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**GENETIC DIVERSITY OF GLUTEN PROTEIN PATTERNS OF WHEAT
CULTIVARS AND THEIR IMPORTANCE IN DOUGH MAKING
PROPERTIES OF FLOUR**

**A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY**

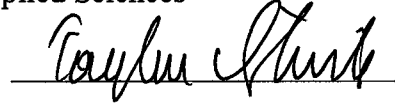
**BY
VELİ CENGİZ ÖZALP**

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DEGREE OF DOCTOR OF PHILOSOPHY
IN
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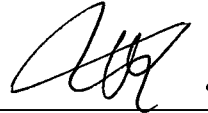


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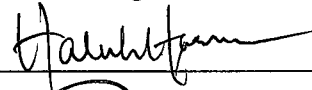
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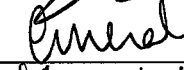
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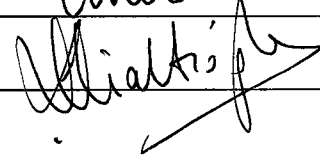
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ABSTRACT

GENETIC DIVERSITY OF WHEAT GLUTEN PROTEINS AND THEIR IMPORTANCE IN DOUGH MAKING PROPERTIES OF FLOUR

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Glutenin protein differences for 13 Turkish Wheat cultivars (Gün 91, Orso, Kır  , Bezostaja, Yayla, Gerek, Atay 85, Sertak, Ak 702, Bolal, Tosun, Dicle 94,   akmak) were assessed using SE-HPLC and SDS-PAGE. The glutenin subunit polymorphism among cultivars was shown by these techniques and tabulated. The dough making quality was evaluated by finding their loaf volumes using SDS sedimentation volume test. ‘Yayla’ had the highest value with a loaf volume of 3.24 ml. The polymorphism between cultivars were further investigated

by PCR amplified fragments using two LMW and one HMW primers. Only HMW glutenin subunits *Ay* gene produced two different patterns.

The rest of the study focused on the silent *Ay* gene such that, the new allele of gene was cloned and expressed in a heterologous yeast system in order to evaluate the influence of the *Ay* product on the dough making quality. For this purpose, the *Ay* gene was cloned from the genome of hexaploid 'Yayla' by PCR primers based on the published sequences. The *Ay* gene sequence was inserted in a pPIC9 expression vector between XhoI and EcoRI sites and was transformed into *P. pastoris*. The expression conditions were optimized to obtain production of 1.2 g protein per liter medium. This production level is about 4 fold as that gained from previous *E. coli* expression studies.

The secretory products were directly collected from culture medium. The identity of the product was verified as *Ay* protein by SDS-PAGE (66 kD) and RP-HPLC (32 min retention time). The influence of the silent gene product on dough making quality was studied by evaluating its effect on loaf volume as determined by SDS-microsedimentation method. Incorporation of *Ay* protein into flour of 13 different cultivars resulted in increased loaf volume indicating a possible improvement in dough making quality. The *Ay* added flour of 'Yayla' showed a significant increase in SDS Sedimentation value compared to that of the flour of 'Yayla'.

Keywords: Wheat, Dough making quality, *Pichia pastoris*, *Ay* gene, Glutenin

ÖZ

BUĞDAY GLUTEN PROTEİNLERİNİN GENETİK FARKLILIKLARI VE EKMEKLİK UN ÖZELLİKLERİNDEKİ ÖNEMİ

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Onüç Türk buğday çeşidi (Gün 91, Orso, Kırac, Bezostaja, Yayla, Gerek, Atay 85, Sertak, Ak 702, Bolal, Tosun, Dicle 94, Çakmak) gluten proteinleri büyüklük dışlama HPLC ve SDS-PAGE elektroforetik yöntemlerle değerlendirildi. Bu iki teknikle çeşitler arasında gluten proteinleri altbirimleri açısından farklılıklar olduğu saptandı. Çalışılan çeşitler arasındaki ekmeklik kalitesi SDS çöktürme testi ile belirlenen kabarıklık hacmi yoluyla yapıldı. Genetik farklılıklar kullanılan primer çiftleri ile PCR yoluyla çoğaltılan parçalar yoluyla incelendi. Düşük moleküler ağırlıklı gluten altbirimleri için tasarlanan primer çifti farklılık saptayamazken, yüksek moleküler ağırlıklı glutenin altbirimleri için tasarlanan

primer çifti ile farklılık bulundu. Bu farklılık *Ay* geni için hazırlanan primer çifti ile bulundu.

Çalışmanın bundan sonraki kısmında '*Ay*' geni ekmeklik buğday '*Yayla*'dan daha önce yayınlanan sekansları kullanarak tasarlanan primerlerle klonlandı. '*Ay*' geni pPIC9 vektörüne *Xho*I ve *Eco*RI arasına yerleştirildi ve *P. pastoris*'e aktarıldı. Ortama yollanan protein kültürden doğrudan toplandı ve SDS-PAGE (66 kD) ve RP-HPLC yöntemleriyle incelendi. Üretim koşulları optimize edilerek 1.2 g/l oranında üretim sağlandı. Bu *E. coli*'den elde edilenin 4 katıdır. İzole edilen '*Ay*' proteininden 0.7 mg 13 buğday çeşidinin unlarıyla 1/7 (w/w) oranıyla karıştırılarak SDS çöktürme testi ile incelendi. Bu karışım unlarının kabarma hacimlerinde yükselme değerlerinin değiştiği gözlemlendi. '*Yayla*' çeşidinin unu SDS çöktürme değerlerinde istatistiksel olarak önemli yükselmeler gösterdi.

Anahtar Kelimeler: Buğday, Hamur Kalitesi, *Pichia pastoris*, *Ay* geni, Glutenin

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LIST OF ABBREVIATIONS

Bp	: Base pair
DTT	: Dithiothreitol
HMWGS	: High Molecular Weight Glutenin Subunit
IPP	: Insoluble Polymeric Protein
kD	: Kilodalton
LMWGS	: Low Molecular Weight Glutenin Subunit
MCS	: Multiple Cloning Site
PAGE	: Polyacrylamide Gel Electrophoresis
RP-HPLC	: Reverse Phase High Pressure Liquid Chromatography
SE-HPLC	: Size Exclusion High Pressure Liquid Chromatography
SPP	: Soluble Polymeric Protein
SDS	: Sodium Dodecyl Sulphide
SSDSV	: SDS Sedimentation Volume
TBE	: Tris-Borate-EDTA Buffer
TCA	: Trichloroacetic acid
TE	: Tris-EDTA Buffer

CHAPTER 1

INTRODUCTION

1.1 General Information

Wheat is one of the most important food sources in the world as human food and feed for livestock. Wheat is part of the *Triticaea* tribe from which three of the major seed crops are produced: wheat, barley, and rye. *Triticaea* are distributed world wide, in temperate regions of the globe. There are annual and perennial forms. Cultivated bread and durum wheats descend from hybridized wild grasses. Durum wheat (*Triticum turgidum*) is a hybrid that occurred spontaneously between an unidentified wild grass and an einkorn (a primitive diploid wheat). Bread wheat (*T. aestivum*), a hexaploid, is the product of a later spontaneous hybridization between a tetraploid wheat and *T. tauschii* (also *Aegilops squarrosa* or "goat grass"), which is still found in the wheat fields of Asia Minor (Figure 1.1). It is possible to speculate that events leading to the formation of current cultivated wheats may have occurred many times in nature, rather than as a single hybridization event (Harlan 1992).

Wheat emerged at early periods of civilization and dispersed worldwide. The early dispersal leading to the current cultivated varieties emerged from an expansion called the Neolithic dispersal (roughly 6000-3000 B.C.). This

Wheat is the most important crop in the Central Anatolian Plateau (CAP) of Turkey. It is the principal Turkish cereal grain domestic consumption item. In terms of value, wheat is the first leading Turkish field crop (Kün, 1988). There are several hundred varieties of wheat produced in Turkey. Generally speaking, wheat is more often grown in arid regions where soil quality is poor.

The wheat kernel is the seed from which the wheat plant grows. Each tiny seed contains three distinct parts that are separated during the milling process to produce flour. The kernel of wheat is a storehouse of nutrients essential to the human diet. Endosperm is about 83 percent of the kernel weight. It is the source of white flour. The endosperm contains the greatest share of the protein in the whole kernel, carbohydrates, iron as well as many B-complex vitamins, such as riboflavin, niacin, and thiamine. Bran is about 14.5 percent of the kernel weight. Bran is included in whole wheat flour and is also available separately. Of the nutrients in whole wheat, the bran contains a small amount of protein, larger quantities of the B-complex vitamins, trace minerals, and indigestible cellulose material also called dietary fiber. Germ about 2.5 percent of the kernel weight. The germ is the embryo or sprouting section of the seed, usually separated because of the fat that limits the keeping quality of flour. Of the nutrients in whole wheat, the germ contains minimal quantities of protein, but a greater share of B-complex vitamins and trace minerals. Wheat germ can be purchased separately and is included in whole wheat flour. The protein fraction of kernel is known to be the determining factor for the breadmaking quality in addition to nutritional value of flour.

All-Purpose flour is the finely ground endosperm of the wheat kernel separated from the bran and germ during the milling process. All-Purpose flour is made from hard wheat or a combination of soft and hard wheat from which the home baker can make a complete range of satisfactory baked products such as yeast breads, cakes, cookies, pastries and noodles. *Enriched All-Purpose Flour* has iron and B-vitamins added in amounts equal to or exceeding that of whole wheat flour. Bleached Enriched All-Purpose flour is treated with chlorine to mature the flour, condition the gluten and improve the baking quality. The chlorine evaporates and does not destroy the nutrients but does reduce the risk of spoilage or contamination. Unbleached Enriched All-Purpose flour is bleached by oxygen in the air during an aging process and is off-white in color. Nutritionally, bleached and unbleached flour are the same. Bread flour, from the endosperm of the wheat kernel, is milled primarily for commercial bakers but is also available at retail outlets. Although similar to All-Purpose flour, it has greater gluten strength and generally is used for yeast breads.

The most of nutritional value of Turkish food is coming from wheat flour. Wheat flour is a good source of complex carbohydrate. Fiber is the indigestible carbohydrate in food, which acts like a broom to sweep the digestive tract. One slice of whole wheat bread contains 1.5 grams of dietary fiber; one slice of white bread contains 0.5 grams of dietary fiber. Wheat foods are moderate sources of incomplete protein. This means that while wheat and other cereal grains may contain all eight of the amino acids necessary for good health, but not all eight are found at adequate levels. However, combining wheat or other cereal grains with

animal proteins or legumes makes the grain protein complete. Within the cereal group, wheat contains more protein than rice or corn. Fats account for 2 to 23 percent of wheat foods, although wheat alone contains very little fat. Most often, the fat content in wheat foods results from fat added in production, such as the oil or shortening found in many baked or fried wheat foods. Bread and pasta products are low-fat. Wheat foods are a good source of thiamine, B-vitamin, niacin (essential for the efficient use of protein by the body), iron (for normal dietary needs), zinc, riboflavin, selenium and magnesium, nutrients essential to good health.

On the basis of information presented by several authors (Hitchcock, 1950; Clayton, 1972; Sakamoto, 1973; Paulis and Wall, 1977; Smith and Lester, 1980), a tree diagram summarizing currently accepted genetic relationships of cereal grains was constructed by Bietz (1982). In this diagram, he classified wheat, barley, and rye in the same subfamily (*Festucoideae*) and tribe (*Triticeae*) of the grass family (*Gramineae*), and this close relationship is reflected in the structures of their prolamine storage proteins. Only in wheat, however, do these proteins form a cohesive mass (gluten). Barley and rye are diploids, each with seven pairs of chromosomes, while wheat species are diploid, tetraploid, or hexaploid.

Total number of chromosome = 42 for hexaploids (used for breadmaking)

= 28 for tetraploids (Durum wheat used for pasta)

= 14 for diploids (primitive wheat)

The last two originated from the hybridization of related wild diploid species. Bread wheat (*Triticum aestivum*) is an allohexaploid with three genomes (each having seven pairs of chromosomes called A, B, and D). The A and D genomes are thought to be derived from a wild diploid wheat (*Triticum* sp.) and the related wild grass *Aegilops squarrosa* (*T. tauschii*), respectively. The origins of the B genome are not known. Durum or pasta wheats (*T. durum*) are tetraploid, with only the A and B genomes. Because the A, B, and D genomes are derived from related diploid species, they have genes that encode related but not identical proteins. The interactions of proteins encoded by the different genomes are important in determining the precise physical and technological properties of dough produced from pasta and bread wheats. Each chromosome consists of a long arm and a short arm joined by a centromere.

1.2 Gluten Proteins of Wheat and Their Polymorphism

The storage proteins of wheat are unique because they are also functional proteins. They do not have enzyme activity, but they are the only cereal proteins to form strong cohesive dough that will retain gas and produce light baked products. This property makes cereals an important protein source after processing into bread, pasta and noodles, breakfast cereals and fermented drink. For all these applications the quality is determined, to a greater or lesser extent, by the gluten proteins, which account for about half of the total grain nitrogen.

The gluten proteins consist of a matrix of LMW and HMW glutenin subunits with gliadins dispersed around. The subunits are held together by disulphide bonds between cysteine residues and hydrogen bonding. The identity and characteristics of gluten proteins are important since the quantity and quality of each subunit determines the end-product quality of the flour.

1.2.1 Classification of Gluten Proteins

Gluten proteins can be easily isolated by removing starch and albumins/globulins by gently working dough under a small stream of water. After washing, a rubbery ball is left, which is called gluten. Traditionally, gluten proteins have been classified into four types according to their solubility (Osborne, 1907) as follows:

- albumins are soluble in water or dilute salt solutions and are coagulated by heat;
- globulins are insoluble in pure water, but soluble in dilute salt concentrations and insoluble at high salt concentrations;
- prolamins are soluble in aqueous alcohol, and
- the glutenins are soluble in dilute acid or bases, detergents, or dissociating (urea) or reducing (beta-mercaptoethanol) agents.

Shewry *et. al.* (1986) proposed an alternative classification based on composition and structure rather than solubility. Wheat seed proteins were classified according to either solubility criteria or chemical (therefore genetic) criteria. It is convenient to divide wheat proteins into monomeric and polymeric

classes depending on whether they consist of single or multiple chain polypeptides. Monomeric proteins comprise two main groups: gliadins and albumin/globulins. Gliadins are storage proteins, whereas albumin and globulins are metabolic and include various enzymes. Gliadins are divided into α -, β -, γ -, and ω -gliadins based on their mobility on 1D acid-PAGE. Molecular weights of gliadins range from 30,000 to 80,000 kD. Albumins (soluble in water) and globulins (soluble in dilute salt solution) are a mixture of low molecular weight compounds. Their amino acid composition is distinctly different from the gluten proteins. Glutenins form the major portion (85 %) of the polymeric proteins. They are found in discrete protein bodies in flour together with gliadins. Glutenins are similar in composition to gliadins. They are made of two main groups of polypeptide chains, High Molecular Weight (HMW) and Low Molecular Weight (LMW) subunits. HMW polypeptides range 80,00-120,000 kD and LMW subunits 30,000-55,000. These sizes were deduced from mobilities in SDS-PAGE, however, it has been found that electrophoretic mobility in SDS-PAGE does not rank HMW subunits exactly according to size.

Most of the enzymes are found in the albumin or globulin groups. Nutritionally, the albumins and globulins have a very good amino acid balance. They are relatively high in lysine, tryptophane and methionine (Pomeranz, 1968). The prolamins were among the earliest proteins to be studied, with the first description of wheat gluten being that of Beccari (MacRitchie, 1998). The prolamins have always been considered to be unique to the seeds of cereals and other grasses and unrelated to other proteins of seeds or other tissues. They have

been given different names in different cereals, such as: gliadin in wheat; avenins in oats; zeins in maize; secalins in rye; and hordein in barley. The gliadins and glutenins are the storage proteins of wheat endosperm and they tend to be rich in asparagine, glutamine, arginine or proline (Abrol *et al.*, 1971; Derbyshire *et al.*, 1976; Kirkman *et al.*, 1982; Larkins, 1975; Spencer and Higgins, 1982) but very low in nutritionally important amino acids lysine, tryptophan and methionine.

