STRUCTURAL CHANGES OF GLIADINS DURING SOURDOUGH FERMENTATION AS A PROMISING APPROACH TO GLUTEN-FREE DIET

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ABSTRACT

STRUCTURAL CHANGES OF GLIADINS DURING SOURDOUGH FERMENTATION AS A PROMISING APPROACH TO GLUTEN-FREE DIET

Gluten intolerance, celiac disease, is an autoimmune disease caused by the ingestion of gluten proteins and the only treatment is the strict gluten-free diet which results in a mucosal recovery. At this point, the variety, availability and low price of gluten-free products on the market are of great importance.

Sourdough is a traditional fermented semi-product that contains lactic acid bacteria (LAB). LAB are capable of metabolizing proteins and peptides with their proteolytic enzymes. It is predicted that gluten proteins are able to be hydrolyzed with the help of this proteolytic mechanisms and also by the wheat proteolytic enzymes.

The objective of this study was to evaluate the changes in the structure of gliadins involved in gluten intolerance during sourdough fermentation with selected LAB. First, sourdough fermentation with selected *Lactobacillus* strains was accomplished. As a control, dough with no inoculation and chemically acidified dough were prepared. During 48 h of fermentation, changes in pH, acidity, LAB concentration and free amino nitrogen content were investigated. SDS-PAGE, 2-D electrophoresis and RP-HPLC were applied to prolamins extracted from each sourdough sample taken at definite time intervals.

The results showed that the inoculated LAB adapted to the dough environment and as they grew, a decrease in pH, and an increase in acidity and free amino nitrogen were observed. According to SDS-PAGE, 2-D electrophoresis and RP-HPLC results, some modifications in the protein patterns were observed in the inoculated doughs. The same alterations occurred in control and chemically acidified doughs, but not as fast as inoculated ones.

ÖZET

GLUTENSİZ DİYET İÇİN GELİŞTİRİLEN EKŞİ MAYA FERMENTASYONU SIRASINDA GLİADİN YAPISINDA OLUŞAN DEĞİŞİKLİKLER

Gluten intoleransı, çölyak hastalığı, genetik olarak yatkın bireylerde buğday depo proteini olan gluten proteini tüketimiyle ortaya çıkan ve ince bağırsak mukozasının hasarıyla karakterize edilen bir intoleranstır. Hayat boyu uygulanacak sıkı bir glutensiz diyetle ince bağırsak mukozası eski sağlıklı haline kavuşmaktadır. Hastaların kaliteli bir yaşam sürebilmesi için glutensiz ürün çeşidinin bol, ürünlerin kolay erişilebilir ve uygun fiyatta olması oldukça önemlidir.

Hamurun fermentasyonu sonucu elde edilen ekşi maya hamuru eski zamanlardan günümüze gelen bir gıdadır ve doğal mikroflora olarak laktik asit bakterileri ve maya içermektedir. Laktik asit bakterilerinin proteolitik aktiviteleri ile gluten proteinlerinin parçalanarak zararsız protein fraksiyonları elde edilebileceği düşünülmektedir.

Bu çalışma seçilmiş laktik asit bakterileri ile gerçekleştirilen ekşi maya fermentasyonu sırasında gliadinlerin yapısında olan değişimleri incelemeyi amaçlamaktadır. İlk olarak buğday unu ile hazırlanan hamura laktik asit bakterileri inokule edilerek ekşi maya fermentasyonu gerçekleştirilmiştir. Kontrol olarak bakteri inokule edilmemiş hamur ve kimyasal olarak asitlendirilmiş hamur hazırlanmıştır. 48 saatte tamamlanan fermentasyon süresince pH, asitlik, laktik asit bakteri konsantrasyonu ve amino nitrojen içeriğindeki değişim incelenmiştir. Fermentasyonun belirli zamanlarında alınan ekşi maya hamuru örneklerinden ekstrakte edilen prolaminlere SDS-PAGE, iki boyutlu elektroforez ve RP-HPLC uygulanmıştır.

Sonuçlara göre fermentasyon ilerledikçe inokule edilen bakterilerin hamur ortamına adapte olduğu gözlenmiş ve sayılarındaki artış neticesinde pH değerlerinde düşüş, asitlik ve serbest amino nitrojen değerlerinde artış gözlenmiştir. SDS-PAGE ve iki boyutlu elektroforez jel görünümlerinde ve RP-HPLC piklerinde fermentasyon ilerledikçe değişiklikler gözlenmiştir. Bu değişiklikler bakteri inokulasyonu yapılan ekşi maya hamurlarında gözlendiği gibi kontrol ve kimyasal olarak asitlendirilmiş hamurlarda da gözlenmiştir.

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LIST OF ABBREVIATIONS

2-DE **:** Two-dimensional Electrophoresis

cm **:** Centimeter

CD **:** Celiac Disease

cfu **:** Colony forming units

FAN **:** Free Amino Nitrogen

h **:** Hour

LAB **:** Lactic Acid Bacteria

Lb. ***:*** *Lactobacillus*

min **:** Minute

µl **:** Microliter

mM **:** Milimolar

M_r **:** Molecular weight

MRS **:** de Man, Rogosa and Sharpe Medium

PBS **:** Phosphate Buffered Saline

pI **:** Isoelectric point

RP-HPLC **:** Reverse-Phase High Performance Liquid Chromatography

RT **:** Room temperature

R_t **:** Retention time (min)

s **:** Second

SD **:** Sourdough

SDS-PAGE **:** Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

ssp. **:** Subspecies

CHAPTER 1

INTRODUCTION

Celiac disease is the most common autoimmune disease in the world. This gluten sensitive enteropathy occurs after the consumption of gluten, and also genetic susceptibility is the prerequisite. The wheat storage protein, gluten, consists of gliadin and glutenin fractions which have amino acid sequences rich in proline and glutamine residues. High proline and glutamine content makes gluten proteins resistant to gastrointestinal digestion enzymes. After reaching to small intestine, gluten activates the immune response and mucosa structure is damaged. The damage causes malabsorption and the problems related with it. In order to treat celiac disease, gluten-free diet has a key and unique role. Following a strict gluten-free diet based on avoidance of gluten containing foods results a recovery of mucosal damage and healthy small intestine. Today, several gluten-free products are on the market. However, they have poor sensory quality and nutritional deficiencies.

Sourdough fermentation technology is a traditional biotechnological approach. Sourdough is a fermented semi-product and it is the mixture of wheat flour and water. Microflora of sourdough mainly consists of lactic acid bacteria, mostly *Lactobacillus* strains, and yeast. As fermentation progresses, the acidity decreases as a result of microbial growth. Endogenous proteolytic enzymes of wheat flour and proteolytic activities of lactic acid bacteria lead to a gluten degradation. This breakdown of gluten could be specific to toxic gliadin and glutenin fragments which trigger celiac disease. It means that sourdough fermentation is probably a promising approach to detoxify gluten. Additionally, sourdough fermentation enhances the texture, flavor and shelf-life of the final product and also it has a significant effect on nutritional value.

In Turkey, the biodiversity of lactic acid bacteria is quite huge. Among these biodiversity, strains capable of degrading gliadins could exist.

In the light of above-mentioned information, the main objective of this study was to investigate the changes in protein degradation during sourdough fermentation that start with the selected *Lactobacillus* strains. The fermentation parameters of sourdough samples were studied and the structural changes of gliadins were determined

by using electrophoretic and chromatographic techniques. It is expected that the findings of this study contribute greatly to the knowledge of the mechanism of biochemical changes and protein degradation that occur during sourdough fermentation.

CHAPTER 2

GLUTEN AND DETECTION METHODS

2.1. Gluten

The gluten proteins are the main grain storage proteins and constitute over half of the total grain protein at maturity (Shewry and Halford, 2002). When flour is mixed with water, gliadin and glutenin form gluten. After the wheat dough is washed to remove water soluble contents and starch, gluten remains as a rubbery mass (Wieser, 2007). Gluten has extensibility and elasticity properties which arise from gliadins and glutenins, respectively, which are the main components of gluten. Gliadins contribute mainly to the viscosity and extensibility. Contrarily, glutenins are responsible for dough strength and elasticity (Wieser, 2007). The visco-elastic properties of gluten allow gases to be retained during fermentation and contribute to leavening (Wieser, 2000). In other words, gluten is the key point of the bread quality.

The cereal seed proteins were classified by Osborne (1907) according to their sequential extraction and differential solubility as albumins which are soluble in water and dilute buffers, globulins which are soluble in saline solutions, prolamins which are soluble in 70–90% ethanol, and glutelins which are soluble in dilute acid or alkali. Although this classification is still commonly used, gliadins and glutenins can be grouped according to their amino acid sequences as sulphur-poor prolamins (ω-gliadins), sulphur-rich prolamins (γ- and α/β- gliadins and low molecular weight (LMW) subunits of glutenin) and finally high molecular weight (HMW) subunits of gliadins (Figure 2.1) (Shewry and Tatham, 1997; Tatham et al., 1985).

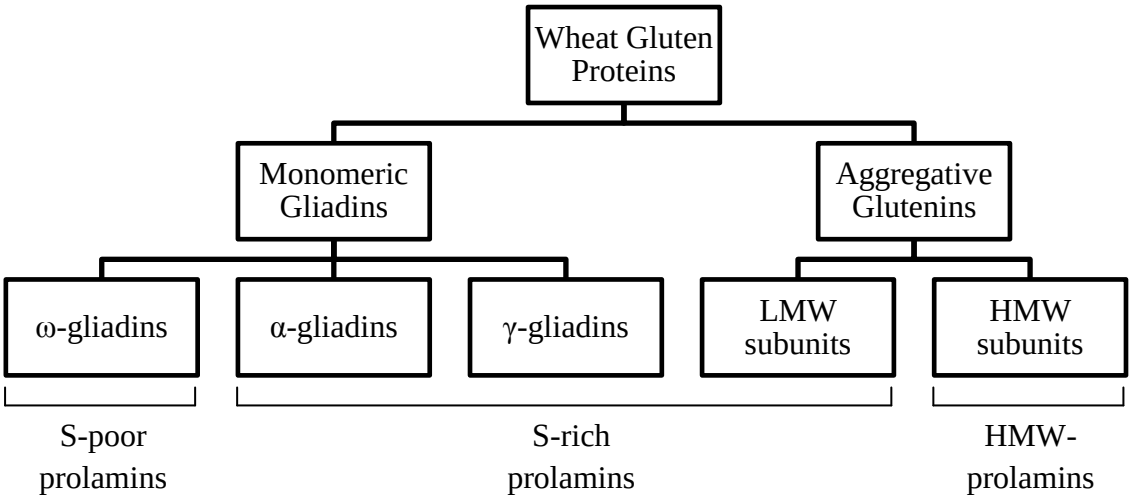


Figure 2.1. Classification of gliadin and glutenin subunits in wheat flour
(Source: Lindsay and Skerritt, 1999)

The existence of distinct regions or domains responsible for structural differences and the repeated regions in amino acids sequences in prolamins constitute the typical structure. They are also rich in proline and glutamine amino acids (Shewry and Halford, 2002). The properties and amino acid compositions of wheat gluten are given in Table 2.1.

Table 2.1. The properties of wheat gluten proteins
(Source: Shewry and Tatham, 1997)

Group	**Subunit Structure**	**% Total fraction**	**M_r**	**Amino acid composition (mol%)**
HMW subunits of glutenin	Polymeric	6-10	65-90,000	30-35 Gln 10-16 Pro 15-20 Gly 0.5-1.5 Cys 0.7-1.4 Lys
ω-gliadins	Monomeric	10-20	40-75,000	40-50 Gln 20-30 Pro 8-9 Phe 0 Cys 0-0.5 Lys
LMW subunits of glutenins **α- and γ-gliadins**	Polymeric Monomeric	70-80	30-45,000	30-40 Gln 15-0 Pro 2-3 Cys <1.0 Lys

2.1.1. Gliadins

The gliadins consist of monomeric proteins which contain no disulphide bonds (the ω-gliadins) or have only intra-chain disulphide bonds (α- and γ-gliadins) (Shewry et al., 1997; Wieser, 2007). Monomeric gliadins which have M_r between 30,000-75,000, have been classified into three groups based on their mobilities at low pH in gel electrophoresis as α/β-, γ-, ω-gliadins in order of decreasing mobility (Tatham et al., 1985; Wieser, 2007).

According to complete amino acid sequences of several α- and γ-gliadins, the structure is divided into a short N-terminal domain, a central repetitive domain and a non-repetitive C-terminal domain (≈300 residues). It was indicated that α-gliadins contain six cysteine residues and form three inter-chain disulfide bonds, on the other hand γ-gliadins contain eight cysteine residues and form four inter-chain disulfide bonds (Tatham and Shewry, 1995). ω-gliadins contain no cysteine residues. While ω-gliadins have high content of β-turns and lack α-helical or β-sheet structure, γ-gliadins have both α-helical and β-sheet conformations (Tatham et al., 1985).

Schematic structures of γ- and α-gliadin are given in Figure 2.2. In γ-gliadin a N-terminal domain contains 12 residues located before a region of repeats based on P-Q-Q-P-F-P-Q. Most cysteine residues are placed at the proline-poor non-repetitive domain. In the case of α-gliadins the repetitive domain consists of repeats based on two motifs: P-Q-P-Q-P-F-P, and P-Q-Q-P-Y. Also, C-terminal domain contains two regions rich in glutamine (poly-Gln regions) (Shewry and Tatham, 1990).

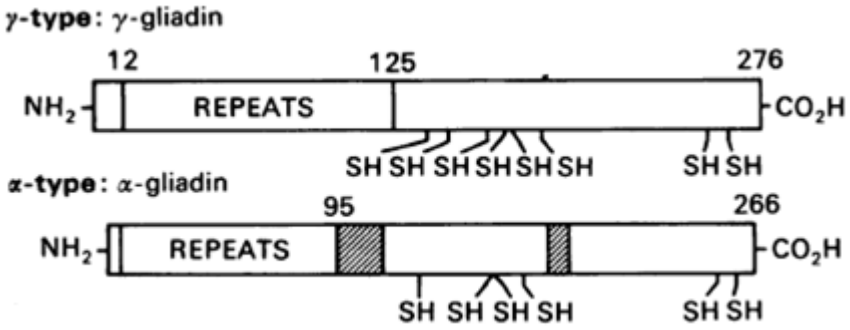


Figure 2.2. Schematic structures of γ- and α-gliadin. SH, positions of cysteine residues; grey areas, poly-Gln regions (Source: Shewry and Tatham, 1990).

2.1.2. Glutenins

The glutenins consist of protein subunits present in polymers which contain inter-chain disulphide bonds. They are insoluble in aqueous alcohols (Shewry et al., 1997).

According to their M_r values, glutenins are considered as x-types which have M_r values of about 83,000-88,000, and y-types which have M_r, values of about 67,000-74,000 (Shewry and Tatham, 1990).

Reduction of these bonds results in subunits which are soluble in alcohol/water mixtures and are classified into two types: high molecular weight (HMW) and low molecular weight (LMW). The latter group is highly complex and still incompletely characterized, but it is clear that some components are closely related to the α-type, β-type and γ-gliadins, while others can be considered to form a discrete group of LMW subunits which can be divided into several types (Lew et al., 1992). Both the gliadins and glutenins can be classified as prolamins as they are soluble in alcohol/water mixtures, either in the native state or after reduction of inter-chain disulphide bonds (Shewry et al., 1997).

2.2. Gluten Detection Methods

Qualitative and quantitative determination of gluten in foods is of great importance for both detecting the contamination and investigating the alteration in its structure. In Codex standard (Codex Alimentarius Commission, 2008), it was exactly stated that;

- "The antibody used should react with the cereal protein fractions that are toxic for persons intolerant to gluten and should not cross-react with other cereal proteins or other constituents of the foods or ingredients.
- Methods used for determination should be validated and calibrated against a certified reference material, if available.
- The detection limit has to be appropriate according to the state of the art and the technical standard. It should be 10 mg gluten/kg or below.
- The qualitative analysis that indicates the presence of gluten shall be based on relevant methods (e.g. ELISA-based methods, DNA methods)."

Enzyme-linked Immunoassay (ELISA) R5 was suggested as the method of gluten determination in Codex standard (Valdés et al., 2003). Immunochemical methods such as ELISA and western-blotting are based on the antibody-antigen interactions and even small amounts of gluten are detected.

PCR techniques are also used and they provide more sensitive results than immunochemical methods (Köppel et al., 1998). However, there is a limiting factor: DNA from hydrolyzed products (beers, syrups, etc.) cannot be detected (Wieser, 2008).

Electrophoretic methods such as SDS-PAGE and two-dimensional electrophoresis are the separation methods widely used. The gluten proteins are separated according to their molecular weights and/or isoelectric points (pI).

Chromatography (e.g. SE-HPLC and RP-HPLC) and mass spectrometry (MALDI-TOF MS, LC-MS, etc.) are also the prefered methods. Mass spectrometry leads more resolved proteins and identification gives precise results.

A certified reference "European Gliadin Reference (IRMM-480)" is available (Codex Alimentarius Commission, 2003). 28 wheat varieties grown in Europe are used in this reference. This material is shown to be soluble, homogenous and stable, and can be used as a reference for immunochemical gliadin and gluten determinations. It was stated that it is available on direct request to IRMM (European Commission, Directorate-General Joint Research Centre [JRC], Institute for Reference Materials and Measurements, Reference Materials Unit, Retieseweg, 2440 Geel, Belgium).

The above-mentioned detection techniques can be used separately or in combination. In this study SDS-PAGE, 2-D electrophoresis and RP-HPLC techniques were applied to gluten proteins. In proceeding sections, these methods were described in more detail.

2.2.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The principle of SDS-PAGE is the separation of polypeptides according to their molecular weights. The polyacrylamide gel contains SDS, an anionic detergent, which forms necklace-like anionic complexes with proteins that masks the charge of the proteins (Ibel et al., 1990). In polyacrylamide gel the proteins are separated according to

their molecular weights under applied current: The higher the molecular weight, the shorter the distance they migrate.

The Laemmli buffer system is the mostly used buffer system for SDS-PAGE (Laemmli, 1970). The glycerol in sample buffer increases the density. As a result, the sample is easily loaded to the well by preventing the leakage of the sample out of the well. Bromophenol blue dye is also included in the sample buffer to follow the sample during electrophoresis and the time for electrophoretic run can be predicted. The buffer system separates proteins at basic pH that prevents aggregation.

Briefly, two types of polyacrilamide gels are prepared. First, separating gel (mostly 12%) is prepared and cast. After the polymerization, a stacking gel (mostly 4%) is poured. A comb is used to obtain wells in which samples are pipette. The protein sample is mixed with sample buffer and is loaded to the well. After all, voltage is applied and migration of proteins occurs (Figure 2.3). At the end of the run, gel is stained and visualized.

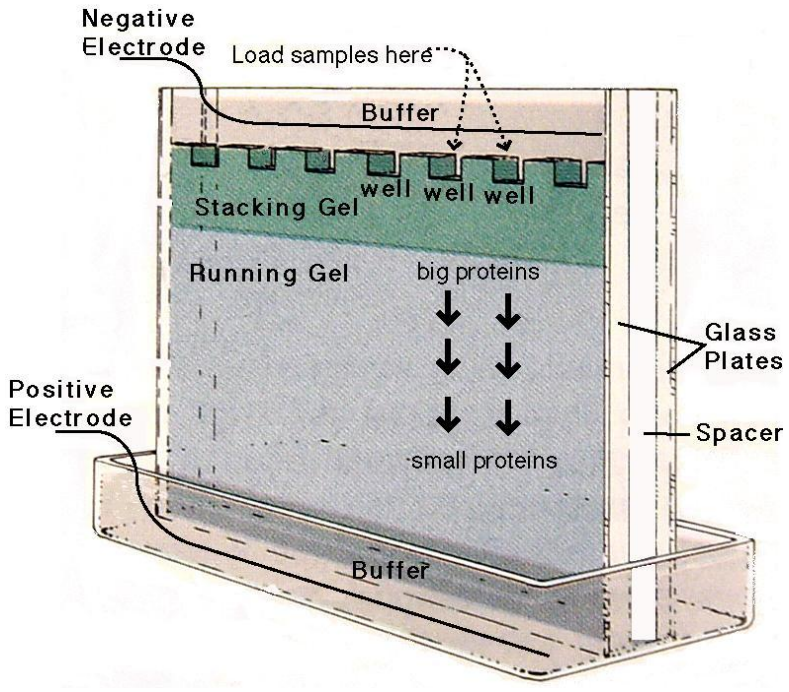


Figure 2.3. The representative picture of SDS-PAGE system
(Source: Georgia Institute of Technology, 2009)

2.2.2. Two-Dimensional Electrophoresis (2-DE)

2-D electrophoresis is carried on in two steps: Isoelectric focusing and SDS-PAGE. After the proteins are separated in immobilized pH gradient (IPG) strips according to their isoelectric point (pI), SDS-PAGE is applied. As a result, the proteins are spread on the gel as spots with more resolution which is the basic advantage of this technique.

2.2.2.1. First Dimension: Isoelectric Focusing (IEF)

In isoelectric focusing technique, proteins are separated according to their pI. The pI is the pH value specific for each protein at which the net charge of protein is zero. A protein is positively charged in a solution at pH values below its pI and negatively charged at pH values above its pI. When a protein is placed in a solution with a pH gradient and an electric field is applied, the protein migrates to the opposite charge. Migration stopped when the protein reached a point where its net charge is equal to zero, in other words a point where its pI is equal to the pH (Figure 2.4). If diffusion occurs from its pI, the protein charge alters and migrates back (Bio-Rad Manual, 2009).

In order to keep the native charges of proteins, they should be suspended in a buffer with low ionic strength. The proteins should be solubilized well, so during focusing aggregation is prevented. The interfering molecules should be removed. For these purposes, protein is mixed with a buffer containing chaotropic reagents, non-ionic or zwitterionic detergents, reducing agents and carrier ampholytes.

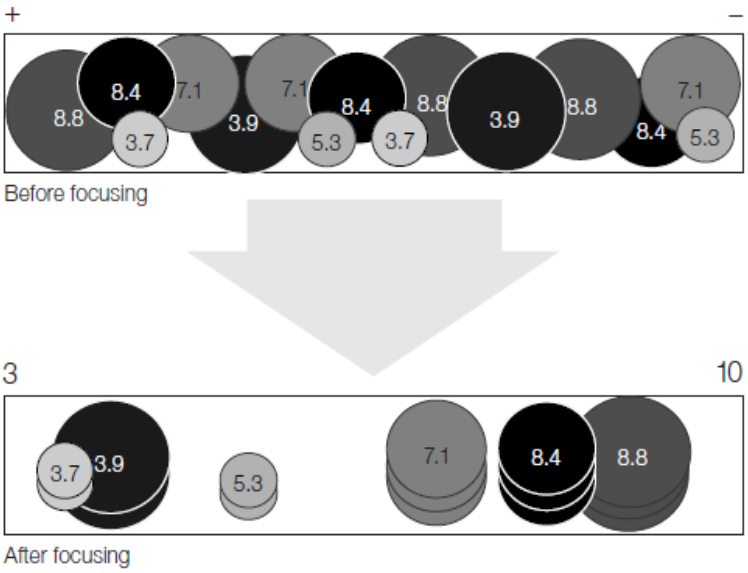


Figure 2.4. The rearrangements of proteins present in a mixture according to their pI values after IEF procedure (Source: Bio-Rad Manual, 2009)

IPG strips with several pH gradients are used in IEF. Samples are loaded to this strips and an appropriate electric field is applied. Then the strips are treated with equilibration buffers (I and II) to reduce disulfide bonds and alkylate the sulfhydryl groups of the cysteine residues. At the same time, proteins are coated with SDS to lose their ionic charge and they are ready for second dimension (SDS-PAGE).

2.2.2.2. Second Dimension: SDS-PAGE

As a second separation procedure, SDS-PAGE is applied to the proteins which were separated by IEF. The SDS-PAGE procedure described previously is applied but with some modifications. Since the sample has already been loaded to IPG strips, there is no need to create wells on polyacrylamide gels. The strip is placed onto the separating gel and electrophoretic run is started. After the run, gel is stained and spots are visualized.

2.2.2.3. Staining of the Polyacrylamide Gel

Several staining procedures are applied to SDS-PAGE and 2-D gels to make the bands and spots visible. Silver and coomassie staining are the most preferred methods. It is known that silver staining is more sensitive than coomassie staining. According to a study in literature about staining of prolamins no single staining method provides complete staining (van den Broeck et al., 2008). It was stated that high amount of protein sample has to be loaded on the gel for coomassie staining. When compared to coomassie staining, Colloidal PageBlue (CBB G-250) staining is very sensitive, but some proteins are stained poorly. It is indicated that silver staining is sensitive but overdevelopment may occur. Another dye, SYPRO Ruby, is also regarded as very sensitive.

After staining the gel is visualized with a device equipped with a charged coupled device (CCD) camera. Image is captured and analyzed with a software package to determine the molecular weights and pIs.

2.2.3. Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Reversed-phase chromatography (RP-HPLC) is used when the mobile phase is more polar than stationary phase. Molecules are separated on the basis of differences in their hydrophobicity; polar compounds elute first. Mobile phases such as methanol-water and acetonitrile-water mixtures are used. However, in separation of proteins or peptides acetonitrile and water containing 0.1% trifluoroacetic acid (TFA) are commonly used. The most widely used columns are C_8 and C_{18}. Increased amount of organic solvent in mobile phase results in reduction in retention time of sample (Hışıl, 2004).

