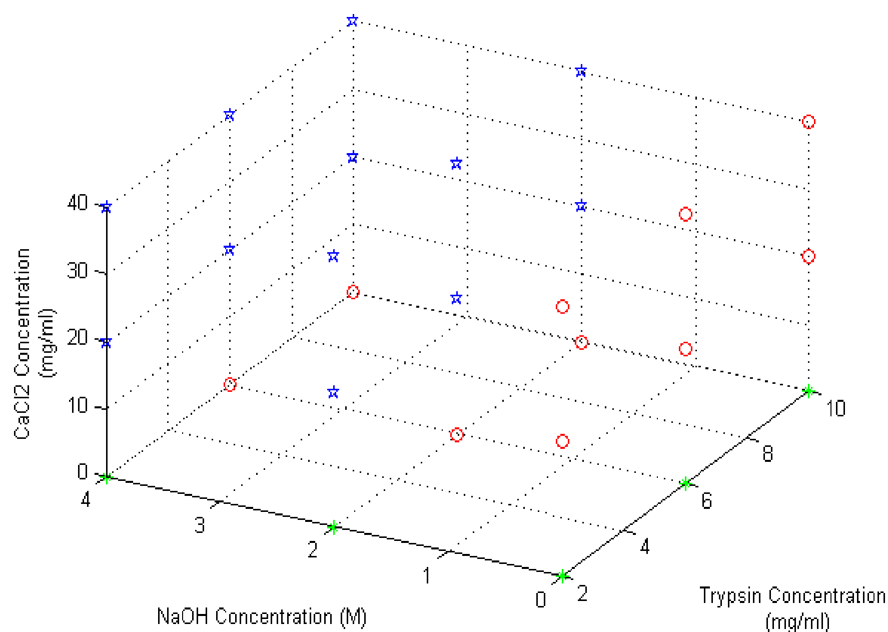


Figure 4.5. The phase behaviour of trypsin as a function of protein concentration, pH and ionic strength (colors in the figure denotes green star : solution, blue star: aggregate, red circle/ring: gelation).

Figure 4.5 shows the total phase behaviour of trypsin under the experimental conditions studied as solution, gelation and aggregate.

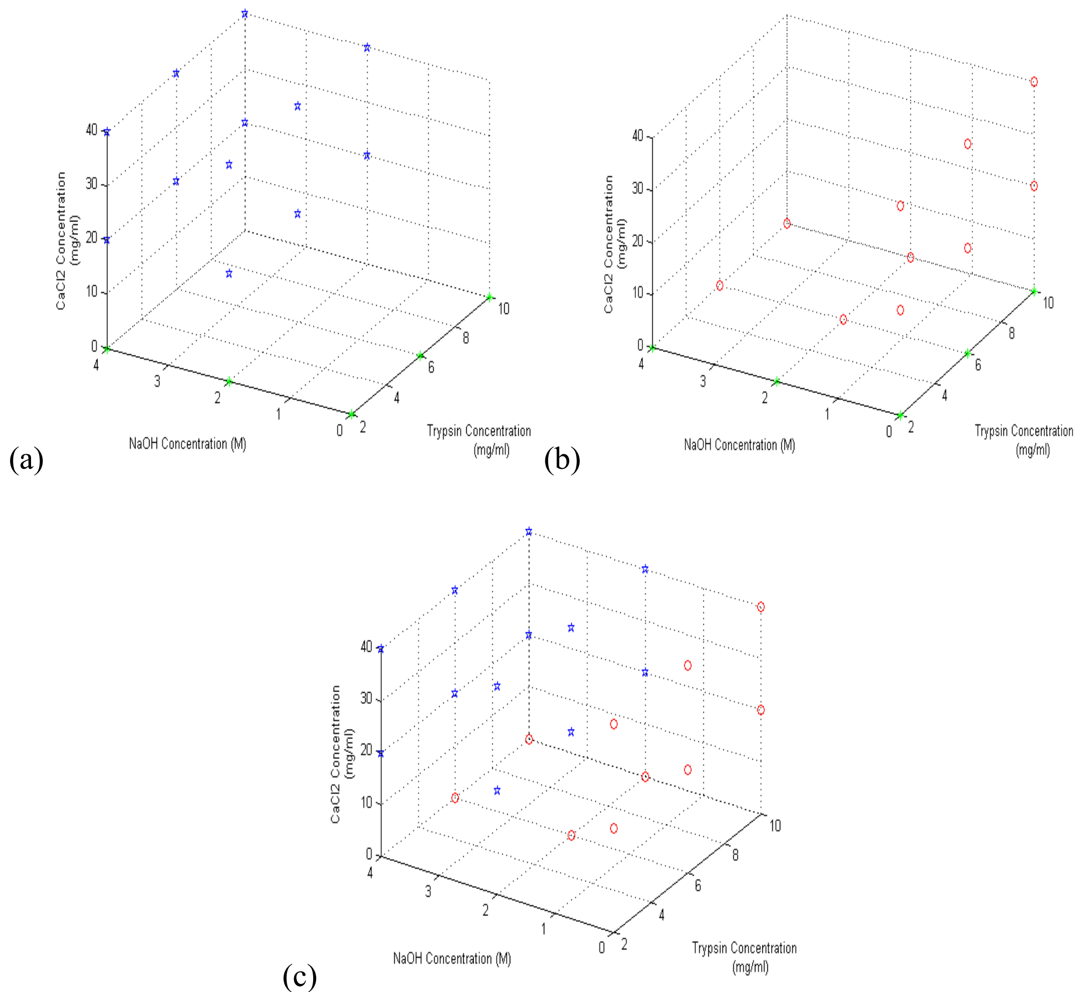


Figure 4.6. (a) The phase behaviour of trypsin as a function of protein concentration pH and pH and ionic strength in the form of solution and aggregation (colors in the figure denotes green star: solution, blue star: aggregate).(b) The phase behaviour of trypsin as a function of protein concentration, pH and ionic strength in the form of solution and gelation (colors in the figure denotes green star: solution, red circle/ring: gelation). (c) The phase behaviour of trypsin as a function of protein concentration, pH and ionic strength in the form of gelation and aggregation (colors in the figure denotes blue star: aggregate, red circle/ring: gelation).

In all trypsin concentrations in Figure 4.6 (a) in the absence of both NaOH and $CaCl_2$ solution phase was observed. At the lowest concentration, in the presence of $CaCl_2$ and in the absence of NaOH, gelation was observed due to $CaCl_2$ binding effect to water. In contrast, in the presence NaOH and in the absence of $CaCl_2$, the solution was obtained due to the insufficient concentration of trypsin. In all trypsin concentration aggregation was observed in the presence of both NaOH and $CaCl_2$.

Gelation was observed in Figure 4.6 (b) in the presence of either NaOH or $CaCl_2$ except the 2 mg/ml trypsin concentration because in the presence of NaOH and in the absence of $CaCl_2$ due to the insufficient trypsin concentration gelation wasn't observed. In all trypsin concentrations, in the absence of both NaOH and $CaCl_2$ solution phase was observed. At the lowest concentration in the presence of $CaCl_2$ and in the absence of NaOH gelation was observed due to $CaCl_2$ binding effect to water. In contrast to this in the presence NaOH and in the absence of $CaCl_2$ solution was observed due to the insufficient concentration of trypsin.