Storage proteins are usually synthesized in very large amounts and consequently need to be stored in a highly concentrated form and in a subcellular compartment in which they are separate from other metabolic processes. This is achieved by a combination of specific solubility properties and deposition into protein bodies (1 – 20 μ m). The protein bodies have been found both in the aleurone cells and the endosperm. The solubility properties are determined by the primary structures of the individual proteins and their interactions by non-covalent forces (notably hydrogen bonds and hydrophobic interactions) and by covalent disulphide bonds. Storage proteins are synthesized on the rough endoplasmic reticulum with a signal peptide which directs the nascent polypeptide into the lumen of the endoplasmic reticulum and is itself removed by proteolytic cleavage. The signal sequences of the storage proteins all display the characteristics for transported proteins in other organisms (Von Heijne, 1983) such as length, hydrophobicity, and the presence of an amino acid with a small uncharged side chain prior to the N-terminus of the mature protein. There is some sequence homology between the signal sequences for closely related zeins (Spena *et al.*, 1982). Thus, it appears that the features of this protein segment that have been

conserved in evolution are the nature of the amino acids and perhaps the tertiary structure of the signal sequence rather than the actual amino acid sequence.

By analogy with animal systems (Kreil, 1981), the function of signal sequences in plant storage proteins is to facilitate the translocation of storage protein into the lumen of the ER as the first step in intracellular transport. Protein folding and disulfide bond formation are considered to occur within the lumen of the endoplasmic reticulum, and may be assisted by molecular chaperones and by the enzyme protein disulfide isomerase respectively (Freedman, 1989; Gething and Sambrook, 1992). The precise mechanism of intracellular transport of storage proteins from their site of synthesis to their site of deposition are still largely unknown.

1.2.2 Gliadins

Gliadins are the monomeric subunits of gluten matrix and are thought to be important in giving the viscous property of the matrix. The gliadins are divided into four groups, called α -, β -, δ -, and ω -gliadins, based on their electrophoretic mobility at low pH (Woychick *et al.*, 1961), but more than 30 components can be separated by two-dimensional electrophoretic procedures. The amino acid compositions of the α -, β -, δ -gliadins are similar to each other and to that of the whole gliadin fraction (Tatham *et al.*, 1990). The omega-gliadins contain little or no cysteine or methionine and only small amounts of basic amino acids. All gliadins are monomers with either no disulphide bonds (omega-gliadins) or

intrachain disulphide bonds (alpha-, beta-, and gamma-gliadins). Although no complete sequences of omega-gliadins have been determined, Kasarda *et al.* (1983) purified a number of individual components from bread and pasta wheat and determined their relative molecular weights by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); and the MWs fell between 44,000 and 74,000, with most above 50,000. The alpha-, beta-, and gamma-gliadins have lower MWs, ranging between about 30,000 and 45,000 by SDS-PAGE and by amino acid sequencing. The latter approach has shown that the alpha- and beta-gliadins are closely related, and both are now usually referred to as alpha-type (in contrast to the gamma-type) gliadins. Although there is some overlap, the alpha-type gliadins generally have lower MWs than gamma-type gliadins.

Genes coding for the gliadin proteins are located on the short arms of groups 1 and 6 chromosomes (Wrigley and Shepherd, 1973; Brown *et al.*, 1981). The group 1 chromosomes control all the omega-gliadins, most of the gamma-gliadins, and a few of the beta-gliadins, whereas genes on the group 6 chromosomes code for all the alpha-gliadins, most of the beta-gliadins, and some of the gamma-gliadins. The genes coding for gliadin proteins occur as a single complex locus on each of the short arms of groups 1 and 6 chromosomes rather than at two or more loci.

In dough formation, the gliadins do not become covalently-linked into large elastic networks as the glutenins but act as a 'plasticiser', promoting viscous

flow and extensibility, which are important rheological characteristics of dough. They may interact through hydrophobic interactions and hydrogen bonds. Ultracentrifugation were used to analyze the gliadin fraction extracted with aqueous ethanol. Although the protein was not homogeneous, a principal non-spherical component with a molecular weight of about 34,500 was present. Diffusion measurements showed gliadin to have a high degree of asymmetry with a calculated axial ratio of 8:1 while Entrikin (1941) using dielectric measurements showed an axial ratio of 13:1. Hydrodynamic studies showed that gliadins had a low intrinsic viscosity, indicating a compact, globular conformation (Taylor and Cluskey, 1962; Wu and Dimler, 1964). Grosskreutz (1961) reported detailed X-ray scattering and electron microscopy studies of two gluten fractions, the first gliadins and the second fraction a major glutenin subfraction called α_1 -gluten (not the same as α -gliadin).

The gliadins appeared to have a doughnut shape, about 340Å in diameter and 90Å thickness, with a central hole of about 100Å in diameter. Grosskreutz (1961) suggested that this shape resulted from the flattening of a hollow, sphere during drying. Kasarda, (1980) divided wheat varieties into two types (Type 1 and Type 2) based on the major features of the gel electrophoretic patterns of their α -gliadins. A sub-fraction of the α -gliadins of Type 1 wheat variety was named A-gliadin which was found to be encoded by genes on chromosome 6A. Other α -gliadins were coded by genes on chromosomes 6A, 6B and 6D. At pH 3, the A-gliadins were found to be partially unfolded and dissociated into

monomers. As the pH was increased, the molecules became compactly folded and aggregated to form microfibrils.

At pH 5 and at an ionic strength of 0.005 M, electron microscopy revealed that the aggregates collected by ultracentrifugation had a microfibrillar structure about 80Å in diameter and up to 3000 or 4000Å long (Kasarda *et al.*, 1967). CD and ORD studies of A-gliadin dissolved in 10⁻⁵ M HCl at pH 5.0 showed that it was made up of 33-34% alpha-helix (Kasarda *et al.*, 1968). The molecule was found to be more stable than most globular proteins. 65% of the helical structure present at 25°C remained when the temperature was raised to 90°C. The effects of heating were reversible when cooled.

The primary structure of alpha-type gliadins can be divided into five different domains (Kasarda *et al.*, 1984). Domain I consists of non-repetitive N-terminal sequences and of repetitive sequences rich in glutamine, proline and aromatic amino acids. Domain II contains a polyglutamine sequence with a maximum of 18 residues of glutamine. Domains III and V are homologous to the corresponding domains of gamma-type gliadins and low MW subunits of glutenin. Domain IV, is unique to alpha-type gliadins and is rich in glutamine but poor in proline. Most alpha-type gliadins contain six cysteine residues, located in domain III (four residues) and V (two residues). Because of the monomeric character of alpha-type gliadins, and the absence of free sulphydryl groups, it has been assumed that the cysteine residues are linked by three intramolecular disulphide bonds (Kasarda *et al.*, 1987). On the basis of the sequence homology to

g-type gliadins and low MW subunits, definitive positions for disulphide bridges have been postulated for alpha-type gliadins (Köhler *et al.*, 1993).

The gamma-type gliadins are single monomeric proteins with only intra-chain disulphide bonds and are considered to be the ancestral type of S-rich prolamin (Shewry *et al.*, 1986). Complete amino acid sequences of several gamma-gliadins have been deduced from genomic and cDNA sequences (Bartels *et al.*, 1986; Okita *et al.*, 1985; Scheets *et al.*, 1985). These sequences showed a clear domain structure, with a non-repetitive sequence of 14 residues at the N-terminus, an N-terminal repetitive domain based on a heptapeptide repeat motif (consensus Pro.Gln.Gln.Pro.Phe.Pro.Gln) and a non-repetitive C-terminal domain which contained all the cysteine residues. Structural studies, using circular dichroism and structure prediction, indicated that the two domains adopt different conformations. Whereas the repetitive domain adopts a beta-reverse turn rich conformation, the non-repetitive domain is rich in alpha-helix (Tatham *et al.*, 1990). Scanning tunnelling microscopy (STM) and small-angle X-ray scattering (SAXS) results indicated that the gamma-gliadins have a compact conformation, with axial ratios of approximately 1.5:1 (Thomson *et al.*, 1992). A SAXS study of an S-poor prolamin, C hordein, with a repetitive motif (consensus Pro.Gln.Gln.Pro.Phe.Pro.Gln.Gln) similar to that of the repetitive domain of the gamma-gliadins (consensus Pro.Gln.Gln.Pro.Phe.Pro.Gln) has been reported (I'Anson *et al.*, 1992). The SAXS data indicated that in solution C hordein behaved as a worm-like chain (an extended conformation) with a high degree of flexibility. The similarity of the repeat motifs would indicate that the N-terminal

domain of the g-gliadin may adopt a similar conformation in solution to that of C hordein.

Small deformation oscillatory measurements on gliadin between 50-70°C showed the elastic modulus (G') to be roughly equal to the viscous modulus (G'') in magnitude, but a large increase in G' was observed at temperatures above 70°C. The increase in the elastic component was attributed to crosslinking reactions occurring among gliadin molecules, resulting in the formation of a network structure. The G' reached a peak at 120°C, and the G'' increased to a plateau value in the temperature range 90-110°C. On further heating G'' fell to a minimum value at 120°C, whereas G' was at its maximum, indicating maximum structure build-up. At this point, the aggregation reaction appeared to have been completed and a highly crosslinked network formed. On increasing the temperature even further, a reduction in G' with a simultaneous peak in G'' was observed. This indicated a softening of the crosslinked gliadin at 130°C.

1.2.3 Glutenin

The HMW glutenin subunits are coded by genes (loci) on the arms of chromosomes 1A, 1B, and 1D. These loci are designated Glu-A1, Glu-B1, and Glu-D1, respectively. Electrophoretic studies have revealed appreciable polymorphism in the number and mobility of the HMW subunits in different wheat cultivars. That is, the genes on the chromosome 1 long arms show multiple allelism. Based on electrophoretic studies, Payne *et al.* (1981) proposed that there

were two main types of subunits, the x type (of high relative molecular weight) and the y type (low MW). This subdivision has been supported by chemical and genetic evidence.

A particular subunit is specified by noting its chromosome, followed by its classification as x or y, and finally a number (designating the protein subunit), this number increasing with decreasing MW. In contrast to the HMW subunits, the LMW glutenin subunits are encoded by genes on the short arms of chromosomes 1A, 1B, and 1D, these loci being designated Glu-A3, Glu-B3, and Glu-D3. The allocation of genes coding for particular proteins has been achieved by use of genetic variants in combination with electrophoresis. Examples of these variants are aneuploids, lines that are deficient in a single chromosome. Much of the pioneering work in this area was conducted by Sears (1969). In simple terms, when a particular chromosome is missing, this may coincide with the disappearance of a certain band or bands in the electrophoretic pattern of the wheat protein, thus identifying the chromosome that carries the genes responsible for the synthesis of the missing proteins.

Glutenins are a family of polypeptides found in the seeds of wheats and related wild grasses. The glutenin proteins make up 35-40% of flour protein and consist of subunits that form large polymers (MWs above 1×10^6 and possibly exceeding 1×10^7) stabilised by interchain disulphide bonds (Field *et al.*, 1983). After reduction, both HMW and LMW subunits were observed by SDS-PAGE. The latter resembled the monomeric alpha-type gliadins in amino acid

composition and had MW in a similar range. The HMW subunit contain significantly less proline and had an MW value from 80,000 to 160,000 by PAGE. Extensive genetic analysis of the HMW subunits by Payne and co-workers (Payne, 1987), established that genes coding for the HMW subunits are located on the long arms of chromosomes 1A, 1B and 1D at complex loci designated Glu-A1, Glu-B1 and Glu-D1 respectively. By contrast, genes for the LMW subunits and the gliadins are located on the short arms of the same chromosomes (designated Gli-A1, Gli-B1 and Gli-D1 respectively). The HMW subunits consist of nonrepetitive domains of 88-104 and 42 residues at the N- and C-termini, respectively, separated by a longer repetitive domain (481-690 residues). Variation in the repetitive domain is responsible for most of the variation in the size of the whole protein, and it is based on random and interspersed repeats of hexapeptide and no peptide motifs, with tripeptides also present in x-type subunits only. Structure prediction indicated that the N- and C-terminal domains are predominantly alpha-helical, while the repetitive domains are rich in beta-turns.

Several models for the structure of wheat glutenin have been proposed. One of the earliest molecular models was that of Ewart (1968). Ewart's latest model shows one disulphide bond between two adjacent polypeptide chains of glutenins, which consist of linear polymers. Ewart pointed out that the rheological properties of a dough are dependent on the presence of rheologically active disulphide bonds and thiol groups as well as on secondary forces in the concatenations (Ewart, 1979).

Kasarda *et al.* (1976) proposed an alternative model in which glutenin has only the intrachain disulphide bonds. They suggested that the intrachain disulphide bonds force glutenin molecules into specific conformations that facilitated interaction of adjacent glutenin molecules through non-covalent bonds, thereby causing aggregation.

Khan and Bushuk (1979) proposed a model of functional glutenin complexes that contained both inter- and intrachain disulphide bonds. On the basis of results from SDS-PAGE, they proposed an aggregate of two types of glutenin complexes, I and II. In their model, glutenin I comprised subunits of molecular weight 6.8×10^4 and lower, held together through secondary forces, such as hydrogen bonds and hydrophobic interactions: glutenin II comprised crosslinked subunits of molecular weights above 6.8×10^4 , linked by interchain disulphide bonds.

Shewry *et al.* (1989) proposed a model for HMW (γ -type subunit 9), which has at the N-terminus, a non-repetitive sequence of 80-100 residues containing three to five cysteine residues. At the C-terminus, there is another non-repetitive sequence of 42 residues including one cysteine, this region being identical for all subunits. The central region of the polypeptide (490-690) consisted of a number of repeated sequences forming beta-turns. From the dimensions of the subunits as determined by hydrodynamic measurements, the beta-turns were thought to be organized into a loose spiral.

1.2.4 High Molecular Weight Glutenin Subunits

The high molecular weight glutenin subunits are critical for dough processing characteristics (Shewry, 1992). The genes are located at Glu-1 loci of each genome (A, B, and D) of *Triticum aestivum*. The HMW-glutenin genes are divided into similar x-type and y-type genes which evolved by duplication.

The HMW-glutenin genes were expressed at high levels, 2 % of seed proteins per gene. In addition individual HMW-glutenins accumulates to different levels. In some wheat cultivars the HMW-glutenin Bx polypeptide (encoded by Glu-B1-1 gene) accumulates to a higher level than the other HMW-glutenins (Marchylo *et. al.* 1992, Kolster *et. al.* 1993). Anderson *et. al.* (1989) suggested a connection between this higher expression and a 54-bp Bx promoter-sequence duplication that includes the “cereal-box” implicated in seed-specific expression (Forde *et. al.* 1985).

Most of the detailed promoter studies were limited by the inability of to transform monocots. The studies used heterologous systems transgenic tobacco (Halford *et. al.* 1989, Thomas and Flavel, 1990) and transient monocot cell assays (Blechl *et. al.* 1994).

1.3 Genetic Strategies for Improvement of Breadmaking Quality

Achieving acceptable end-use quality for milling and baking is an important objective of wheat breeding programs. On average, it requires 10-14 years for a new wheat variety to be developed and enter the commercial market place. Genetic analysis and correlation studies showed that the breadmaking quality of wheat can be manipulated through the differences in quality and quantity of glutenin proteins. Research about glutenin proteins can be applied directly to optimize processing the commercial product or indirectly to breed wheat varieties having properties that match specific end-use requirements.

The quality of flour involve numerous physical and chemical variables. The breadmaking potential of wheat is largely derived from the quantity and quality of its protein content. Genetic and environment each play important roles in determining protein quality. Protein quantity is influenced mainly by environmental factors, while the quality of the protein is genetically determined. In wheat varieties that are grown under comparable environmental conditions, a high quality wheat will produce good bread over a fairly broad range of protein levels, but a poor quality wheat will generally result in low quality bread even if the protein content is higher.