In the case of RP-HPLC separations of gliadins, since it is less hydrophobic than others, it is known that ω-gliadins elute first than α, β, and γ-gliadins (Figure 2.5). As a result, each gliadin group is separated by RP-HPLC as a unique subgroup and quantification is accomplished easily (Bietz, 1983; Wieser et al., 1998). Column temperature has a great effect on separation efficiency and separations are carried out at higher temperatures such as 70°C (Huebner and Bietz, 1987).

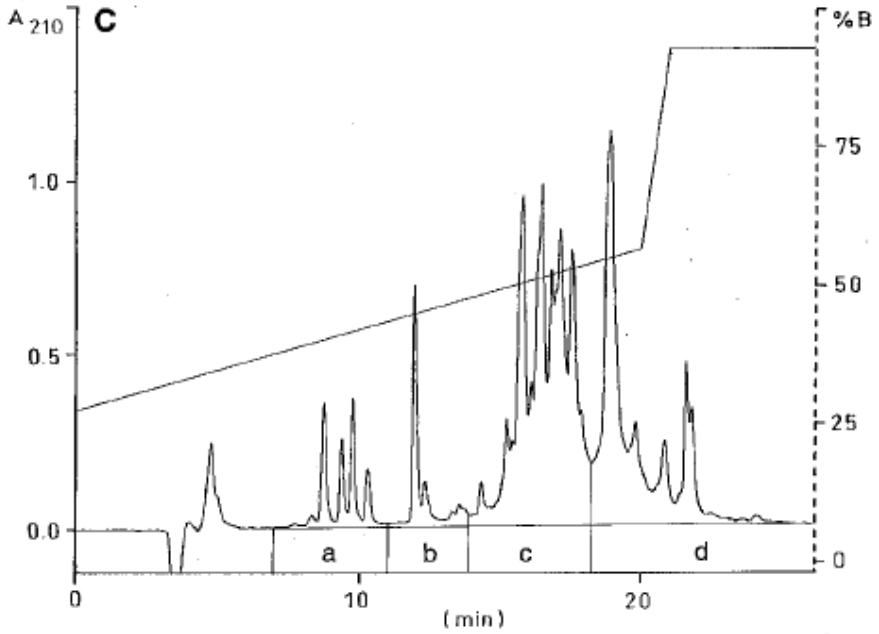


Figure 2.5. RP-HPLC chromatogram of gliadin extract. a-b, ω-gliadins; c, α-gliadins; d, β-gliadins (Source: Wieser et al., 1998).

RP-HPLC is a high resolution technique, frequently resolving more components than alternative electrophoretic techniques. Although the equipment is costly, this technique gives fast, sensitive, reproducible results and it is easy to automate (Bietz et al., 1984).

CHAPTER 3

CELIAC DISEASE

3.1. Introduction

Celiac disease (CD), gluten sensitive enteropathy, is a chronic inflammatory disease characterized by atrophy of the villi on the small intestine mucosa, and is induced in genetically susceptible people by the ingestion of proteins present in gliadins in gluten, secalins in rye and hordeins in barley (Di Sabatino and Corazza, 2009). The situation of avidins in oat is still controversial. Gliadins, secalins and hordeins include high amount of proline (≈ 20%) and glutamine (≈ 38%) residues, contrarily avidins have only ≈ 10% of proline residues (Auricchio et al., 2004). Some studies in the literature show that after oat consumption no symptoms were observed and the results of biopsies indicate that the mucosa was undamaged (Dissanayake et al., 1974; Holm et al., 2006). On the other hand, occurrence of abdominal bloating, increased intestinal symptoms and also villous damage after oat consumption were also reported (Lundin et al., 2003; Peräaho et al., 2004). In general, the limited amounts of pure and uncontaminated oats are considered as harmless (Rashid et al., 2007).

3.2. Mechanism and Diagnosis

Celiac disease is caused by genetic and environmental factors. It was reported that specific histocompatibility leukocyte antigen (HLA) class II genes known as HLA-DQ2 and HLA-DQ8 are present in most celiac disease patients (Catassi and Fasano, 2008b). The effects of consumption of wheat, barley and rye, genetic susceptibility and some other environmental factors such as breast feeding period and infections are shown in Figure 3.1.

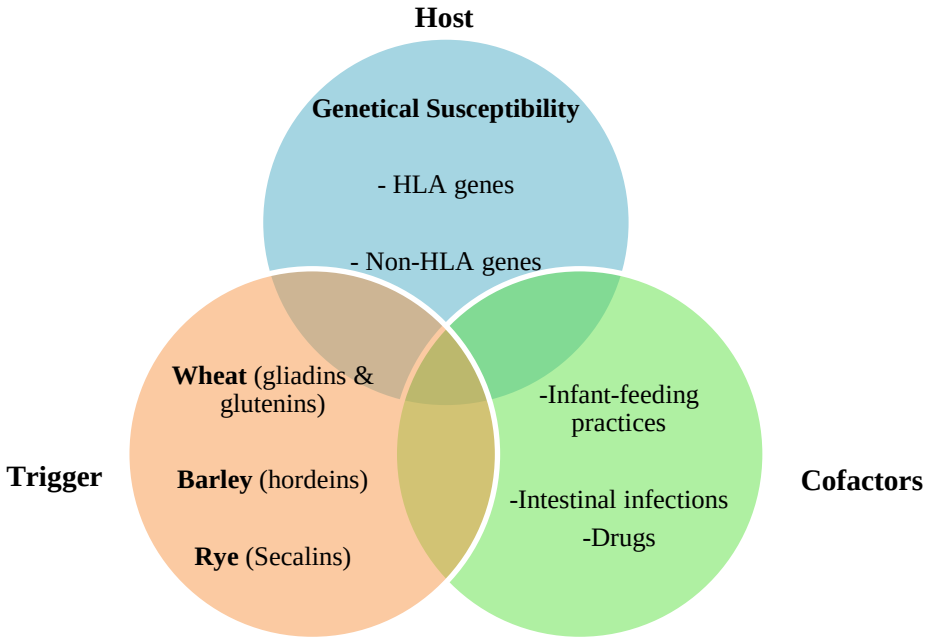
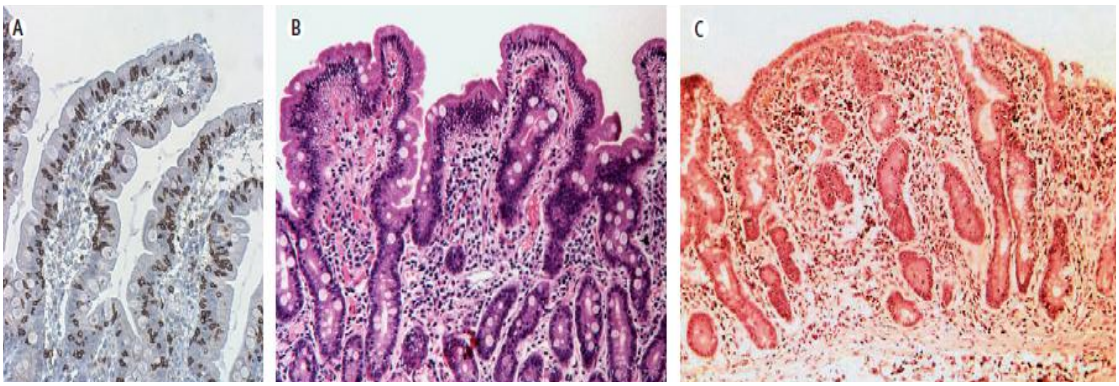


Figure 3.1. The factors involved in celiac disease
(Source: Di Sabatino and Corazza, 2009).

As stated in Chapter 1, gliadin has high proline and glutamine content. As a result, enzymes of the human digestive tract which lack postproline cleaving activity have a partial effect on gliadin (Lindfors et al., 2008; Stepniak et al., 2006). In the small intestine, deamidation of glutamine by the tissue transglutaminase (tTG) occurs and it results in the formation of glutamic acid. The gliadin peptides have an increased affinity for the human leukocyte antigen (HLA) molecules after deamidation. Intestinal T cells, which release proinflammatory cytokines, become active with the interaction between gliadin peptides and HLA molecules. The enterocytes are damaged, lamina propria infiltration of inflammatory cells occurs, the proliferation of the intestinal crypts is increased, and villous atrophy occurs (Figure 3.2) (Catassi and Fasano, 2008a; Di Sabatino and Corazza, 2009). In Figure 3.3, the mechanism is shown in the detail.

Another mechanism which is unrelated to T cell activation was also reported. Presence of biologically active sequences which are not recognized by T cells (e.g. A gliadin 31–43) cause an activation of innate immunity. These are able to interfere with cellular growth and they increase the activity of tyrosine kinase receptors (Auricchio et al., 2004).



(a) (b) (c)

Figure 3.2. Representative pictures of villus structures during different periods of celiac disease. (a) Non-atrophic lesion (b) Atrophic with shortened villi (c) Atrophic with no longer detectable villi (Source: Corazza et al., 2007; Di Sabatino and Corazza, 2009).

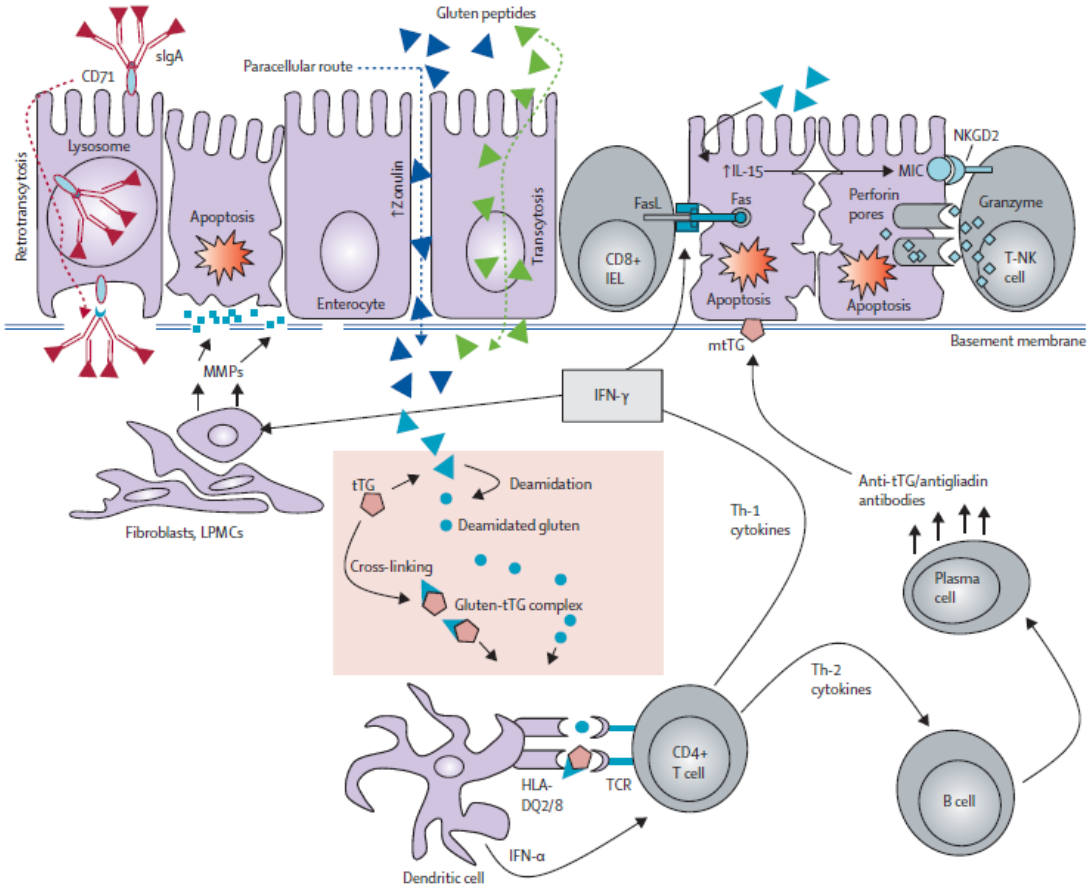


Figure 3.3. The schematic representation of the mechanism of mucosal damage in celiac disease (Source: Di Sabatino and Corazza, 2009).

CD has an incidence of 1 of 100–550 people in the European population (Fasano and Catassi, 2001). The symptoms are varied greatly between individuals. Malabsorption, abdominal pain and bloating, chronic diarrhea, growth failure, iron-deficiency anemia, nonspecific arthritis, depression and low bone mineral density are included as some of the symptoms (Mearin, 2007). Since these problems are not specific to disease and could remind other health problems, diagnosis could take time. On the other hand, in some cases there are no symptoms. As a result, the diagnosed cases consist small amount of the overall patients (Figure 3.4).

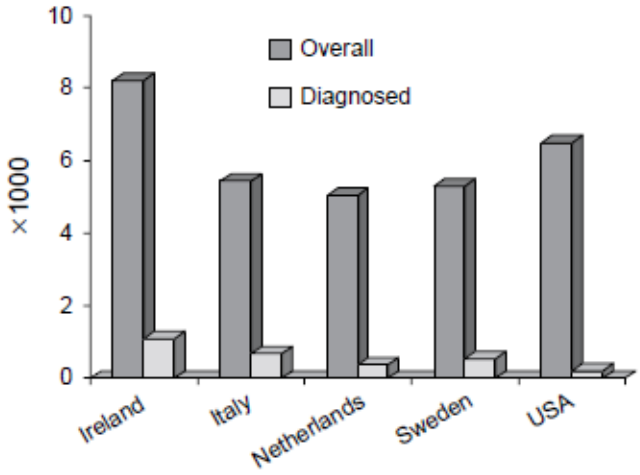


Figure 3.4. The prevalence of celiac disease in different countries. White bars represent clinically diagnosed cases, gray bars represents overall cases (Source: Catassi and Fasano, 2008a).

The iceberg model is very helpful to indicate the current state of the clinical spectrum (Figure 3.5). In the figure, the top of the iceberg consists of individuals with diagnosed CD which are following a gluten-free diet. Below the water level, the patients with undiagnosed CD exist. They may remain unidentified because they have no symptoms or the symptoms are not linked with CD. Finally, at the bottom of the iceberg, the potential CD patients are present. They are genetically susceptible, but the disease does not develop (Gallagher et al., 2004; Mearin, 2007).

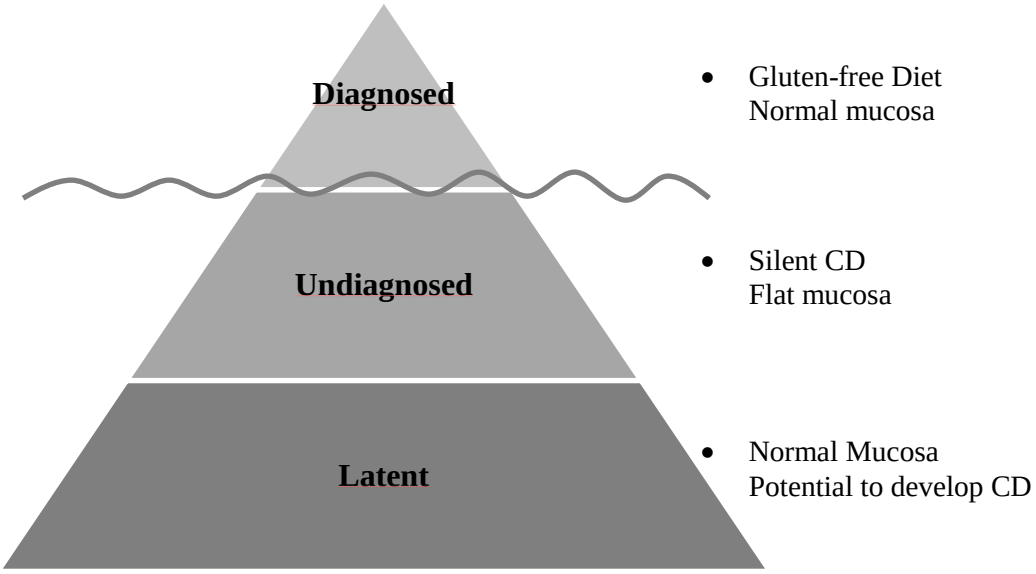


Figure 3.5. Iceberg Model

The diagnosis procedure consists of the detection of associated serum antibodies and autoantibodies such as anti-gliadin and most recently, anti-transglutaminase. In order to confirm the obtained result, small bowel biopsy which is provided by capsule or oral endoscopy is required (León et al., 2005).

It is known that celiac disease has an association with Type I Diabetes Mellitus, Dermatitis Herpetiformis, autoimmune thyroiditis, vasculitis, autoimmune alopecia and hepatitis etc. which are also autoimmune diseases (León et al., 2005).

3.3. Toxic Fragments of Gluten Proteins

As stated previously gluten contains proteins, especially gliadins, rich in proline and glutamine content. The toxicity of gliadins results from the peptides generated after the hydrolysis of gliadins by gastrointestinal enzymes. Tertiary structures of gliadins are not responsible for the toxicity (Wieser and Kieffer, 2001). The main reasons which make gliadin toxic are;

- Low digestibility
- Presence of epitopes for intestinal T cells
- Higher affinity to HLA molecules after deamidation with tissue transglutaminase

- Presence of biologically active sequences not recognized by T cells (Auricchio et al., 2004).

The N-terminal domain of α-gliadins is the main activator of celiac disease and the peptide sequences indicated as toxic were found as PSQQ and QQQP * (Wieser, 1995). Some fragments indicated as toxic are 31–43 fragment of α-gliadin (Marsh et al., 1995), oligopeptide corresponding to amino acids 31-49 of A-gliadin (Sturgess et al., 1994), 33-mer of α2-gliadin (residues 57 to 89) (Shan et al., 2002), 26-mer peptide from γ-5 gliadin (Shan et al., 2005), peptide 9 (^{41}QPYPQPQPFP^{50}) located in the domain I of α- and α/β-gliadins and peptide 42 (^{206}LGQGSFRPSQ^{215}) only in α-gliadin (Lahdeaho et al., 1995). Fraser et al. (2003) stated that each patient demonstrated a reaction to peptide G8 (corresponding to residues 56–75 of α-gliadins). Since the patients were HLA DQ2 positive, none was HLA DQ8 positive, it cannot be concluded that peptide G8 is toxic to all CD patients.

3.4. Gluten-free Diet and Product Market

The strict withdrawal of gluten from the diet is of great importance for celiac disease patients, because the only treatment is gluten-free diet. Following a strict gluten-free diet leads to a mucosal recovery and healthy small intestine.

In Codex Alimentarius (Codex Alimentarius Commission, 2008), gluten-free foods described as dietary foods;

"a) consisting of or made only from one or more ingredients which do not contain wheat (i.e., all *Triticum* species, such as durum wheat, spelt, and kamut), rye, barley, oats or their crossbred varieties, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer,

b) consisting of one or more ingredients from wheat (i.e., all *Triticum* species, such as durum wheat, spelt, and kamut), rye, barley, oats or their crossbred varieties, which have been specially processed to remove gluten, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer."

Additionally, the foods specially processed to reduce the gluten content to a level between 20 and 100 mg/kg were also defined as "Foods consist of one or more ingredients from wheat (i.e., all *Triticum* species, such as durum wheat, spelt, and

kamut), rye, barley, oats or their crossbred varieties, which have been specially processed to reduce the gluten content to a level above 20 up to 100 mg/kg in total, based on the food as sold or distributed to the consumer".

In the same standard, it was stated that "Oats can be tolerated by most but not all people who are intolerant to gluten. Therefore, the allowance of oats that are not contaminated with wheat, rye or barley in foods covered by this standard may be determined at the national level".

In practice, the amount of gluten tolerated varies from person to person. In literature, the limit could not be strictly stated until now and several literature studies state different limits. Generally, gluten intake less than 10 mg per day is probably accepted as harmless and it is so low when compared to the Codex standard (Akobeng and Thomas, 2008). It shows us that the current level stated in Codex does not seem to consider the threshold in practice, particularly in terms of low gluten containing foods (20-100 ppm). On the other hand, it is more appropriate to state the threshold as total gluten intake instead of gluten concentration in food products (Akobeng and Thomas, 2008; Mearin, 2007)

At this point, patients come across several difficulties. Gluten is the main ingredient of many food products and also breads and pastries are widely consumed. The available gluten-free products on the market are limited. The available products are more expensive, and they are generally starch-based and poor in quality when compared to their counterparts. Contamination of naturally gluten-free foods, residual gluten in gluten-free wheat starch, mislabeling of foods constitute the other important problems. It is hard to follow a gluten-free diet because of these reasons. The allergen warnings must be written on the packages. The social pressure is also another important factor which forces patients, especially young ones, to consume gluten containing foods (Catassi and Fasano, 2008b; Leffler et al., 2008; Lerner, 2010; Mearin, 2007).

All over the world, the gluten-free products constitute a growing market. Several companies produce gluten-free products such as breads, pasta, cookies, biscuits etc. In Turkey, the products on the market predominantly comprise of imported products. Gluten-free flour mixes and bread (Sinangil, Sinangil Gıda San. Tic. ve Paz. A.Ş., Ankara Halk Ekmek A.Ş.-METVAK A.Ş. and İstanbul Halk Ekmek A.Ş.), a gluten-free biscuit (Pronot, Eti Gıda Sanayi ve Ticaret A.Ş.) and a gluten-free muesli (Doğa Bitkisel Ürünler San. ve Tic. A.Ş.) are the only products produced in our country. The imported products have higher costs, almost 10-fold, which makes them less affordable.

As a result of the high cost of the products, the patients are in difficulty to adhere their gluten-free diet.

3.5. Novel Therapies

Since gluten-free diet has several negative outcomes, alternative techniques to the treatment of celiac disease are also investigated.

Enzymatic hydrolysis of gliadins with oral enzyme supplements and lactic acid bacteria is one of the important approaches. As an oral supplementation a prolyl endoprotease from *Aspergillus niger* was identified. This enzyme is active at stomach pH and is resistant to low pH and digestion by pepsin (Stepniak et al., 2006). This enzyme degrades gluten peptide and intact glutens and also eliminates T cell stimulatory properties of a pepsin/trypsin digest of gluten. LAB is the other option to degrade gluten enzymatically. Sourdough draws attention, because it contains both gluten and LAB.

Blocking HLA-DQ2/DQ8 is another aspect under consideration. It leads to blocking of the T-cell response specifically whereas it could not be able to remove non-HLA-DQ-mediated Tcell responses (Mearin, 2007). Alternatively, the selective inhibition of tTG could be utilized but the effects of this procedure to other important biological mechanisms are unclear (León et al., 2005).

There are many studies about the genetic modification of gluten to eliminate the biologically active peptide sequences in gliadins (León et al., 2005). Site-directed mutagenesis of wheat has an advantage that the baking quality is not affected. However, it is difficult in practice, because of the frequent repeated amino acid sequences. On the other hand, specific epitopes could be identified which makes it possible to develop antigenic peptides (Catassi and Fasano, 2008b).

CHAPTER 4

SOURDOUGH FERMENTATION

4.1. Introduction

Sourdough is a fermented semi-product that is the mixture of wheat flour and water and it contains lactic acid bacteria. Sourdoughs are classified into three types (Figure 4.1). Type I sourdough is the traditional one, which is produced with the continuous refreshments. Type II sourdoughs are mostly in liquid form and are used to start a new sourdough fermentation. Type III sourdoughs are in the dried form which is ready to use in production (Böcker et al., 1995; De Vuyst and Neysens, 2005). Process parameters such as fermentation time, temperature, inoculum amount and also the properties of wheat flour affect the efficiency of sourdough fermentation process. These factors are illustrated in Figure 4.2.