Aggregation was observed, independent from trypsin concentration, in the presence of both NaOH and $CaCl_2$ in Figure 4.6 (c) gelation was observed in the presence of either NaOH or $CaCl_2$ but the lowest trypsin concentration has an exception related to the insufficient trypsin concentration.

4.3. The Effect of Sucrose on Trypsin Gelation Time

Carbohydrates are one of the food ingredients and are also commonly used in food industry. In addition, carbohydrates are added into the food formulations to improve their gelation properties (Yemisi et al., 2007). Since sucrose was one of the important food additives in carbohydrate nature, it was used to determine its effects on trypsin gelation.

4.3.1. The Effect of Sucrose Concentration on Gelation Time

To observe the effect of sucrose on gelation time gelation experiments were carried out using sucrose at 5 mg/ml trypsin and 0.1 M NaOH concentrations. The sucrose concentrations used were 10 mg/ml, 20 mg/ml and 30 mg/ml as seen in Figure 4.7.

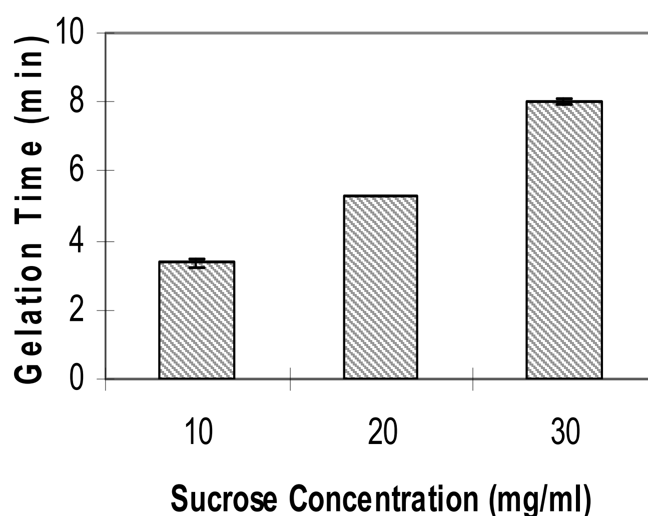


Figure 4.7. The dependence of trypsin gelation time on sucrose concentrations (5 mg/ml trypsin and 0.1 M NaOH were used for the concentrations of 10 mg/ml, 20 mg/ml and 30 mg/ml sucrose in the absence of $CaCl_2$ at room temperature).

In Figure 4.7 it was observed that increasing sucrose concentrations caused an increase in gelation time. Carbohydrates affect gelation time. They were generally found to increase the gelation time of proteins (Christ et al., 2005; Semenova et al., 2002; Bryant et al., 2000).

4.3.2. The Effect of Trypsin Concentration on Gelation Time with Sucrose

The effect of sucrose addition on gelation time was investigated with different trypsin concentrations at the sucrose concentration of 20 mg/ml. Similarly, the NaOH concentration was kept constant at 0.1 M.

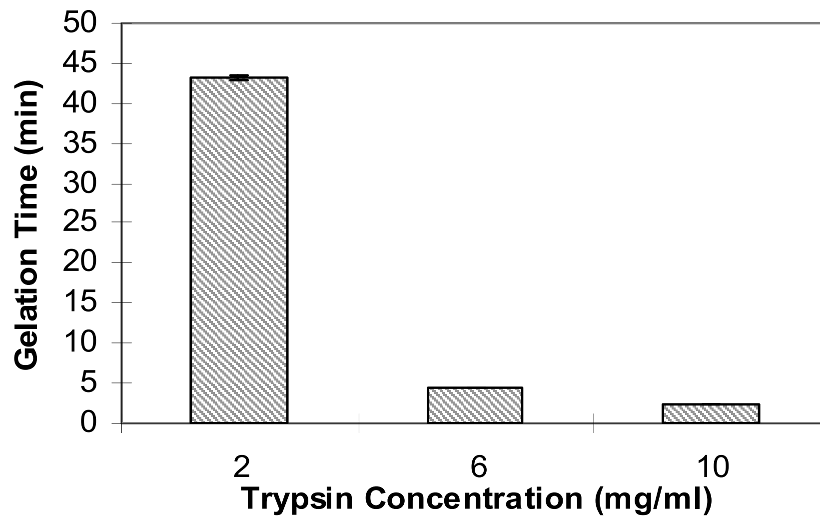


Figure 4.8. The gelation time with different trypsin concentrations (20 mg/ml sucrose and 0.1 M NaOH were used for the trypsin concentrations of 2 mg/ml, 6 mg/ml and 10 mg/ml in the absence of $CaCl_2$ at room temperature).

It was observed that gelation time increased with decreasing trypsin concentrations (Figure 4.8). Sucrose presence was not found to affect the general trend of trypsin concentration effect on gelation time when Figure 4.1 is considered.

4.3.3. The Effect of NaOH Concentration on Gelation Time with Sucrose

The effect of sucrose addition to gelation time was investigated with different NaOH concentrations (5 M, 2.5 M, 1 M, 0.5 M, 0.01 M and 0.001 M) at the sucrose concentration of 30 mg/ml. Similarly, the trypsin concentration was kept at 5 mg/ml.

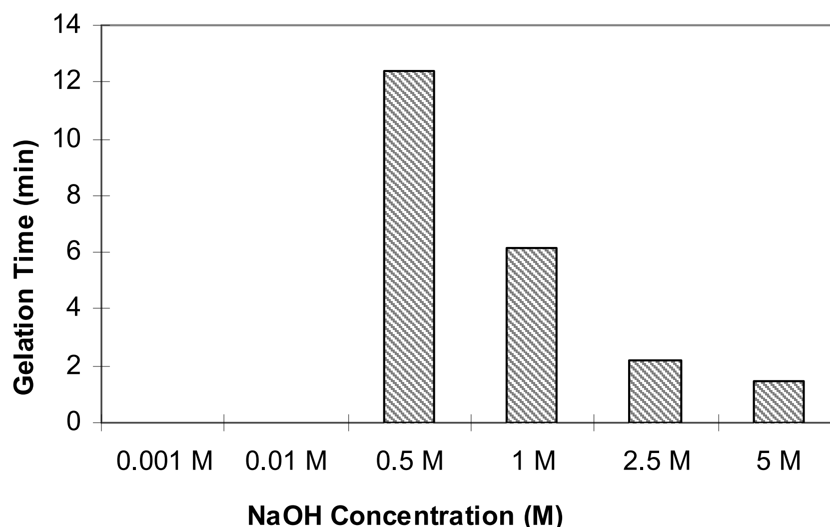


Figure 4.9. The gelation time with different NaOH concentrations (5 mg/ml trypsin and 30 mg/ml sucrose were used for the NaOH concentrations of 0.001 M, 0.01 M, 0.5 M, 1 M, 2.5 M and 5 M. In the absence of $CaCl_2$ at room temperature).

Gelation was observed in all samples except for 0.01 M and 0.001 M NaOH concentrations. In 0.01 M and 0.001 M NaOH concentrations, the samples remained in the solution phase (Figure 4.9). Also, the same behavior was observed in trypsin gelation related with 0.01 M and 0.001 M NaOH concentrations without sucrose (Figure 4.2). It was observed that gelation time decreased with increasing NaOH concentrations as in the trypsin gelation without sucrose. Sucrose presence was not found to affect the general trend of NaOH concentration effect on gelation time when Figure 4.2 is considered.