The determination of contribution of a specific subunit to the bread-making quality can be assayed in several ways. One approach is the sequential

same nature, but enriched in monomeric gliadins or in the polymeric glutenin (MacRitchie, 1987). Genetic variants of wheat provide glutens that differ mainly by one or several LMW or HMW glutenin subunits. There are several type of genetic variants. Near-isogenic lines differ by the deletion or substitution of one or several genes of given chromosome. Translocation lines differ by a whole chromosome or a chromosome arm. Genetic lines are powerful tool in investigating glutenin subunits in doughs, the remaining flour composition being considered unchanged (MacRitchie, 1990). The use of wheat genetic variants has shown the effect of modifications on the glutenin subunit genes to always be cumulative (Benedettelli *et. al.* 1992) and sometimes additive.

1.3.1 Overexpression Studies in Transgenic Wheat

The breadmaking quality of wheat is related to the viscoelastic behavior of dough, which is due to its protein network, gluten. Determination of contribution of the different components to the rheology of functional gluten can be assessed in 2 major ways. Sequential extraction of gluten by dilute acid yields fractions with components of the same nature, but enriched in monomeric gliadins or in the polymeric glutenin. Genetic variants of wheat provide glutens that differ mainly by one or several low- or high-molecular weight glutenin subunits. The use of wheat genetic variants has shown the effects of modifications on the prolamin genes to always be cumulative (Bennedetelli *et. al.*, 1992), and sometimes

additive for technological parameters. The importance of the quantity and quality of HMW glutenin subunits on the extractability of storage protein and on the baking quality flours was also confirmed (Benedetelli *et. al.*, 1992).

The high molecular weight (HMW) subunits of glutenin gluten proteins have been shown to play a major role in determining the level of gluten visco-elasticity. Furthermore, genetic studies have shown that differences in some of the properties of these subunits are associated with variation in the gluten visco-elasticity and hence the breadmaking quality of wheat cultivars (Shewry *et. al.* 1997). It is suggested that the contribution of HMW-glutenin to visco-elasticity results from two main protein structural features: location of cysteines residues (the basis of the cross-linked gluten matrix) in the terminal non-repetitive domains of the polypeptide, and a central domain composed of repeats of short peptide motifs comprising 77-84 % of the polypeptide in published sequence (Shewry *et. al.* 1992). The total number of prolamines in wheat endosperm varies greatly but as many as 100 distinct proteins are found. This makes it difficult to study the contribution of individual proteins to rheological properties. One approach is to express cloned wheat prolamine genes in heterologous systems from which single prolamines can be isolated and studied (HMW-glutenins in bacteria, Galili, 1989; α -gliadins in yeast, Blechl *et. al.* 1992; α -gliadins in bacteria; γ -gliadins in yeast). These systems can be combined with recombinant DNA technology to make modifications in polypeptides and to construct simple structure peptides for studies. Such approaches have been used to study several polypeptides related to elasticity.

1.3.2 Expression in Heterologous Systems

Recombinant protein expression is the fundamental technique in biomolecular research and the Biotech industry. Overproduction of proteins for structural and functional studies and for industrial applications are the application areas of this technique. These techniques rely on the universality of the Genetic Code, the relative similarity of the translational machinery ribosome.

There are many different protein expression systems in prokaryotes and eukaryotes. Expression in *E. coli* is the typical system for prokaryotes and yeast for eukaryotic systems. Several yeast genes were found to complement bacterial genes when they are placed in *E. coli* vectors without modification. These include:

HIS3 = *hisB* in *E. coli*

LEU2 = *leuB* in *E. coli*

URA3 = *pyrF* in *E. coli*

TRP1 = *trpC* in *E. coli*

This permitted cloning of these yeast genes by complementation very early on and they are the standard set of yeast selectable markers today. (Carbon, 1993)
The *E. coli* RNA polymerase apparently recognize weak promoter like elements in the yeast DNA, not necessarily in the yeast promoter itself.

1.3.2.1 Expression in *E. coli*

One aspect of expression in *E. coli* is secretion. It is possible to add a signal sequence onto a protein so it will be secreted into the periplasm. The problem is that *E. coli* normally does not secrete proteins in the medium. They usually export them to the periplasm or the outer membrane, so it is difficult to get them to be completely secreted to the medium. *Bacillus subtilis* can secrete proteins to the medium. The advantages of doing this are great, since the secreted protein will be nearly the only one in the medium, purification is much simplified. Also proteolysis is reduced. Sometimes this can be a serious problem with proteins made and retained in the cytosol. One other advantage is that proteolytic processing of the leader will generate an N-terminal without formyl methionine that is the starting amino acid in *E. coli*. Careful planning of the fusion joint between the desired protein and the leader sequence can give the native protein sequence after cleavage. Finally, proteins in *E. coli* are not able to form disulfide bonds easily because of the strong reducing nature of the cytoplasm. Eukaryotic proteins with many Cys residues would benefit from export to the periplasm or medium where they could form the correct disulfide bonds more readily (Nagarajan, 1990).

It is easier to get a normally secreted protein to be secreted in *E. coli*. These tend to be smaller proteins and they have evolved to be passed through a membrane. Non-secreted proteins are harder. Since *E. coli* cannot do the proper

post translational modifications to eukaryotic proteins, it may be best to go right to the *Pichia pastoris* yeast secretion system for eukaryotic proteins.

1.3.2.2 Expression in Yeast

Yeasts, as host organisms for the production of heterologous proteins, can be manipulated by the application of molecular genetics and are simple to culture. They also perform post-translational protein modification as do most eukaryotes. One of the common yeast used for the production of recombinant proteins was *Saccharomyces cerevisiae*. Yeast can be grown rapidly on simple media and to high cell density, and its genetics are more advanced than any other eukaryote, so that it can be manipulated almost as readily as *E. coli* (Romanas *et. al.*, 1992).

Several features are important factors that makes yeast an excellent expression system. Yeast is easy to grow. It has a doubling time of 1.5-2 hours at 30 °C. Yeast has a Haploid /Diploid life cycle. It can be grown as either one. This allows mutation of essential genes that would be lethal in the haploid strain. One allele can be mutated and then sporulate the yeast to get haploid cells. The 4 spores can be tested for a 2:2 inheritance pattern that would result in two viable spores and two non-viable spores for an essential gene. Genes from different strains can be combined by mating and allowing them to segregate during meiosis. Almost any combination can be achieved in this way, unless the genes are very closely linked. Yeast can grow by fermentation on glucose or by respiration on carbon sources such as glycerol/ethanol. This is not possible for

higher eukaryotes. Because of this ability, yeasts are ideal organisms for the study of mitochondrial proteins needed for respiration. Mutations in these genes are not lethal when the cells are grown on dextrose (glucose). Many features important for higher eukaryotes are conserved in yeast. DNA replication RNA synthesis and processing Protein synthesis Protein targeting to organelles and their translocation across membranes nuclear pores/ mitochondria/ ER/ peroxisomes etc.

Vectors are needed that can be grown in both yeast and *E. coli*. This is important, because all the basic techniques used in *E. coli* can be exploited. It is much easier to grow large amounts of plasmid DNA and do constructions of novel plasmids in *E. coli*. To be able to move the plasmids back and forth, it is necessary to have the essential features needed by each organism on the shuttle vectors. These include the origin of replication and antibiotic resistance marker(s) for *E. coli*. In yeast all vectors will need a selectable marker that is mutated or deleted in the host. The usual strains used in the experiments have about 6 selectable markers. These include *His3*, *Ura3*, *Trp1*, *Leu2*, *Ade2* and *Can1*. These are in the biosynthetic pathways for histidine, uracil, tryptophan, leucine and adenine. *Can1* confers sensitivity to canavanine, an arginine analog that gets incorporated in proteins and is lethal to cells. *Can1* is an arginine permease that allows canavanine to enter cells. The *Can1* based plasmids can be used in plasmid shuffling experiments where one version of a gene (usually the wild type WT) is replaced by another version (the mutant) by selecting for the loss of the WT vector on canavanine. This technique is required if the original gene is an essential gene and the cell cannot survive without it. The chromosomal copy can

be deleted and replaced by the WT plasmid version. The WT can then be exchanged for a mutant version by adding canavanine. A similar method is done with *URA3* and a drug called 5-FOA (fluoro orotic acid) FOA is converted to a toxin by *URA3* so the presence of *URA3* on a plasmid is lethal in the presence of FOA. In this way a *URA3* plasmid can be chased out of yeast. FOA is very expensive and is used on very small plates.

There are three types of yeast vectors for three different purposes. Integrative plasmids for introducing a gene into a yeast chromosome, centromeric plasmids that contain a yeast centromere and are low copy, episomal plasmids called 2 micron from the 2 micron circles seen in some yeast strains. These are high copy vectors in yeast (20-100 copies per cell).

The most important property a yeast strain in the presence of defects in several genes that can be used as selectable markers on plasmid shuttle vectors. Most strains are not wild type for this reason.

The genotype of yeast is a listing of the relevant genes that are not wild type in a given strain. These are listed in a special manner to indicate the mutated gene and how it has been modified. The mating type is also given as either MATa or MATalpha. Yeast have two mating types, and strains that are the same mating type cannot be mated. A typical strain description is given below.

JLY1053 (MATa, *aac1::LEU2*, *aac2::HIS3*, *his3-11,15*, *trp1-1*, *ura3-1*, *can1-100*, *ade2-1*, *leu2-3,112*)

The JLY1053 is the strain name. In this case JL stands for Janet Lawson and Y stands for yeast. It is common to name yeast in this way with initials followed by Y and a number. (*aac1::LEU2* indicates that the *aac1* gene has been inactivated by inserting the selectable marker *LEU2* into the gene. The lower case letters indicate a non-functional mutant gene; *his3-11,15* indicates that the *HIS3* gene is a mutant in this strain and so it can be used as a selectable marker. The specific mutations are indicated by the numbers 11 and 15. This particular mutant has two modifications).

Another feature often indicated is a deletion. This can be a partial or complete deletion and it is shown by the delta symbol as in *his3-delta1*.

There are several methods to transform yeast that are frequently used. One is the Li Acetate procedure and the other is electroporation. Li Acetate is the most common method, because it does not require an expensive electroporator. The Lithium acetate procedure was developed as a more convenient way to transform yeast cells than the protoplast method whereby the yeast cell wall was digested to make the cells more permeable. It was known that Ca^{+2} ions could be used in making competent *E. coli*, but this did not work with yeast (Ito, 1983). This method has been refined by Dan Gietz et al. (1992).

The procedure involves starting with a yeast culture at about 0.8 OD₆₀₀ after they have just gone through two doubling times. This insures that the cell wall on most of the yeast is incomplete. The yeast are washed and incubated with Li acetate for one hour at 30 °C. Plasmid DNA is added and PEG (polyethylene glycol) is added in Li acetate buffer. A carrier of denatured single stranded DNA is added to improve efficiency of transformation. After 30 minutes, the cells are heat shocked at 42 °C for 15 minutes and then they are washed and plated on selective media

Yeast can be grown on rich media or dropout media, where one or more components have been left out. This is done for selection of plasmids that contain genes for synthesis of these components. For example histidine will be left out of the media if a *HIS3* plasmid is being used. Rich media is generally YPD or 1% yeast extract, 2% peptone, 2% dextrose. For solid media, 2% agar is included. The dextrose (or glucose) is for growth by fermentation and functional mitochondria are not required. Another rich media is YPG or YPGE. These are the same as YPD except the carbon source is now non-fermentable (3% glycerol or 3% glycerol/3% ethanol). Yeast must have functioning mitochondria to grow on YPG or YPGE. The dropout media does not contain yeast extract. Instead it has yeast nitrogen base without amino acids. It is not necessary to add 19 amino acids. Only those required by the yeast strain need be added. This kind of medium is referred to as Synthetic Complete media or SC. When a component is left out it is called SC-leu-trp or whatever components are missing.

Homologous recombination is the ability of complementary sequences to align and exchange fragments in a double crossover event. There is exact base to base exchange in this reaction. The frequency of homologous recombination is much greater in yeast than in higher eukaryotes. Therefore it has been exploited and is one of the most important tools in yeast genetics. Homologous recombination works best with linear fragments of DNA introduced into yeast. This is true because the ends of the fragments are highly recombinogenic. But circular plasmids can also undergo homologous recombination. The precise targeted deletion of genes in yeast and their replacement with selectable markers is dependent on this phenomenon. In higher eukaryotes, targeting of genes to known locations is one of the greatest problems for gene therapy. In yeast it is relatively easy to do.

An alternative to just putting the selectable marker into the gene at a restriction site is to digest the cut DNA with an exonuclease like Bal31. This can eat away much of the coding sequence of YFG before you blunt end ligate the selectable marker. It is advantageous to remove as much of the coding sequence as possible unless you are only interested to know what the phenotype is when you disrupt a gene. There is always the possibility that only the N-terminal is required for function, so a disruption that does not remove the N-terminal may still be active.

Deletions are better than disruptions, but they are harder to make. In a deletion, all the coding sequence of YFG is removed. The logic is the same,

homologous recombination swaps out a new construction you have introduced for the wild type gene on the chromosome. In a deletion, you have designed the linear piece to have 5' and 3' untranslated sequence from YFG bracketing the selectable marker gene. This can be done most easily if existing restriction sites allow removal of all the coding region and ligation of the marker without any more work. Most of the time this is not possible. If you have to construct a fragment with a marker bracketed by untranslated regions from YFG, the best way to do it is by PCR with addition of restriction sites at the junctions. These three pieces will have to be assembled into a vector one at a time and then the fragment can be cut out and used in a transformation.

For expression of yeast genes in yeast, to determine the effects of mutations, it is generally best to use the native gene's promoter in a CEN Plasmid so expression is similar to the wild type gene. However, there are a variety of promoters to choose for various purposes.

The Gal 1,10 promoter This promoter is inducible by galactose. It is frequently valuable to be able to turn expression of the gene on and off so it can be followed the time dependent effects of expression. The Gal 1 gene and Gal 10 gene are adjacent and transcribed in opposite directions from the same promoter region. The regulatory region containing the UAS sequences can be cut out on a DdeI Sau3A fragment and placed upstream of any other gene to confer galactose inducible expression and glucose repression.

PGK, GPD and ADH1 promoters. These are high expression constitutive promoters. PGK stands for phosphoglycerate kinase, GPD for glyceraldehyde 3 phosphate dehydrogenase, ADH1 for alcohol dehydrogenase

1.3.2.2.1 Expression in *Pichia Pastoris*

The methylotropic yeast *Pichia pastoris* has been developed as an expression system for the efficient production of foreign proteins (Cregg, 1993). A number of genes have been heterologously expressed in *P. Pastoris* by using the promoter of the alcohol oxidase 1 (*AOX1*) gene of the yeast. *AOX1* is the first enzyme in the methanol-utilization pathway and is strongly regulated at the transcriptional level (Couderc, 1980). The proteins expressed includes a variety of proteins both soluble (Clare, 1991; Laroche, 1994; Ridder, 1995) and integral (Weis, 1995) ones. *Pichia* Expression Kit is an Invitrogen licensed kit developed by Salk Institute Biotechnology. *Pichia Pastoris* is an eucaryote and hence it has all the advantages of eucaryotic expression systems as protein processing, folding, and postranslational modifications. It is also easy to handle as *E. coli* or *Sachoromyces cerevisiae*. It is faster, easier and cheaper than other eucaryotic expression systems (Cregg, 1993).

Escherichia coli has toxic cell wall pyrogens and mammalian cells may contain oncogenic or viral DNA, so that products derived from these organisms must be tested more extensively. Yeast can be grown rapidly on simple media, to high cell density, and its genetics are more advanced than any other eukaryote, so

that it can be manipulated almost as readily as *E. Coli* (Romanas, 1992). However, because of the problems encountered using it, *S. cerevisiae* has not proven to be ideal as a foreign gene expression host. One of these problems is that the promoters used for expression are either weak, not well regulated, or not easily regulated in the fermenter. As a result, expression strains which look promising in the laboratory are not productive when moved to a commercial scale. Other problems with *S. cerevisiae* are associated with secretion. Foreign proteins secreted from *S. cerevisiae* are often at too low a concentration to be commercially attractive. In addition, route through their secretory pathway can result in the addition of oligosaccharide chains. Possibly as a consequences of hyperglycosylation many foreign proteins targeted for secretion from *S. cerevisiae*.. can not pass through the cell wall and remain trapped in the periplasmic space (Cregg, 1989).