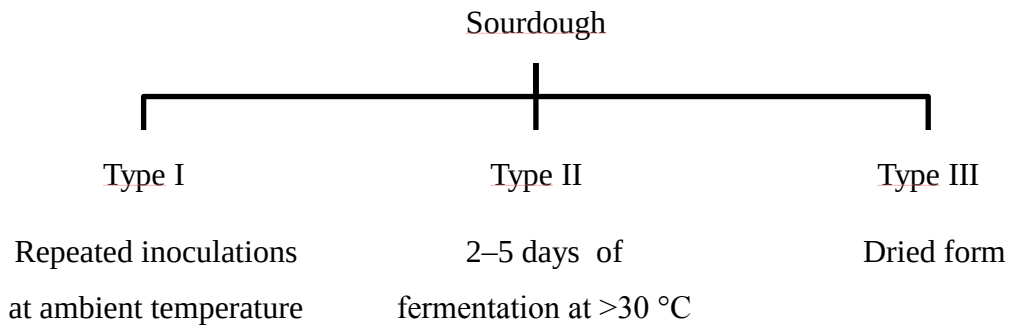


Figure 4.1. Classification of sourdoughs

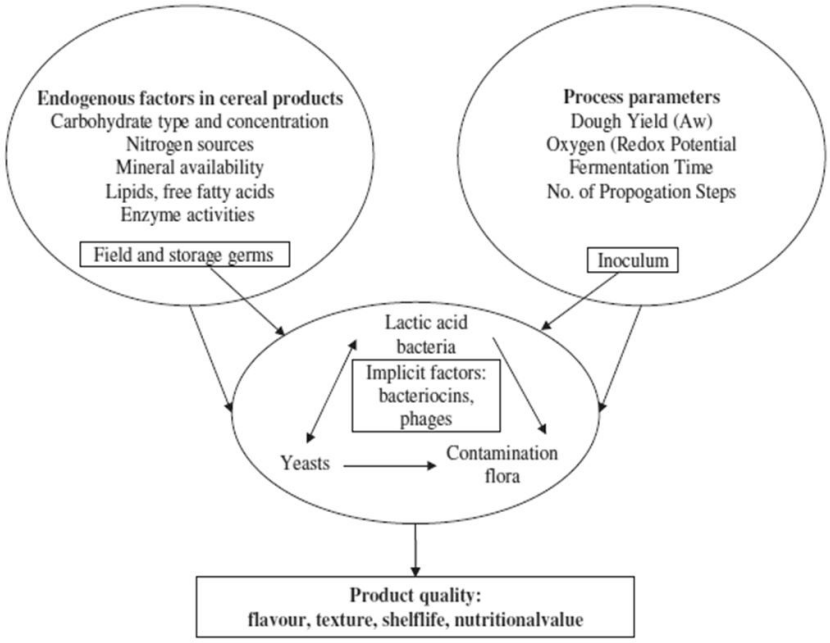


Figure 4.2. Factors effecting the growth and metabolic conditions of the sourdough microflora and the quality of sourdough bread (Source: Hammes and Ganzle, 1998).

4.2. Positive Effects of Sourdough Fermentation

Several changes occur during sourdough fermentation due to the lactic acid bacteria metabolism and wheat flour enzymes. Positive changes in nutritional value, flavor, texture and shelf-life of the final product are summarized in Figure 4.3.

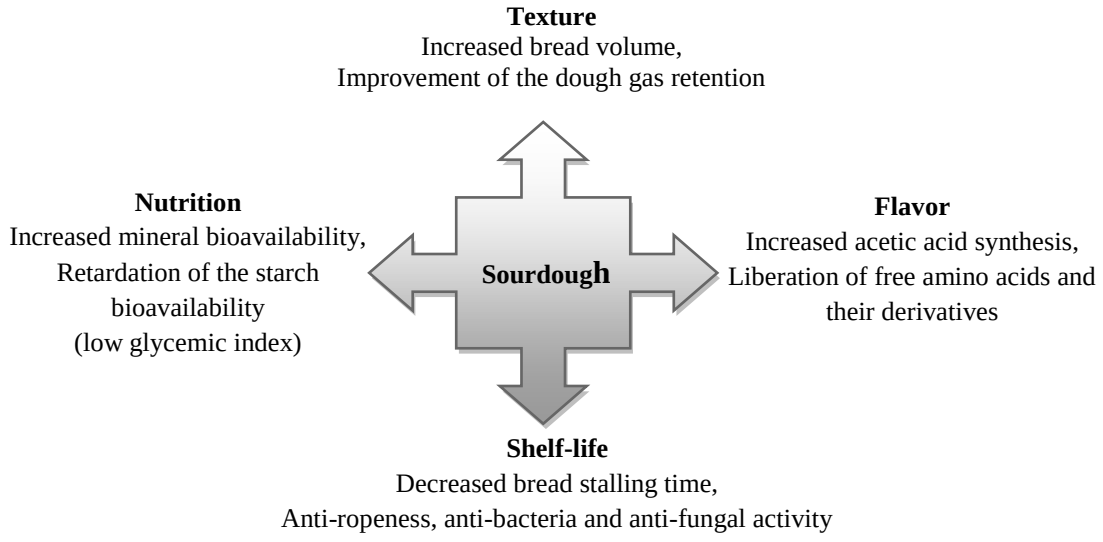


Figure 4.3. Effects of sourdough fermentation on nutrition, texture, flavor and shelf-life.

4.2.1. Nutrition

Phytic acid binds minerals in grains. In grain and sourdough microflora, phytase activity is present and this activity is correlated with increasing acidity. Since the acidic conditions occur during sourdough fermentation, mineral bioavailablity is enhanced with the degradation of phytic acid by phytases (Moroni et al., 2009; Poutanen et al., 2009). Glycemic index of wheat flour also is lowered with the reduction of starch digestibility. There are alterations in the amounts and bioaccessibilities of phenolics, vitamins and sterols with the sourdough fermentation. Health is positively affected by the formation of exopolysaccharides (glucan, fructans and gluco- and fructo-oligosaccharides) which have prebiotic properties. Some other metabolites produced by LAB cause an increase in dietary fibre content (Hammes and Ganzle, 1998; Poutanen et al., 2009).

4.2.2. Texture and Shelf-life

Sourdough fermentation has several effects on texture (Arendt et al., 2007). During sourdough fermentation, there occurs a loss of firmness and elasticity in dough (Clarke et al., 2004). As a result of dextran production by some LAB, improvements in freshness, mouthfeel and softness of baked product are provided (Lacaze et al., 2007). Starch retrogradation and also staling are delayed with the acidity increase due to LAB activity which cannot be achieved with the chemical acidification (Corsetti et al., 1998; Corsetti et al., 2000). In a recent study involved in the evaluation of gluten-free breads, it was shown that the biologically acidified gluten-free breads were softer than the chemically acidified counterparts after 5 days (Moore et al., 2008).

Formation of antimicrobial and antifungal substances such as organic acids, CO_2, ethanol, hydrogen peroxide, diacetyl, fatty acids, phenyllactic acid, reuterin etc. were also reported in some studies (Dalié et al., 2010; Gänzle and Vogel, 2003; Moore et al., 2008). These substances are of great importance in terms of preservation of products and extended shelf-life.

4.2.3. Flavor

The liberation of amino acids and production of alcohols, aldehydes, ketones, acids, esters, ether derivates, furan derivates, hydrocarbons, lactones, pyrazines, pyrrol derivates, and sulfur compound contribute to the flavor development during sourdough fermentation. Diacetyl, acetaldehyde, and hexanel produced by homo-fermentative LAB and ethyl-acetate, alcohols, and aldehydes produced by hetero-fermentative are the main flavor components (Gobbetti et al., 2008).

4.3. Sourdough Lactic Acid Bacteria

Sourdough microflora consists of several LAB and yeasts (Table 4.1). Although obligately homofermentative and facultatively or obligately heterofermentative *Lactobacillus* strains are typical sourdough LAB. *Leuconostoc, Weissella*, and *Pediococcus* species are also frequently present (De Vuyst and Neysens, 2005). The most commonly isolated LAB are *Lb. sanfranciscensis, Lb. plantarum* and *Lb. brevis*. *Lb. pontis, Lb. reuteri, Pediococcus pentosaceus, Leuconostoc mesenteroides, Lb. delbrueckii* ssp., *Lb. casei, Lb. alimentarius, Lb. fermentum, Lb. rossiae* have also been identified. The most common yeasts are *Saccharomyces cerevisiae, Saccharomyces exiguus, Candida holmii, C. krusei, Pichia norvegensis* and *Hansenula anomala* (De Vuyst and Neysens, 2005; Gobbetti, 1998).

There are basic differences between sourdough LAB with respect to their reductive capacity. Glucose is metabolized by homofermentative and facultative heterofermentative strains through Emden-Meyerhoff pathway, whereas pentose-phosphate pathway was used by heterofermentative lactobacilli for hexose metabolism (Vermeulen et al., 2006).

Table 4.1. The classification and microflora of sourdoughs
(Source: De Vuyst and Neysens, 2005)

Type I	Type II	Type III
Obligate heterofermentative	**Obligate heterofermentative**	**Obligate heterofermentative**
Lb. sanfranciscensis	*Lb. brevis*	*Lb. brevis*
Lb. brevis	*Lb. fermentum*	
Lb. buncheri	*Lb. frumenti*	**Facultative heterofermentative**
Lb. fermentum	*Lb. pontis*	*Lb. plantarum*
Lb. fructivorans	*Lb. panis*	*P. pentosaceus*
Lb. pontis	*Lb. reuteri*	
Lb. reuteri	*Lb. sanfranciscensis*	
W. cibaria	*W. confusa*	
Facultative heterofermentative	**Obligate homofermentative**	
Lb alimentarius	*Lb. acidophilus*	
Lb. casei	*Lb. delbrueckii*	
Lb. paralimentarius	*Lb. amylovorus*	
Lb. plantarum	*Lb. farciminis*	
	Lb. johnsonii	
Obligate Homofermentative		
Lb. acidophilus		
Lb. delbrueckii		
Lb. farciminis		
Lb. mindensis		
Lb. amylovorus		

4.4. Proteolysis

Proteolytic enzymes can be classified as proteinases and peptidases. Proteinases hydrolyze proteins into peptides and peptidases catalyse the breakdown of peptides to amino acids or act on specific peptide bonds (Gänzle et al., 2008). In sourdough, lactic acid bacteria and wheat flour endogenous enzymes contribute to proteolysis in sourdough fermentation. In Figure 4.4, the whole proteolysis mechanism is illustrated schematically. With the growth of LAB, acidity increases and some changes occur in gluten network. Significant increase in positive net charge and electrostatic repulsion and the reduction in disulfide bonds lead to an increase in the gluten solubility (Clarke et al., 2004; Thiele et al., 2002). Also, under acidic conditions, cereal enzymes become

active. Aspartic proteinases and carboxypeptidases are in the major proteinase group in both wheat and rye flours (Loponen et al., 2004; Tuukkanen et al., 2005). Germinated rye grain contains mainly aspartic and cysteine proteinase activities that are especially active at pH 3.8 (Brijs et al., 2002). Since gluten proteins become more susceptible to degradation, primary proteolysis of proteins to peptides takes place (Gänzle et al., 2008; Loponen et al., 2004). After the peptides form, they are also hydrolyzed to amino acids by LAB (secondary hydrolysis). LAB proteolytic system consists of an extracellular proteinase, peptide transport systems, and intracellular peptidases (Figure 4.5) (Guedon et al., 2001). Proteins are degraded by a cell wall-associated extracellular proteinases and peptides are taken into the cell via transport systems. After all, peptides are degraded with several intracellular peptidases. LAB possess endopeptidase, aminopeptidase, tripeptidase, dipeptidase and proline iminopeptidase activities (Casey and Meyer, 1985; Christensen et al., 1999; Rollán and Font de Valdez, 2001).

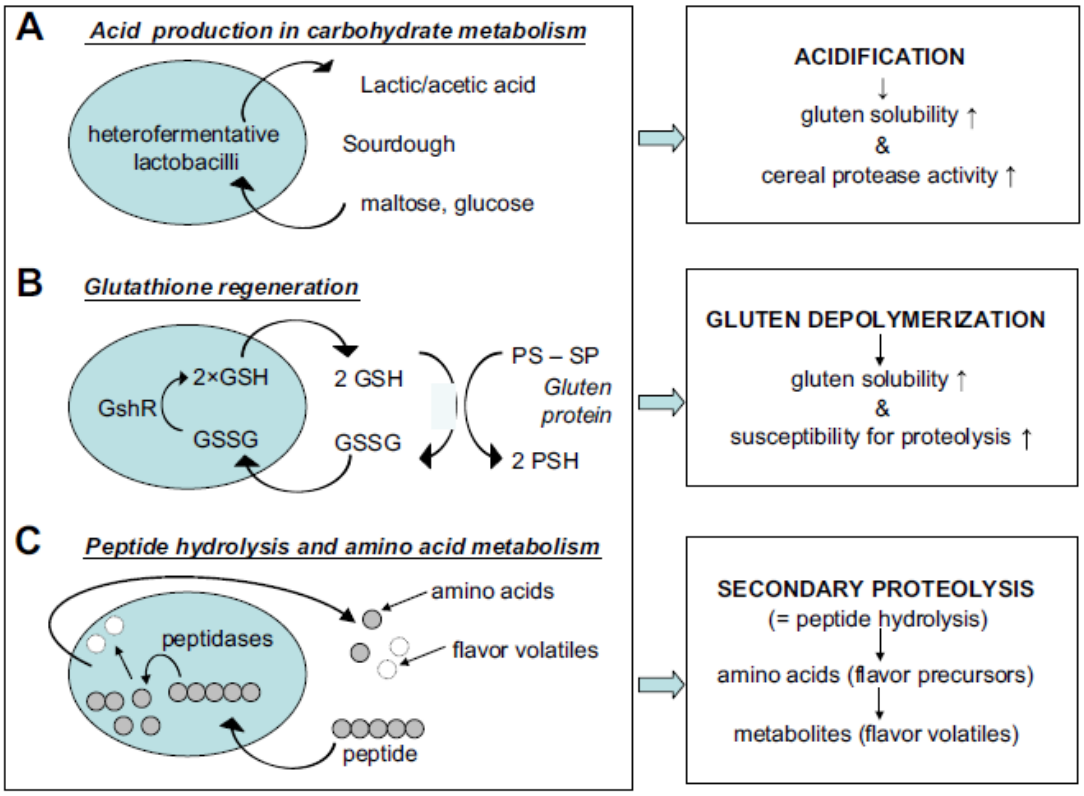


Figure 4.4. Proteolysis in sourdough
(Source: Gänzle et al., 2008)

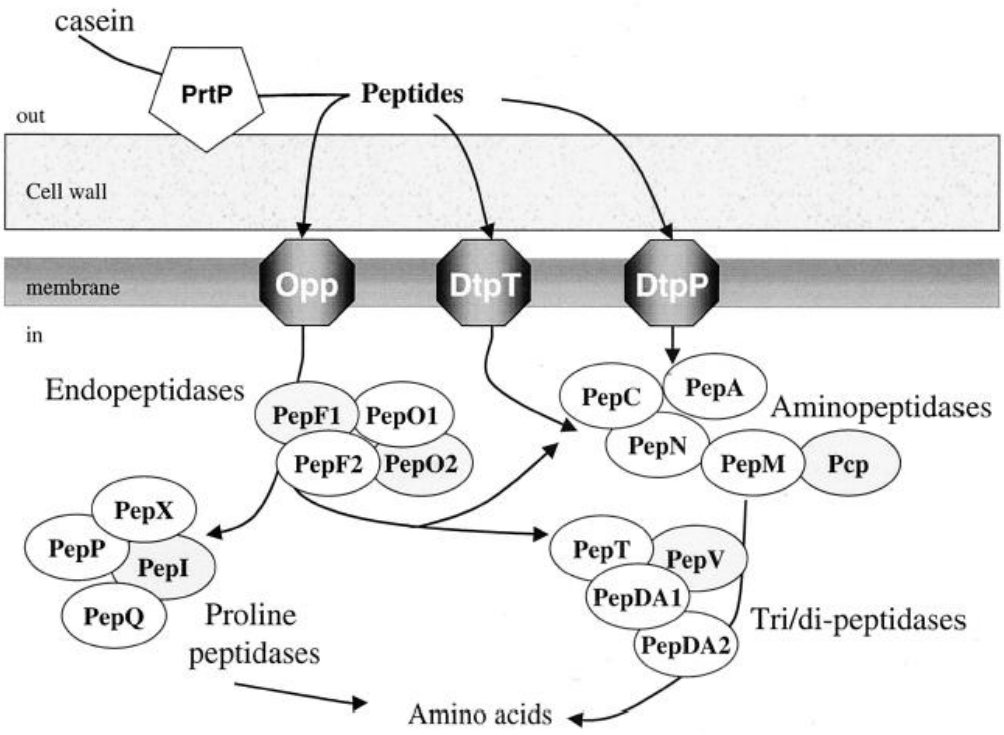


Figure 4.5. Schematic representation of the *Lb. lactis* proteolytic system. The cell wall proteinase (pentagon), three transport systems (hexagon), and 18 intracellular peptidases (oval) are represented in their relative locations in the cell (Source: Guedon et al., 2001).

In literature, there are several studies about the investigation of proteolysis during sourdough fermentation. It was demonstrated that albumins, globulins, and gliadins, except glutenins, were hydrolyzed during sourdough fermentation by *Lb. alimentarius* 15M, *Lb. brevis* 14G, *Lb. sanfranciscensis* 7A, and *Lb. hilgardii* 51B (Di Cagno et al., 2002). Fragment 31-43 of A-gliadin was hydrolyzed after 4 h of treatment by enzyme preparations of lactobacilli. Liberation of free amino acids was observed during fermentation. On the basis of these results, wheat sourdough bread was produced with the same *Lactobacillus* species (Di Cagno et al., 2004). The amount of wheat flour was decreased to 30% by mixing with oat, millet, and buckwheat flours. Almost complete hydrolysis of wheat gliadins was achieved while prolamins from oats, millet, and buckwheat were affected less or not at all. The hydrolysis by the lactobacilli also concerned oligopeptides such as fragment 62-75 of A-gliadin and the 33-mer peptide. A comparison with a chemically acidified dough or with a dough started with baker's yeast alone showed that the hydrolysis was due to the proteolytic activity of sourdough

lactobacilli and that prolamin fractions were not affected during dough fermentation with yeast. The above-mentioned LAB were also used to ferment durum wheat semolina (Di Cagno et al., 2005). By using 2-D electrophoresis, it was shown that 92 of the 130 durum wheat gliadin spots were hydrolyzed almost totally during fermentation. According to R5-Western blot, pasta fermented with lactobacilli still contained 1045 ppm of gluten, and it was stated that pasta formulations include 20% of freeze-dried fermented durum wheat semolina can provide safe threshold. Rizzello et al. (2007) employed different *Lb. sanfranciscensis* strains, characterized by marked peptidase activity towards Pro-rich peptides, and fungal proteases together with the LAB used previously by Di Cagno et al. (2002) during wheat long time fermentation. The results showed that gluten level was detected less than 20 ppm, and this means that wheat flour hydrolysis was achieved at its maximum when compared with the other researches explained above. It was shown that freeze-dried preparation of VSL#3 (VSL Pharmaceuticals) containing *Streptococcus thermophilus, Lb. plantarum, Lb. acidophilus, Lb. casei, Lb. delbrueckii* spp. *bulgaricus, Bifidobacterium breve, B. longum* and *B. infantis* hydrolyzes completely the α2-gliadin-derived epitopes 62–75 and 33-mer (De Angelis et al., 2006). When used individually, strains belonging to VSL#3 preparation were less effective in causing hydrolysis. Since the acidification and related redox potential were found to affect the solubility, polymerization and hydrolysis of the polypeptides, all the results of the above studies were compared to those of a chemically acidified dough. Tuukkanen et al. (2005) showed that during rye sourdough fermentation rye secalins are degraded even without any microorganisms, especially under acidic conditions. This result declares that the endogenous proteolytic enzymes of rye, aspartic proteinases, effectively hydrolyze rye secalins when LAB contribute to sourdough chemistry by producing organic acids. Hartmann et al. (2006) investigated whether proteases isolated from germinating seeds were able to degrade celiac-toxic proline- and glutamine-rich peptides. It was shown that these proteases cleave toxic gliadin peptides into non-toxic fragments with less than nine amino acids. Single prolamin types (ω-, α-, γ-type) and glutelin types (high- and low-molecular-weight subunits) showed a similar degree of degradation. Loponen et al. (2009) also showed that in sourdoughs that were prepared using germinated rye, the rye prolamins were efficiently hydrolyzed to levels that might be tolerated by celiac patients. It was indicated that more than 99.5% of the rye prolamins were hydrolyzed in germinated rye sourdoughs when compared to that of unfermented rye flour. The use of germinated rye

in sourdoughs may avoid the technological problem caused by complete elimination of gluten, because the water binding and gas retention are mediated by pentosans which are not affected by proteolysis (Gänzle et al., 2008).

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

5.1.2. Lactic Acid Bacteria

In sourdough formulations, three different LAB strain were used: *Lactobacillus acidophilus* NRRL-B 1910 obtained from Agricultural Research Service Culture Collection (NRRL, U.S.); *Lactobacillus casei* D4 and *Lactobacillus delbrueckii* ssp. *bulgaricus* TY30 isolated previously by the research group in Food Engineering Department of IZTECH from Comlek cheese and Toros yogurt, respectively (Bulut, 2003; Erkuş, 2007).

5.1.2. Chemicals

Chemicals used in this study are listed in Appendix A.

5.1.3. Reagents and Solutions

Preparations of reagents and solutions used in this study are given in Appendix B.

5.2. Methods

5.2.1. Wheat Flour Analysis

5.2.1.1. Protein Content

Protein content of wheat flour was determined according to Kjeldahl method described in AACC International Method 46-12.01 with some modifications in the amounts of chemical solutions used (AACC, 1976). 1 g of wheat flour was placed in a digestion tube. 13 ml sulfuric acid, a catalyst tablet containing 3.5 g potassium sulphate and 3.5 mg selenium, and a spatula of anti-foaming agent were added. A blank containing only these reagents were also prepared. All the tubes were digested at 420°C for 5 hours (Kjeldatherm and Turbosog, C. Gerhardt GmbH & Co. KG, Germany). After digestion period the tubes were cooled at room temperature and placed in the distillation-titration unit (Vapodest 50s, C. Gerhardt GmbH & Co. KG, Germany). After addition of 50 ml of deionized water, distillation with 80 ml of 32% sodium hydroxide was carried on and the distillate was accumulated in a beaker that contains 30 ml of boric acid. Titration with 0.1 N HCl was performed and the amount of consumption was recorded. The protein content (% protein in dry matter) was calculated according to equation 5.1 with the conversion factor of 5.7 (F_{HCl}, factor of HCl).

$$\%\ \text{Protein} = \frac{(\text{sample-blank})_{\text{ml HCl}} \times F_{HCl} \times 0.014 \times 5.7}{\text{weight of sample (g)}} \times \frac{100}{(100 - \%\ \text{Moisture})} \qquad (5.1)$$

5.2.1.2. Moisture Content

Moisture content of wheat flour was determined according to ICC Standard No. 110/1 (ICC, 1960a). 5 g of wheat flour was weighed in a metal dish and placed in an incubator for 2-2.5 h until the constant weight was reached. After cooling in a desiccator, weight was recorded. Calculation was done according to equation 5.2.

$$\% \text{ Moisture} = \frac{\text{loss in weight (g)}}{\text{initial sample weight (g)}} \times 100 \quad (5.2)$$

5.2.1.3. Ash Content

Ash content of wheat flour was determined according to ICC Standard No. 104/1 (ICC, 1960b). 3 g of wheat flour sample was weighed into a crucible. Ethanol was added that was enough to cover the surface of the sample and a pre-burning was applied with the help of a lighter until the flame was disappeared. Sample was placed into a muffle furnace (Protherm, Turkey) and was incinerated at 900 °C until the sample residue became light gray-white. After cooling in a desiccator, weight was recorded. Ash content was calculated as in equation 5.3.

$$\% \text{ Ash} = \frac{\text{weight of residue (g)}}{\text{sample weight (g)}} \times 100 \quad (5.3)$$

5.2.2. Preparation of Lactic Acid Bacteria for Sourdough Fermentation

5.2.2.1. Preparation of Subcultures

100 µl of each lactic acid bacteria were taken from frozen glycerol stocks and inoculated to 5 ml of MRS Broth. Broths containing *Lb. acidophilus* NRRL-B 1910 and *Lb. casei* D4 were incubated at 37°C for 24 h, and *Lb. delbrueckii* ssp. *bulgaricus* TY30 was incubated at 42°C for 24 h. Subculture was done for each LAB and these overnight incubated Lactobacillus cells were used for sourdough fermentation.

5.2.2.2. Catalase Test

Lactic acid bacteria are catalase negative bacteria. Catalase test was performed in order to ensure that the bacteria used in sourdough fermentation were LAB. Catalase activity was determined by adding 1-2 drops of hydrogen peroxide solution (3%) to the colonies grown overnight on MRS agar plates. Colonies without gas formation indicate that bacteria are catalase negative.

5.2.3. Sourdough Fermentation

The LAB cells of each strain were harvested via centrifugation at 5000 rpm for 15 min at 4°C. After washing with PBS (pH 7.4), LAB cells were suspended in water and the cell concentrations were adjusted to 2 McFarland unit with a McFarland densitometer (DEN-1, BioSan, Latvia) and were used in fermentation process. The densitometer was previously calibrated by suspending $2x10^8$ cfu/ml of *Lb. acidophilus* NRRL-B 1910 cells in PBS. That measurement on densitometer corresponds to 2 McFarland unit.

200 grams of wheat flour were mixed manually with stated amounts of tap water and 30 ml of 2 McFarland unit of each LAB suspension to produce 300 g of dough (Table 5.1) (Di Cagno et al., 2002). Each LAB has the final concentration of $2x10^7$ cfu/g dough. Dough samples (LA, LC, M2, M1, C and CAD) were incubated in a glass beaker covered with foil at 37°C for 48 h (Figure 5.1). Differently, the dough inoculated with only *Lb. delbrueckii* ssp. *bulgaricus* TY30 (LD) was fermented at 42°C for 48 h. A dough contains no bacterial inoculation (C) was used as control. Another dough without bacterial inoculation acidified to pH 4.22 at 5.5^{th} h of fermentation (CAD) was employed to observe the effects of acidity. Acidification was provided with a lactic and acetic acid mixture (4:1, v/v).