4.3.4. The Effect of $CaCl_2$ Concentration on Gelation Time with Sucrose

The effect of $CaCl_2$ addition to gelation time was investigated with different sucrose concentrations (0 mg/ml, 10 mg/ml, 20 mg/ml and 30 mg/ml) at the $CaCl_2$ concentration of 40 mg/ml. Similarly, the trypsin concentration was kept at 6 mg/ml and 2 M NaOH was used.

It was observed that gel with sucrose showed the same behavior with the gel without sucrose in $CaCl_2$ concentration of 40 mg/ml and aggregation was obtained in all sucrose concentrations. It can be pointed out that aggregation was independent from sucrose concentration, but it depends on $CaCl_2$ presence because in the absence of sucrose aggregation was observed.

4.4. The Trypsin Gel Resolubilization Studies

Resolubilization experiments were carried out to observe the trypsin gel resolubilization behaviour in different kinds of solutions. In all samples 5 mg/ml trypsin and 0.1 M NaOH were used. After gelation 0.4 ml deionized water, isopropanol, water-isopropanol mixture, and starting solution were added onto trypsin gel and waited for resolubilization.

4.4.1. The Resolubilization of Trypsin Gel

After trypsin gel formation process completed, trypsin gel without sucrose resolubilization behaviour was tested with different solutions such as deionized water, alcohol, water alcohol mixture, and starting solution (Table 4.2).

It was observed that trypsin gel easily dissolved in water and starting solution.

Table 4.2. The resolubilization behaviour of trypsin gels.

Solution	Hours		
	1 Hour	3 Hours	7 Hours
Starting Solution	+ - -	+ + -	+ + +
Water	+ - -	+ + -	+ + +
Isopropanol	+ - -	+ - -	+ + +
Isopropanol-Water Mixture	+ - -	+ + -	+ + +

(The symbols in the table denotes for +++ totally dissolved, ++_ nearly totally dissolved, +_ _ a little dissolved, _ _ _ not dissolved)

4.4.2. The Resolubilization of Trypsin Gel with Sucrose

After trypsin gel formation process was completed, trypsin gel with sucrose resolubilization behaviour was tested with different solutions such as deionized water, alcohol, water alcohol mixture, and starting solution.

Table 4.3. The resolubilization of trypsin gels with sucrose

Solution	Hours		
	1 Hour	3 Hours	7 Hours
Starting Solution	+ - -	+ + -	+ + +
Water	+ - -	+ + -	+ + +
Isopropanol	+ - -	+ - -	+ + +
Isopropanol-Water Mixture	+ - -	+ + -	+ + +

(The symbols in the table denotes for +++ totally dissolved, ++_ nearly totally dissolved, +_ _ a little dissolved, _ _ _ not dissolved)

Generally the same behaviour was observed in trypsin gel with sucrose with the trypsin gel without sucrose. Also trypsin gel with sucrose was easily dissolved in water and starting solution like trypsin gel without sucrose.

4.5. Trypsin Stability Studies

The stability experiments play an important role in understanding the structure of trypsin in the gelation process. Specific activity measurements were performed to compare the stability of the original trypsin with the trypsin gel and sucrose added trypsin gel. For comparison the specific activities of the original trypsin and the trypsin after gelation and resolubilization process were measured for both gels with and without sucrose.

4.5.1. Stability Studies of Trypsin after Gelation with and without Sucrose in Different NaOH Concentrations

The specific activities of original trypsin, trypsin gel and trypsin gel with sucrose were showed on the Figure 4.10. According to the specific activity measurements which were shown in Figure 4.10 after gelation there was decreases both in the activity of trypsin gel and trypsin gel with sucrose when compared with that of the original trypsin. This occurs because during gelation process, the trypsin gel and trypsin gel with sucrose lose their activities due to the structural changes during gelation process. In addition, it was observed that there was no strong effect of sucrose in retaining the specific activity of trypsin in the gelation process.

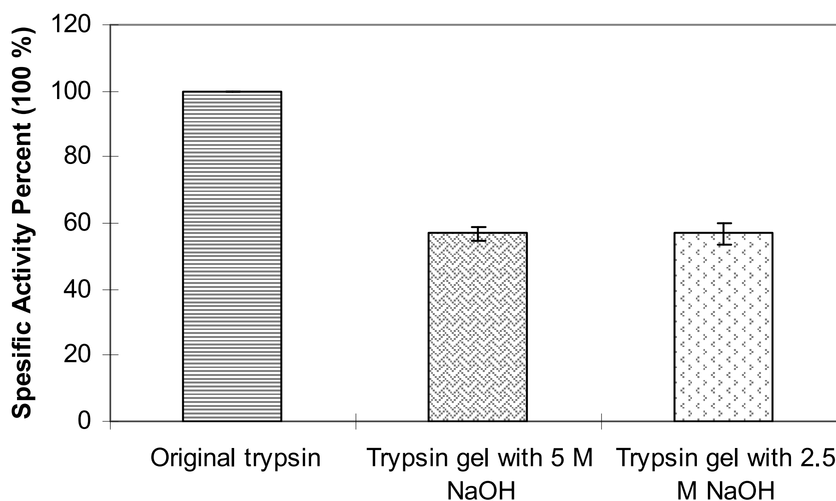


Figure 4.10. The specific activity percentages of original trypsin, trypsin gel and trypsin gel with sucrose.

4.5.2. Trypsin Stability of Trypsin Gel with different NaOH Concentrations

The specific activity percentage of 2.5 M and 5 M NaOH added trypsin gel specific activity comparements were shown in the Figure 4.13. It can be said that NaOH concentrations do not have an effect on the specific activity of trypsin after gel formation. As it is seen in Figure 4.11 the specific activities of trypsin in gels with different NaOH concentrations had nearly the same specific activities.

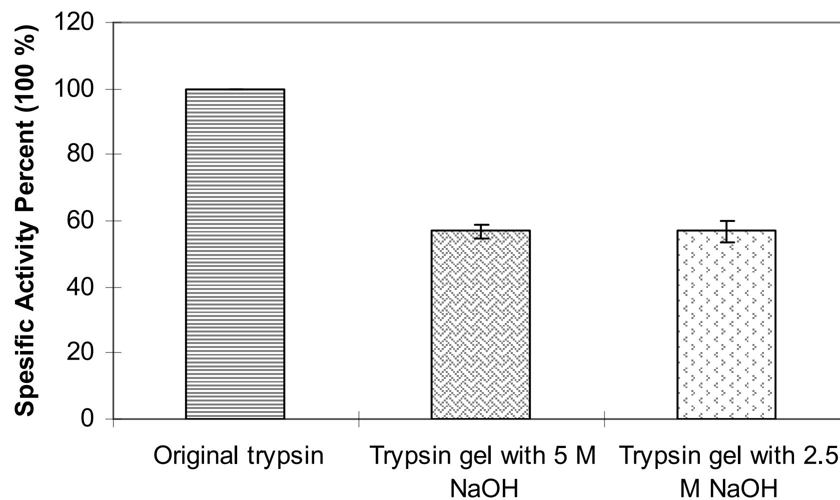


Figure 4.11. The specific activity of original trypsin and trypsin gels in two different NaOH concentrations.