Pichia pastoris has been developed as a host in response to problems encountered using *S. cerevisiae* for heterologous expression (Buckholz and Gleeson, 1991; Romanas 1992). *Pichia pastoris* has many of the advantages of higher eukaryotic expression hosts such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or *S. cereviasiae*. It is easier, faster and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels. As a yeast, it shares the advantages of molecular and genetic manupilations with *Saccharomyces*, and it has the added

advantage of 10- to 100-fold higher heterologous protein expression levels. These features make *Pichia* very useful for protein expression (Cregg *et. al.*, 1993).

P. pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. It is reported that the first yeast using methanol as the sole carbon source. The pathway begins with the oxidation of methanol to formaldehyde and hydrogen peroxide, a reaction catalyzed by the enzyme alcohol oxidase (AOX). Formaldehyde can be oxidized further to formate and ultimately to carbon dioxide, by a dissilatory pathway which generates energy. This is called C1 pathway (Anthony, 1982).

Critical to the *P. Pastoris* system is the observation that many of the C1 pathway enzymes are present at higher levels in methanol-grown cells than in cells grown on other carbon sources. From the perspective of developing a system for heterologous expression, the most interesting of the enzymes is AOX, the first enzyme in the methanol-utilization pathway. AOX is undetectable in cells cultured on carbon sources such as glucose, glycerol or ethanol, but constitutes up to 30 % of total soluble protein in methanol-grown cells (Couderc and Baratti, 1980). It was suggested that AOX synthesis would be regulated at the transcriptional level.

One essential element in any expression system is the ability to transfer and maintain DNA sequences of interest in the cell. Transfromation method for *P. pastoris* are similar to those for *S. cerevisiae* and are based on auxotrophic mutant

hosts and vectors which contain complementary biosynthetic genes (Cregg *et. al.*, 1985). Vectors are transformed into *P. pastoris* using modified versions of the spheroplast generation-calcium chloride-polyethylene glycol method, the whole cell-lithium salt method or electroporation (Cregg *et. al.* 1985).

Gene insertion events at the *AOX1* (GS115) locus arise from a single crossover event between the locus and any of the three *AOX1* regions on the vector; the *AOX1* promoter, the *AOX1* transcription termination region (TT), or sequences even further downstream of *AOX1* (3' *AOX1*). This results in the insertion of one or more copies of the vector upstream or downstream of *AOX1*. The phenotype of such a transformant is His⁺Mut⁺. This event could also happen at the 5' *AOX1* regions of the plasmid and genome with the resulting insertion positioned 5' to an intact *AOX1* locus. This also occurs with non-linearized plasmid and plasmid that religates.

In either GS115 (Mut⁺) or KM71 (Mut^s), gene insertion events at the *his4* locus arise from a single crossover event between the *his4* locus in the chromosome and the *HIS4* gene on the vector. This results in the insertion of one or more copies of vector at the *his4* locus. Since the genomic *AOX1* locus is not involved in this recombination event, a His⁺ transformant has the same Mut phenotype as the parent strain.

The second essential element in an expression system is the promotor employed to derive heterologous gene expression. Ideally, the promoter should

meet three requirements. The first and most obvious requirement is that it should be efficiently transcribed so that a high level of expression is possible. The second requirement is that the promoter should be tightly regulated so that foreign protein production strains can be in an expression-off mode. The third is that it should be possible to induce promoter expression cheaply and easily in large-volume, high density, fermentor cultures, using existing fermentation technology (Cregg *et. al* , 1989). A unique attraction of the *P. pastoris* expression system is the promoter, which is derived from the *P. pastoris* alcohol oxidase I gene (*AOX1*). The *AOX1* gene was isolated from a cDNA library of RNA from methanol-grown, and not ethanol-grown, cell. *AOX1*-encoding cDNAs were identified by comparing the predicted amino acid sequence of the 5' end of these cDNAs with the known 5'-terminal sequence of the *AOX1* protein (Ellis *et. al.*, 1985). A genomic clone of *AOX1* which included its promoter was isolated and characterized. Evidence that *AOX1* is regulated by the carbon source at the level of transcription came from RNA-DNA filter hybridization results (Tschopp *et. al.*, 1987).

Further studies of AOX genomic clones revealed that the *P. pastoris* genome also contains a second functional alcohol oxidase gene, *AOX2*. It encodes a protein that is 97 % identical to and has approximately the same specific activity as that of *AOX1* (Cregg *et. al.*, 1989). However, in *P. pastoris* cells growing on methanol, *AOX1* is responsible for all but a minor fraction of the total AOX protein. Thus, it is the *AOX1* promoter that is incorporated into most *P. pastoris* expression vectors (Cregg *et. al.*, 1993).

A wide variety of heterologous proteins have been secreted from yeast and this approach offers certain advantages over intracellular production. Many proteins are naturally secreted and often only can adopt their correct conformation by folding within the secretory pathway. Thus, secretion is used mainly for the production of correctly-folded, naturally-secreted proteins (Romanas *et. al.*, 1992).

1.4 Wheat Quality Assays

Wheat quality can be defined as the suitability of the grain to the intended processing and product manufacture. This includes criteria such as milling performance, dough rheology, baking quality. The concepts of flour quality and flour strength are difficult to define concisely. This is largely because flour quality is expressed by a variety of chemical and physical properties of dough, none of which serves as an adequate index by itself, or is independent of other variables. Thus, different physical and chemical testing methods, different baking test procedures, and different dough processing treatments, when applied to the same flour, will yield results that may lead to widely divergent conclusions as to the flour's quality. Moreover, the end use to which a flour is to be put enters importantly into any evaluation of its quality.

Biochemical methods, such as electrophoresis, monoclonal antibody tests for protein composition, wet and dry gluten contents, SDS-sedimentation volume,

and pigment content are used to predict some of the characteristics. The SDSS test is commonly used as an indicator of gluten quality. Methods determining rheological properties are alveograph, farinograph, mixograph, and viscoelastograph. Protein content has also been used as a rough indicator of flour quality. Protein comprises all the nitrogenous substances present in flour and other food materials and is estimated by the Kjeldahl and other methods that are specific for nitrogen. Gluten, on the other hand, represents the end product obtained when a small piece of dough is worked and washed in water. The specific subunits of Glutenin and gliadins were determined showing specific quality characteristics.

SDS-Sedimentation volume test uses the ability of flour dispersed in an aqueous solution of SDS to form a proteinaceous gel when centrifuged at high speed. The sedimentation test introduced by Axford et. al. (1978), and its predecessor, the Zeleny sedimentation test (Zeleny, 1947), have been widely used as small-scale tests of breadmaking quality in wheat breeding programs. The measurement of gluten strength have been involved the mixograph or the farinograph. These instruments record the characteristics of a flour-water mixture as it is worked into a dough, and also the tolerance of dough to mixing. Gluten strength has also been measured by a gluten-stretching method with viscoelastograph for monitoring breadmaking quality.

Commercial wheat assays test quality from 700 g of flour to evaluate potential performance. Wheat breeding programs make early-generation quality

selections based on: dough-strength testing devices such as mixograph; determination of flour protein content; and some measures of aggressive protein quality by procedures such as the SDS sedimentation (SDSS) test.

1.5 Aim of the Study

The objectives of this study can be summarized as follows:

- a) To investigate the gluten protein polymorphism of 13 Turkish wheat cultivars at protein and genomic level,
- b) To clone the silent *Ay* gene from cultivar 'Yayla',
- c) To construct a yeast expression vector and express the product of cloned *Ay* gene in a heterologous system (*P. pastoris*),
- d) To isolate and evaluate the influence of *Ay* gene product on dough making properties of the flours of 13 Turkish cultivars.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

Seeds of *Triticum aestivum* cv Gün 91, Orso, Kır  , Bezostaja, Yayla, Gerek, Atay 85, Sertak, Ak 702, Bolal, Tosun, and *Triticum durum* cv Dicle 94,   akmak used in this study were obtained from Eski  ehir Wheat Research Center. The seeds were grown on vermiculite-soil mixture in a growing chamber at 23 ± 5   C with a photoperiod of 16 hours light and 8 hours dark for ten days.

2.1.2 Chemicals

The chemicals used in this study were commercially available from Merck, Sigma and Fluka. Protein markers were obtained from BioRad.

2.2 Methods

2.2.1 Fractionation of Flour Proteins

The reduced and unreduced fractions were isolated as sequentially. Grains were milled on a Brabender Quadraplax experimental mill into straight grade-flour and stored at 4 °C in eppendorf tubes. Total polymeric proteins were separated into two groups on the basis of their solubility in 2 % SDS-Tris buffer (pH=8.1) (without and with reducing agent, 1 % DDT) and these were indicated as soluble (SPP) and insoluble (IPP) polymeric proteins for HPLC analysis. The proteins of flour samples were extracted with 500 µl of sample buffer (2 % SDS, 5 % 2-mercaptoethanol, 0.001 % pronin Y, 10 % glycerol, and 0.063 M Tris-HCl pH 6.8) by incubating 1 hours at 60 °C with occasional mixing and centrifuged at 13 000 rpm for 5 min. The supernatant was kept as the soluble fraction and the pellet were further extracted with the sample buffer with DDT to obtain insoluble fraction. The protein content of samples were determined by Bradford protein determination method (Bradford, 1976) and were fractionated on polyacrylamide gel electrophoresis. 10 µl of sample was loaded in the analysis.

2.2.2 Electrophoresis of Flour Samples

SDS-PAGE of reduced and unreduced fractions was performed according to the method of Laemmli (1970) using 10 % gels. Electrophoresis were performed in mini gel apparatus of Biorad at 200 V (constant voltage) until the

tracking dye, Pyronin Y had reached the bottom of gel. The acrylamide gel solution acrylamide (9.85 %), bis-acrylamide (0.15 %), SDS (0.1 %), and Tris-HCl pH 8.8 (37.6 %). The protein samples were dissolved in sample buffer (2 % SDS, 5 % 2-mercaptoethanol, 0.001 % pronin Y, 10 % glycerol, and 0.063 M Tris-HCl pH 6.8) and 5 μ l sample corresponding to 5 μ g of protein were loaded in wells. The broad range standard proteins (200, 116, 97.4, 66, 45, 31, 21.5, 14.5 kD) from BioRad were used as marker. The proteins were visualized by staining with coomassie blue according to Payne *et. al.* (1979).

2.2.3 HPLC Analysis of Flour Samples

Soluble and insoluble protein fractions were also analyzed by using a HPLC system (Beckman) according to Ciaffi *et. al.* (1993). A Waters protein-pak 300 SW column for reverse phase and SEC-4000 column for size exclusion were used. Absorption of eluents was recorded at 210 nm.

The SPP (Soluble Polymeric Protein) samples were fractionated by SE-HPLC into three peaks of decreasing size-range (peak 1-3 in Figure 3.1) representing soluble polymeric proteins, gliadins and albumin/globulins. The SPP were extracted by crashing a single freeze-dried grain in 500 μ l extraction buffer (1 M Tris (pH=8.1), 0.1 % SDS) and 15 μ l of the sample was applied into HPLC. The reduced protein extracts were fractionated by SE-HPLC into similar three peaks containing predominantly high and low MW glutenin subunits (Caffi, 1993). Proteins were run on a SEC-4000 column, the elution buffer was 0.1 %

Tris, 0.01 % SDS buffer. The column was kept at 25 °C and the flow rate was 0.5 ml/min. Analysis by SDS-PAGE of collections corresponding to the areas shown in Figure 3.1 were done for the sample 'Bezostaja'.

2.2.4 SDS-Sedimentation Test

SDS-Flour samples were tested for bread-making quality by an modified micro procedure of SDS-Sedimentation test of Axford *et. al.* (1978). The procedure of Sapirstein (1999) was followed as indicated below. The volumes of material which sediments after mixing 0.7 g flour with 15 ml of 1.5 % SDS was measured after an incubation of 5 hours at room temperature and centrifugation at 13000 rpm for 15 min. The volume of the gel formed was recorded as the SDS sedimentation volume. This procedure was also carried out for testing the effect of *Ay* gene product on breadmaking quality of flour of each genotype. The mixture flour of each genotype prepared by adding 0.1 g of *Ay* gene product and 0.6 g of flour to prepare a 1/7 (w/w) ratio mixtures which were tested for bread-making quality by the SDS-Sedimentation test as described above. Control experiments were done by using 10 mg of secreted proteins of parent plasmid transformed cell cultures. The experiments were designed to obtain 4 measurements from the samples of four different expression cultures. The data for SDS Sedimentation value were statistically evaluated by using SPSS for Windows.

2.2.5 DNA Isolation

Genomic DNA was prepared using the following procedure as described by Campenhout, *et. al* (1995): 10 mg of fresh leaf tissue from 10 days old seedlings was put in a 1.5 ml eppendorf tube, frozen in liquid nitrogen and ground with a sealed disposable pipette tip that had been placed onto an automatic screwdriver. One ml of warm (60 °C) extraction buffer (100 mM Tris-HCl pH 9.5, 1.4 M NaCl, 20 mM EDTA, 2 % CTAB, 0.2 % thioglycerol, 2 % PVP, 0.1 % vitamin C) was added followed by 400 µl chloroformisoamylalcohol (24:1). This mixture was incubated in a water bath at 60 °C for 10 min. After centrifugation (5 min at 14 000 rpm), the supernatant was treated with 100 µg RNase A for 15 min at 37 °C. After the addition of 500 µl isopropanol, DNA was precipitated on ice for 10 min and centrifuged for 10 min at 14 000 rpm in a Beckman Centrifuge. The pellet was washed with 1 ml cold 70 % Ethanol and dissolved in 100 µl TE-buffer (10 Mm TRIS-HCl, 1mM EDTA, pH=8.0) and stored at -20 °C until use.

2.2.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis were done in a Bio-Rad horizontal electrophoresis unit in 1X TBE buffer (0.089 M Tris, 0.089 M Boric acid, 2 mM EDTA, pH=8.0) according to standard procedures (Sambrook, 1989). The DNA markers used were Hind III and EcoRI digested λ (21226, 5148, 4268, 3536, 2027, 1904, 1584, 1375, 947, 831, 564) or Hind III digested λ (23130, 5216, 6557, 4361, 2322, 2027, 504). 0.8 % gels were run under 80 V at room

temperature for 2-3 hours. The bands were visualized by ethidium bromide staining under UV light.

2.2.7 Polymerase Chain Reaction

The PCR reactions contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, 1 µM of each primer, 2 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norvall, Conn.) and 50 ng genomic DNA in a total volume of 100 µl. The reaction mixture was overlaid with mineral oil and subjected to 1 cycle of 1 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 59 °C, 90 s at 72 °C on a Perkin Elmer thermoblock. Aliquots of amplification products (10 µl) was electrophoretically analyzed on 1.5 % agarose gels in 1XTBE buffer following standard procedures (Sambrook *et. al.* 1989). Ethidium bromide stained gels were visualized under UV light and photographed with an eye-shot of Promega. Oligonucleotides used as primers were synthesized on the basis of *Ay* glutenin gene previously published and have the following sequence.

2.2.8 Primers Used for PCR

The primers used for polymorphism were designed according to Compenhout (1995). The primer set has been synthesized in Iontek-Bursa. The sequences were as follows:

O15: TTTGCCCTCATCGCCGTTGT

O13: TGCTGTTGAGGTTGTTGGAAAGA

P15 GCGCAAATTTACAGCAACA

P13 TGGCACACTAGTGGTGGTTTC

For cloning complete *Ay* gene, a primer set were constructed on the basis of published sequences (Forde, 1985). XhoI and EcoRI sites were used in the expression plasmid construction. Since the sequence between XhoI and SnaBI encoded the KEX-2 site (Glu-Lys-Arg-X) and needed for cleavage of signal sequence after secretion, this sequence were recreated in the primers for efficient cleavage of *Ay* protein from signal sequence. The sequences used for amplification of *Ay* gene from genomic DNA of 'Bola' were as follows:

CEN1

5'-TCA GCT CGA GAA AAG AGA AGG TGA GGC CTC TAA GCA A-3'

CEN2

5'-ACG AAT TCT CAC TGG CTA GCC GAC AAT GCG TCG-3'

2.2.9 Cloning PCR Products

The final extension step from the amplification protocol was omitted to leave blunt-end PCR products. The PCR products were purified by QIAquick Gel Extraction Kit (Qiagen) after running in 1 % agarose gel. The fragment isolated were directly ligated into pGEMT vector. Ligation was carried out according to Sambrook (1989) as follows: a reaction volume of 20 µl contained 200 ng PCR

product, 80 ng of pGEMT vector, 2 units of T4 DNA ligase (Promega), 3.3 mM Tris acetate buffer pH 7.9, 1 mM Mg-acetate, 6.6 mM K-acetate, 0.05 dithiothreitol. This ligation mixture were incubated at 4 °C overnight. Half of it was used to transform *E. coli* strain JB101.