Table 5.1. Sourdough formulations

Code	Wheat flour (g)	Water (ml)	LAB Cell suspension, $2x10^8$(ml)		
			Lb. acidophilus **NRRL-B 1910**	***Lb. casei*** **D4**	***Lb. delbrueckii*** **ssp.** ***bulgaricus*** **TY30**
LA	200	70	30	-	-
LC	200	70	-	30	-
LD	200	70	-	-	30
M1	200	40	-	30	30
M2	200	10	30	30	30
C	200	100	-	-	-
CAD	200	100	-	-	-

The prepared sourdough samples were represented with their codes as below;

LA : Dough fermented with *Lb. acidophilus* NRRL-B 1910

LC : Dough fermented with *Lb. casei* D4

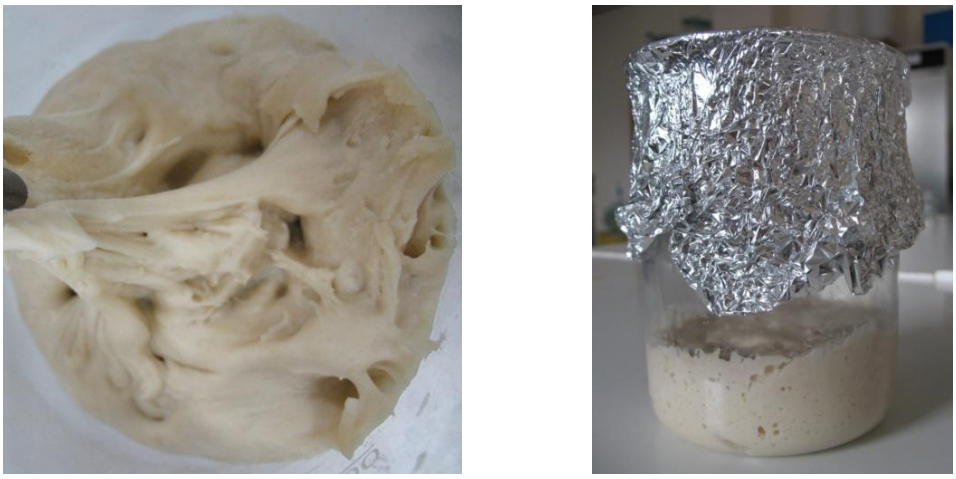
LD : Dough fermented with *Lb. delbrueckii* ssp. *bulgaricus* TY30

M1 : Dough fermented with *Lb. casei* D4 and *Lb. delbrueckii* ssp. *bulgaricus* TY30

M2 : Dough fermented with *Lb. acidophilus* NRRL-B 1910, *Lb. casei* D4 and *Lb. delbrueckii* ssp. *bulgaricus* TY30

C : Dough contains no bacterial inoculation (Control)

CAD : Acidified dough



(a) (b)

Figure 5.1. Sourdough samples. (a) Before fermentation (b) During fermentation in a glass beaker with a foil cover.

5.2.4. Determination of Fermentation Parameters

In order to follow the progress of fermentation, pH and total titratable acidity were measured and numbers of LAB were counted for each sourdough sample.

5.2.4.1. Determination of pH and Total Titratable Acidity (TTA)

The pH and TTA of sourdough samples at 0, 3, 6, 24 and 48 h of fermentation were determined (Tuukkanen et al., 2005). 10 g of sourdough sample was taken and after addition of 90 ml of ultra pure water, homogenization was done by using a bar blender for 40 s. pH of this homogenate was measured via a pH meter with a glass electrode (Hanna Instruments, U.S.). TTA was determined by titration with standardized 0.1 N NaOH by using phenolphthalein as indicator. The volume (ml) of 0.1 N NaOH which was consumed till the sample became colorless was the TTA value.

5.2.4.2. Lactic Acid Bacteria Cell Count

The LAB concentrations of 0, 3, 6, 24 and 48 h fermented sourdoughs were determined. 10 g of sourdough sample was suspended in 90 ml of sterile deionized water and homogenized with a stomacher for 60 s. Serial dilutions were prepared in peptone water. 1 ml of proper dilutions was added to petri dishes and MRS agar was poured according to double layer technique. Plates were incubated for 48 h at 37 and 42°C, the latter was for sample LD, and colonies on plates which contain a range of 30-300 colonies were counted.

5.2.5. Free Amino Nitrogen (FAN) Content

The amino nitrogen concentrations of sourdough samples were analyzed according to ninhydrin method with some modifications (ASBC, 1992). 5 mL of 200 mM sodium phosphate (pH 8.0) was added to 375 mg of sourdough sample. It was further extracted at room temperature for 1 h with vortex mixing occasionally, and then was centrifuged at 10000xg for 10 min. The supernatants were diluted with sodium phosphate buffer: Supernatants of 0, 3 and 6 h fermented sourdoughs were diluted at a ratio of 1:1 (extract volume/buffer volume); supernatants of 24 and 48 h fermented sourdoughs were diluted at a ratio of 1:2 and 1:3, respectively.

100 µl of ninhydrin color reagent was mixed with 200 µl of the diluted supernatant and placed in a boiling water bath, then this mixture was incubated for 16 min. After cooling at 20°C for 20 min, 0.5 mL of KIO_3 solution was added, and mixed. Within 30 min, the absorbance was measured at 570 nm against sodium phosphate buffer (Varioskan Flash, Thermo Scientific, U.S.). A standard calibration curve was plotted with glycine as a standard. The amino nitrogen contents of samples were calculated according to this calibration curve. The results were expressed as mg glycine/L.

FAN data were analyzed by analysis of variance (ANOVA) and Tukey's test at 5% significance level was used for comparison of means (MINITAB 14, Minitab Inc., U.S.).

5.2.6. Fractional Extraction of Wheat Proteins

Wheat protein fractions were extracted sequentially from sourdough samples according to modified procedure of a method described by Osborne (Osborne, 1907; Weiss et al., 1993). Sourdough samples taken at 0, 24 and 48 h of fermentation were used in extraction. 1 g of dough sample was extracted with 50 mM Tris-HCl buffer (pH 8.8) for 1 h at 4 °C with occasionally vortex mixing and centrifuged at 20,000xg for 20 min (Allegra 25R, TA-14-50 Rotor, Beckman Coulter, U.S.). The supernatant which was composed of albumin and globulin was removed. The pellet was treated with 4 ml of 75% ethanol (v/v) for 2 h at 25°C in an orbital shaker at 100 rpm and centrifuged. The supernatant which contains gliadins was taken. The pellet was extracted with 4 ml of SDS-DTT buffer for 2 h at room temperature with occasional vortex mixing and was centrifuged to get supernatant which contains glutenin fraction. The pellet obtained at each extraction step was extracted twice and the supernatants of these steps were discarded. Gliadin and glutenin extracts were stored at -80°C until further analyses.

5.2.7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was applied to gliadin and glutenin extracts. It was carried out according to Laemmli, (1970) and all solutions used in this analysis were given in Appendix B. PROTEAN II XL (20x22 cm) system (Bio-Rad, U.S.) was used. 12% separating gel was poured between the glass plates which were arranged by using 1 mm spacers. After separating gel was polymerized, 4% stacking gel was poured and 25 well comb was inserted. The gliadin extracts were mixed with sample buffer in a ratio of ≈1:9, according to nanodrop (NanoDrop 8000, Thermo Scientific) measurements (Appendix E) and were heated in a boiling water bath for 5 min. Molecular weight marker with a 10-170 MW range (Fermentas, Canada) was used. After loading 10 µl/well of each sample and 2 µl of marker to the wells, the gels were placed in tank and 1x running buffer was loaded. The lid of the tank was closed and the system was connected to a power supply (EC 3000 XL, Thermo Scientific, U.S.) and a cooling unit (PolyScience, U.S.). The electrophoretic run was carried out at a constant current of 32 mA for 30 min and 48 mA for 5 h at 10°C. In order to separate individual bands more

effectively, a 15% separating gel was also poured and electrophoresis was done under the same conditions as described before but the electrophoretic run was carried out for 8 h. After the run was over, gel was taken and put into fixer solution.

It was planned to use European Gliadin Reference (IRRM-480) as a standard. However, it was stated that the gliadin reference was no longer available and there were no plans to replace or reproduce it (Joanna Ostrowska from Institute for Reference Materials and Measurements, personal communication, April 13, 2010). As a result, no gliadin standard could be used in SDS-PAGE and also in further gliadin analysis.

5.2.8. Two-Dimensional Electrophoresis (2-DE)

Two-dimensional electrophoresis was carried out into two steps: Isoelectric focusing and SDS-PAGE.

5.2.8.1. First Dimension: Isoelectric Focusing (IEF)

First dimension consists of rehydration and sample application to IPG strips, isoelectric focusing and equilibration steps.

5.2.8.1.1. Rehydration and Sample Application

Immobilized pH gradient (IPG) strips, nonlinear pH 3-10 and 17 cm, were used in 2-DE. Gliadin extract, which contains 30 µg of protein, was mixed with rehydration buffer. 400 µl of each extract+rehydration buffer and also 2-D SDS-PAGE Standards (Bio-Rad, U.S.) were pipetted as a line along the edge of a channel in rehydration/equilibration tray. It was important to avoid bubble formation, which may interfere with the even distribution of sample in the IPG strip. After loading the sample, the cover sheet of the IPG strip was peeled using forceps and placed on the sample, gel side down. After the strip was incubated at room temperature for 1 h, 3 ml of mineral oil was poured on the strip to prevent evaporation during rehydration and the plastic lid of the rehydration/equilibration tray was covered. The strips were rehydrated overnight at 25 °C.

5.2.8.1.2. Separation According to pI

Paper wicks for each end of the channels of focusing tray were placed to cover the wire electrode. 10 µl of nanopure water was pipetted onto each wick to wet them. The rehydrated strips were allowed the mineral oil to drain on a filter paper by holding them vertically and they were transferred to a focusing tray by maintaining the gel side down. The "+" end of the strip should be positioned at the same side with the tray marked as "+". 3 ml of mineral oil was added onto the strips by avoiding any bubble formation and the tray lid was covered. The focusing tray was placed into the PROTEAN IEF cell (Bio-Rad, U.S.) and IEF was initiated after focusing conditions were programmed as shown in Table 5.2, at 20 °C (Di Cagno et al., 2002). The mineral oil was removed and the strips were transferred to rehydration/equilibration tray, gel side up. If the strips were used later, the covered tray was wrapped in with plastic film and stored at -80 °C.

Table 5.2. IPG Strip Focusing Conditions

	Start Voltage	**End Voltage**	**Time/Volt-Hours**	**Ramp**
Step 1	0 V	300 V	1 h	Linear
Step 2	300 V	500 V	3 h	Linear
Step 3	500 V	2000 V	4 h	Linear
Step 4	2000 V	8000 V	32000 V-h	Linear
Step 5	8000 V	500 V	99 h	Linear

5.2.8.1.3. Equilibration

6 ml of equilibration buffer I was added for one strip and the rehydration/equilibration tray was placed on an orbital shaker and was shaked at 40 rpm for 10 min. After incubation, equilibration buffer I was removed and 6 ml of

equilibration buffer II was added to each strip and shaking was carried out at 40 rpm for 10 min. Equilibration buffer II was discarded. SDS-PAGE was further applied to the strip.

5.2.8.2. Second Dimension: SDS-PAGE

For the second dimension, only 12% separating gel was prepared as defined previously and no stacking gel was used. After the separating gel was cast and allowed to polymerize, the low-melt overlay agarose was pipetted to the space above the gel. The IPG strip was dipped into the 100 ml graduated cylinder containing 1x running buffer to remove excess equilibration buffer and placed onto the back glass plate of the SDS gel. With the help of forceps, the IPG strip was pushed carefully, avoiding air bubble formation, along the space among the plates until it reached the top of the SDS gel. Although 2-D SDS-PAGE Standards were used as a separate gel, molecular weight marker (Fermentas) was also blotted on a very small piece of filter paper and pushed to the top of the SDS gel near the strip for all sourdough samples. After agarose was solidified, gel sandwiches were snapped onto the cooling core and the reservoirs was filled with 1x running buffer. Electrophoresis was carried out at a constant current of 48 mA for 5.5 h at 10°C. Gel was taken and put into fixer solution.

5.2.9. Silver Staining of SDS-PAGE AND 2-D Gels

Silver staining procedure was used for both staining of SDS-PAGE and 2-D electrophoresis gels. The steps of silver staining procedure were shown in Figure 5.2. Orbital shaker was utilized during the staining procedure. It was important that times at all stages should be exact. The compositions of the solutions used for the silver staining are given in Appendix B.

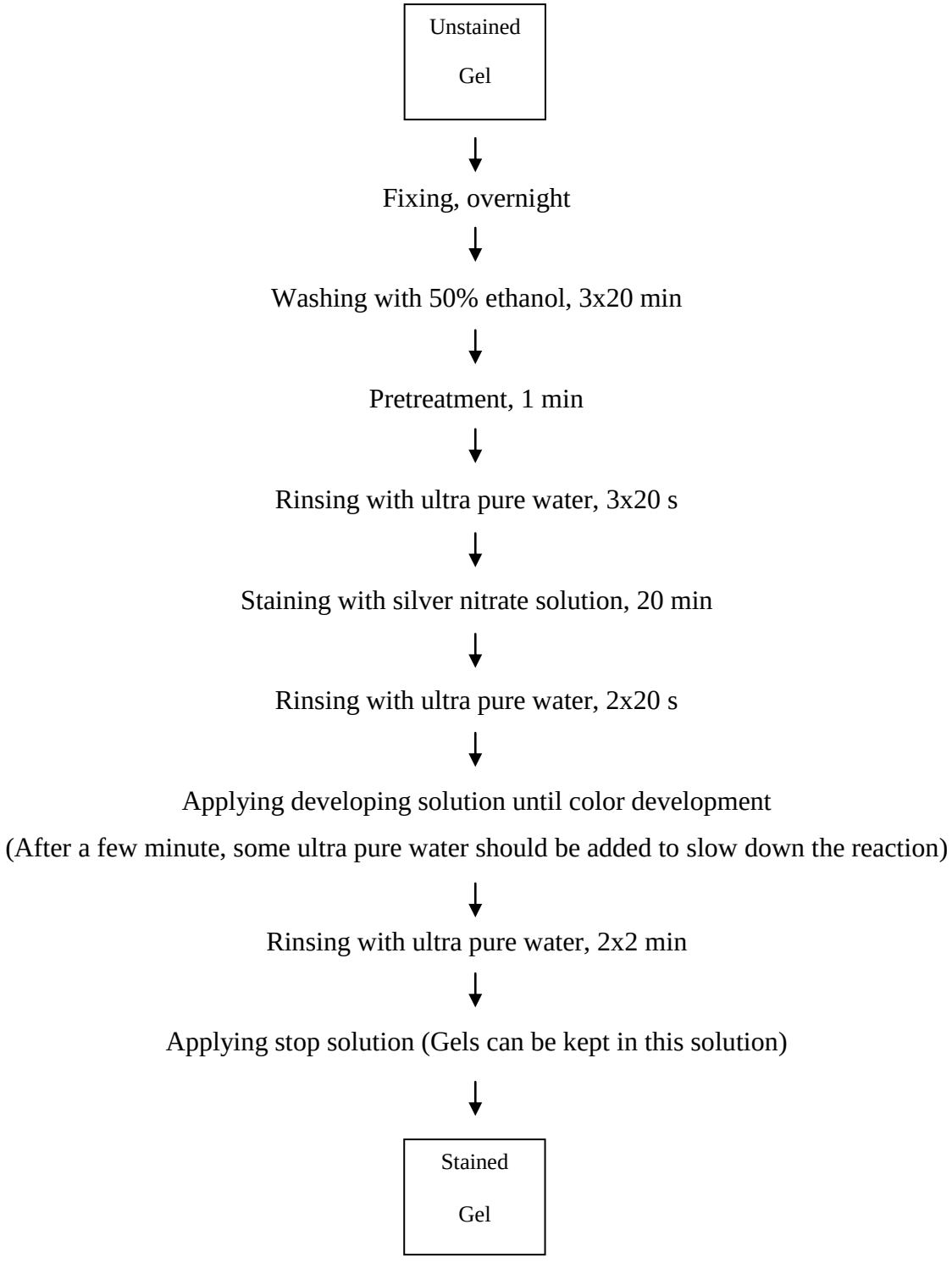


Figure 5.2. Silver staining procedure applied to SDS-PAGE and 2-DE gels.

5.2.10. Visualization of Stained Gels

The silver stained gels were monitored by using an imaging system (VersaDoc 4000 MP, Bio-Rad, U.S.). Analysis of SDS-PAGE and 2-D gels were performed by using Quantity One 1-D Analysis Software (Bio-Rad, U.S.) and Bio 2-D Software (Bio-Rad, U.S.), respectively. Molecular weights of protein bands of SDS-PAGE gels and for 2-D gels in addition to molecular weights pI values for protein spots were determined.

5.2.11. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC analysis of gliadins was done according to Qian et al. (2008) by using Perkin Elmer (PE) series 200 autosamplers, PE series 200 pump (Norwalk CT 06859), PE series 200 column heater, PE series 200 EP diode array detector (DAD) HPLC system. 15µl of filtered (0.45µm) gliadin extract was injected to 5µm, 15 cm x 4.6 mm Zorbax SB300-C18 Reverse-Phase column, (Agilent Technologies, U.S.) and analyzed for 120 min using a linear acetonitrile gradient from 24 to 50% over 108 min. Column temperature was 60°C and 210 nm was used to monitor column effluent. The compositions of mobile phase solutions were given in Appendix B.

CHAPTER 6

RESULTS AND DISCUSSIONS

6.1. Wheat Flour Analysis

It is important to validate that sourdough fermentation was performed by using a standard raw material, wheat flour. For this purpose, protein, moisture and ash contents of wheat flour were determined.

6.1.1. Protein Content

Protein content is of great importance in terms of nutritional value and technological aspects. Protein content of the wheat flour was found as 10.78 % of dry matter. In Codex Alimentarius, it was stated that the protein content of wheat flour must be minimum 7.0 % on a dry weight basis (Codex Alimentarius Commission, 1985). The wheat flour which was the raw material used in sourdough fermentation has protein content higher than this stated limit.

6.1.2. Moisture Content

The moisture content of wheat flour is of great importance in terms of microbial growth which affects the shelf-life. Moisture content of the wheat flour was calculated as 11.92 %. In Codex Alimentarius, the upper limit of wheat flour moisture content was stated as 15.5% (Codex Alimentarius Commission, 1985). According to this standard, the wheat flour which was used in sourdough fermentation has appropriate moisture content.

6.1.3. Ash Content

Ash content gives information about the mineral content of the wheat flour. Wheat flours were divided into three groups on the basis of their ash contents: Type 550, type 650 and type 850 which have ash contents as 0.55%, 0.65% and 0.85% on dry matter basis, respectively. Ash content of the wheat flour used in this study was determined as 0.54 % of dry matter (type 550).

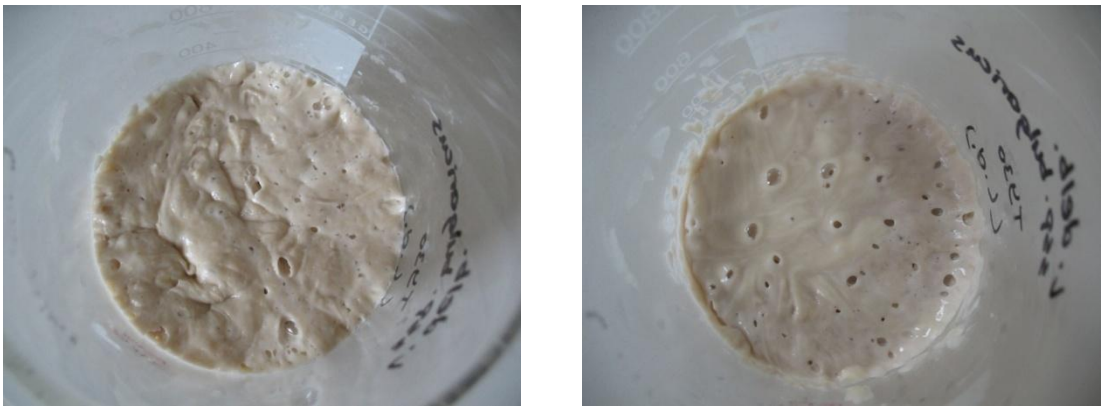
6.2. Catalase Activity

LAB lacks catalase enzyme which breaks down hydrogen peroxide and leads to formation of water and oxygen. This property is really useful in identification and assuring that no contamination exists. The LAB used in sourdough fermentation showed no catalase activity.

6.3. Sourdough Fermentation

As previously indicated in Chapter 4, sourdough fermentation enhances the nutritional value, texture and flavor of the final product and also improves the shelf-life. These positive changes are due to the microbial growth and wheat flour enzymes. Sourdough microflora consists of several LAB and yeasts. By the inoculation of additional LAB, some differences are likely to occur. In addition, sourdough fermentations with mixed cultures of selected strains were also performed because there could be synergistic effect of the strains (De Angelis et al., 2006). Also, to evaluate the changes in dough environment, fermentation parameters (pH, TTA and LAB concentration) were followed.

Sourdough fermentations were accomplished under the stated conditions. It was noticed that the doughs lost their consistency and liquefied as fermentation progressed (Figure 6.1)



(a) (b)

Figure 6.1. Sourdoughs fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* at (a) 24 h (b) 48 h of fermentation.

6.3.1. Fermentation Parameters

During sourdough fermentation, changes in the pH and TTA values occurred as expected, which is the indication of the adaptation of the LAB to wheat sourdough environment.

6.3.1.1. pH Profile

Changes of pH values in time scale for sourdough fermentation is given in Figure 6.2. As seen in this figure, during the whole sourdough fermentation process, there was a decreasing trend of the pH values. The pH values of the doughs which were in the range of 6.12-6.24 at the beginning of fermentation, decreased to 3.84-3.52 range in LAB inoculated doughs after 48 h. At 24 h, all inoculated doughs, except sample LD, seems to reach the values similar to those at 48 h. Since the pH dropped to only 4.55 at 24 h, it could be said that the slowest pH decrease was observed in sample LD due to the low growth rate of this strain. The pH decrease was faster in samples LA and M2 which contain *Lb. acidophilus* NRRL-B 1910. In control dough, the pH values at 24 h and 48 h of fermentation were 5.10 and 3.94, respectively. During the period after chemical acidification, only a small change was observed in the pH of CAD. It is important to mention that although there was a drop, pH of control dough was higher

than the inoculated doughs, particularly LA, LC, M1 and M2, at 24 h. But at 48 h, while there was not a significant drop in LA, LC, M1 and M2, the pH of control dough decreased to the level similar to other dough samples. These results show that the pH decrease in control dough was due to its own microflora. On the other hand, the pH of chemically acidified dough remained almost at the same level between 6 and 24 h. It is likely that the reduction of pH by the acid addition inhibits the natural growth of bacteria in wheat flour microflora. The artificial pH decline does not fit to the gradual decline that bacteria are used to adapt.

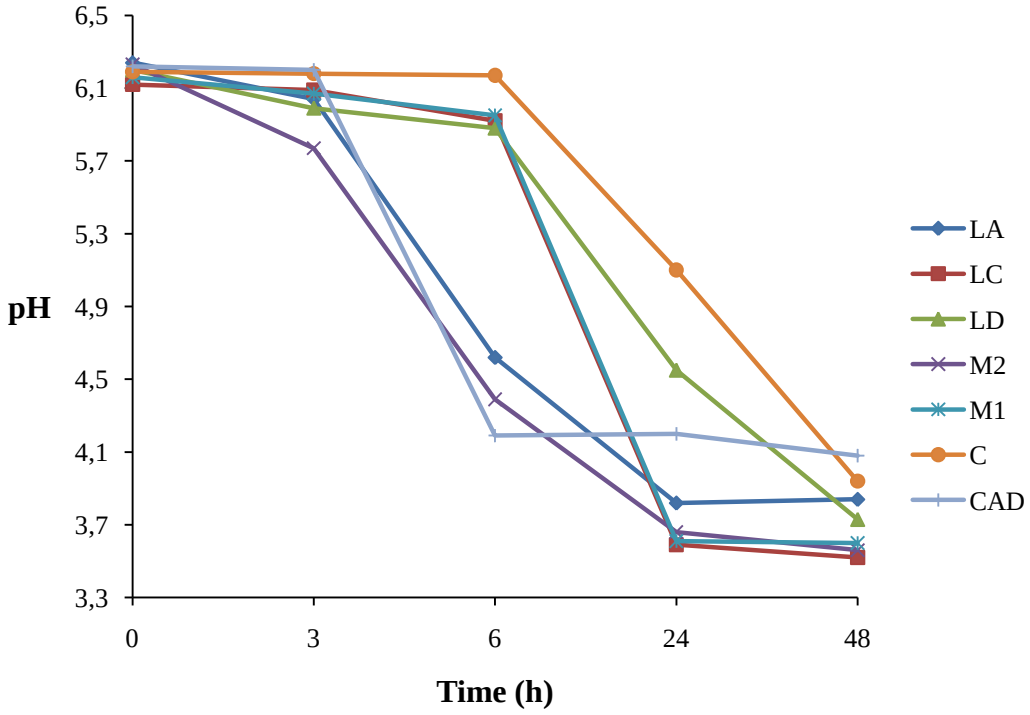


Figure 6.2. Changes in pH values of sourdough samples during fermentation.