4.6. FTIR (Fourier Transform Infrared) Spectroscopy Studies

FTIR analyses were applied to observe the secondary structural changes of the samples after gelation and aggregation process take place. FTIR analysis allows to comparing the secondary structural changes of the original trypsin, trypsin gel, trypsin aggregate and trypsin gel with sucrose.

In protein, studies for protein secondary structure analysis Amide I band region (between 1600 cm^{-1} and 1700 cm^{-1}) was taken into consideration.

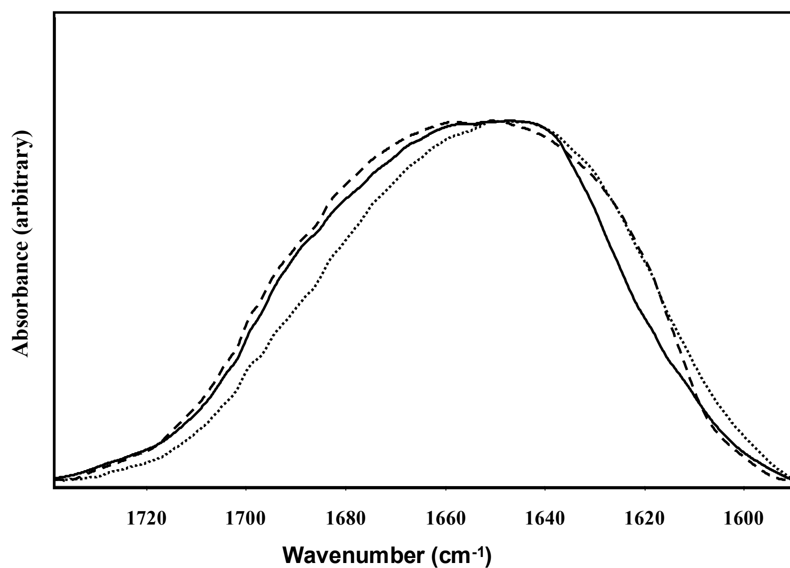
4.6.1. The Effect of Gelation on the Secondary Structure of Trypsin with and without Sucrose

The band assignments of the original trypsin, trypsin gel, trypsin aggregate and trypsin gel with sucrose were carried out in regions between 1700-1600 cm^{-1} which corresponded to the amide I band region. According to spectrums of original trypsin, trypsin gel, and trypsin gel with sucrose the peaks were shown on Figures 4.14. The Amide I band assignments of the spectrums are shown on Table 4.4 and the secondary structural assignments of the second derivative spectra were carried out according to Table 4.5.

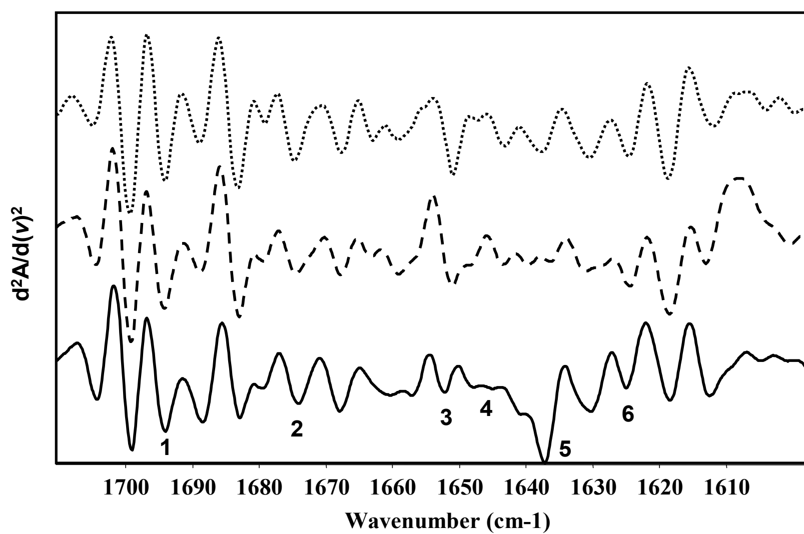
Samples spectrums were selected and their average spectrum was calculated. Later the average spectra were interactively baselined between 1740-1590 cm^{-1}. Finally, normalization was performed and second derivatives were taken on five points in the ranges of 1700-1600 cm^{-1} in the Amide I region.

Table 4.4. The band assignments of secondary structure sub-bands under Amide I band in 1700-1600 cm^{-1} region (Source: Garip et al., 2010).

Peak Number	Mean Frequencies/cm^{-1}	Assignment
1	1694	Antiparallel beta sheets
2	1674	Turns
3	1652	Alpha helix
4	1643	Random coil
5	1637	Beta sheets
6	1625	Aggregated beta sheets



a)



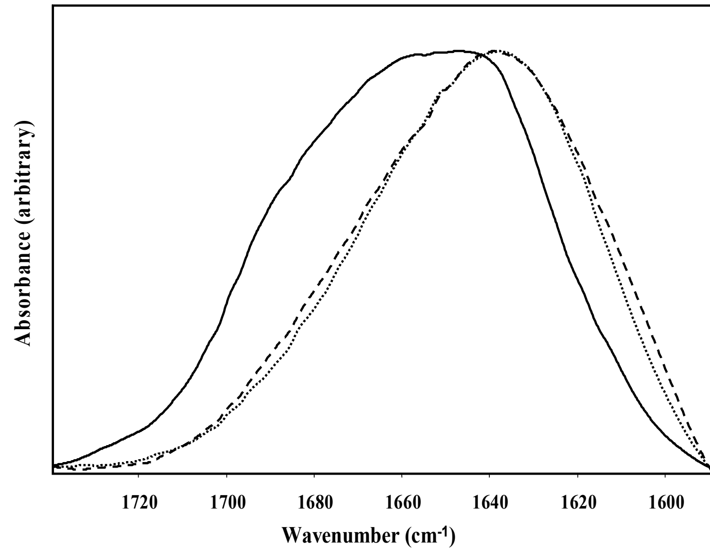
b)

Figure 4.12. The average (a) absorbance and (b) second derivative spectra of original trypsin, trypsin gel and trypsin gel with sucrose in 1700-1600 cm^{-1} region (black line shows original trypsin, dashed line shows trypsin gel, dotted line shows trypsin gel with sucrose).

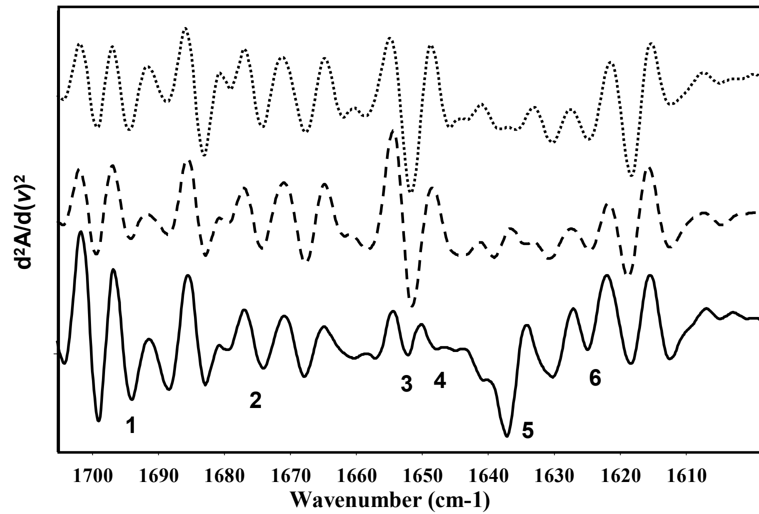
As seen in Figure 4.12 (a) the gel and gel with sucrose showed changes in the secondary structure. However, the most significant changes were observed when the second derivative spectra were taken into consideration. As seen in Figure 4.12 (b) the beta sheets appeared at 1637 cm^{-1} are found to be the most affected from gelation process. The trypsin gel was found to have lower beta sheet structure. However, the sucrose addition appeared to preserve the beta sheet structure. In addition, the antiparallel beta sheet structure, aggregated beta sheet, turns and random coiled structures were found to be left unaffected from the gel formation in the both gels. The alpha helix structure was found to be larger in the gelation process with sucrose and was left unaffected by the gelation process without sucrose.