2.2.10 Plasmid Construction

PGEMT-*Ay* vector and pPIC9 plasmid were digested with EcoRI and XhoI sequentially. Twenty µl reaction volume contained 50 ng DNA and 2 units of EcoRI restrictive enzyme. The mixture was incubated for 1 hours at 37 °C. The concentration of NaCl in the medium were increased to 5 mM and 2 units of XhoI enzyme was added according to manufacturer's advice. The mixture was further incubated for 20 hours at 37 °C . The reaction contents were fractionated at 1 % agarose gels and the restricted *Ay* gene fragment and pPIC9 plasmid were isolated from gel with QIAquick gel extraction kit and ligated into compatible sites of *Pichia* shuttle vector pPIC9 between 5'-promoter and 3'-terminator of the alcohol oxidase gene (*AOX1*) (Figure 2.1). pPIC9 vector provides the α -mating factor signal for secretion and the *HIS4* gene for selection of the recombinant yeast clones.

773
ACAGCAATAT ATAAACAGAA GGAAGCTGCC CTGTCTTAAA CCTTTTITTT TATCATCATT ATTAGCTTAC
|
5' AOX1 Primer Site (866-876)
TTTCATAATT GCGACTGGTT CCAATTGACA AGCTTTTGAT TTAAAGACT TTTAACGACA ACTTGAGAGAG
|
Factor (949-1218)
ATCAAAAAC AACTAATTAT TCGAAGGATC CAAACG ATG AGA TTT CCT TCA ATT TTT ACT GCA
Met Arg Phe Pro Ser Ile Phe Thr Ala
GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT
Val Leu Phe Ala Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp
GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT
Glu Thr Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp
TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG TTT ATA
Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu Phe Ile
|
Factor Primer Site (1162-1172) Xba I
AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT CTC GAG AAA AGA
Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser Leu Glu Lys Arg
Signal cleavage (1204) SmaBI EcoRI AyrI NotI
GAG GCT GAA GCT TAC GTA GAA TTC CCT AGG GCG GCG GCG AAT TAA TTGCGCTTAG
Glu Ala Glu Ala Tyr Val Glu Phe Pro Arg Ala Ala Ala Asn ***
ACATGACTGT TCCTCAGTTC AAGTTGGGCA CTTACGAGAA GACCGTCTT GCTAGATTCT AATCAAGAGG
|
3' AOX1 Primer Site (1327-1347)
ATGTCAGAAT GCCATTGGCC TGAGAGATGC AGGCTTCATT TTGATACIT TTTTATTGT AACCTATATA
|
AOX1 mRNA 3' end (1418)
GTATAGGATT TTTTGTGCA

Figure 2.1. The detail of pPIC9 multiple cloning sites.

The construct were sequenced before transformation to ensure the correct reading frame for secretion and an ATG in the proper context for translation initiation. The primers 5' *AOX1* sequencing primer (5'-GACTGGTTCCAATTGACAAGC-3') and 3' *AOX1* (GCAAATGGCATTCTGACATCC-3') sequencing primer pair were used for sequencing. The 5' primer hybridizes the MCS in the *AOX1* promoter region allowing the determination of 5' *AOX1*-gene of interest junction and confirms that the ORFs are preserved. 3' primer hybridizes 3' of MCS in the 3' *AOX1* (TT) region allowing the determination of the 3' *AOX1*-Ay junction. Nucleotide

sequence analysis was carried out by using the chain terminator method (Sanger *et. al.*, 1977).

2.2.11 Strains and Yeast Cultures

E. coli strain DH5 α was used for transformation and propagation of the recombinant plasmid. *E. coli* was grown in LB medium at 37 °C . The *P. pastoris* strain GS115 deficient in histidinol dehydrogenase activity (*his4*) was used in the experiments. Yeast strain was grown in YPD medium (1 % yeast extract, 2 % peptone and 2 % dextrose) at 30 °C by vigorous shaking.

2.2.12 Expression in *Pichia pastoris*

The transformation of *Pichia pastoris* strains with plasmid construct and the expression of *Ay* gene were based on the procedures as described Invitrogen *Pichia* Expression Kit Manual (Version J) with the indicated modifications.

2.2.12.1 Transformation into *E. coli*

The construct plasmid (1 μ l from ligation mixture) were first transformed into competent *E. coli* and grown overnight. The transformants were selected for ampiciline resistance present on pPIC9 plasmid according to standard procedures (Sambrook, 1989). 100 μ g/ml ampicilin were used for selection of resistant

colonies. Ten resistant colonies were picked up and grown overnight in liquid LB medium with 100 µg/ml ampicilin. Glycerol stocks were prepared for later use.

2.2.12.2 Midiprep Plasmid Isolation

The plasmids (*Ay*-PIC9 construct and pPIC9) from *E. coli* were extracted by QIAgen Midi plasmid isolation kit. A single colony from a freshly streaked selective plate and inoculated to a culture of 2-3 ml LB medium containing ampiciline. Overnight grown cells were diluted into 100 ml LB medium and further grown overnight. The cells were collected by centrifuging at 4000 rpm for 6 min. in a Beckman centrifuge. The pelleted cells were alkalized with lysis buffer (P1 as supplied by manufacturer) and 5 units of RNase A was added. 10 ml of Buffer P2 was added, mixed thoroughly and gently by inversion and incubated at room temperature for 5 min. 10 ml of buffer P3 were added, mixed by inversion and incubated on ice for 20 min. The content was centrifuged at 20000 g in a Beckman JA-17 rotor (12000 rpm) for 30 min at 4 °C and the supernatant containing plasmid DNA was recentrifuged at the same speed for 15 min. The supernatant was infiltrated in QIAgen tip-500, equilibrated with 10 ml buffer QBT and washed three times with 30 ml buffer QC. The DNA was eluted with 15 ml buffer QF and was precipitated by addition of 10.5 ml isopropanol at RT and centrifuged immediately at 15000 g for 30 min. at 4 °C in a Beckman JS-13 rotor (9500 rpm). The pellet was washed with 5 ml room temperature 70 % ethanol and recentrifuged. The air-dry pellet was redissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH=8). The Total DNA obtained was determined by UV

spectrophotometer at 260/280 nm readings and the amount of DNA was calculated as approximately 50 µg.

2.2.12.3 Linearization of Construct for Transformation

The PIC9-*Ay* construct in the correct orientation were linearized with *Sal*I to isolate His⁺-Mut⁺ transformants of strain GS115. Both construct and parent PIC9 vector were linearized. A small portion of digest was analyzed by agarose gel electrophoresis to confirm complete digestion of the plasmid. The digest was extracted with phenol:chloroform:isoamylalcohol (25:24:1) and ethanol precipitate. The pellet was resuspended in 20 µl of TE buffer. The content was immediately used for transformation.

2.2.12.4 Spheroplasting

Cell wall of yeast prevents uptake of DNA. To enable yeast to take up DNA, cell wall must be partially removed. Zymolase is a β-Glucanase that hydrolyses the glucose polymers with β-1,3 linkages in the cell wall. It is crucial not to overdigest since this would cause lysis of cells. Zymolase digestion is monitored with the sensitivity to SDS.

Pichia strain GS115 cells were streaked onto YPD plates incubated at 29 °C for two days. Single colonies were inoculated in 10 ml YPD liquid medium a 50 ml conical tubes and grown overnight at 29 °C with vigorous shaking (300

rpm). Three 500 ml flask with 200 ml YPD medium were inoculated with 5, 10 and 20 μ l cells and incubated with vigorous shaking at 29 °C. The cells were harvested from the flask with OD₆₀₀ between 0.2 and 0.3 by centrifugation at 1500 g for 6 min (Beckman JS-13 rotor). The pellet were washed by resuspending in sterile water, in SED, and then in 1 M Sorbitol. The cells were resuspended in 20 ml of SCE. 7.5 μ l zymolase (2.25 units lytic activity) was added and swirled gently and incubated in 30 °C. 200 μ l aliquot of cells were taken after 2 min and added in 800 μ l of 5 % SDS. This was repeated at times 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 min. The OD₈₀₀ of each tube were read to calculate % of spheroplasting with the following equation:

$$\% \text{ spheroplasting} = [100 - (\text{OD}_{280} \text{ at time } t / \text{OD}_{280} \text{ at time } 0) \times 100]$$

70 % spheroplasting time were determined as 25 min and used for the transformations.

2.2.12.5 Transformation of *Pichia* Cells

100 μ l cells and 10 μ g of plasmid were used in the transformations. DNA and Cell mixture as prepared were incubated at room temperature for 10 min. One ml of PEG/CAT solution were added. After another 10 min incubation at room temperature, the tubes were centrifuged at 750 g for 10 min at room temperature. The pellet were resuspended in 150 μ l of SOS medium and incubated at room temperature for 20 min. 850 μ l of 1 M Sorbitol added and the cells were plated in RDB plates with top agarose to prevent the cells from lysis. The plates were incubated for 4 days at 29°C.

2.2.12.6 His⁺-Mut⁺ Selection

Hundred transformant colonies were simultaneously plated in MM and MD agar (1.34 % yeast nitrogen base, 0.00004 % biotin, and 0.5 % methanol). To differentiate between Mut⁺ and Mut^s controls (GS115/His⁺Mut^s Albumin and GS115/His⁺Mut⁺ β -Gal) were plated onto MD and MM plates. After two days of incubation at 29 °C, the plates were scored for colonies growing normally on MD plates but showing little or no growth on MM plates. Those clones that suffered homologous recombination with *AOX1* sequence (slow growing in MM agar) were selected.

2.2.12.7 PCR Analysis of *Pichia* Integrants

The integration of *Ay* gene in *Pichia* genome were checked by PCR amplification of yeast genomic DNA using 5' *AOX1* and 3' *AOX1* primer sequences (Haning, J. *et. al.*, 1997). Cells from growing colonies were transferred with a sterile micropipet tip into 50 μ l TE (10 mM Tris, 1 mM EDTA, pH 8.0), 0.1 % SDS, vortexed for a few seconds. Five μ l of the solution was used as template in a 50 μ l PCR reaction. PCR amplification were performed on a Hybaid Thermocycler with 2 units of Promega Gold Taq and 40 pmol of each primers. Twentyfive cycles of denaturation at 94 °C for 2 min, 1 min annealing and elongation at 94 °C and 55 °C with 45 seconds denaturing time in the first cycle and 200 seconds elongation in the last cycle.

2.2.12.8 Optimization of *Pichia* Protein Expression

To test for the most productive clones, colonies were grown in 10 ml of buffered liquid BMGY medium (1 % yeast extract, 2 % peptone, 90 mM potassium phosphate, pH 5.6, 1.34 % yeast nitrogen base, 0.00004 % biotin, and 1 % glycerol) at 30 ° for 4 days. Cells were collected with centrifugation at 3000 rpm (Beckman JS-13 rotor) for 6 min and gently resuspended in 2 ml of buffered liquid BMMY medium (same as BMGY medium but containing 0.5 % Methanol instead of 1 % Glycerol) and cultured for another two days to induce the expression of recombinant protein. The production of the clones were monitored after 5, 24, and 48 hours by electrophoretic analysis of the supernatant on 12 % SDS-PAGE gels as described below.

2.2.12.9 Expression and Isolation of *Ay* Protein

Shake-flask cultures were grown at 30 °C for 3 days in BMGY medium (20-30 absorbance units at 600 nm: 20 g of cells (dry weight/liter). Cells were collected by centrifugation at 1500 x g, gently resuspended in 200 ml of BMMY medium and cultures for another two days to induce the production of *Ay* protein. The culture medium was separated from cells by centrifugation at 1500 g (Beckman JS-13 rotor) for 6 min.

2.2.12.10 Identification and Characterization of Expressed Ay Protein

2.2.12.10.1 SDS-PAGE Analysis of Ay Protein

One ml of culture aliquot were directly taken from flask-shake culture. The content were centrifuged at 4000 rpm for 5 min. The supernatant was made 10 % in TCA and 70 % in acetone at the final concentration and incubated for 1 hour at –20 °C. The content was centrifuged at 13000 rpm (Beckman JS-13 rotor) for 10 min and the pellet was resuspended in SDS extracting buffer (62.5 mM Tris, 4 % SDS, 10 % glycerol, 0.001 % bromophenolblue, 2 % 2-mercaptoethanol adjusted to pH 6.8 with HCl). The SDS-PAGE procedure was the same as described above.

2.2.12.10.2 Reverse-phase HPLC Analysis

Reverse-phase HPLC analysis was done in 300 SW column (C8, 300 Å pore size, 5 µm particle size, 25 x 4 mm i.d.) and 4 x 4 cm precolumn were used. Elution of proteins were carried out according to Wieser *et. al.* (1994). A multistep linear gradient (water and Can, each containing 0.1 % TFA) was used to separate proteins. 80 µl of supernatant from cell culture (as described below) were loaded onto HPLC column in one injection. The gradient was increased from 24 to 39 % Can over 15 min. The gradient then increased to 45 % Can over 6 min. and then further increased to 55 % over the next 2 min. The column was rinsed for two 7 min. with 75 % Can and then held at initial conditions. The temperature of

column was maintained at 45 °C. The flow rate was 0.5 ml/min to facilitate analysis of collected fractions. Separation was followed at 220 nm.

2.2.12.10.3 N-terminal sequence analysis

After electrophoretic analysis by SDS-PAGE, N-terminal sequence analysis was performed by blotting the SDS-PAGE gel on polyvinylidene difluoride membranes, followed by the direct analysis on a Beckman LF3000 Protein Sequencer.



CHAPTER 3

RESULTS

3.1 HPLC Analysis of Gluten Proteins

Size Exclusion chromatography for total seed proteins of 10 hexaploid and 3 durum wheat cultivars were analyzed using Beckman SEC-4000 column. Three main peaks were determined for each of the genotypes. These peaks were frequently reported to be useful in predicting the breadmaking properties of flour (MacRitchie and Lafiandra, 1998). In the solubilized part, fraction 1 corresponds to polymeric proteins (molecular weights > 100 kD), fraction 2 to gliadins (with MW between 30 and 80 kD) and fraction 3 to albumin/globulin proteins (with MW between 20 and 30 kD).