Previous findings in various literature also confirm our results. In one of the study, final pH of sourdough samples were found as between the values of 3.9 to 3.7 (Di Cagno et al., 2004). After 24 h of sourdough fermentations with probiotic preparations, the pH was reached to 3.7 to 4.0 (De Angelis et al., 2006). It was observed that fermented doughs had the pH decrease from ≈ 6.5 to 4.5–5.0 after 8 h and at the end of 24 h pH was in the range of 3.5-4.0 (Thiele et al., 2002). In another study of the same

research group, samples were taken at 6 and 24 h which had pH ranges of 4.5-5.5 and 3.6-3.8, respectively (Thiele et al., 2004).

6.3.1.2. Total Titratable Acidity

Changes in TTA values are given in Figure 6.3. The total titratable acidity values of all LAB inoculated doughs, which were between 1.71-1.93 range initially, reached to 7.17-12.63 and 13.49-17.34 range after 24 and 48 h of fermentations, respectively. In accordance with the results of pH measurements, the acidity increased slowly in sample LD. In control dough, the TTA values at time 24 and 48 h of fermentation were 6.96 and 12.74, respectively. For chemically acidified dough, after the acidification, there was an increase in acidity but only ≈ 6 units. It should be indicated that although there was an acidity increase, acidity of control dough was lower than the inoculated doughs, especially LA, LC, M1 and M2 at 24 h. But at 48 h, while there was a slight increase in LA, LC, M1 and M2, the acidity of control dough increased to the level similar to other dough samples. These results show that the acidity increase in control dough was due to its own microflora. On the other hand, the acidity of chemically acidified dough shifted slightly. It is likely that the increase in acidity by the acid addition inhibits the natural growth of bacteria in wheat flour microflora.

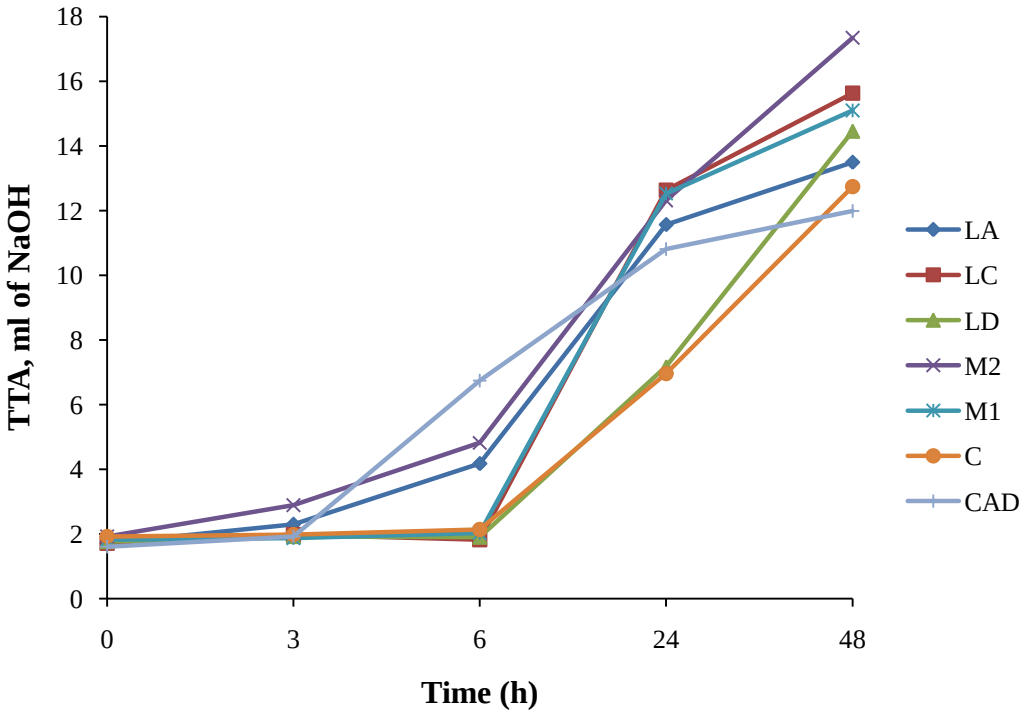


Figure 6.3. Changes in TTA values of sourdough samples during fermentation.

In previous studies, it was stated that the rye sourdough was reached to a TTA value of 17 ± 2.5 at the end of 24 h fermentation (Loponen et al., 2009).

6.3.1.3. Lactic Acid Bacteria Cell Count

As seen in Table 6.1 and Figure 6.4, LAB population of inoculated doughs was counted as ≈ $7.7x10^7$-$1.3x10^9$ cfu/g dough at the end of 24 h of fermentation. After 24 h, LAB concentrations of samples started to decrease except in the sample LD. The enumeration results of control and chemically acidified dough show that there occurred a LAB growth which constituted the microflora of wheat flour and the concentrations reached $2.7x10^3$, $3.2x10^7$ and $3.8x10^8$ cfu/g for control sample and $1.0x10^2$, $1.4x10^6$ and $3.2x10^7$ for CAD at 6, 24 and 48 h, respectively.

Table 6.1. LAB counts of sourdough samples during fermentation (cfu/g).

t (h) / Sample	Initial Time	After Inoculation	3	6	24	48
LA	0	$1.12x10^6$	$1.45x10^7$	$7.85x10^7$	$3.95x10^8$	$3.50x10^7$
LC	0	$6.25x10^6$	$7.80x10^6$	$3.00x10^7$	$1.66x10^9$	$9.53x10^8$
LD	0	$9.90x10^5$	$3.25x10^5$	$2.15x10^6$	$7.70x10^7$	$1.44x10^8$
M1	0	$6.75x10^6$	$1.92x10^7$	$2.00x10^7$	$1.27x10^9$	$9.70x10^8$
M2	0	$7.65x10^6$	$2.93x10^7$	$2.86x10^8$	$1.11x10^9$	$5.35x10^8$
C	0	0	0	$2.72x10^3$	$3.21x10^7$	$3.82x10^8$
CAD	0	0	0	$1.05x10^2$	$1.44x10^6$	$3.24x10^7$

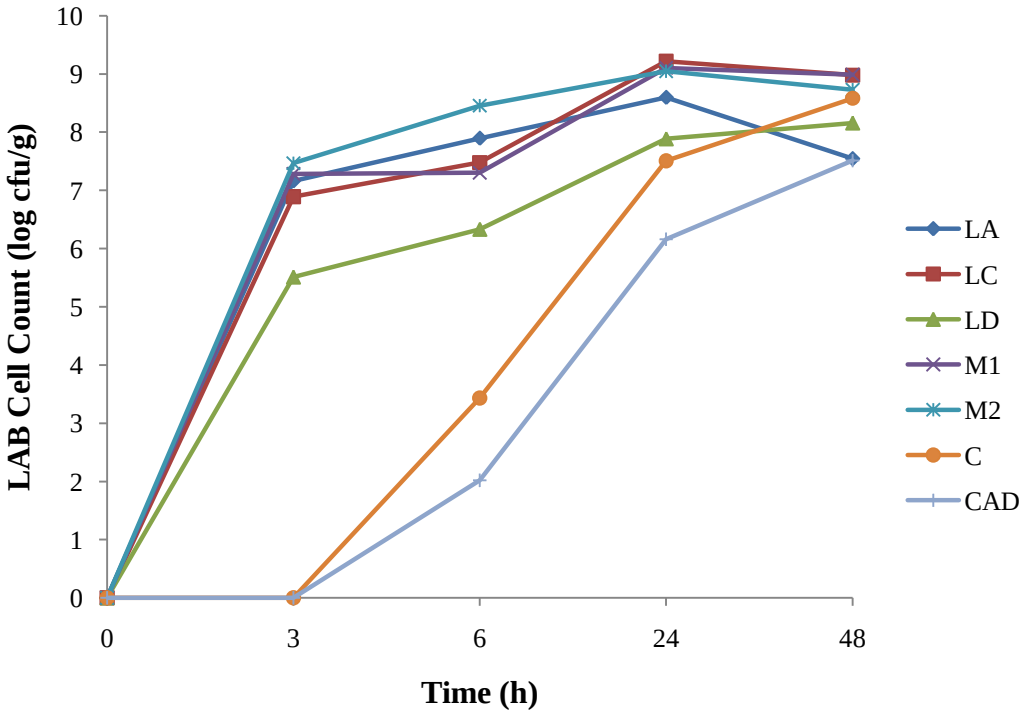


Figure 6.4. LAB counts of sourdough samples during fermentation (log cfu/g).

In a previous study in literature, the LAB content of sourdoughs at the end of 24 and 48 h were found ≈ 10^9 cfu/g sourdough (Di Cagno et al., 2005; Di Cagno et al., 2004; Di Cagno et al., 2002).

6.4. Free Amino Nitrogen (FAN) Content

The ninhydrin method gives information about the quantity of amino nitrogen available to yeast during fermentation and the amino nitrogen remains after the fermentation process in brewing technology. Amino acids, ammonia and end group α-amino nitrogen in peptides and proteins which were formed during hydrolysis of protein were measured by this method. It is also considered that the FAN value provides information on the extent of the proteolysis which occurs during the sourdough fermentation (Loponen et al., 2007).

Although a glycine solution (2 mg FAN/L) was used for determination of FAN value in the original method (ASBC, 1992), it was considered that preparing a calibration curve was more appropriate. The results were given as mg glycine/L. Similarly, in a previous study in literature, FAN values were determined by using a calibration curve and the results were stated as mmol of glycine/L (Thiele et al., 2002).

A standard calibration curve was plotted with a R^2 value of 0.9931 (Appendix C) and changes in FAN values of sourdough samples are given in Appendix D. The FAN values, which were in the range of 55.37-69.86 mg glycine/L at the beginning of fermentation, reached to the range of 201.78-274.43 mg glycine/L after 24 h of fermentation (Figure 6.5). FAN content of all sourdough samples increased as fermentation progressed, especially after 6 h. The FAN concentration was highest in dough LA and the lowest in sample LD. This could be because the acidity was developed faster in LA which extended the time of exposure to acidity that leads to proteolysis. Control dough (C) also resulted in FAN concentration near to that of LA.

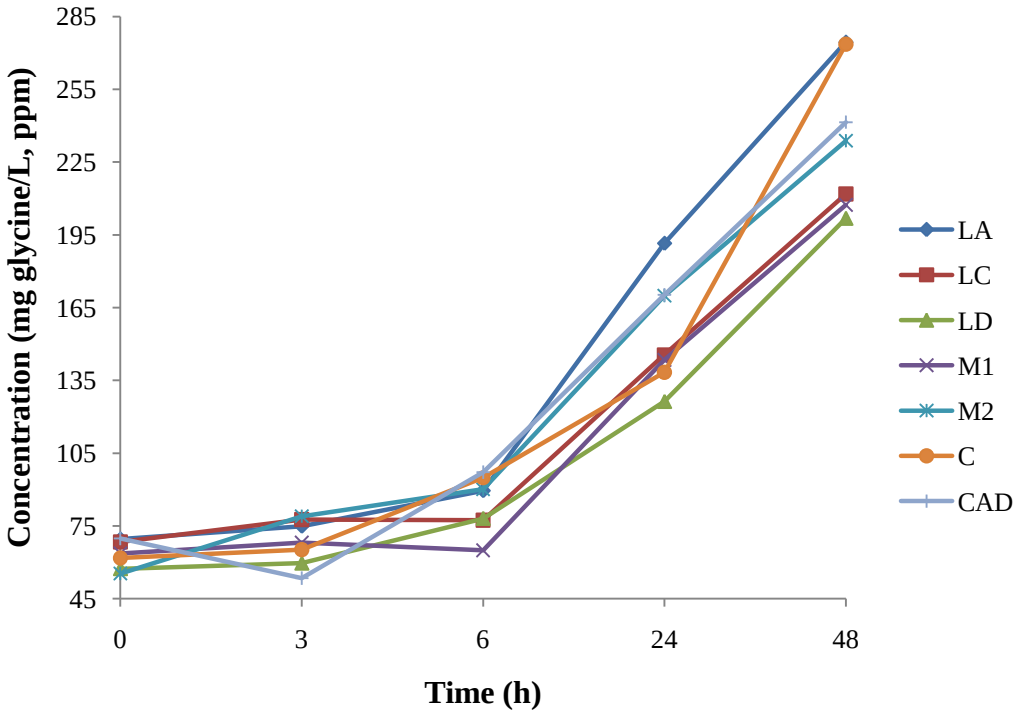


Figure 6.5. Changes in FAN concentrations of sourdough samples during fermentation.

It is obvious that the FAN contents of sourdough samples are related with the acidity of samples. In inoculated doughs (LA, LC, LD, M1 and M2) as LAB grow, the acidity and FAN content increased. In CAD and C, also the FAN contents correlate with acidity. In conclusion, the proteolysis correlates well with acidity development.

It was concluded that comparison of FAN data in terms of the changes in FAN content during definite time intervals provides more information. After 6 h of fermentation, the FAN contents increased, therefore the slopes of the curves for time intervals 6-24 h and 24-48 h were compared, separately. According to data analysis, effect of the types of sourdough samples was found significant ($p < 0.005$). When the slopes of the lines between 6 and 24 h of fermentation were compared, significant differences were investigated between all samples. During this fermentation period, a significant difference between control dough and inoculated doughs, except LD, was observed. Also, no significant differences were observed between CAD and LC, CAD and LD, and M1 and M2. The comparison of the slopes of 24-48 h time interval reveals that all FAN values differs from control dough, whereas no significant difference found between CAD and inoculated dough samples.

In one of the previous studies, it was found that the release of amino nitrogen is correlated with the growth of the organisms (Thiele et al., 2002). The amino acid concentration started to decrease together with the growth of yeasts; contrarily, a linear increase in amino acid levels were observed in doughs fermented with lactobacilli. The acidified dough also had FAN concentration similar to that of doughs fermented with lactobacilli, indicating the proteolytic activity of these bacteria is negligible compared with the wheat flour proteolytic activity. These results were confirmed later with another study which concluded that no remarkable differences were obtained between sourdoughs and neutral or acid control doughs (Thiele et al., 2004). Other work which investigated the amino nitrogen contents showed that FAN values were higher in lactobacilli-fermented rye doughs when compared to chemically acidified rye doughs. Sourdoughs in which germinated rye was used had the amino nitrogen contents about 5-fold greater than that of rye sourdoughs (Loponen et al., 2009).

6.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to investigate protein hydrolyzation during sourdough fermentation, SDS-PAGE was applied to gliadin and glutenin extracts of each sample. In SDS-PAGE, the proteins are separated according to their molecular weights. The principle of this technique was explained in detail in Chapter 2.

6.5.1. SDS-PAGE Gel of Glutenins

The silver stained SDS-PAGE gel of glutenin fractions of all sourdough samples is shown in Figure 6.6.

It was known that glutenins are grouped as LMW-, y-HMW- and x-HMW-glutenins which have molecular weights of 36,000–44,000, 90,000–102,000 and 104,000–124,000, respectively (van Eckert et al., 2010). LMW-glutenin subunits also classified as B- (Mr 42-51,000), C- (Mr 30-40,000) and D-LMW-glutenin subunits (Mr 55-70,000) (Lindsay and Skerritt, 1999).

After 24 h of fermentation, in CAD and all inoculated doughs except LD, the band with a molecular weight of ≈ 36.39 kDa disappeared and another band at 26.79 kDa lost its intensity (Figure 6.7). By the time these bands disappeared, new bands with molecular weights around 26-23 kDa and a band at 37.47 kDa formed. The same changes observed in sample LD and C, but more slowly, at 48 h. Additionally, some of the bands between 35-23 kDa lost their intensities and degraded to small fragments after 48 h. According to these results, it is likely to say that the alteration was related with acidity and could be attributed to wheat flour enzymes.

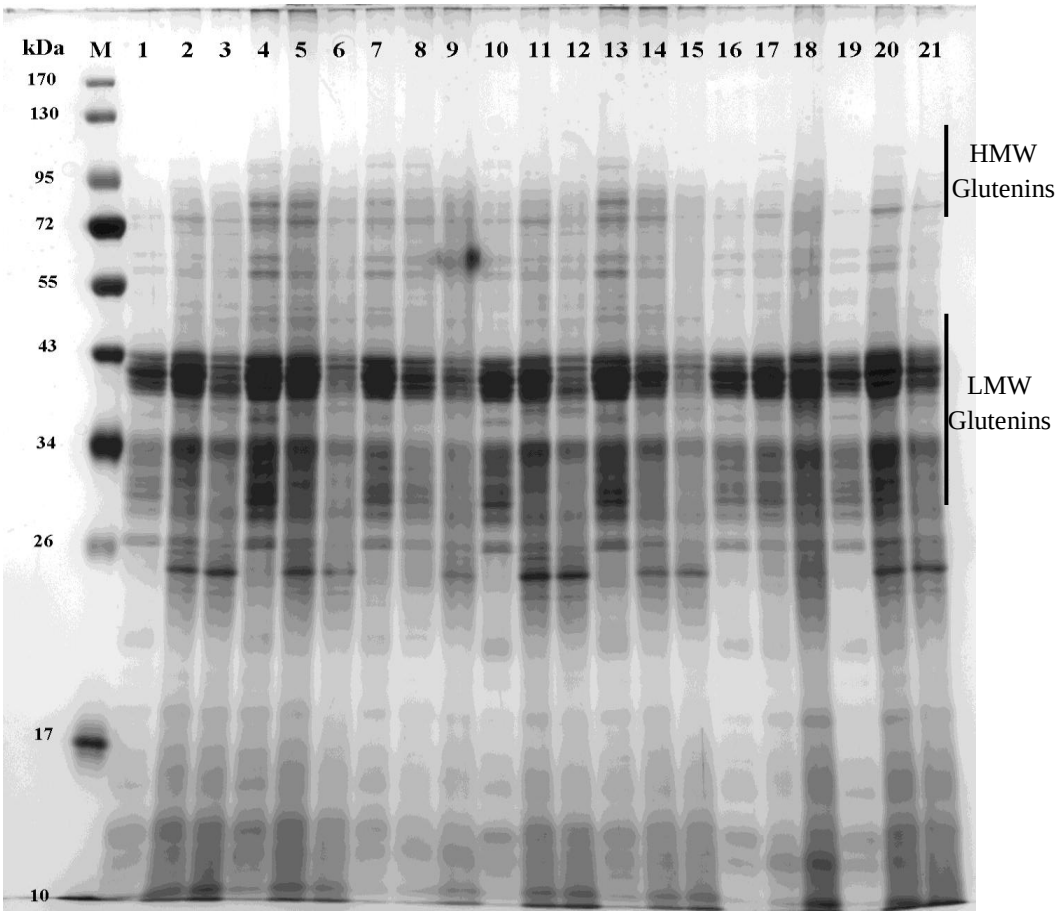


Figure 6.6. SDS-PAGE gel of glutenin fractions from sourdough samples. M represents the molecular weight marker; Lanes 1, 2, 3 correspond to sample LA at 0, 24 and 48 h; Lanes 4, 5, 6 correspond to sample LC at 0, 24 and 48 h; Lanes 7, 8, 9 correspond to sample LD at 0, 24 and 48 h; Lanes 10, 11, 12 correspond to sample M2 at 0, 24 and 48 h; Lanes 13, 14, 15 correspond to sample M1 at 0, 24 and 48 h; Lanes 16, 17, 18 correspond to sample C at 0, 24 and 48 h; Lanes 19, 20, 21 correspond to sample CAD at 0, 24 and 48 h.

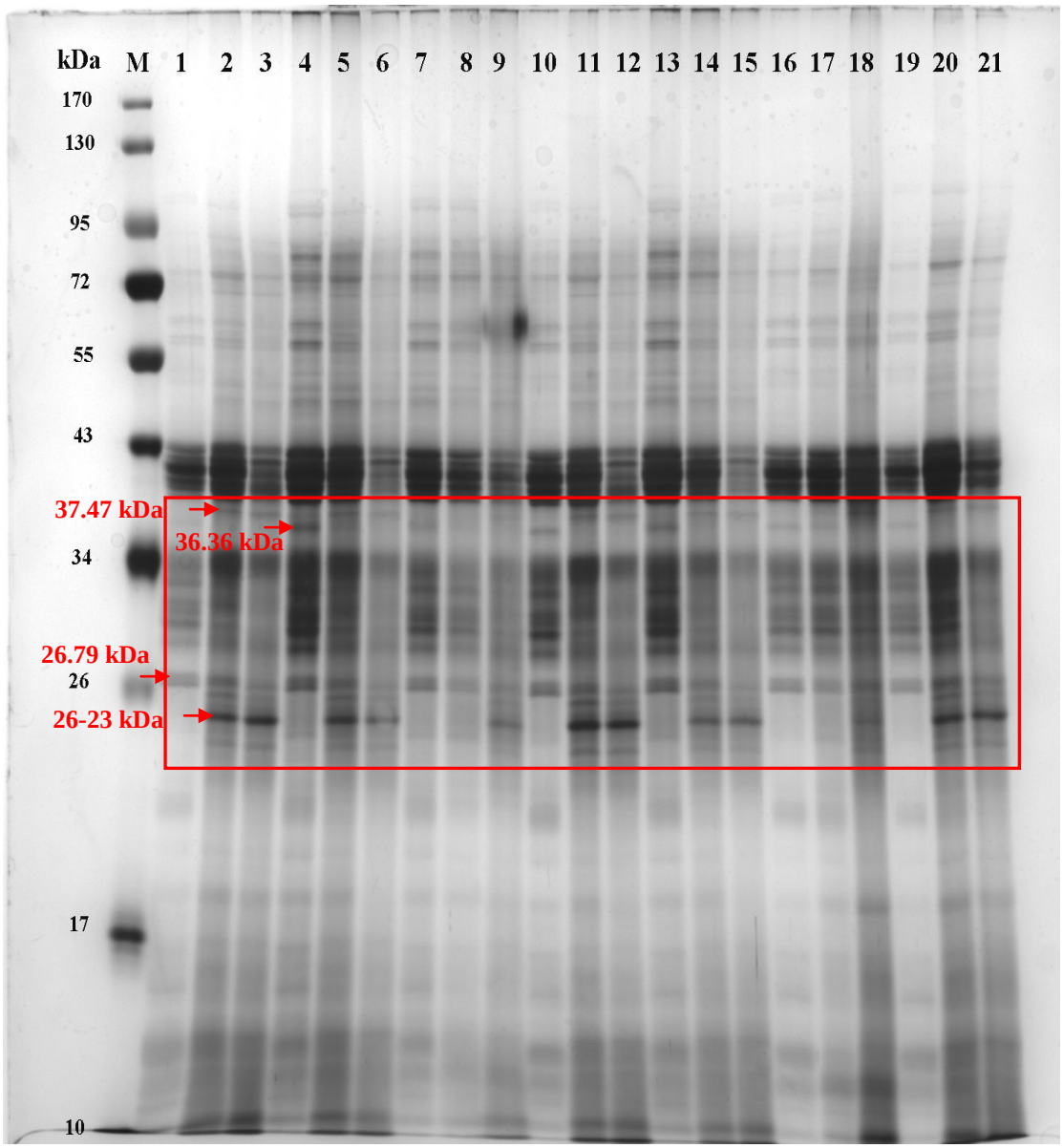


Figure 6.7. SDS-PAGE gel of glutenin fractions with molecular weights of disappeared and formed bands. M represents the molecular weight marker; Lanes 1, 2, 3 correspond to sample LA at 0, 24 and 48 h; Lanes 4, 5, 6 correspond to sample LC at 0, 24 and 48 h; Lanes 7, 8, 9 correspond to sample LD at 0, 24 and 48 h; Lanes 10, 11, 12 correspond to sample M2 at 0, 24 and 48 h; Lanes 13, 14, 15 correspond to sample M1 at 0, 24 and 48 h; Lanes 16, 17, 18 correspond to sample C at 0, 24 and 48 h; Lanes 19, 20, 21 correspond to sample CAD at 0, 24 and 48 h.

6.5.2. SDS-PAGE Gel of Gliadins

The silver stained SDS-PAGE gel of gliadin fractions of all sourdough samples was shown in Figure 6.8. The SDS-PAGE results of gliadins extracted from samples which were taken at the beginning (0 h) of sourdough fermentation shows gliadin bands in the 42-29 kDa range. It is known from previous studies and literature that gliadins are divided into three groups according to their mobilities in SDS-PAGE gel as ω-gliadins (50,000-75,000), γ-gliadins and α/β-gliadins (Mr 30,000-45.000) (Lindsay and Skerritt, 1999; Shewry et al., 1997; Weiss et al., 1993). According to this information, it could be said that, our gliadin extracts contain γ-gliadins and α/β-gliadins. The absence of ω-gliadins could be due to the extraction procedure. The SDS-PAGE gel also contains bands which have lower M_rs such as 21,000- 10,000. This is because small amounts of polymers related to the glutenins are also present in the alcohol-extractable gliadin fraction. They differ from the alcohol-unextractable glutenins in having lower molecular weights and higher contents of LMW subunits. They are called as "aggregated gliadins" or "low molecular weight glutenin" (Shewry and Tatham, 1997).