4.6.2. The Effect of Aggregation on the Secondary Structure of Trypsin with and without Sucrose

The band assignments of the original trypsin, trypsin aggregate and trypsin aggregate with sucrose were carried out in regions between 1700-1600 cm^{-1} which corresponded to the amide I band region. According to spectrums of original trypsin, trypsin aggregate and trypsin aggregate with sucrose the peaks were shown on Figures 4.15. Sample spectrums were selected and their mean spectrum was calculated. After mean calculations of the samples baseline was taken between the ranges of 1740-1590 cm^{-1}. Later, normalization was performed and the second derivatives were taken on five points in the ranges of 1700-1600 cm^{-1} in the Amide I region.



a)



b)

Figure 4.13. The average (a) absorbance and (b) second derivative spectra of original trypsin, trypsin aggregate and trypsin aggregate with sucrose in 1700-1600 cm^{-1} region (black line shows original trypsin, dashed line shows trypsin aggregate, dotted line shows trypsin aggregate with sucrose).

As seen in Figure 4.13 (a) the aggregate and aggregate with sucrose showed changes in secondary structure. The aggregate spectra showed a significant band shift in the average spectra compared to the trypsin control spectrum. The aggregate spectra were similar to each other. However, the most significant changes were observed when the second derivative spectra were taken into consideration. As seen in Figure 4.13 (b) the β-sheets appeared at 1637 cm^{-1} are found to be the most affected from aggregation process. The trypsin aggregate was found to have lower β-sheet structure. However, the sucrose addition did not appeare to preserve the β-sheet structure in the aggregate phase. Similarly the antiparallel β-sheet structure was adversely affected by the aggregation without sucrose case. However, the antiparalell β-sheets were found to be unaffected in the aggregates with sucrose. In addition the turns, aggregated β-sheet and random coiled structures were found to be left unaffected from the aggregate formation in the both cases. The α-helix structure was found to be larger in the aggregation process with and without sucrose. This is an unexpected situation since in the most of the protein aggregation studies the α-helix amouts were found to decrease in favor of β-sheet structures (Wang, 2005). This is the situation where in most of the neurological diseases and other metabolic diseases where β-sheets were the structures leading to the aggregation processes both in vivo and in vitro conditions (Honson et al., 2007; Huang et al., 2000; Morgan et al., 2004; Serpell, 2000; Uversky and Fink, 2004). As opposed to the aforementioned studies in this study trypsin aggregates were found to have lower beta sheet structures with higher α-helix content.

4.7. Iodoacetamide (IAA) Studies

Iodoacetemide (IAA) is used as the disulfide bond blocking agent in protein related analyses. Iodoacetamide effects disulfide bonding by thiol blocking (Figure 4.14). As thiols blocked, disulfide bonding can not take place (Alting et al., 2000). In proteins, disulfide bonds are formed by the introduction and removal of the thiol-disulfide exchange reaction (Hansen and Winther, 2009).

In proteins exposed to adverse conditions such as high pressure, temperature, chemicals cause the exposition of sulfhdryl and cystine groups to environment and make them reactive in formation of disulfide bonds (Visschers and de Jongh, 2005).

Figure 4.14. Iodoacetamide bonding to S^-.
(Source: Poglar, 1979)

Since trypsin contains six intramolecular disulfide bonds (Figure 4.15), the possible role of intra and inter-moleculer disulfide bond formation in the gelation process between trypsin molecules was investigated. IAA was used to study its possible effect on disulfide bond formation as a function of gelation time.

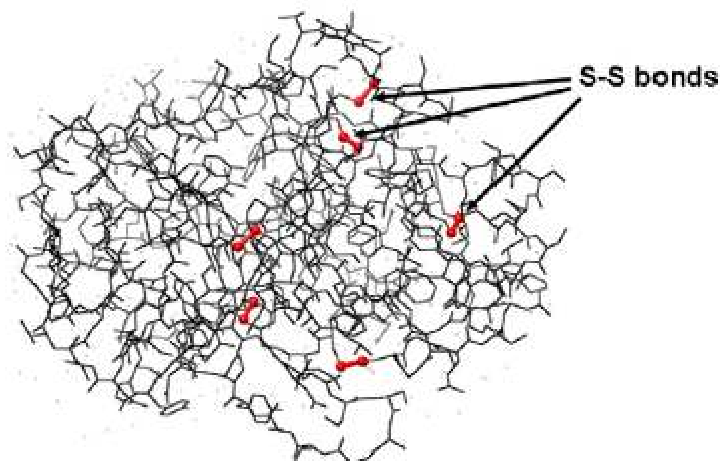


Figure 4.15. Disulfide bonds in bovine pancreatic trypsin.
(Source: Okur et al., 2011).

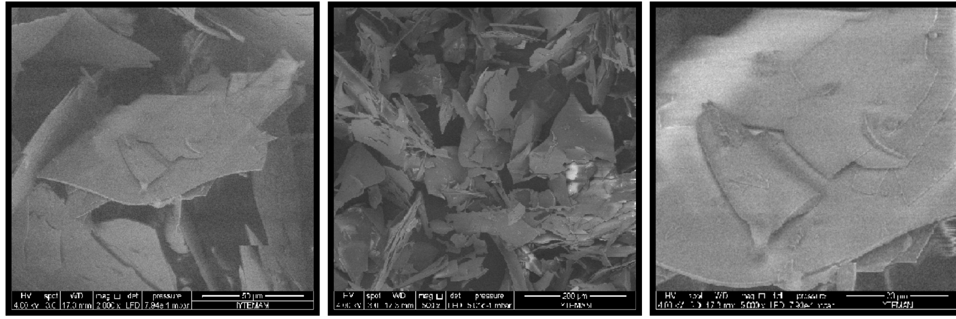
Table 4.5. Gelation time in the presence and absence of IAA

IAA presence / Gel Type	No IAA	1 M and 2 M IAA
Trypsin gel	2.5 minutes	More than 40 minutes
Trypsin gel with sucrose	4 minutes	More than 50 minutes

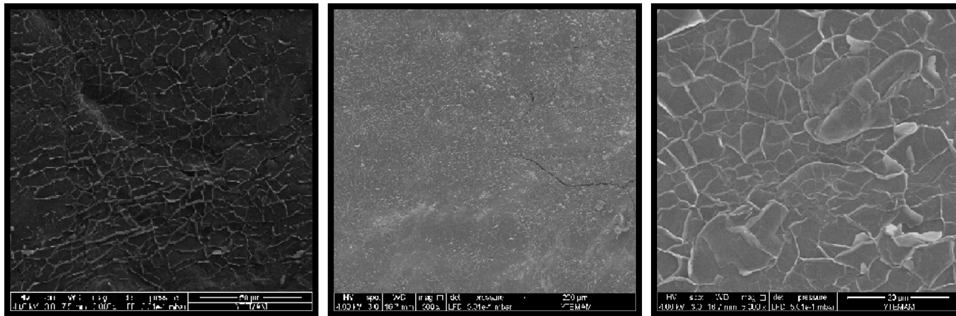
It was observed that in the presence of IAA, gelation was delayed and also soft gels were formed (Table 4.5) Disulfide bond formation increases gel hardness (Visschers and de Jongh, 2005). As the disulfide bond were blocked by the IAA soft gels were obtained. The results of the experiments indicated the possible role of intermolecular disulfide bonds formed during the gelation process.