The analysis are based on a sequential extraction of unreduced and reduced fractions of total seed proteins. Typical elution profile obtained when flour extracts were applied to a TSK 4000-SW column is shown in Figure 3.1. The elution times were marked in one of the HPLC profile of Bezostaja to illustrate the areas of each fraction. Many differences were observed between cultivars as the unreduced and reduced fractions. The peaks were divided into three distinct areas absorbing at 214 nm. Previous studies determined the limits

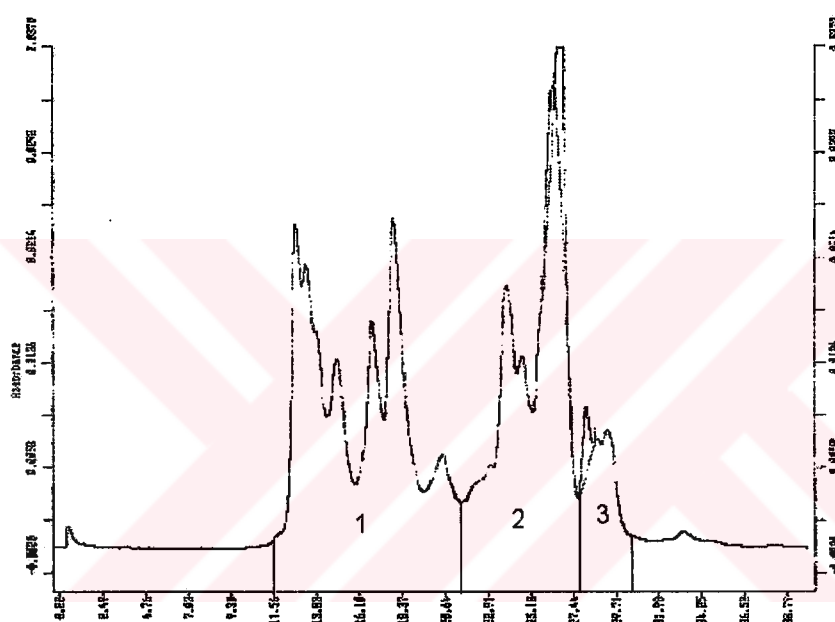


Figure 3.1. HPLC Elution profile of unreduced proteins of flour of Bezostaja extracted with Tris-SDS buffer on a TSK 4000-SW column. Column solvent was 0.1 M Tris buffer (pH=8.1) containing 0.1 % SDS. Three main peaks in increasing elution time corresponding to polymeric proteins (1), gliadins (2), and albumin/globulins (3).

between peaks (MacRitchie and Lafiandra, 1998). Fraction 1 (F1) was defined as the elution between 11.50 and 21.00 min., fraction 2 (F2) between 21.00 and 27.60 min, fraction 3 (F3) between 27.60 and 30.08 min. The Elution times show an inverse correlation between retention times and molecular weight of the subunit. F1 contained polypeptides ranging from 30 to over 200 kD. Most of the polypeptides from F1 were in 40-80 kD range as expected. F2 contained lower molecular weight polypeptides compared to fraction 1 (20-50) and F3 contained still lower molecular weight polypeptides (10-30 kD). The validity of these areas was confirmed by collecting the eluents from HPLC for one example and separating them in SDS-PAGE (Figure 3.2).

Results of SE-HPLC analysis of flour samples of 13 genotypes are given in Appendix. The fraction areas as shown in Figure 3.1 were determined for each genotype and tabulated in Table 3.1. The fraction areas were represented as F1, F2 and F3 for the corresponding peak 1, 2, and 3 respectively. F1/F2 ratio were calculated from the values. Table 3.1 also give the quality value as reported by Kün (1985). This is a categorization of bread wheat to represent the standard for commercial value. The quality were categorized into 3 classes depending on color, structure, and weight of grain, external ingredients and humidity value. This categories were assigned by Turkish Office of Cereals depending on Turkish wheat standards (TS 2974).

The F1 fraction ranged from 8.14 for Orso to 11.77 for Dicle95 with a correlation coefficient of -0.36 to the quality value. The F2 fraction ranged from

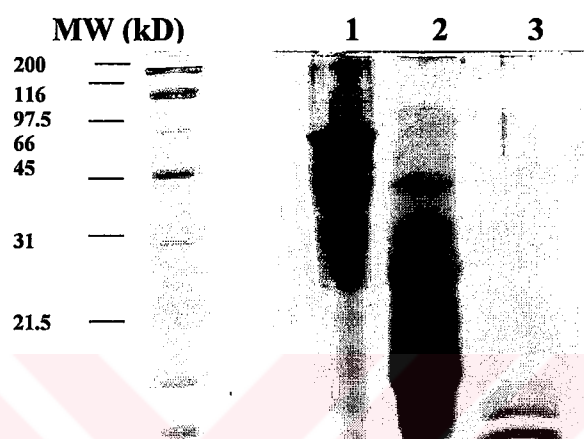


Figure 3.2. SDS-PAGE (11 %) result of HPLC fractionated samples of elution of sample Bolal. The bands were visualized by Coomassie Blue staining. Lane1, 2, and 3 represents proteins present in peaks 1, 2 and 3 of Figure 3.1, respectively.

Table 3.1 HPLC fraction areas as estimated from chromatograms of 13 wheat genotypes. The values were the mean values of three replicates. The quality values are from K  n (1985). F1 and F2 fractions are given as cm².

Genotypes	F 1	F 2	F 3	F1/F2	Quality Value
G��n 91	7.98��0.012	8.18��0.104	3.45��0.198	1.025	��
Orso	8.14��0.060	7.43��0.078	3.53��0.174	1.096	3
Kıra��	8.48��0.041	8.15��0.064	3.94��0.195	1.041	1
Bezostaja	8.91��0.058	7.94��0.147	3.43��0.172	1.122	1
Yayla	9.14��0.143	7.52��0.048	3.56��0.206	1.215	1
Gerek	9.54��0.075	8.73��0.086	4.75��0.138	1.093	2
Atay 85	9.68��0.055	7.35��0.084	3.85��0.295	1.317	��
Sertak	9.96��0.049	9.37��0.071	4.66��0.183	1.063	��
Ak 702	10.05��0.129	8.42��0.162	3.84��0.207	1.194	��
Bolal	10.38��0.070	7.99��0.066	4.21��0.235	1.299	2
Tosun	10.82��0.998	7.69��0.083	4.16��0.153	1.407	1
��akmak	11.35��0.153	8.57��0.107	4.25��0.169	1.324	��
Dicle 95	11.77��0.067	7.18��0.083	3.33��0.151	1.639	1

7.18 for Dicle95 to 9.37 for Sertak with a correlation coefficient of 0.10 to the quality value. The F3 fraction ranged from 3.33 for Dicle95 to 4.75 for Gerek with a correlation coefficient of 0.21 to the quality value. The F1/F2 ratio ranged from 1.041 for Kırac to 1.639 for Dicle95 with a correlation coefficient of -0.35. The durum wheat genotypes (Çakmak and Dicle95) had highest F1 value. Their F2 and F3 values were also higher than that of most of the bread wheat genotypes. The F2 and F3 values of Çakmak was higher than that of Dicle genotype and consequently Dicle had a higher F1/F2 ratio compared to the other durum genotype Çakmak.

3.2 SDS Sedimentation Volume Determination

The sedimentation volumes of genotypes were given in Table 2. The flour loaf volume values were determined by a SDS microsedimentation test (Sapirstein and Suchy, 1999). Among the genotypes used in this study 'Yayla' had the highest SDS Sedimentation volume with 3.24 ml. The fractions obtained in SE-HPLC analysis had highest correlation with SDS sedimentation volume in the F1/F2 ratio with correlation coefficient of 0.41. The correlation coefficient of SDS sedimentation value with F1 fraction was 0.26. F2 and F3 fractions did not significantly correlated. The correlation coefficient with F2 was 0.05 and with F3 was -0.04. highest SDS-Sedimentation value with 3.2 and Ak-702 had the lowest value with 1.2. Other genotypes had values between these values. The general quality classification as described by Kün (1985) correlated with this values.

Table 3.2. The loaf volumes of genotypes as determined by SDS microsedimentation volume.

Genotypes	SDS Sedimentation Volume (ml)	Quality Value
Gün 91	2.1±0.040	–
Orso	1.9±0.043	3
Kıraç	2.8±0.041	1
Bezostaja	2.12±0.050	1
Yayla	3.24±0.072	1
Gerek	1.89±0.066	2
Atay 85	2.02±0.128	–
Sertak	2.54±0.054	–
Ak 702	1.27±0.148	–
Bolal	1.43±0.078	2
Tosun	2.20±0.032	1
Çakmak	2.32±0.270	–
Dicle 95	3.15±0.136	1

3.3 Comparison of HPLC Fractions and SDSSV Values

Previous studies showed that distinct differences in sizes of corresponding peaks were observed for samples known to differ widely for baking strength (MacRitchie and Lafiandra, 1998). Figure 3.3 shows that the SDS Sedimentation Volume (SDSSV) and F1/F2 ratio of HPLC analysis are correlated. This variation between genotypes are highly significant. Correlation has been obtained between HPLC peak area and technological property, loaf volume ($r=0.52$).

The loaf volumes were more closely associated with the soluble fraction F1/F2 ratio ($r=0.41$) than with F1 ($r=-0.26$), F2 ($r=0.05$), F3 ($r=-0.05$). Both F1 and F2 were positively correlated with loaf volume. The highest correlation coefficient were observed for F1/F2 ratio.

3.4 Electrophoretic Analysis of Gluten Proteins

The SDS-PAGE procedure has been used to identify the glutenin proteins of wheat varieties in many studies (Hoseney, 1969; MacRitchie, 1980; Preston and Tipples, 1980). Basic research is being carried out to develop prediction tests for wheat quality evaluation based on electrophoretic mobilities of glutenin subunits.

Glutenins are composed of polypeptides, cross-linked by disulphide bonds. These polypeptides can be divided in two groups according to their

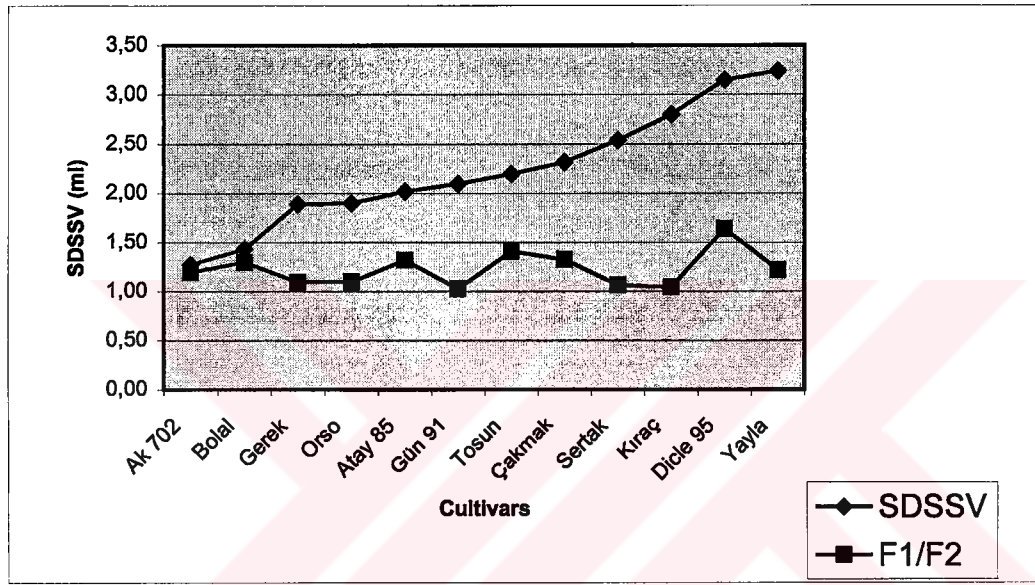


Figure 3.3. The comparison of SDSSV (Table 3.2) and F1/F2 ratio of HPLC analysis (Table 3.1).

molecular weight: low molecular glutenin subunits (LMW-GS) with molecular weights between 30-55 kD and high molecular weight glutenin subunits (HMW-GS) between 90-140 kD.

The subunit composition of 13 wheat genotypes were analyzed by SDS-PAGE after extracting the total seed proteins. The extraction procedure was designed to obtain sequentially the unreduced fractions and reduced fractions which were analyzed to obtain their subunit compositions separately. The banding nomenclature were based on the numbering of individual subunits within each glutenin group as shown schematically in Figure 3.4. The range of proteins were first separated according to their MW. The ranges were known to be approximately in the following MWs: A1 (High Molecular Weight Glutenin Subunits): >80 kD; A2 (ω -gliadins): 80-51 kD; A3 (Low Molecular Weight Glutenin Subunits): 51-40 kD; A4 (α -, β -, and γ -gliadins): 40-28 kD; A5 (Albumins, globulins): <28 kD.

SDS-PAGE band patterns of gluten proteins form soluble fractions is shown in Figure 3.5. The analysis of gels presented in Figure 3.5 was shown in Table 3.3. Each band in the Figure 3.4 were evaluated as present (+), absent (-) or strong expression (+++).

HMW Glutenin subunit group showed an intense band 2 for 'Çakmak' in the soluble fraction. There were three banding patterns. The first one was the absence of band 2 (two genotypes Gün and Ak). The second one was absence of

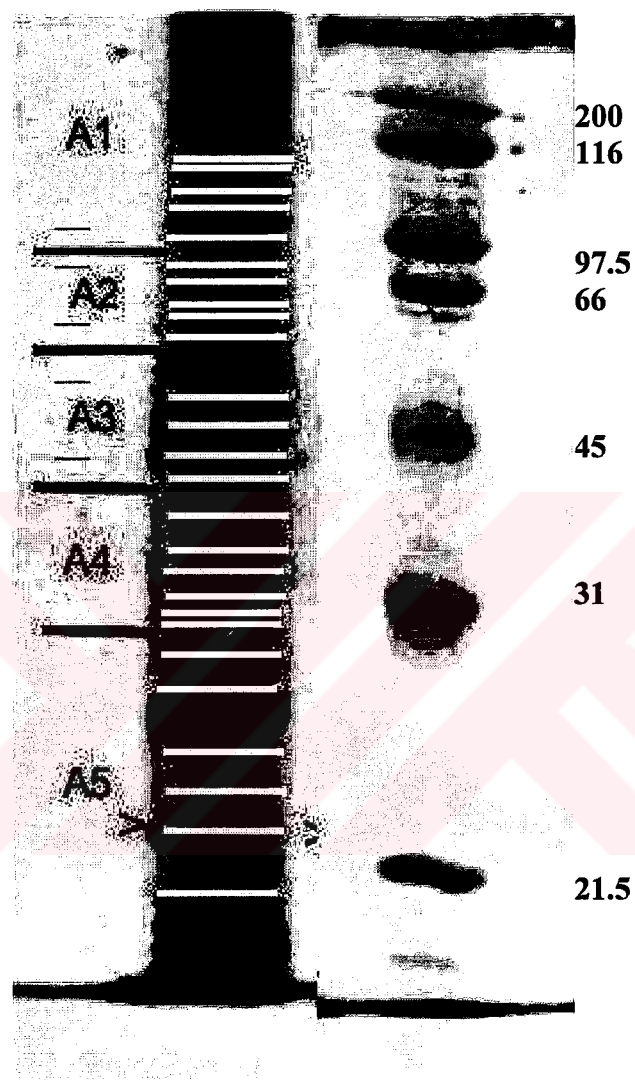


Figure 3.4. Schematic representation of glutenin subunit groups. Nineteen bands were represented within 5 groups (A1-A5).

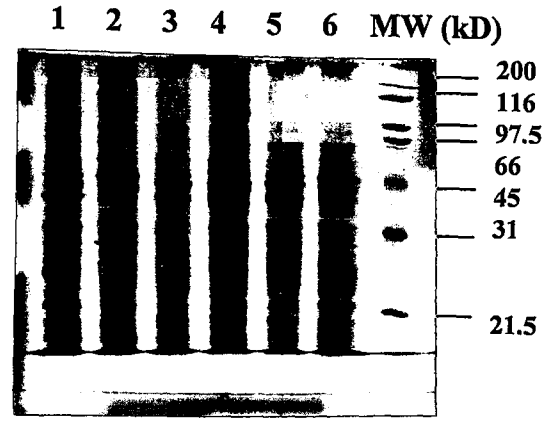


Figure 3.5. SDS-polyacrylamide-gel (10 %) electrophoresis of glutenin subunits of solouble fractions. The band numbering was as given in Figure 3.4. Lane 1: Yayla, 2: Sertak, 3: Gün, 4: Çakmak, 5: Bezostaja, 6: Ak, 7: Bolal, 8: Dicle, 9: Orso, 10: Atay, 11:, 12: Gerek , Tosun, 13: Kıraç.

Table 3.3. SDS-PAGE band patterns of gluten proteins from soluble fractions

(Figure 3.5). A (+) sign indicates the presence of the band. (+++) indicates the presence of a denser band, (-) sign indicates the absence of the band. The fractions are as indicated in Figure 3.4.