The gliadins are stacked between 42-29 kDa region and it is hard to recognize individual bands. As stated, 15% separating gel was used with 8 h electrophoresis in addition to 12% gel to overcome this problem. The obtained gel is shown in Figure 6.9. Although the bands were separated better, no significant differences when compared to 12% gel were observed. So, the band evaluation was done on 12% acrylamide gel.

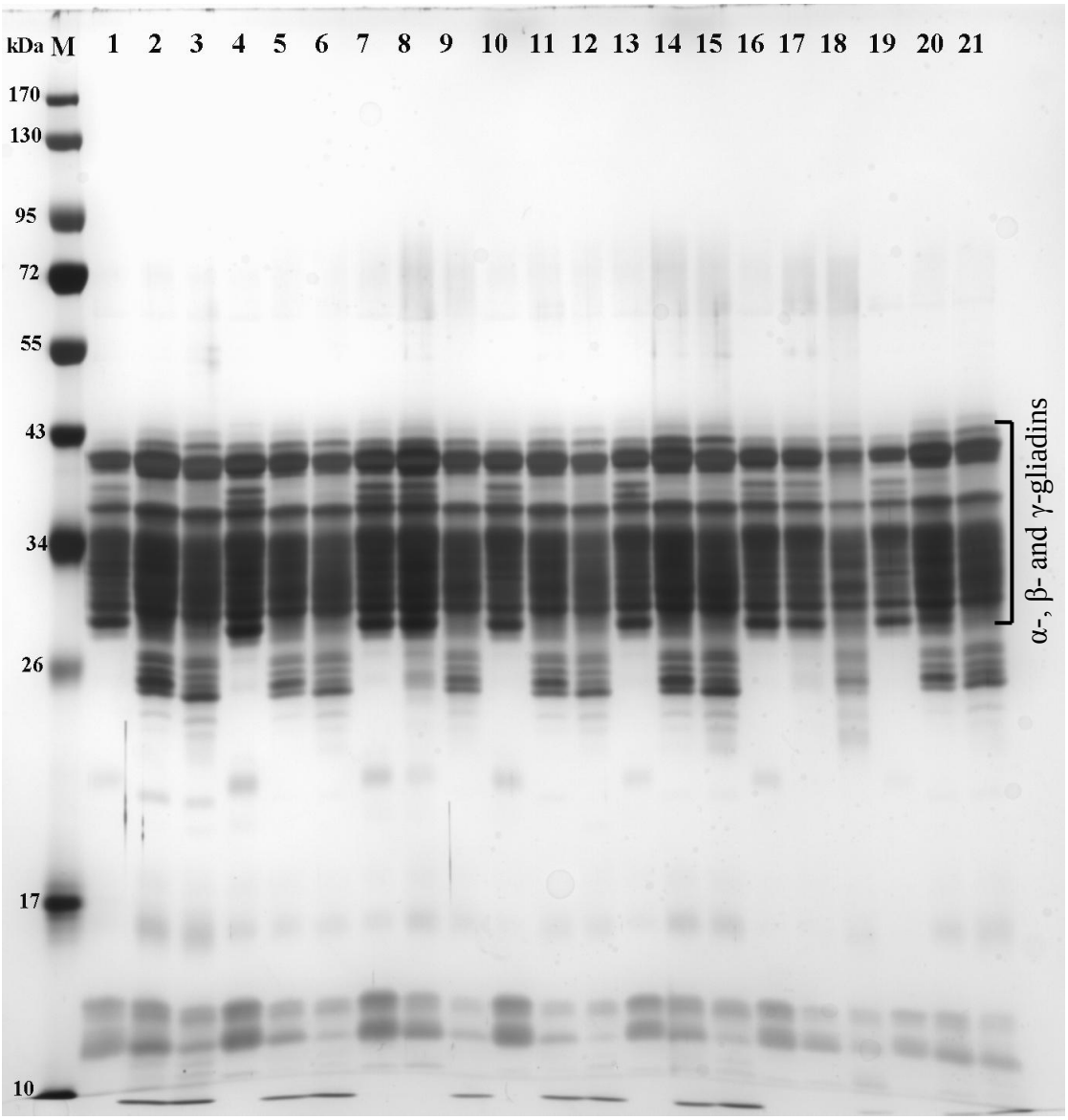


Figure 6.8. SDS-PAGE gel (12%) of gliadin fractions from sourdough samples. M represents the molecular weight marker; Lanes 1, 2, 3 correspond to sample LA at 0, 24 and 48 h; Lanes 4, 5, 6 correspond to sample LC at 0, 24 and 48 h; Lanes 7, 8, 9 correspond to sample LD at 0, 24 and 48 h; Lanes 10, 11, 12 correspond to sample M2 at 0, 24 and 48 h; Lanes 13, 14, 15 correspond to sample M1 at 0, 24 and 48 h; Lanes 16, 17, 18 correspond to sample C at 0, 24 and 48 h; Lanes 19, 20, 21 correspond to sample CAD at 0, 24 and 48 h.

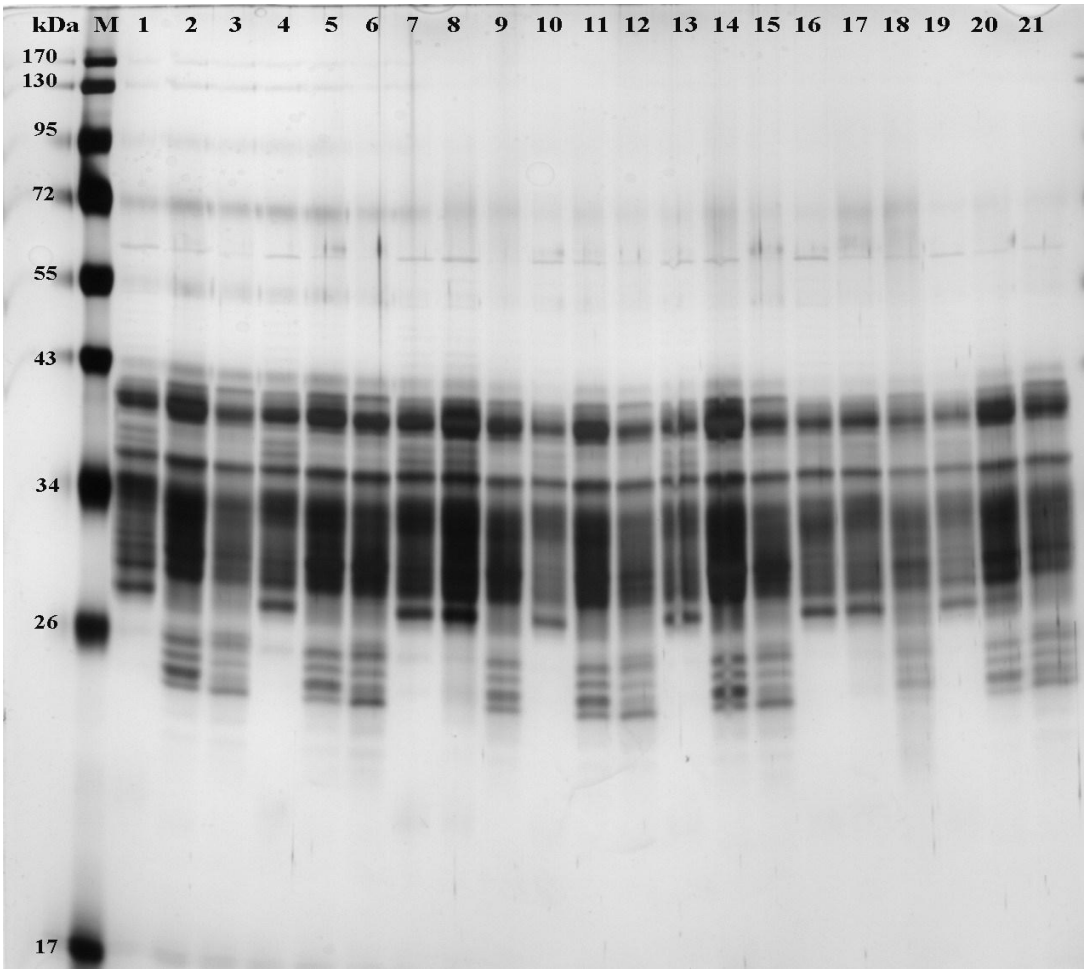


Figure 6.9. SDS-PAGE gel (15%) of gliadin fractions from sourdough samples. M represents the molecular weight marker; Lanes 1, 2, 3 correspond to sample LA at 0, 24 and 48 h; Lanes 4, 5, 6 correspond to sample LC at 0, 24 and 48 h; Lanes 7, 8, 9 correspond to sample LD at 0, 24 and 48 h; Lanes 10, 11, 12 correspond to sample M2 at 0, 24 and 48 h; Lanes 13, 14, 15 correspond to sample M1 at 0, 24 and 48 h; Lanes 16, 17, 18 correspond to sample C at 0, 24 and 48 h; Lanes 19, 20, 21 correspond to sample CAD at 0, 24 and 48 h.

In all gliadins, which were extracted from inoculated sourdough samples taken at 0 h of fermentation, the bands at 38 and 37 kDa existed (Figure 6.10) With the progress of fermentation, changes occurred in the protein structure and these two bands disappeared. When looking at the bands at 24 h, except the sample LD, bands with molecular weights of 38, 37 and 28 kDa disappeared, on the other hand, 4 new bands with molecular weights in the range of 27-25 kDa formed. The newly formed bands were considered as the degradation products of disappeared bands. The same changes were observed in sample LD at 48 h, but more slowly. In control dough, the same

changes occurred as in the LD, but only 2 new bands formed (27 and 25 kDa) instead of 4 bands. CAD had also the same band appearance as the inoculated doughs. Although time differs, these changes occurred during sourdough fermentation in all samples and also in chemically acidified and control doughs. Because of this reason, the disappearance and formation of bands could be attributed to protein degradation which is not specific to any bacterial species used. The enzymes present in wheat flour such as aspartic proteinases and serine carboxypeptidases which are active at acidic pH ranges, 3.0-4.5 and 4.0-6.0, respectively, are likely to be responsible for the degradation (Loponen, 2006).

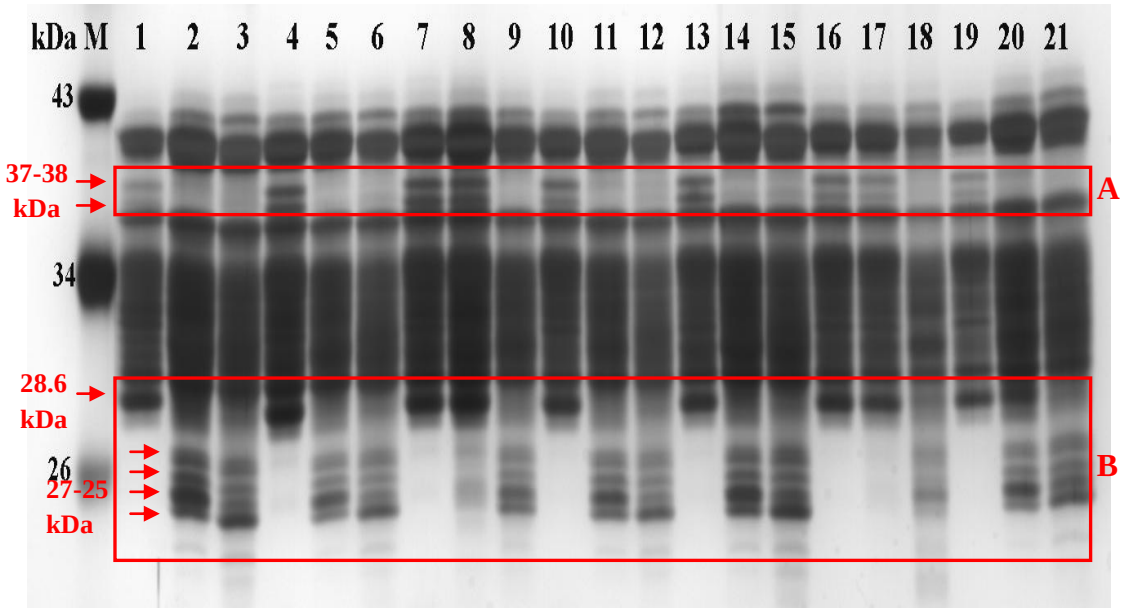


Figure 6.10. SDS-PAGE gel of gliadin fractions with molecular weights of disappeared and formed bands. M represents the molecular weight marker; Lanes 1, 2, 3 correspond to sample LA at 0, 24 and 48 h; Lanes 4, 5, 6 correspond to sample LC at 0, 24 and 48 h; Lanes 7, 8, 9 correspond to sample LD at 0, 24 and 48 h; Lanes 10, 11, 12 correspond to sample M2 at 0, 24 and 48 h; Lanes 13, 14, 15 correspond to sample M1 at 0, 24 and 48 h; Lanes 16, 17, 18 correspond to sample C at 0, 24 and 48 h; Lanes 19, 20, 21 correspond to sample CAD at 0, 24 and 48 h.

6.6. Two-Dimensional Electrophoresis (2-DE) of Gliadins

Two-dimensional electrophoresis was applied only to gliadin extracts, because of their significantly higher content of toxic fractions in comparison to glutenins. Some toxic fragments were mentioned in Section 3.3. According to SDS-PAGE results, 2-D electrophoresis was applied to 9 different gliadin extracts. The samples of LC (0 h), LA (24 h), LC (24 h), M1 (24 h), M2 (24 h) and LD (48 h) were analyzed together with C (0 h), C (24 h) and CAD (24 h) (Figure 6.11). The selection of these samples was due to the SDS-PAGE results: for each sample, the gliadin extracts, at the time when their band modifications were observed in SDS-PAGE, were further analyzed by 2-DE.

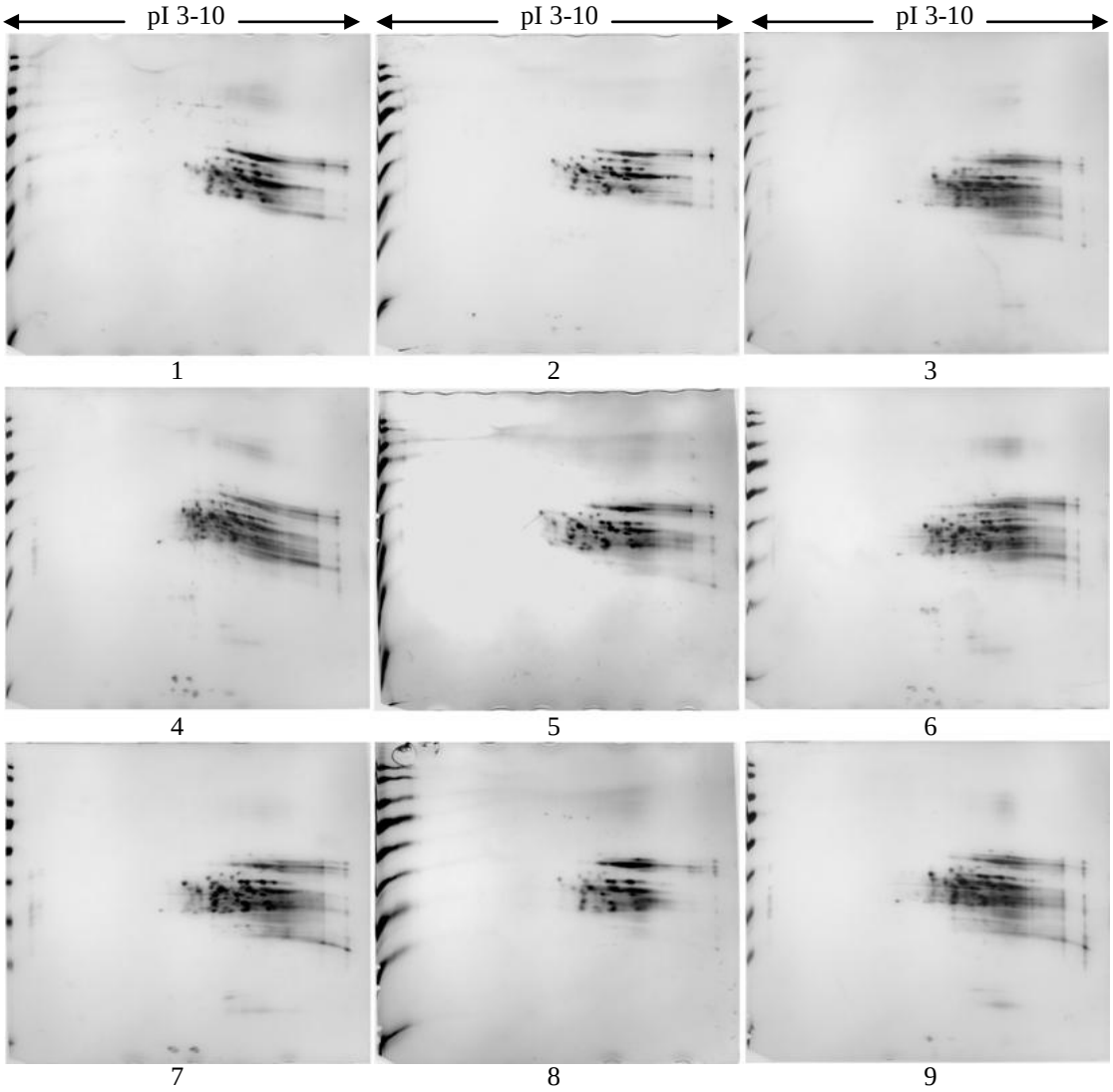
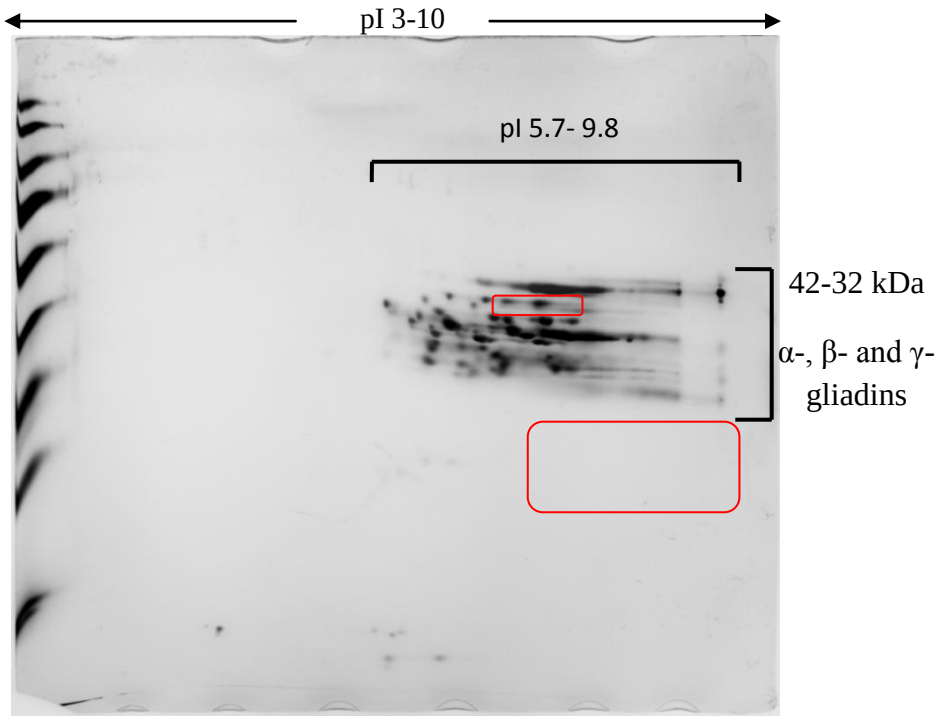


Figure 6.11. 2-D gels of gliadin fractions from sourdough samples. (1) C at 0 h (2) LC at 0 h (3) CAD at 24 h (4) C at 24 h (5) LC at 24 h (6) LA at 24 h (7) M1 at 24 h (8) M2 at 24 h (9) LD at 48 h.

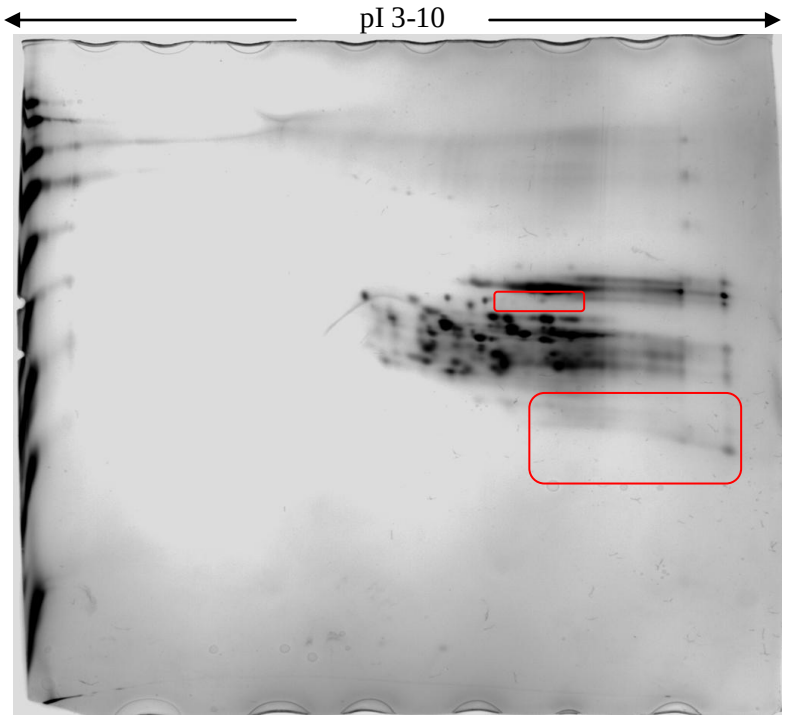
Figures 6.12 shows the 2-D gel of gliadins extracted from sample LC at 0 h and 24 h, M1 at 24 h and LD at 48 h in detail. In our 2-D gels, gliadin spots located mostly in 42-30 kDa range and at pI 5.7-9.8. About 51 spots were detected on 2-D gels of gliadin extracts of samples taken at the beginning of fermentation (0 h). According to a previous study, the α-, β- and γ-gliadins **(**M_r 35,000-50,000) are located at the more basic end of the gel ≈ pI from 6.5 to 9, whereas the ω-gliadins are present in pI 4-7 range (Weiss et al., 1993). In accordance with SDS-PAGE results, it could be said that our extracts contains α-, β- and γ-gliadins. In Figure 6.13 the disappeared and formed spots were circled in red and were indicated with the arrows. After 24 h of fermentation some spots disappeared: two spots (spot 1 and 2) with molecular weight of ≈ 39 kDa and pI of 6.4 and 6.7; one spot (spot 3) with molecular weight of ≈ 38 kDa and pI of 7.1. Also newly formed very small spots which were distributed in two wide regions (region 4) one with the range of ≈ 30-29 kDa and pI of 6.5-8.9 and the other in the range of ≈ 28-27 kDa and pI of 8.9-9.5 were observed.

As the gliadins separated according to both their molecular weights and isoelectric points, 2-DE provides more information about degraded fractions. One protein band in SDS-PAGE was separated into several spots. For instance, two of the protein bands located at 37-38 kDa, were divided nearly to 7-8 spots in 2-D gel.

The results of 2-DE agreed with SDS-PAGE findings. The degradations occurred in all gliadins and were parallel with acidity increase. The spot disappearance and formation occurred in LA, LC, M1, M2 and CAD after 24 h, in control and LD after 48 h. Changes occurred in all samples and also in chemically acidified and control doughs. The disappearance and formation of the bands are probably due to the effect of acidification. LAB gradually shifts the pH to ≈ 3.5-4.0 and during this period wheat flour proteolytic enzymes reach their optimum pH values. Activated enzymes catalyze the breakdown of proteins to peptides. Since no different band alteration occurred in chemically acidified dough, it is likely that protein degradation is not specific to any bacterial species used.