4.8. Scanning Electron Microscopy (SEM) Studies of Trypsin Gelation and Aggregation

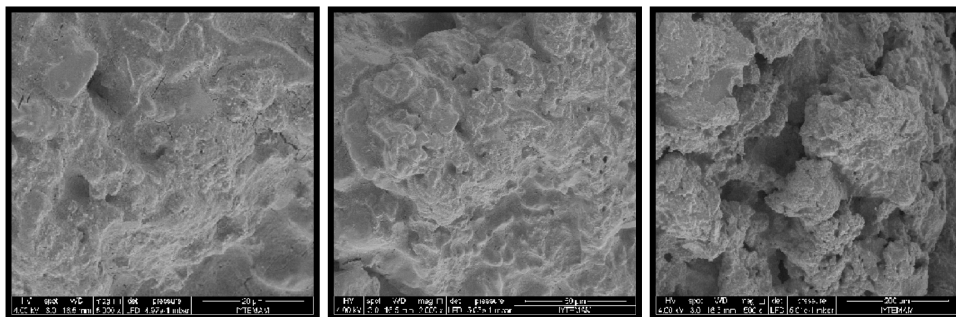
SEM images were taken to observe the morphologic effect of the gelation process on trypsin. SEM images of original trypsin, trypsin gel and trypsin aggregate were taken to investigate the differences comperatively. The gels and aggregates were air dried at room temperature for one day before their SEM images taken. SEM images of the original trypsin, trypsin gel and trypsin aggregate were taken in the magnifications of 20 µm, 50 µm and 200 µm.



a) 20 µm 50 µm 200 µm



b) 20 µm 50 µm 200 µm



c) 20 µm 50 µm 200 µm

Figure 4.16. SEM images of a) original trypsin, b) trypsin gel, c) trypsin aggregate in the magnification of 20 µm, 50 µm and 200 µm.

As seen in Figure 4.16, original trypsin flakes were observed as thin sheets under the SEM. In the SEM images of the gel after drying appears to have a smoother surface appereance. As seen in the same figure, trypsin aggregates appear to have an amorphous structure.

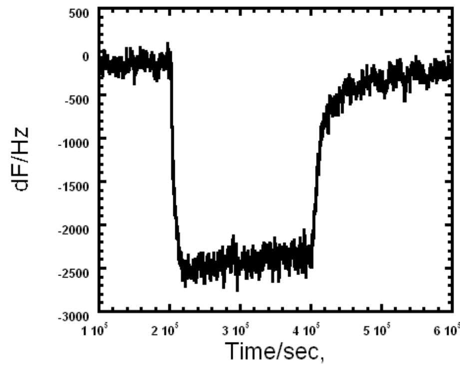
4.9. Quartz Crystal Microbalance (QCM) Studies with Trypsin Thin Film

The trypsin gel was found to form a thin layer on gold substrate of QCM probes in a previous study (Okur et al., 2011). Proteins were shown to interact via disulfide bonds with gold molecules (Losev et al., 2004). Similarly, in our study disulfide reshuffling should cause the free sulfides to form new interactions/bonding with gold molecules found on the gold surface of the QCM probes. In addition the thin layer was found to adsorb and desorb water molecules from its near environment. Therefore the thin layer was shown to be sensitive to humidity changes.

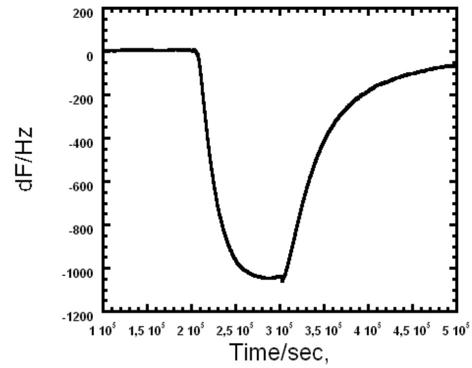
QCM is an appropriate device in transducing chemical and biochemical signal sensing. As a biosensor, it is commonly used in thin film sensing abilities (Davis and Higson, 2005).

Thin films can be explained as “two dimensional” material, where the thickness, the third dimension, gets so small (from 1 μ to 1 nm) where there is a considerable increase in the surface-to-volume ratio (up to 10^6) (Tvarozek et al., 1998). Thin films of bio-polymers and proteins are highly interested related with their chemical and physical properties as being kind of biosensor (Tharanathan, 2003).

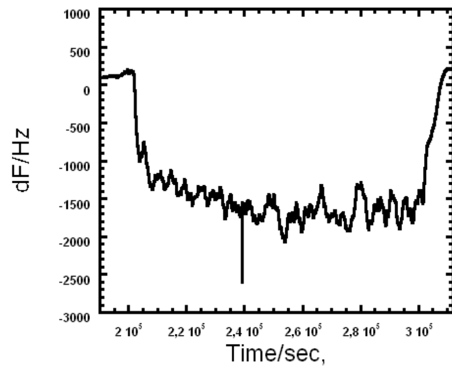
By coating QCM probe with trypsin thin film, the resonance frequency changes were determined by QCM after sensing analysis of benzene (C_6H_6), carbon monoxide (CO), carbon dioxide (CO_2), dichloromethane (CH_2Cl_2), hydrogen peroxide (H_2O_2) and propanol gases.