Fraction	Ak702	Atay	Bezost.	Bolal	Çakmak	Dicle	Gerek
A1 (HMWGS)							
Band1	+	+	+	+	+++	+	+
Band2	-	+	+	+	-	+	+
Band3	+	+	+	+	-	+	-
Band4	+	+	+	+	-	+	+
A2 (ω -gliadins)							
Band1	+	+	+	+	-	+	+
Band2	-	+	+	+	+++	+	+
Band3	+	-	+	-	+	-	+++
Band4	+	+	+	+	+	+	+
Band5	+	-	+	+	+	+	-
A3 (LMWGS)							
Band1	-	-	+	+	+	+	-
Band2	+	+	+	+	+	+	+
Band3	+	+++	+	+++	+	+	+++
Band4	+	+	+	+	+	+	+++
A4 (α,β,γ -gliadins)							
Band1	+	+	+	+	-	+	+
Band2	+	+	+	+	-	+	+
Band3	+	+	+	+	-	+	+
Band4	+	-	+	+	+++	+	-
Band5	+	+	+	+	+	+	+
Band6	+	+	+	+	+	+	+

Table 3.3. (Continued)

Fraction	Gün	Kıraç	Orso	Sertak	Tosun	Yayla
A1 (HMWGS)						
Band1	+	+	+	+	+	+
Band2	-	+	+	+	+	+
Band3	+	+	+	+	+	+
Band4	+	+	+	+	+	+
A2 (ω -gliadins)						
Band1	+	+	+	-	-	+
Band2	+	+	+	+	+	+
Band3	+	-	-	+	+	+
Band4	+	+	+	+	+	+++
Band5	+	+	+	+	+	+
A3 (LMWGS)						
Band1	+	+	+	+++	+	+++
Band2	+	+	+	+	+	+
Band3	+	+	-	+	-	+
Band4	+	+	+	+	+	+
A4 (α,β,γ -gliadins)						
Band1	+	+	-	+	+	+
Band2	+	-	+	+	+	+
Band3	+	+	+	+	+	+
Band4	-	+	+	+	+	+
Band5	+	+	+	+	+	+
Band6	+	+	+	+	+	+

band 2, 3, and 4 (Çakmak). The last pattern was presence of all the four bands (Atay, Bezostaja, Bolal, Dicle, Gerek, Kırış, Orso, Sertak, Tosun, Yayla). The band 1 was present in all the genotypes. Band 2 was present in all genotypes except Gün, Ak, and Çakmak. Band 3 was absent in Çakmak and Gerek. Band 4 was absent only in Çakmak.

The ω - gliadin group showed intense band 2 for 'Gün', 'Çakmak' and 'Sertak' in soluble fractions. There were seven banding patterns. The first one had all the five bands (two genotypes Gün and Bezostaja). The second one was absence of band 2, 3, and 4 (Çakmak). The last pattern was absence of band 3 (Atay, Bolal, Dicle, Kırış, Orso). The third pattern was absence of band 1 (Sertak and Tosun). Ak did not have band 2, Çakmak had band 1, Gerek band 5 and Yayla had an overexpressed band 1. The band 1 was present in all genotypes except Sertak, Tosun, and Çakmak. Band 2 was absent in only Ak. Band 3 was absent in Kırış, Orso, Atay, Bolal, and Dicle. Band 4 was present in all genotypes. Band 5 was absent in Atay and Gerek.

LMW glutenin subunits were found to be most variable as to accumulated subunits. Atay, Bolal, Gerek, Sertak and Yayla had denser bands in LMW region after electrophoresis of soluble fraction. LMW Glutenin subunit group showed intense band 2 for 'Sertak', 'Tosun' and 'Yayla'; band2 for 'Ak702' and 'Bezostaja'; band 3 for 'Bolal' and Gerek' in soluble fraction. There were six banding patterns. The first one had all the four bands (Gün, Kırış, Bezostaja, and Dicle). The second one was absence of band 3 (Orso and Tosun). The third pattern was

absence of overexpressed band 1 (Sertak and Yayla). Ak did not have band 1, Atay and Gerek band 1. The band 1 was present in all genotypes accept Ak, Atay and Gerek. Band 2 was present in all genotypes. Band 3 was absent in Orso and Tosun. Band 4 was present in all genotypes.

The α -, β -, γ -gliadins group showed intense band 4 for 'Çakmak', 'Sertak' and 'Yayla' in soluble fraction. There were five banding patterns. The first one did not have band 4 (Gün, Atay, and Gerek). The second one was presence of all bands (Sertak, Tosun, Yayla, Ak, Bezostaja, Dicle). Kırac did not have band 2, Orso band 1, Çakmak band 1, 2, and 3. The band 1 was present in all genotypes accept Orso and Çakmak. Band 2 was absent in Kırac and Çakmak. Band 3 was absent in Çakmak. Band 4 and band 5 was present in all genotypes.

SDS-PAGE band patterns of gluten proteins form insoluble fractions is shown in Figure 3.6. This fraction was prepared by including reducing agent DDT to the pellet. The analysis of gels presented in Figure 3.6 was shown in Table 3.4.

HMW Glutenin subunit group showed an overexpressed band1 in insoluble fraction of Çakmak.. There were four banding patterns. The first one was the absence of band 1 and 3 (Gün and Ak). The second one had all the genotypes (Kırac, Orso, Sertak, Yayla, Atay, Bezostaja, Bolal). Third one was absence of band 3 (Tosun, Dicle, Gerek). Çakmak did not have band 2, 3, and 4. The band 1 was present in all the genotypes accept Ak and Gün. Band 2 was

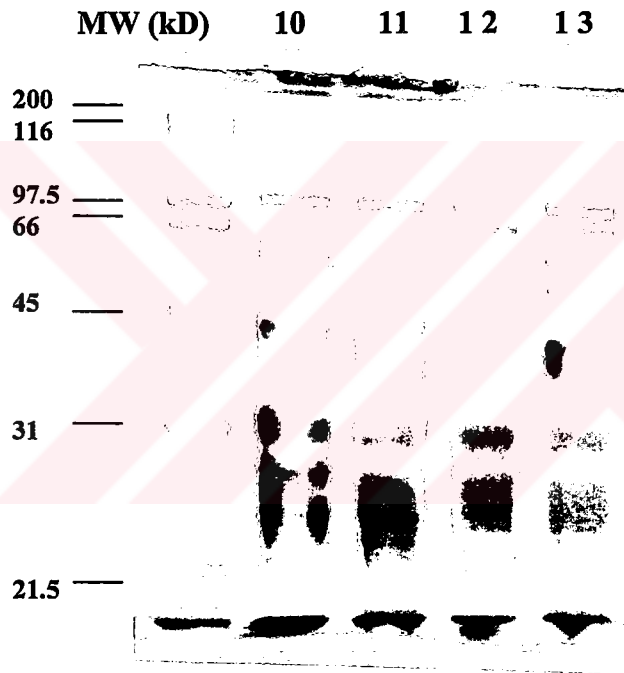
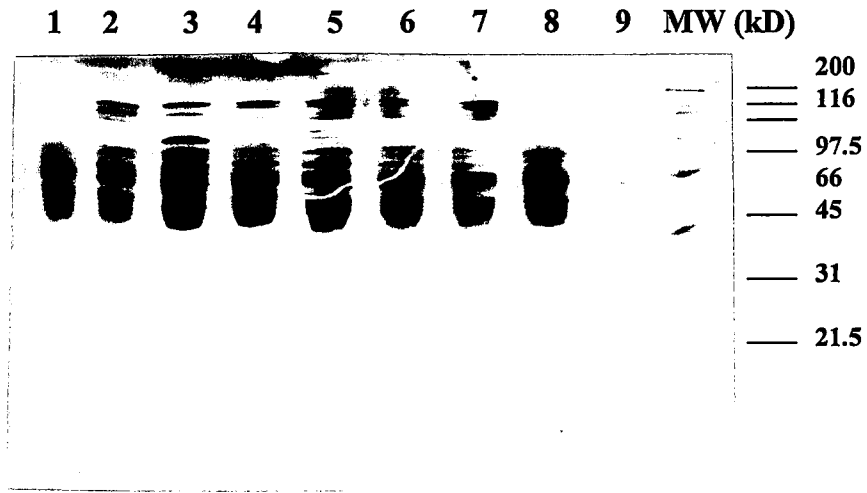


Figure 3.6. SDS-polyacrylamide-gel (10 %) electrophoresis of glutenin subunits of insoluble fraction. The band numbering was as given in Figure 3.4. Lane 1: Ak, 2: Gün, 3: Çakmak, 4: Sertak, 5: Yayla, 6: Tosun, 7: Bezostaja, 8: Atay, 9: Gerek, 10: Bolal, 11: Dicle, 12: Orso, 13: Kıraç.

Table 3.4. SDS-PAGE band patterns of gluten proteins from insoluble fractions

(Figure 3.6). A (+) sign indicates the presence of the band. (+++) indicates the presence of a denser band, (-) sign indicates the absence of the band. The fractions are as indicated in Figure 3.4.

Fraction	Ak702	Atay	Bezost.	Bolal	Çakmak	Dicle	Gerek
A1 (HMWGS)							
Band1	-	+	+	+	+++	+	+
Band2	+	+	+	+	-	+	+
Band3	-	+	+	+	-	-	-
Band4	+	+	+	+	-	+	+
A2 (ω -gliadins)							
Band1	-	+	+	+	+	+	+
Band2	-	-	+	+	+++	-	+
Band3	+	+	+	-	+	+	+
Band4	+	+	-	+	+	+	+
Band5	+	+	+	+	+	+	-
A3 (LMWGS)							
Band1	-	-	-	-	+++	-	-
Band2	-	+	+	+	+	-	+
Band3	+	+	+	+	+	+	+
Band4	+	+	+	+++	+	+	+
A4 (α,β,γ -gliadins)							
Band1	+	+	+	+	-	+	+
Band2	+	+	+	+	-	+	+
Band3	+	+	+	+	-	+	+
Band4	+	-	+	+	+++	+++	-
Band5	+	+	+	+	+	+	+
Band6	+	+	+	+	+	+	+

Table 3.4 (Continued)

Fraction	Gün	Kıraç	Orso	Sertak	Tosun	Yayla
A1 (HMWGS)						
Band1	-	+	+	+	+	+
Band2	+	+	+	+	+	+
Band3	-	+	+	+	-	+
Band4	+	+	+	+	+	+
A2 (ω -gliadins)						
Band1	+	+	+	-	-	+
Band2	-	+	+	+	+	+
Band3	+	-	-	+	+	+
Band4	+	+	+	+	+	+
Band5	+	+	+	+	+	+++
A3 (LMWGS)						
Band1	+	+	+	+++	+	+
Band2	+	+	+	+	+	+++
Band3	+	+	+	+	-	+
Band4	+	+	+++	+	+	+
A4 (α,β,γ -gliadins)						
Band1	+	+	+	+	+	+
Band2	+	-	+	+++	+++	+
Band3	+	+	+	+	+	+++
Band4	+	+	+	+	+	+
Band5	+	+++	+	+	+	+
Band6	+	+	+	+	+	+

present in all genotypes except Çakmak. Band 3 was absent in Gün, Tosun, Ak, Çakmak, Dicle, and Gerek. Band 4 was absent only in Çakmak.

ω - gliadin group showed overexpressed band 3 for 'Çakmak' in insoluble fraction. There were eight banding patterns. The first one was absence of band 2 (Gün, Atay, and Dicle). The second one was absence of band 3 (Kıraç, Orso, and Bolal). The third pattern was absence of band 1 (Sertak and Tosun). Ak did not have band 1 and 2, Gerek band 5, Bezostaja band 4. Yayla had an overexpressed band 5 and Çakmak band 2. The band 1 was absent in Ak, Sertak, and Tosun. Band 2 was present in all genotypes except Gün, Ak, Atay, and Dicle. Band 3 was absent in Kıraç, Orso, and Bolal. Band 4 was absent only in Bezostaja. Band 4 was present in all genotypes except Gerek.

LMW Glutenin subunit group showed overexpressed band 1 for 'Çakmak' and band 2 for 'Yayla' in insoluble fraction. Atay, Bolal, Gerek, Sertak and Yayla had denser bands in LMW region after electrophoresis of soluble fraction. LMW Glutenin subunit group showed intense band 2 for 'Sertak', 'Tosun' and 'Yayla'; band 2 for 'Ak702' and 'Bezostaja'; band 3 for 'Bolal' and Gerek' in soluble fraction. There were seven banding patterns. The first one had all the four bands (Gün and Kıraç). The second one was absence of band 1 (Sertak and Çakmak). Tosun did not have band 3, Ak and Dicle band 1 and 2. Orso had overexpression of band 4, Yayla band 2. The band 1 was absent in Ak, Atay, Bezostaja, Bolal, Dicle and Gerek. Band 2 was present in all genotypes except Ak and Dicle. Band 3 was absent in Tosun. Band 4 was present in all genotypes.

The α -, β -, γ -gliadins group showed overexpressed band 4 for 'Çakmak', 'Dicle' and 'Yayla'; band 5 for 'Kıraç' in insoluble fraction. There were six banding patterns. The first one had all the bands (Gün, Orso, Ak, Bezostaja, and Bolal). The second one was overexpression of band 5 (Sertak and Tosun). Kıraç did not have band 2. Sertak and Tosun had overexpressed band 2. Çakmak and Dicle had overexpressed band 4. The band 1 and band 3 was present in all genotypes except Çakmak. Band 2 was absent in Kıraç and Çakmak. Band 5 and band 6 was present in all genotypes.

3.5 DNA Polymorphism in Gluten Genes

The polymorphism of gluten proteins were also studies at the genomic level by using PCR based methods. Six hexaploid wheat genotypes were used in the analysis. In PCR analysis of genomic DNA, Campenhout *et. al.* (1989) constructed three sets of sequence specific primers based on published LMW glutenin gene sequences of hexaploid wheat. In this study, the set O were used to scan the six Turkish hexaploid wheat genotypes to determine polymorphism. The primers were designed within the coding region such that their PCR products should contain the whole repeat domain and a large part of C-domain. For this set, the predicted length of the reaction product was 695 bp based on a 'Cheyenne' cultivar (Okita *et. al.* 1985). A band of this size was the most prominent band and present in all cultivars. The primer set O as described in Okita (1985) (O15:TTTGCCCTCATCGCCGTTGT and O13:TGCTGTTGAGGTTGTTGGAAAGA, on Glu-A3) resulted in a single band

corresponding to 695 bp for all the genotypes as shown in Figure 3.7. Another primer set P (P15: GCGCAAATTTACAGCAACA, P13: TGGCACACTAGTGGTGGTTTC, on Glu-A3 location) were used and there were no polymorphism either (Figure 3.8).

The primers for complete *Ay* gene produced a different amplification pattern between the 6 wheat cultivars (Figure 3.9). The primers were designed from published sequences to obtain the complete sequence of *Ay* gene. This gene is located on A genome and part of HMW glutenin subunit location. The amplified fragment was approximately 1850 bp. for Gün95, Gerek and Tosun. 'Bolal' and 'Yayla' gave slightly higher molecular weight bands with approximately 1900 bp.

3.6 Construction of the Expression Vector and Transformation into *Pichia pastoris*

The *Ay* gene sequence first digested out from pGEM-T chimeric vector with XhoI and EcoRI and ligated into *Pichia* transformation vector pPIC9 between sites Xho I and Eco R1 (Figure 3.10). In the resulting vector, the gene was under the control of *AOX1* (Alcohol Oxidase I) promoter which is induced by methanol. The recombinant construct and the parent vector (control) were digested with SalI prior to transformation of the strain GS115 to obtain recombinant clones with different phenotypes concerning methanol utilization.

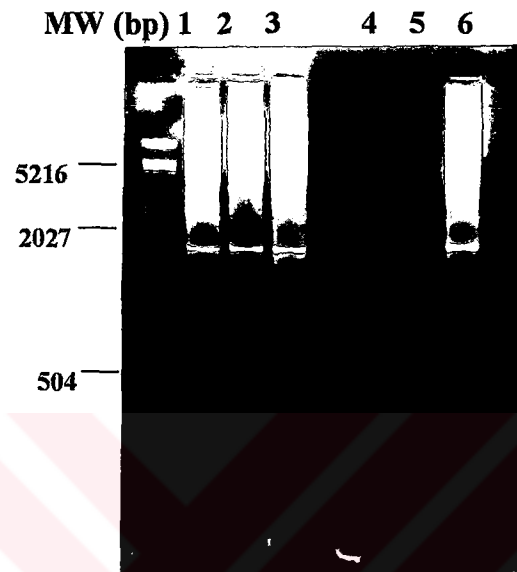


Figure 3.7. Agarose gel (0.8 %) of PCR amplified fragments of 6 genotypes using primer set O13-O15. Okita (1985) reported that this set produced a 695 bp fragment in hexaploid genomes corresponding to part of LMW glutenin subunit. The genotypes were Yayla (1), Bolal (2), Gerek (3), Tosun (4), Bezostaja (5) and Gün95 (6).