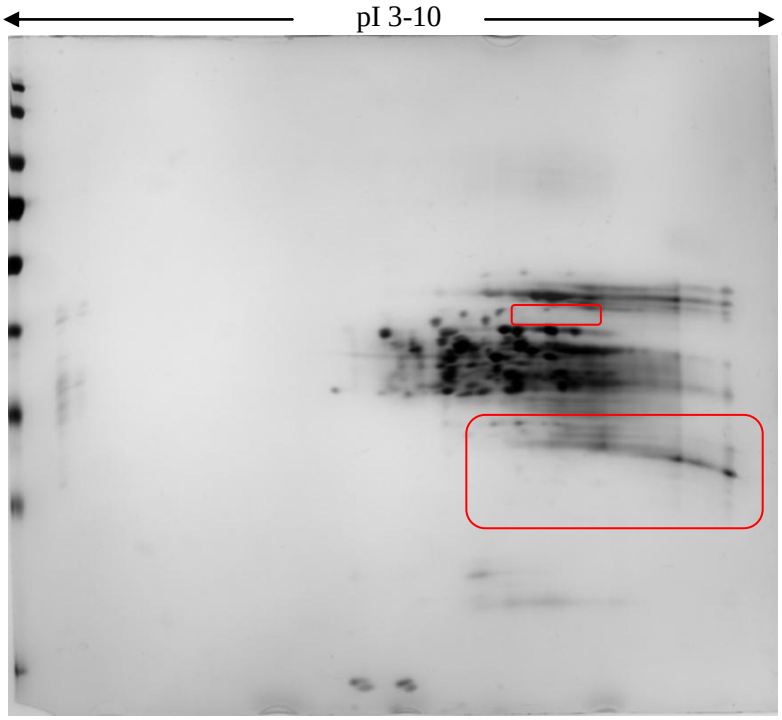
(a)



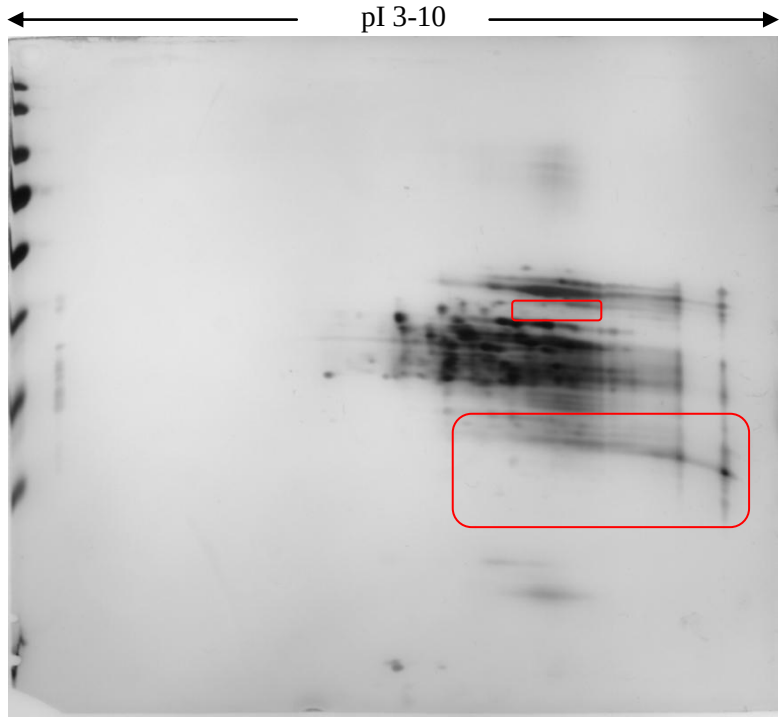
(b)

Figure 6.12. 2-D gels of gliadin extracts from (a) LC at 0 h (b) LC at 24 h (c) M1 at 24 h and (d) LD at 48 h.

(cont. on next page)



(c)



(d)

Figure 6.12 (cont.).

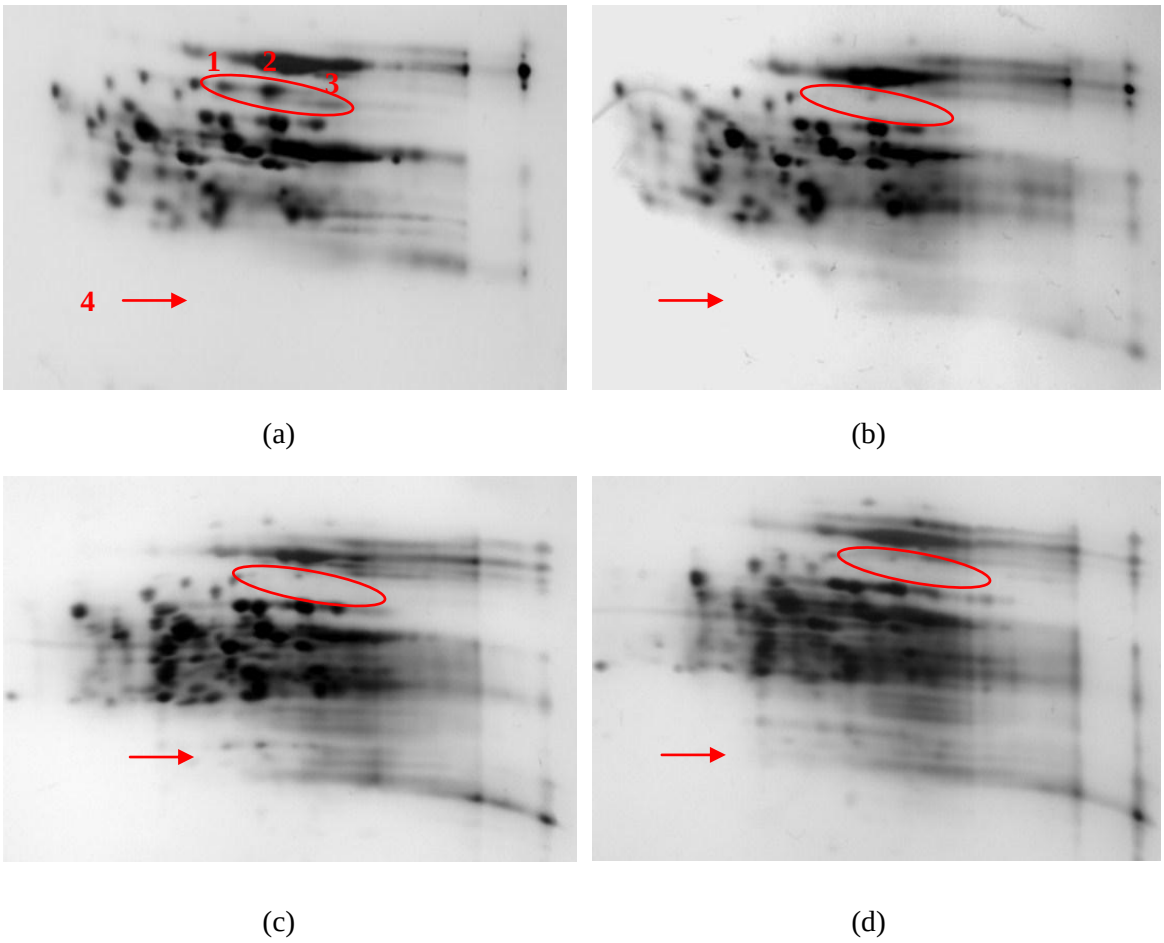


Figure 6.13. Enlarged views of 2-D gels of gliadin extracts from (a) LC at 0 h (b) LC at 24 h (c) M1 at 24 h and (d) LD at 48 h.

6.7. RP-HPLC of Gliadins

It was previously found that gliadins are eluted based on their surface hydrophobicity, so ω-gliadins which have lower surface hydrophobicities elute first. α and γ-gliadins are eluted after ω-gliadins (Bietz, 1983). It is also important to state that, the aqueous alcohol extracts also contain aggregated gliadins (ethanol-soluble glutenin). Together with α- and γ-gliadins, these fractions are also eluted (Wieser et al., 1998). From quantitative point of view, α-gliadins are generally predominant and are followed by γ- gliadins and ω- gliadins (Wieser, 2000).

RP-HPLC of gliadins was carried out in order to confirm SDS-PAGE and 2-DE results. All gliadin extracts from sourdough samples taken at times 0, 24 and 48 h were analyzed by using RP-HPLC. In Figure 6.14, the RP-HPLC profile of control dough at 0 h was shown. According to peak assignments in Qian et al. (2008), it is likely to say that α- and γ-gliadins form the distinctive peaks.

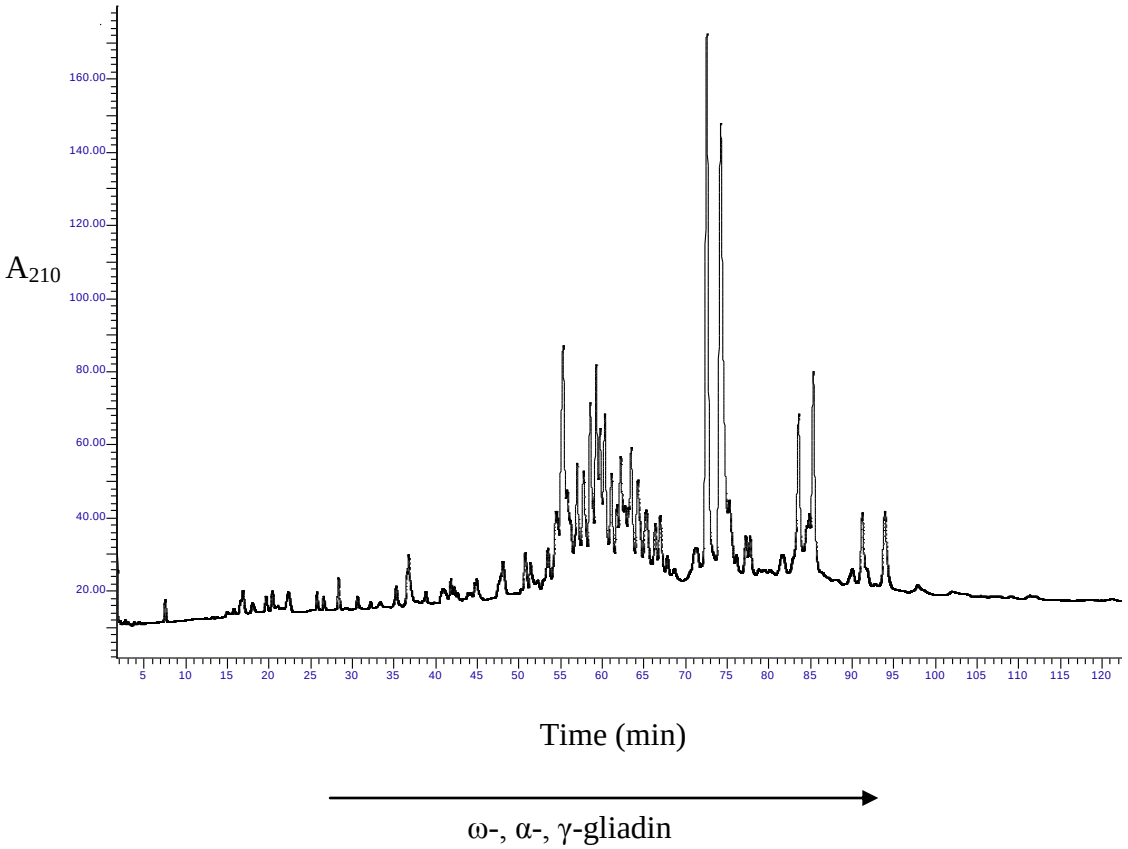


Figure 6.14. RP-HPLC results of gliadins from control dough (C) at 0 h.

The chromatograms of all gliadin samples were given in Appendix F. Since similar changes were observed, only the representative chromatograms of gliadin extracts of sample LC (0, 24 and 48 h) were given in Figure 6.15. In all dough samples, the peak appearance of gliadin fractions underwent some significant changes, which occurred due to the structural changes of proteins, as fermentation progressed. The main alterations in chromatograms of samples LA, LC, M1 and M2 were observed after 24 h and 48 h of fermentations. The peaks 3 (R_t=83.58) and 4 (R_t=85.45) disappeared, also

peaks 5(R_t=91.45) and 6 (R_t=94.22) lost their intensities after 24 h. Samples LA, LC, M1 and M2 further had additional changes such as the disappearance of peaks 1(R_t=66.26), 2 (77.16 and 77.92), 5(R_t=91.45) and 6 (R_t=94.22) at 48 h. In control dough, CAD and LD no distinctive changes were investigated after 24 h. At the end of 48 h of fermentation, the peaks 3 (R_t=83.58) and 4 (R_t=85.45) disappeared, also peaks 1(R_t=66.26), 2 (77.16 and 77.92), 5(R_t=91.45) and 6 (R_t=94.22) lost their intensities. These results were in accordance with SDS-PAGE and 2-DE results. On the other hand, RP-HPLC seems more versatile than electrophoretic methods. While no differences in bands and/or spots were observed in gel for LC at 24 and 48 h in electrophoresis gels, the disappearance of peaks 2, 5 and 6 could be observed in RP-HPLC chromatograms (Figure 6.15).

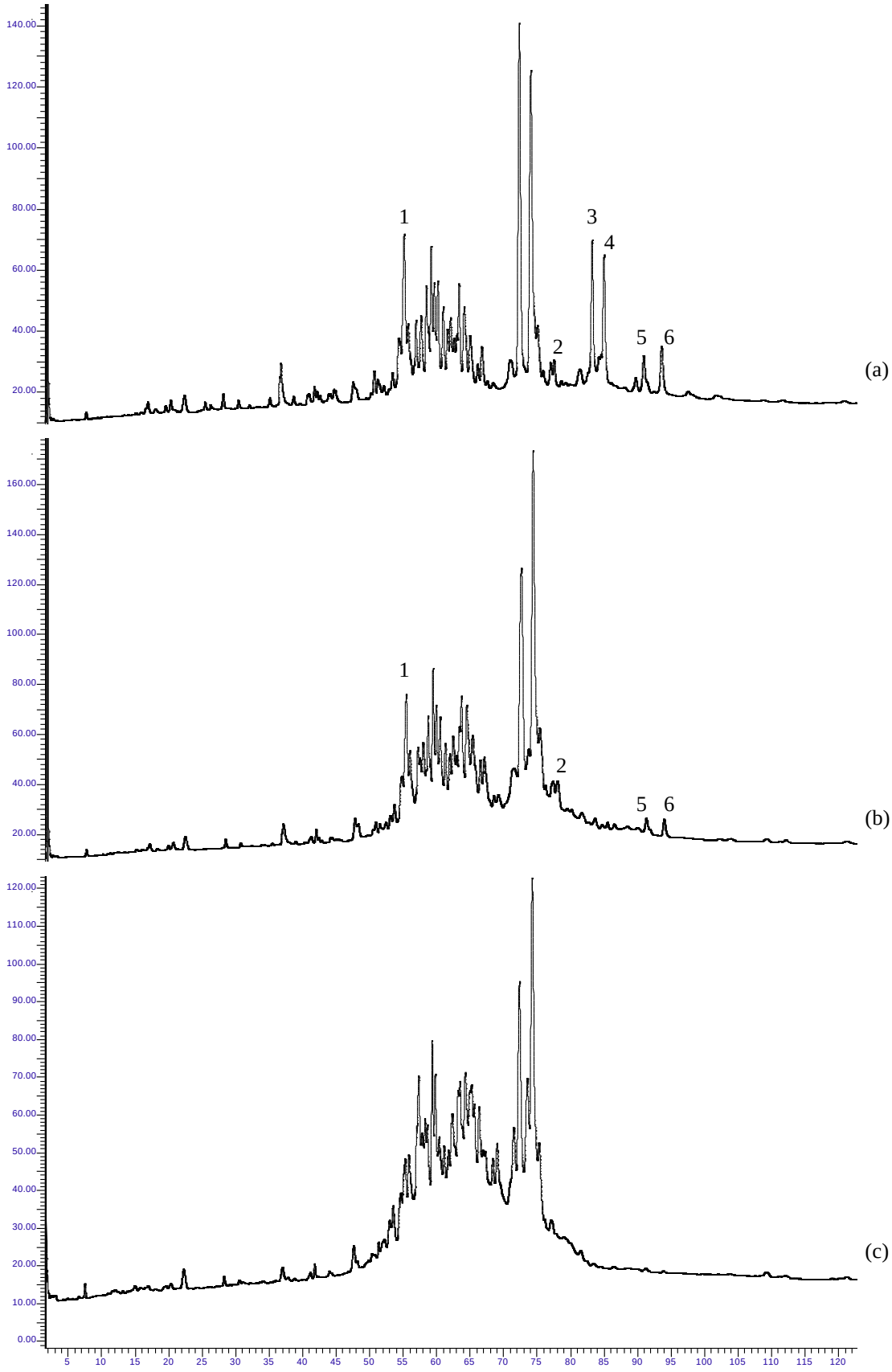


Figure 6.15. RP-HPLC results of gliadins extracted from sourdough sample LC at (a) 0 h (b) 24 h and (c) 48 h.

CHAPTER 7

CONCLUSIONS

Celiac disease, gluten enteropathy, which has a prevalence of about 100-550 people in European population, occurs in genetically susceptible individuals as a result of ingestion of gluten proteins present in wheat, rye and barley. The current treatment of CD is a gluten-free diet throughout life which results in mucosal recovery of damaged villus structure of small intestine. Sourdough is a fermented semi-product contains both gluten and lactic acid bacteria. Since lactic acid bacteria and wheat enzymes have proteolytic activities, degradation of gluten proteins are observed during sourdough fermentation. As a result, sourdough fermentation can be used for degradation of toxic fragments in gluten.

In this study, sourdough fermentation was performed with selected lactic acid bacteria, and fermentation parameters and changes in gliadin structure were investigated. *Lactobacillus acidophilus* NRRL-B 1910, *Lactobacillus casei* D4, *Lactobacillus delbrueckii* ssp. *bulgaricus* TY30 were used individually and as mixed cultures in wheat flour sourdough fermentation. Also, control dough without bacterial inoculation and chemically acidified dough were employed. Total titratable acidity, pH and lactic acid bacteria count were determined during sourdough fermentation. Gliadin and glutenin fractions extracted from sourdough samples were analyzed via SDS-PAGE, two-dimensional electrophoresis and RP-HPLC.

After the end of fermentation period, the acidity and pH of the sourdough samples reached to 13.49-17.34 and 3.84-3.52 range, respectively. LAB population was enumerated as 10^7-10^9 cfu/g dough. Free amino nitrogen levels increased nearly 4-fold. According to SDS-PAGE results, band modifications related with gliadin hydrolysis observed. As a result of hydrolysis, new bands formed and previously existed bands disappeared. Moreover, changes of the spot pattern in 2-D gels and qualitative changes in RP-HPLC chromatograms agreed with these results. Results of 2-D electrophoresis provided information about both molecular weight and pI of the gliadin fractions. Also, it should be concluded that RP-HPLC could be considered as a sensitive technique and is useful in determining the changes during fermentation period. Since no strain related

specific changes were discovered, the degradations occurred in gliadins during sourdough fermentation was resulted from the activation of the wheat flour endogenous proteolytic enzymes. The lactic acid bacteria contribute to this degradation by producing acidity and enhancing the breakdown.

On the basis of these results, the complete characterization of degraded gliadins could be done with mass spectrometry techniques. Also western-blotting technique can be utilized to investigate if the hydrolyzed fragments contain toxic parts. Further studies could include,

- Using novel sourdough lactic acid bacteria isolates during fermentation and preliminary screening of proteolytic activity of isolated strains.
- Addition of proteolytic enzymes to lactic acid bacteria inoculated sourdough formulations.
- After removal of toxic fragments and yield a safe product that is suitable for the consumption of celiac disease patients, evaluation of quality characteristic of both sourdough and obtained final product could be performed.

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APPENDIX A

CHEMICALS

	Chemical	Code
1	2-D SDS-PAGE Standards	Bio-Rad 161-0320
2	Acetic Acid	Merck 1.00056
3	Acetonitrile	Sigma-Aldrich 34851
4	Acrylamide	Sigma A8887
5	Agar	Fluka 05039
6	Ammonium Peroxodisulfate (APS)	Merck 1.01201
7	Bio-Lyte 3/10 Ampholyte	Bio-Rad 163-2094
8	Boric Acid	Amresco 0588
9	Bromophenol blue	Amresco 0449
10	Catalyst	C. Gerhardt GmbH & Co. KG
11	CHAPS	Sigma-Aldrich C3023
12	D(-)-Fructose	AppliChem A3688
13	di-Sodium Hydrogen Phosphate	Merck 1.06580
14	DTT	AppliChem A2948
15	Ethanol	Riedel-de Haën 32221
16	Formaldehyde (37%)	Amresco 0493
17	Glycerol	Merck 1.04093
18	Glycine	Amresco 0167
19	Iodoacetamide	Sigma I6125
20	IPG Strip 3-10	Bio-Rad 163-2009
21	Lactic Acid	Aldrich W261114
22	Low melting Agarose	Prona Reducta
23	Methanol	Riedel-de Haën 24229

	Chemical	Code
24	Mineral oil	Bio-Rad 163-2129
25	MRS Broth	Merck 1.10661
26	N, N' -Methylene-bis Acrylamide	Sigma M2022
27	Ninhydrin	Fluka 33437
28	Peptone from casein	AppliChem A2210
29	Phenolphthalein	Riedel-de Haën 33518
30	Potassium Carbonate	Fluka 60110
31	Potassium Iodate	Merck 1.05051
32	Potassium Phosphate	Riedel-de Haën 04243
33	Prestained protein ladder	PageRuler, Fermentas #SM0671
34	SDS	Merck 8.17034, Sigma L4390
35	Silicon Antifoaming agent	Merck 1.07743
36	Silver Nitrate	AppliChem A0536
37	Sodium Hydroxide	Sigma-Aldrich 06203
38	Sodium Phosphate Dibasic	Riedel-de Haën 04276
39	Sodium Phosphate Monobasic	Riedel-de Haën 04270
40	Sodium thiosulfate pentahydrate	Riedel-de Haën 13479
41	Sulfric Acid	Fluka 84721
42	TEMED	Merck 1.10732
43	Tert-amyl alcohol	Merck 8.06193
44	Trifluoroacetic acid (TFA)	Fluka 91707-10x1ml
45	Tris base	Sigma T6066
47	Urea	AppliChem A1049
48	Wheat Flour	Sinangil
49	β-mercaptoethanol	AppliChem A1108

APPENDIX B

REAGENTS & SOLUTIONS

B.1. Phosphate Buffered Saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

The above chemicals were dissolved in 800 ml of deionized water and pH was adjusted to 7.4. The final volume is completed to 1 L with deionized water. After sterilization at 121°C for 15 min, it was stored at RT.

B.2. Phenolphthalein (0.01%)

0.5 g phenolphthalein was completed to 50 ml with 95% ethanol and mixed thoroughly.

B.3. 0.1 N NaOH (Standardized)

First of all 0,1 N NaOH and 1 N HCl were prepared. 0,1 N NaOH was prepared by dissolving 4 g of NaOH in 1 L of deionized water. 1 N HCl was prepared by taking 41.445 ml of 37% HCl and completed the final volume to 500 ml with deionized water.

0.2 g, 0.3 g and 0.4 g of Na_2CO_3 were weighed into three different Erlenmeyer flasks and dissolved in 100 ml of deionized water. 2-3 drops of methyl orange were added to each flask and titration was carried out with 1 N HCl until the pink color developed. To check the color stabilization flasks were heated to release CO_2. If the pink color is not stable, titration was repeated until the permanent pink color was

reached. The volume of HCl used was recorded. The F factors of these three titrations (F_1, F_2 and F_3) were calculated according to equation B.1.

$$F = \frac{T \times 1000}{S \times N \times 53} \quad \text{(B.1)}$$

In this equation T is the Na_2CO_3 used (g), S is the HCl (ml) and N is the normality of HCl.

F values were found as F_1 is 0.9676, F_2 is 0.9434, F_3 is 0.9865 and mean of the F values ($F_{mean,HCl}$) was calculated as 0.9683.

1 N HCl was diluted to 0.1 N HCl and 10 ml and 20 ml of it were titrated with prepared 0.1 N NaOH by using 1-2 drops of methyl orange as indicator until the pink color was disappeared. The consumed amounts (ml) of NaOH were recorded. F_1 and F_2 were calculated according to equation B.2 as 0.9563 and F2= 1,185, respectively.

$$F_{HCl} \times N_{HCl} \times V_{HCl} = F_{NaOH} \times N_{NaOH} \times V_{NaOH} \quad \text{(B.2)}$$

The mean of F_1 and F_2 ($F_{mean,NaOH}$) was calculated as 1.0707. This result gives the factor value of 0.1 N NaOH.

B.4. MRS Broth

52.2 g of MRS broth medium was dissolved in 1 L of deionized water and autoclaved at 121°C for 15 min.

B.5. MRS Agar

52.2 g of MRS broth medium was dissolved in 1 L of deionized water. Additionally, 15 g of agar was added and autoclaved at 121°C for 15 min.

B.6. Peptone Water

1 g peptone from casein was dissolved in 1 L of deionized water and autoclaved 121°C for 15 min.

B.7. 200 mM Sodium Phosphate Buffer

200 mM sodium phosphate monobasic was prepared by dissolving 0.480 g NaH_2PO_4 in 20 ml of distilled water. 200 mM sodium dibasic was prepared by dissolving 0.284 g Na_2HPO_4 in 100 ml distilled water. They were mixed in a ratio that the final pH reaches 8.0.

B.8. Ninhydrin Solution

5.0 g of $Na_2HPO_4.2H_2O$, 6.0 g of KH_2PO_4, 0.3 g of fructose, and 0.5 g of ninhydrin was dissolved in 100 mL of deionized water. It was stored at 4°C and in the dark until the analysis (Maximum 2 weeks).

B.9. Potassium Iodate Solution

2 g of KIO_3 was dissolved in 1 L of 40% (v/v) ethanol.

B.10. Glycine Standard

A 20000 ppm stock solution was prepared. The glycine standards were prepared in the 5-100 ppm range by diluting the stock solution.

B.11. SDS-DTT Buffer

50 M Tris-HCL with pH 8.8 was prepared to obtain 1%SDS, 0.5% DTT.

B.12. SDS-PAGE AND 2-DE Reagents

B.12.1. Separating and Stacking Gel

Separating and stacking gels were prepared according to Table B.1.