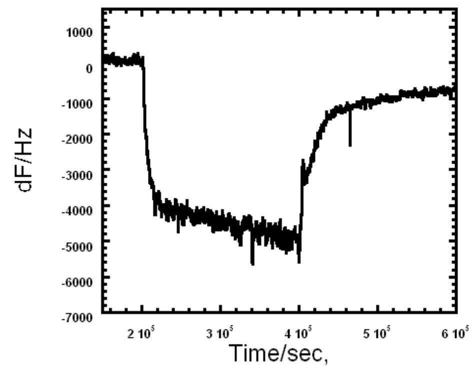
a) Benzene (C_6H_6)



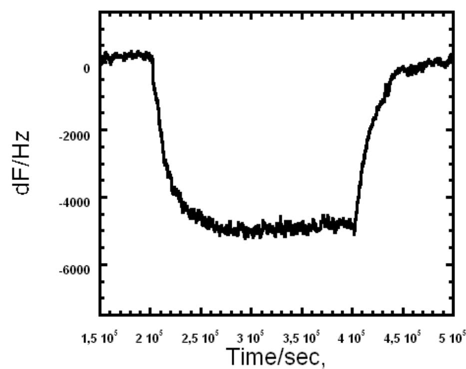
b) Carbon monoxide (CO)



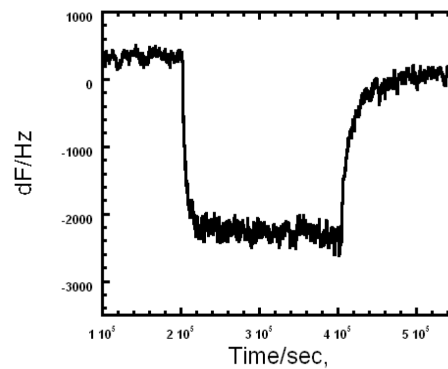
c) Carbon dioxide (CO_2)



d) Dicloromethane (CH_2Cl_2)



e) Hydrogen peroxide (H_2O_2)



f) Propanol (CH_3CH_2OH)

Figure 4.17. The adsorbtion ability of trypsin thin film to gases a) benzene, b) carbon monoxide, c) carbon dioxide d) dichloromethane, e) hydrogen peroxide f) propanol.

Benzene as an industrially important chemical. It (C_6H_6) is a colorless or light yellow liquid chemical at room temperature and highly flammable. It is produced by different human activities (lubricants, detergents, dyes) and natural processes (e.g. forest fires and volcanoes). Benzene disrupts cells function and they can not work properly. It mainly effects blood and causes decrease in red blood cells which resulted in anemia. Also, it causes leukemia and cancer of the blood producing organs (Centers for Disease Control and Prevention, 2011).

Carbon monoxide (CO) is a toxic, odorless and colorless gas which is produced by chimneys, automobile exhausts, tobacco smoke and by many other sources. It inhibits O_2 intake and forms carboxyhemoglobin. Higher concentrations of carbon monoxide can be fatal (United States Environmental Protection Agency). Carbon dioxide (CO_2) is emitted by carbon cycle or by human activities (e.g. burning of fosil fuels). Also CO_2 is given to atmosphere by the plants activities. A higher level of CO_2 is harmful for human beings (United States Environmental Protection Agency, 2011).

Dichloromethane (DCM) (CH_2Cl_2) is a volatile organic and colorless liquid chemical. DCM is generally used as paint remover agents but also it is used as solvent and cleaning agent in chemical manufacture (textile, metal and plastics), pesticides industries and in many other areas. It is commoly used as a water disinfectant agent.
Higher concentrations of DCM cause liver problems and increase cancer possibilities (United States Environmental Protection Agency, 2011).

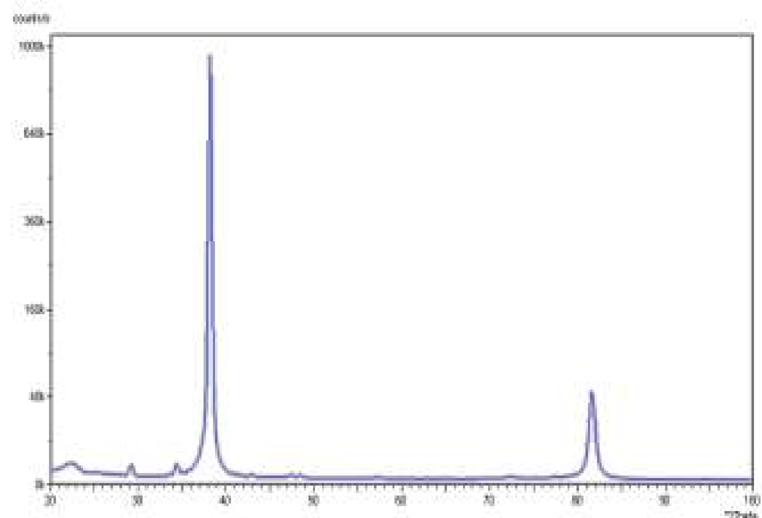
Hydrogen peroxide (H_2O_2) is a colorless liquid with bitter taste. Hydrogen peroxide is a powerful oxidizing agent. It is commonly used in households, in medicinal applications and as a clothes and hair bleach. High levels of H_2O_2 causes irritation in eyes, skin, respiratory airway and throat (Agency for Toxic Substances & Disease Registry, 2011). Propanol (CH_3CH_2OH) is a colorless liquid and also is a kind of alcohol.

Trypsin thin film showed sensing ability to all of the gases (Figure 4.17) which were used in the QCM studies. From the biotechnological aspect, trypsin thin film sensing ability to different gases can be used as a biosensor property. Nonetheless, the drawback of the trypsin thin film is to have sensing affinity to all gases which were used during this study. Biosensors have to be specific for the one of the gases. But being sensitive to all gases is not good for a biosensor.

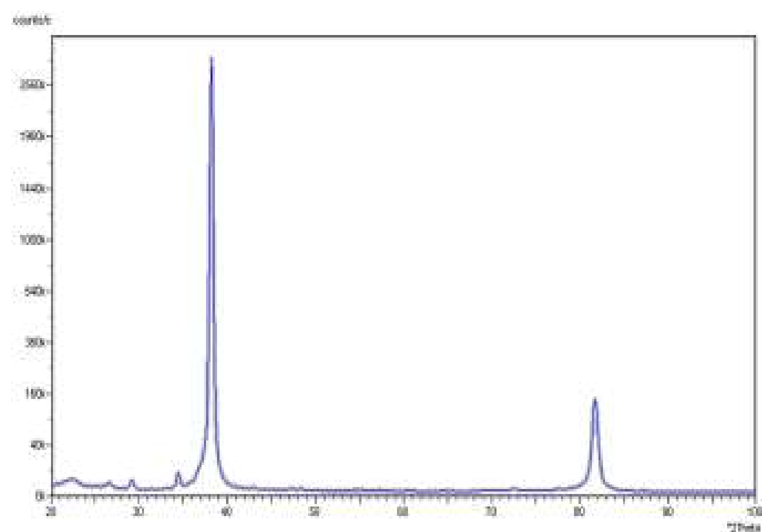
4.10. Grazing Incidence X-Ray Diffraction (GI-XRD) Studies of the Trypsin Thin Film

XRD analysis allows us to observe if our sample contains crystalline structures and the determination of crystalline structure of the samples analyzed. The analysis of the thin films x-ray diffraction techniques were developed on the primary beam entrance to the sample under very small angles of incidence. In other words, this technique is known as GI-XRD which depends on grazing degree incidence x-ray diffraction. The small entrance angle causes the path traveled by the x-rays to significantly increase and the structural information contained in the diffractogram to stem primarily from the thin film (Birkolz, 2006).

According to earlier reports, the trypsin thin film formed on the QCM probe appeared to have regular structures under Atomic Force Microscopy (Okur et al., 2011). Therefore GI-XRD analyses were carried on 2Theta between 20^0-100^0 degrees and in the grazing degree of 0 to investigate the crystalline structure formation of the trypsin thin film on the QCM probes and the results are depicted in Figure 4.18.



a)



b)

Figure 4.18. GI-XRD graphs of (a) empty QCM probe and (b) trypsin thin film on a QCM probe ($*2\theta=20^0$-100^0).

When the the diffractograms of the empty QCM probe and the QCM probe with QCM probe with the thin trypsin film there is no additional peaks which would possibly originate from the protein film. Therefore, it was concluded that there were no crystalline structures in the trypsin thin film according to GI-XRD analyses (Figure 4.18). In addition, original trypsin samples were also controlled for the presence of any crystalline structures and were found to have no crystalline structures (data not shown).

CHAPTER 5

CONCLUSION

In this study bovine pancreatic trypsin was used to investigate the structural and functional characterization of the observed cold gelation process. In addition, the aggregation was also observed under some experimental conditions. It was investigated that the gelation and aggregation process parameters extensively effect gelation time and also determines whether gelation or aggregation to take place. Gelation time was determined as a function of the variables namely, trypsin concentration, NaOH concentration and $CaCl_2$ concentration. Also, the effect of sucrose addition to the gelation time was investigated. At the same NaOH concentration (in the absence of $CaCl_2$), the gelation time increased as the protein concentration decreased. In the presence of sucrose it was observed that gelation time increased as protein concentration decreased. As sucrose concentration increased, the gelation time increased as well. At the low NaOH concentrations gelation was not observed due to the insufficient concentration of NaOH. As NaOH concentration increased in contrast, gelation time decreased. Gelation with the sucrose addition under different NaOH concentrations showed the same behaviour with the gels without sucrose.

In the presence of $CaCl_2$, in gels with and without sucrose, up to a concentration gelation was observed. However, after a concentration rather than gelation aggregation was observed. In brief, it can be concluded that protein concentration, NaOH concentration, $CaCl_2$ concentration and sucrose addition have effects on both gelation and the obtained phase.

As a result of trypsin stability studies, it could be claimed that there was a specific activity loss in bovine pancreatic trypsin after gelation process which can be related with the structural changes in the molecule.

In the resolubilization studies, it was observed that trypsin gel had dissolving affinity to starting solution and water which are the main components of gel the formation process. In the starting solution and water the gels were dissolved easily and in a short time than the other solutions (Isopropanol and isopropanol-water mixture).

Considering the FTIR experiments, the secondary structure of bovine pancreatic trypsin was not preserved in trypsin gel and trypsin aggregate through the gelation and aggregation processes. It was observed that the β–sheet structure, as evidenced in the original trypsin at the 1637 cm^{-1} band, disappeared in the trypsin gel and aggregate. Increases in the α–helix structure in 1651 cm^{-1} in trypsin gel with sucrose and aggregate with and without sucrose were observed. Different from the original trypsin, a shift was observed in the 1652 cm^{-1} band and between the 1656 cm^{-1} and 1658 cm^{-1} band, in the α–helix region. Also, different from the original trypsin, not so clear but a shift was observed in the 1648 cm^{-1} band, which is related with the random coil region.

IAA (Iodoacetamide) addition was observed to delay gelation and block the disulfide bonding. Therefore, it can be concluded that the protein gelation is the result of intermolecular disulfide bonding rather than intermolecular β-sheet structure formation.

The SEM analyses showed that there were morphological changes among original bovine pancreatic trypsin, gel and aggregate after gelation and aggregation processes. Original trypsin appeared as regular formations whereas dried trypsin gel had shrinks and wrinkles on the surface,and trypsin aggregate owned amorphous and particulate structure.

The gas adsorption tendency of the protein film formed after gelation was demonstrated as a potential biotechnological application of the gelation process. In the QCM studies it was observed that bovine pancreatic trypsin thin film had sensing ability for all the gases studied (benzene, carbon monoxide, carbon dioxide, dichloromethane, hydrogen peroxide and propanol. The thin film adsorption property was not observed to be gas spesific for the gasses tried.

Through the GI-XRD studies it was observed that the trypsin thin film did not contain any crystalline structures.

In future, trypsin gel can be used in controlled release studies. In addition, three dimensional tissue-cell culture studies can be carried out. Different alcohols and additives can be used to improve the gel characteristics. The adsorption-desorption kinetics for different gases can be studied to enlighten the gas-protein film interactions. The potential of the thin films to be used as gas sensors should be studied as well.

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APPENDIX A

CHEMICALS USED IN EXPERIMENTS

CHEMICAL	CODE
Trypsin	BioChemika 93610
Isopropanol	Riedel-Haën 24137
Sucrose	Amresco 03355
Calcium chloride	AppliChem A3652
BAEE (N_{α}-Benzoyl-L-arginine ethyl ester hydrochloride)	Sigma B4500
Tris	Amresco 0826
Iodoacetamide (IAA)	Sigma I6125
Potassium bromide (KBr)	Sigma 07302TB

APPENDIX B

EXPERIMENTAL PROCEDURES

B.1. Starting Solution

1 ml of 1 N HCl was added into 1L of volumetric flask. Later deionized, distilled H_2O was added after filtered from 0.22 μm filter to 1L volume.

B.2. Tris Buffer

12.1 gr Tris and 2.22 gr $CaCl_2$ was dissolved in nearly 950 ml of deionized, distilled H_2O. By using pH 7 and pH 10 standards the pH meter calibration was checked. The pH of the buffer solution was adjusted to pH 7.6 ± 0.01 at 25 C by adding 6 N HCl. The ultimate volume is completed to 1L. Later by using 0.22 μm filter the buffer was filtered.

B.3. Substrate Solution

0.1714 gr of BAEE (n-Benzoyl l-Arginine Ethyl Ester) was dissolved in 100 ml of Tris Buffer.

B.4. Enzyme Solution (for Standard Curve Formation)

100 mg of trypsin was dissolved in 100 ml of starting solution and stirred at speed setting until protein dissolved. Enzyme solution may be diluted in 1/20 for using in Trypsin-BAEE assay. But in this study 7.2 mg trypsin was used for 7.2 ml starting solution.

B.5. The Specific Activity of Trypsin Gel

10 mg/ml of trypsin was used for gel preparation according to trypsin gel procedure. After trypsin gel formed for dissolution 0.25 ml of starting solution was added onto trypsin gel and waited for gel transition from gel phase to sol phase. Later 0.2 ml of dissolved trypsin gel was added into 1.8 ml of starting solution and trypsin dissolved gel was diluted in the 1/10 proportion. The absorbance measurements were carried out for 278.0 nm and 253.0 nm, for the 20 mg/ml trypsin gel ΔA_{min} and $C_{trypsin}$ measurements like original trypsin.

B.6. The Specific Activity of Trypsin Gel with Sucrose

5 mg/ml of trypsin was used for trypsin gel with sucrose formation. Like trypsin gel 0.25 ml of starting solution was added for dissolution of trypsin gel with sucrose and 0.2 ml of dissolved sample was taken and added into 1.8 ml starting solution for dilution in 1/10 proportion. The absorbance measurements were done at 278.0 nm and 253.0 nm for ΔA_{min} and $C_{trypsin}$ measurements like original trypsin and trypsin gel for specific activity calculations.

B.7. The Specific Activity of Trypsin Gels with Different NaOH Concentrations

Except NaOH concentrations all the procedure is the same with trypsin gel process. After gel formation 0.25 ml starting solution was added onto gels for dissolution. Later 200 µl dissolved gel was added into 1800 µl starting solution and the dissolved gels were diluted in 1/10 proportion. Later their readings were performed at 278.0 nm and 253.0 nm absorbance for the specific activity calculations.