Table B.1. The compositions of separating and stacking gels

	Separating Gel, 12%	**Stacking Gel, 4%**
Acrylamide/bis (30% T, 2.67% C Stock)	40.0 ml	1.3 ml
Distilled Water	33.5 ml	6.1
1.5 M Tris-HCl, pH 8.8	25.0 ml	-
0.5 M Tris-HCl, pH 6.8	-	2.5 ml
10% (w/v) SDS	1.0	100 µl
10% ammonium persulfate (fresh)	500 µl	50 µl
TEMED	50 µl	10 µl
Total Monomer	100 ml	10 ml

The volume of Acrylamide/Bis stock required for the desired total monomer concentration with the following formula: volume 30% T, 2.67% C Stock= (x % T) x (3.33).

B.12.2. Acrylamide/Bis (30%T, 2.6%C)

Acrylamide	146 g
N,N'-Methylene-bis Acrylamide	4 g

These two components were dissolved in 500 ml with distilled water, filtered through 0.45 µm filter and stored at 4 °C. Maximum shelf life is 30 days.

B.12.3. 1.5 M Tris-HCl, pH 8.8

54.45 g Tris base was dissolved in 150 ml of distilled water, pH was adjusted to 8.8 and the volume was completed to 300 ml. It was stored at 4°C.

B.12.4. 0.5 M Tris-HCl, pH 6.8

6 g Tris base was dissolved in 60 ml of distilled water, pH was adjusted to 6.8 and the volume completed to 100 ml. Stored at 4 °C.

B.12.5. SDS (10%, w/v)

10 g SDS was dissolved in 60 ml distilled water with gentle stirring at 37 °C and the volume was completed to 100 ml.

B.12.6. Ammonium Persulfate (10%, w/v)

100 mg ammonium persulfate was dissolved in 1 ml distilled water.

B.12.7. Sample Buffer

SDS reducing buffer: 62.5 mM Tris-HCl, pH 6.8, 20% Glycerol, 2% SDS, 5% β-Mercaptoethanol)

Distilled water	3.0 ml
0.5 M Tris-HCl, pH	1.0 ml
Glycerol	1.6 ml
10% SDS	1.6 ml
β-Mercaptoethanol	0.4 ml
0.5% (w/v) bromophhenol blue (in water)	0.4 ml

B.12.8. 5x Electrode (Running) Buffer

Tris base	45.0 g
Glycine	216.0 g
SDS	15.0 g

All components were dissolved in 3L of distilled water and stored at 4°C. pH should not be adjusted with acid or base. 300 ml of 5x stock was diluted with 1.2 L distilled water for one electrophoretic run.

B.12.9. Rehydration/Sample Buffer

8 M Urea	4.8 g
CHAPS	0.2 g
DTT	77.13 mg
1x Bio-lyte 3/10 ampholyte	100 µl

The above ingredients were dissolved in 10 ml of ultra pure water. Final solution contained 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% 3/10 ampholyte.

B.12.10. Equilibration Buffer I

6 M Urea	3.6 g
%2 SDS	0.2 g
0.375 M Tris-HCl, pH 8.8	2.5 ml
%20 Glycerol	2 ml
130 mM DTT	200 mg

All the ingredients were dissolved in 10 ml of ultra pure water.

B.12.11. Equilibration Buffer II

6 M Urea	3.6 g
%2 SDS	0.2 g
0.375 M Tris-HCl, pH 8.8	2.5 ml
%20 Glycerol	2 ml
135 Mm Iodoacetamide	250 mg

All the ingredients were dissolved in 10 ml of ultra pure water. Iodoacetamide was added freshly.

B.12.12. Overlay Agarose

25 mM Tris	3.0285 g
192 mM Glycine	1.4413 g
0.1% SDS	0.1 g
Bromophenolblue	10 µl
0.5% low melting agarose	0.5 g

B.13. Silver Staining Solutions

B.13.1. Fixer

50 ml of methanol, 36 ml of acetic acid and 150 µl of 37% formaldehyde were added to 300 ml ultra pure water.

B.13.2. 50% Ethanol

500 ml of ethanol and 500 ml ultra pure water were mixed.

B.13.3. Pretreatment Solution

0,08 g of sodium thiosulfate was added to 400 ml of ultra pure water and mixed.

B.13.4. Silver Nitrate Solution

0,8 g of silver nitrate was dissolved in 400 ml of ultra pure water and 150 µl of 37% formaldehyde was added.

B.13.5. Developing Solution

9 g of potassium carbonate was dissolved in 400 ml of ultra pure water and mixed with 8 ml of pretreatment solution and 300 µl of 37% formaldehyde.

B.13.6. Stop Solution

200 ml of methanol and 48 ml of acetic acid were mixed and volume was completed to 400 ml with ultra pure water.

B.14. RP-HPLC Solvents

Solvent A: 0.1% trifluoroacetic acid (TFA) in acetonitrile

Solvent B: 0.1% TFA in water

APPENDIX C

STANDARD CALIBRATION CURVE FOR FAN MEASUREMENT

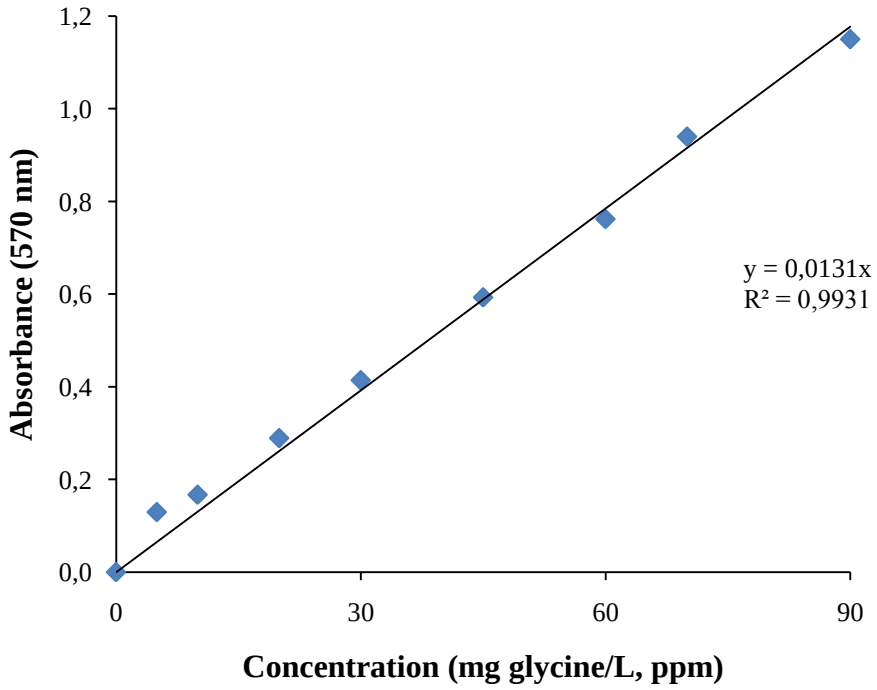

Figure C.1. Standard calibration curve for FAN content analysis

APPENDIX D

DATA OF FAN ANALYSIS

Table D.1. Absorbances and concentrations of samples for FAN analysis

Sample Code	Time (h)	Absorbance (570nm)		Absorbance (Mean)	Concentration (ppm)	Concentration (ppm) x dilution factor*
LA	0	0.4484	0.4625	0.4554	347.653	69.53061
	3	0.4618	0.5188	0.4903	374.261	74.85214
	6	0.5753	0.5975	0.5864	447.638	89.52763
	24	0.8247	0.8481	0.8364	638.457	191.5371
	48	0.8794	0.9184	0.8989	686.202	274.4806
LC	0	0.4409	0.4553	0.4481	342.061	68.41221
	3	0.4998	0.5168	0.5083	388.009	77.60183
	6	0.4950	0.5185	0.5067	386.816	77.36321
	24	0.6279	0.6431	0.6355	485.115	145.5344
	48	0.6800	0.7075	0.6937	529.580	211.8319
LD	0	0.3703	0.3812	0.3758	286.832	57.36641
	3	0.3844	0.3967	0.3906	298.139	59.62771
	6	0.5012	0.5218	0.5115	390.448	78.08954
	24	0.5427	0.5601	0.5514	420.929	126.2787
	48	0.6490	0.6726	0.6608	504.444	201.7776
M1	0	0.4208	0.4133	0.4171	318.375	63.67504
	3	0.4375	0.4548	0.4462	340.577	68.11542
	6	0.4186	0.4324	0.4255	324.806	64.96122
	24	0.6170	0.6361	0.6265	478.269	143.4807
	48	0.6684	0.6898	0.6791	518.390	207.3559
M2	0	0.3554	0.3701	0.3628	276.914	55.38275
	3	0.5144	0.5207	0.5175	395.072	79.01435
	6	0.5802	0.6004	0.5903	450.605	90.12092
	24	0.7289	0.7552	0.7421	566.474	169.9421
	48	0.7503	0.7811	0.7657	584.505	233.8018
C	0	0.3941	0.4154	0.4048	308.993	61.79855
	3	0.4194	0.4356	0.4275	326.331	65.26626
	6	0.6145	0.6283	0.6214	474.354	94.87076
	24	0.5939	0.6142	0.6041	461.123	138.3368
	48	0.8786	0.9136	0.8961	684.056	273.6224
CAD	0	0.4577	0.4575	0.4576	349.291	69.85817
	3	0.3446	0.3554	0.3500	267.203	53.44069
	6	0.6222	0.6508	0.6365	485.877	97.17542
	24	0.7252	0.7618	0.7435	567.550	170.265
	48	0.7710	0.8106	0.7908	603.643	241.4571

*: Dilution factors are 2 (for samples taken at 0, 3 and 6 h), 3 (for samples taken at 24 h) and 4 (for samples taken at 48 h).

APPENDIX E

NANODROP RESULTS OF GLIADINS

Table E.1. NanoDrop measurements of gliadin extracts (mg/ml)

Sample	Time (h)	1. measurement (mg/ml)	2. measurement (mg/ml)	Mean (mg/ml)
LA	0	1.02	1.49	1.26
	24	6.48	6.16	6.32
	48	5.00	4.39	4.70
LC	0	1.92	1.90	1.91
	24	3.37	3.01	3.19
	48	2.05	1.73	1.89
LD	0	5.19	4.65	4.92
	24	4.74	4.21	4.48
	48	3.48	3.38	3.43
M1	0	3.19	2.13	2.66
	24	3.87	3.47	3.67
	48	2.84	2.77	2.81
M2	0	1.49	1.49	1.49
	24	5.67	5.21	5.44
	48	5.58	3.79	4.69
C	0	1.31	3.24	2.28
	24	1.24	2.36	1.80
	48	3.75	2.88	3.32
CAD	0	1.57	1.17	1.37
	24	2.69	3.83	3.26
	48	3.00	3.04	3.02

APPENDIX F

RP-HPLC CHROMATOGRAMS OF GLIADIN EXTRACTS

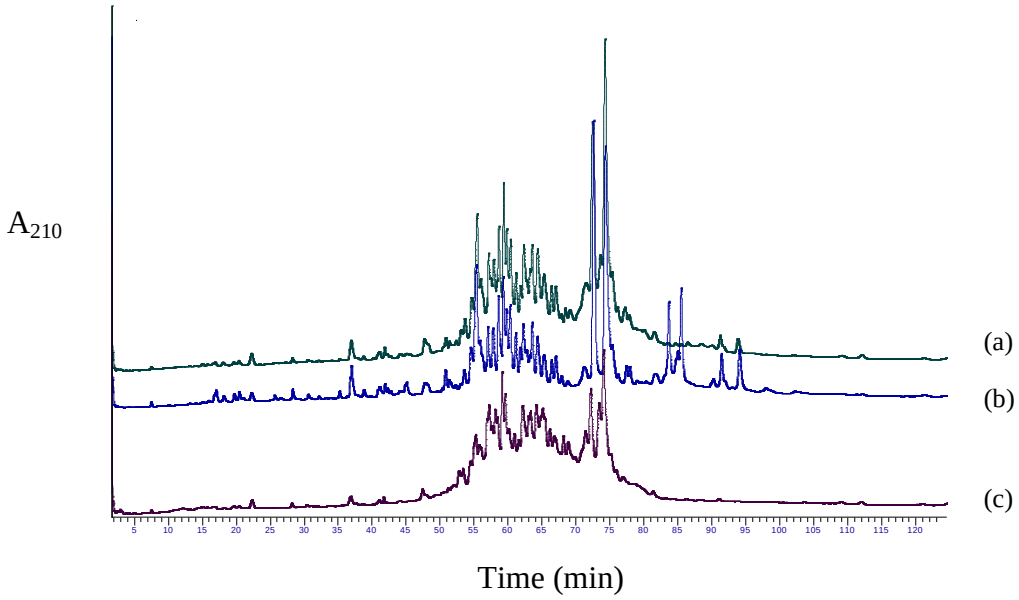

Figure F.1. RP-HPLC chromatograms of sample LA at fermentation times of (a) 24 h (b) 0 h (c) 48 h.

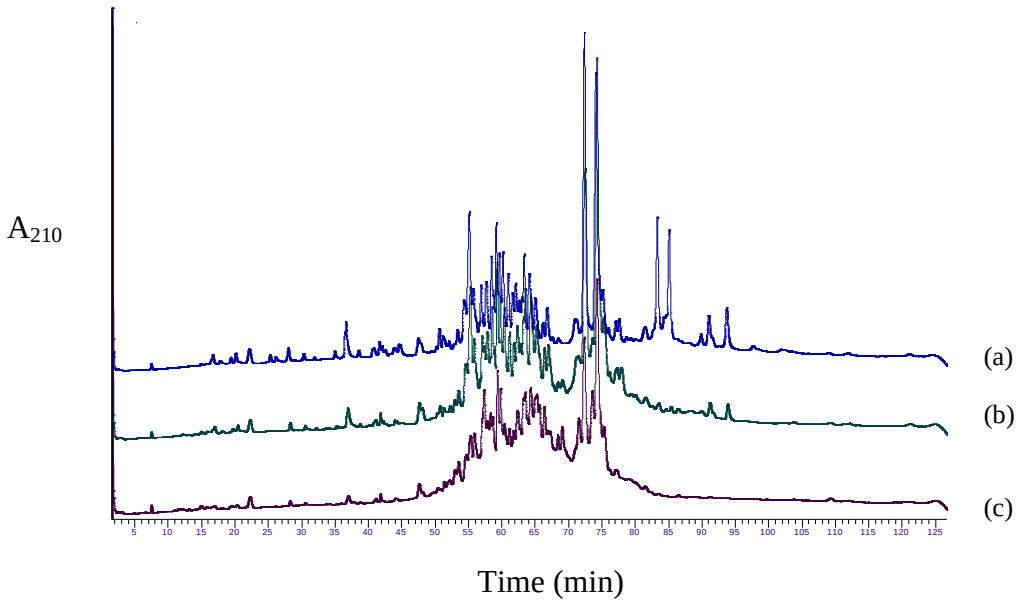

Figure F.2. RP-HPLC chromatograms of sample LC at fermentation times of (a) 0 h (b) 24 h (c) 48 h.

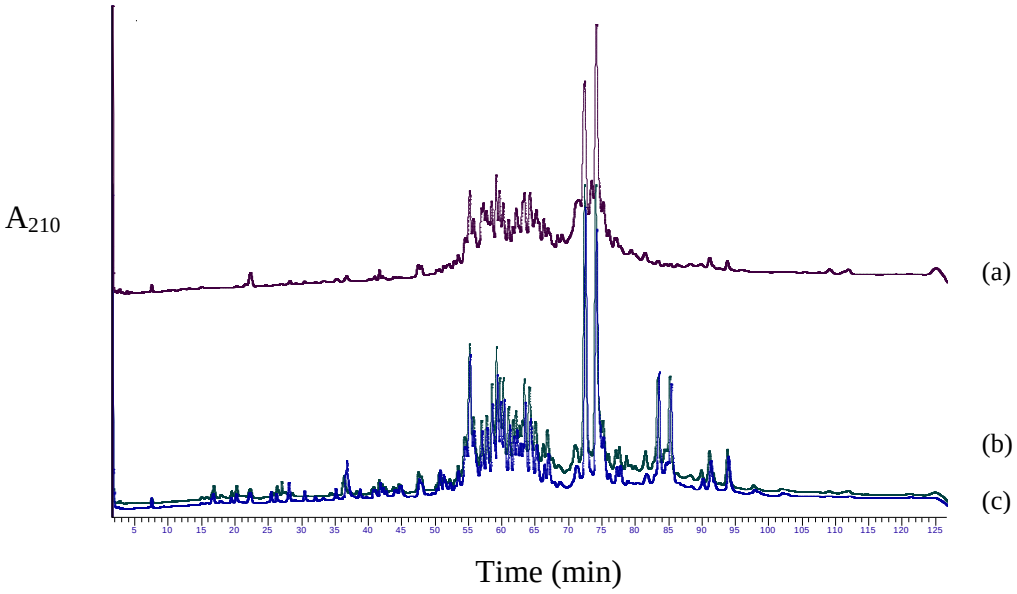

Figure F.2. RP-HPLC chromatograms of sample LD at fermentation times of (a) 48 h (b) 24 h (c) 0 h.

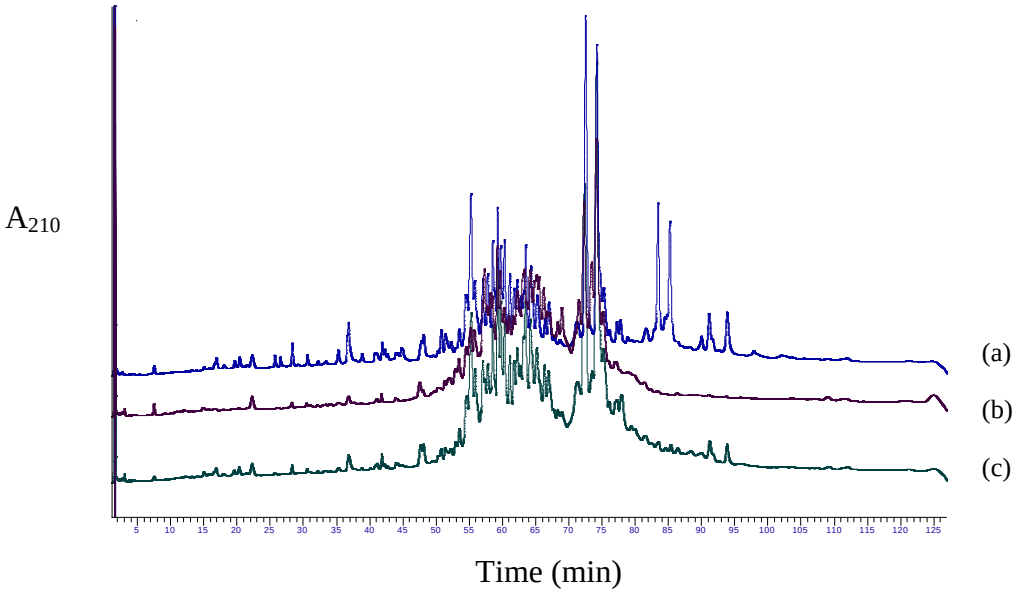

Figure F.4. RP-HPLC chromatograms of sample M1 at fermentation times of (a) 0 h (b) 48 h (c) 24 h.

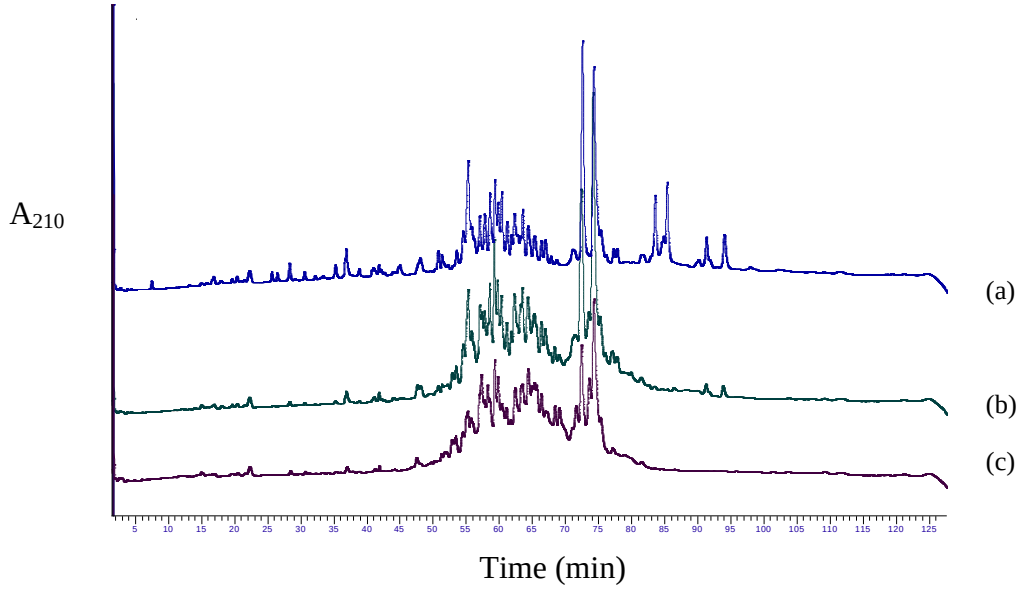

Figure F.5. RP-HPLC chromatograms of sample M2 at fermentation times of (a) 0 h (b) 24 h (c) 48 h.

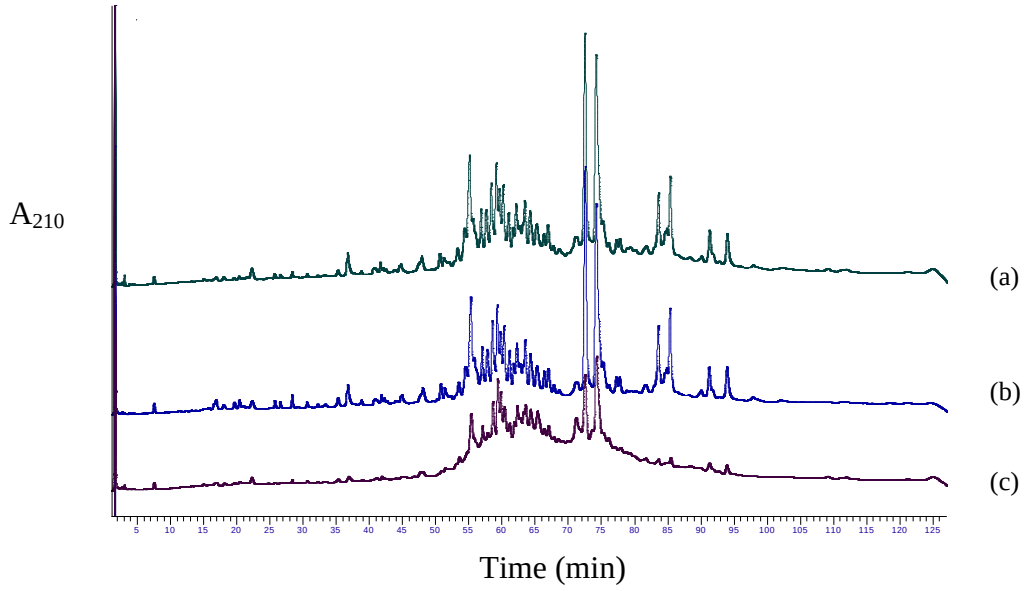

Figure F.6. RP-HPLC chromatograms of sample C at fermentation times of (a) 24 h (b) 0 h (c) 48 h.

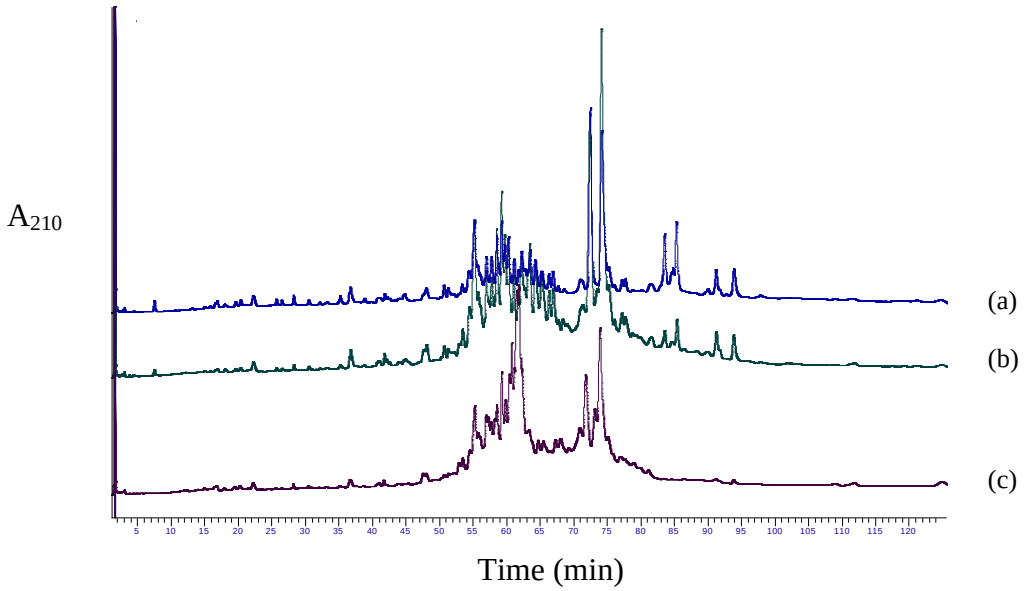

Figure F.7. RP-HPLC chromatograms of sample CAD at fermentation times of (a) 0 h (b) 24 h (c) 48 h.