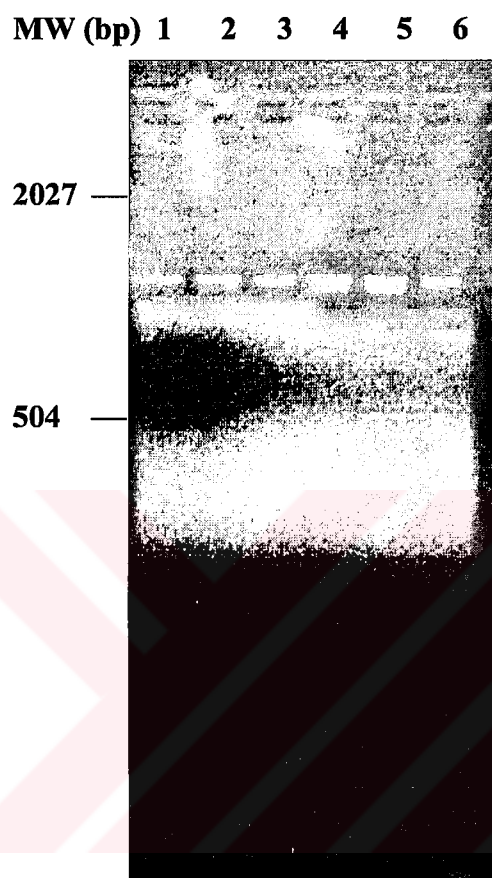


Figure 3.8. Agarose gel (0.8 %) of PCR amplified fragments of 6 genotypes using primer set P. This set produced a 984 bp fragment in hexaploid genomes corresponding to part of LMW glutenin subunit. The genotypes were Yayla (1), Bolal(2), Gerek (3), Tosun (4), Bezostaja (5) and Gün95 (6).



Figure 3.9. Agarose gel (0.8 %) of PCR amplified fragments of 6 genotypes using primer set CEN1-CEN2. Forde (1985) reported that this set produced a 1864 bp fragment in hexaploid genomes corresponding to complete fragment of Ay gene of HMW glutenin subunit. The genotypes were Bolal (1), Gerek (2), Tosun (3) , Gün95 (4), Yayla (5) and Bezostaja (6).

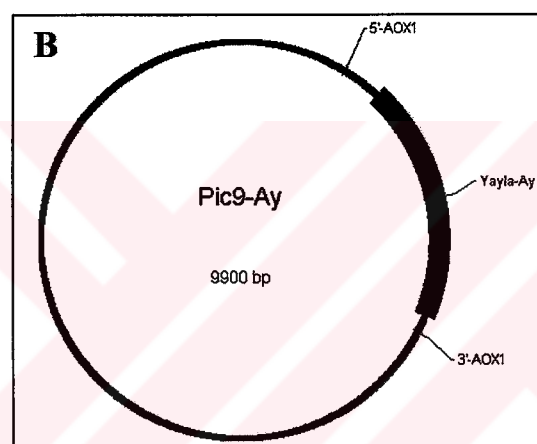
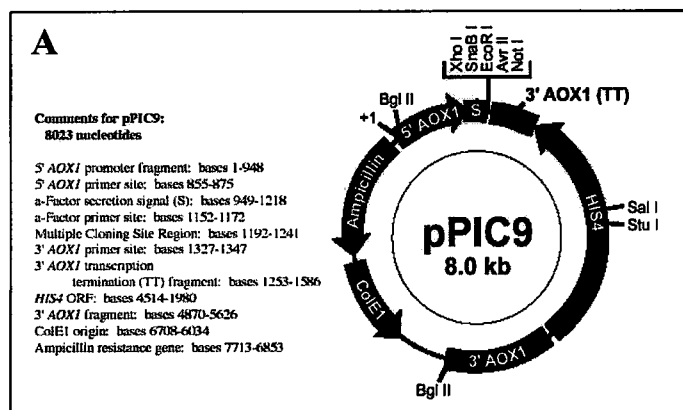


Figure 3.10. A) The map of pPIC9 vector (Invitrogen *Pichia* Expression Manual (J)) **B)** Expression plasmid constructed for heterologous expression of the High Molecular Weight Glutenin Subunit Ay in *P. pastoris*. 5'AOX1 promoter of *P. pastoris* alcohol oxydase 1 gene, Ay, PCR amplified Ay gene from 'Yayla' genome; TT, 3'AOX1 transcription termination fragment; HIS4, open reading frame of the HIS4 gene; 3' fragment of the AOX1 gene; SalI, BglII, positions of the restriction enzymes, which were used for homologous recombination of the construct into the genome of *P. pastoris*.

The Sal I digestion was preferred since His⁺-Mut⁺ recombinants were aimed in GS115 strain. The construct were linearized with SalI to favor His⁺-Mut⁺ recombinants in GS115. As a control, parent plasmid pPIC9 was linearized with the same enzymes. This control plasmids with no insert was used to check for background expression. Transformation of the *P. pastoris* GS115 strain with the linearized vectors was carried out by spheroplast method. The transformed cells were plated with top agarose to protect them from lysis prior to selection. After 3 days first His⁺ transformants appeared. Transformation efficiency was 10⁴ His⁺ transformants for µg DNA (Figure 3.11). The transformants were screened for histidinol dehydrogenase (His⁺) phototrophy by plating on a dextrose-based medium without histidine supplementation. The growth of spheroplasted cells on RDHB plates showed that the removal of cell wall did not reduce the viability of cells (Figure 3.11-A). The control plate were streaked with everything except yeast cells and there was no growth (Figure 3.11-B). The RDB plates for pPIC9-*Ay* plasmid transformed (Figure 3.11-C) and parent pPIC9 (Figure 3.11-D) showed colonies indicating successful transformations.

The colonies from His⁺ GS115 transformants were screened for Mut⁺ or Mut^s phenotype on MM plates. Nearly 20 % of the His⁺ clones from the transformation showed reduced growth on methanol as the sole carbon source (slow growing Mut) indicating the integration of the expression cassette into the *AOX1* gene (Figure 3.12).

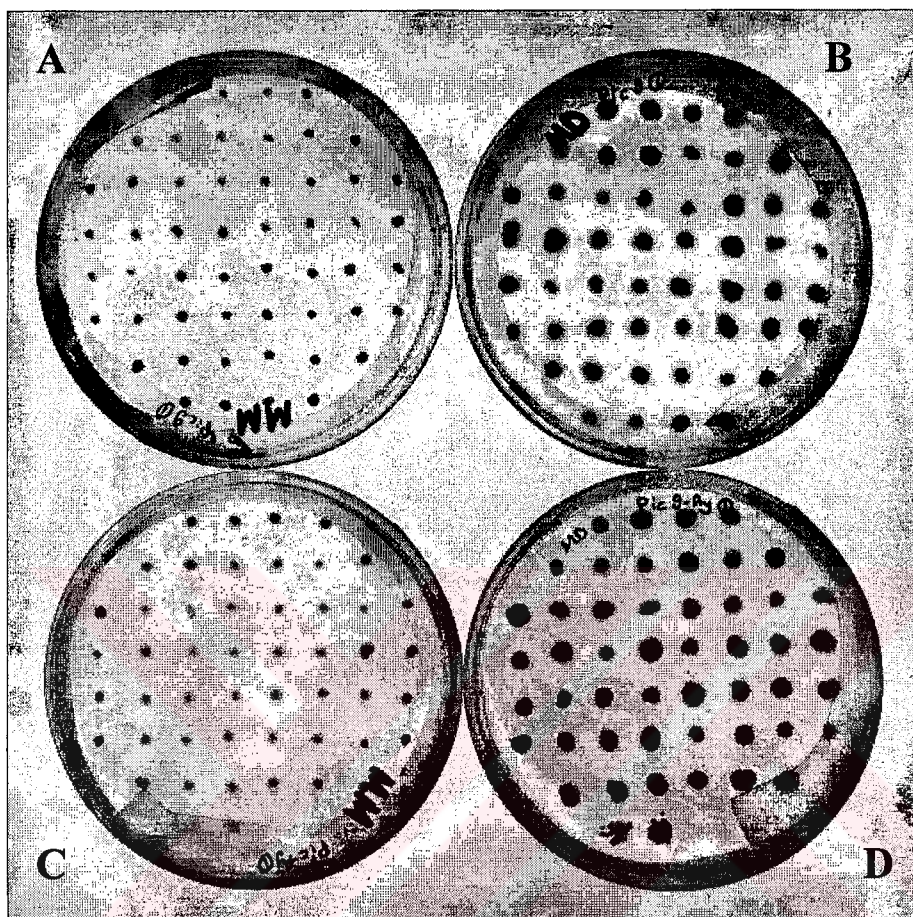


Figure 3.12. Mut selection of transformed colonies. The colonies were plated on MM and MD medium. Upper plates (A, B) are GS115 Albumin and lower plates (C, D) are the pPIC9-Ay construct transformed cells (Plate D of Figure 3.11).

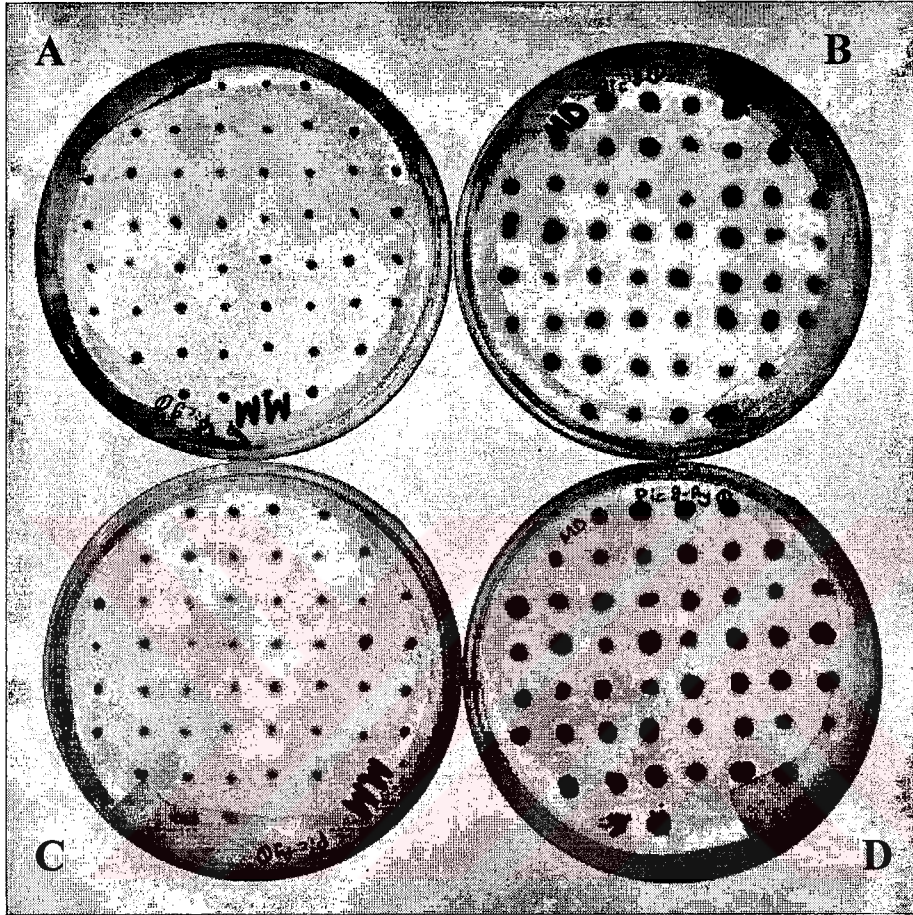


Figure 3.12. Mut selection of transformed colonies. The colonies were plated on MM and MD medium. Upper plates (A, B) are GS115 Albumin and lower plates (C, D) are the pPIC9-Ay construct transformed cells (Plate D of Figure 3.11).

The transformants were isolated into liquid cultures of 2 ml and they were replica plated for later uses. Control transformants were also isolated for checking background expression. The isolates grown for overnight were confirmed for the transformation with the *Ay* gene by PCR amplification on the DNA samples isolated from transformed yeast cells (Figure 3.11, lower-right plate). Amplification of the *Ay* gene was carried out either with the 5'-*AOX1* primer paired with 3'-*AOX1* primer. The sequences and amplification regions of primer sets were shown in Figure 3.13. Mut⁺ integrants must produce two bands. One corresponds to the size of *Ay* gene (1.85 kb), the other to the *AOX1* gene (approximately 2.2 kb). If Muts integrants were screened, only the band corresponding to *Ay* gene will be observed. Parent plasmid PIC9 will produce 492 bp for 5'-*AOX1* primer and 195 bp for α -factor primer. Appearance of ghost bands was reported to be insignificant.

The verification of the introduction of *Ay* DNA fragment into the genome of the strain GS115 was demonstrated by PCR from genomic DNA of the clones with *Ay* specific primer pair which was amplifying 1.2 kb fragment of the gene.

3.7 Expression of Yayla-*Ay* Gene in *Pichia pastoris*

The colonies of GS115 strain transformed by pPIC9-*Ay* construct were induced with methanol to secrete the *Ay* gene product, and detected in the medium by SDS-PAGE. The secreted protein was approximately 66 kD which corresponds to a slightly higher molecular weight polypeptide than the expressed *Ay* gene

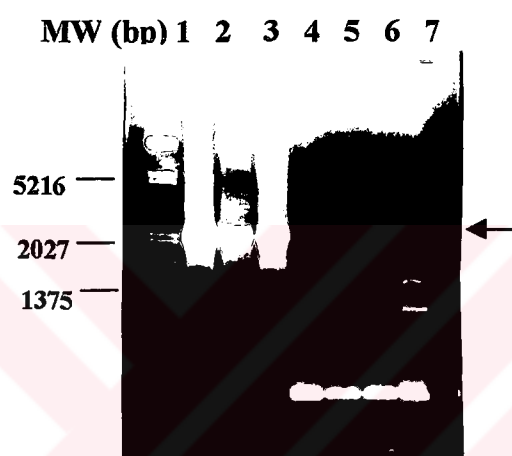


Figure 3.13. Confirmation of integration of Ay in p>PIC9 plasmid Agarose gel (0.8 %) of PCR amplified fragments of genomic DNA of transformed *Pichia* cells. Amplification were carried out with primers set 5'-AOX1 and 3'-AOX1. Lanes are 7 different colonies from the lower-right plate of Figure 3.11. The arrow indicates the amplified fragments containing Ay gene.

Products in literature (D'Ovidio, 1996). The protein amount in supernatant was determined by Bradford protein determination method and presented in Figure 3.14. The amount of protein in the medium increased over 1.2 mg/ml up to 80 hours of expression and then, start to decrease to 0.6 mg/ml after 196 hours. Time course analysis of the same aliquots with SDS-PAGE was presented in Figure 3.15. The secretion of *Ay* gene product increased in density up to 60 hours and start to decrease after 75 hours.

The protein products were also analyzed with RP-HPLC as shown in Figure 3.16. The *Ay* gene product was observed in 31.8 min retention time. The peak at this time was in higher amount compared to the other peaks.

3.8 Overexpression and Isolation of *Ay* protein

The *Ay* product were expressed in shake-flask for 72 hours and the protein content of the medium were isolated by TCA precipitation. The precipitated content was analyzed by SDS-PAGE as shown in Figure 3.17. The comparison of the secreted proteins from *Ay*-transformants and parent plasmid transformants showed that there was a strong band at 66 kD only in the aliquots of *Ay* transformed cultures (Lane 1 and Lane 2 in Figure 3.17). The background polypeptides exist, but in lower quantity compared to the band at 66 kD. The comparison of the strong band with the molecular weight of *Ay* protein as secreted from *E. coli* revealed that the Yayla-*Ay* protein had a higher molecular weight (Lane 1 and Lane 3 in Figure 3.17). The protein products were also

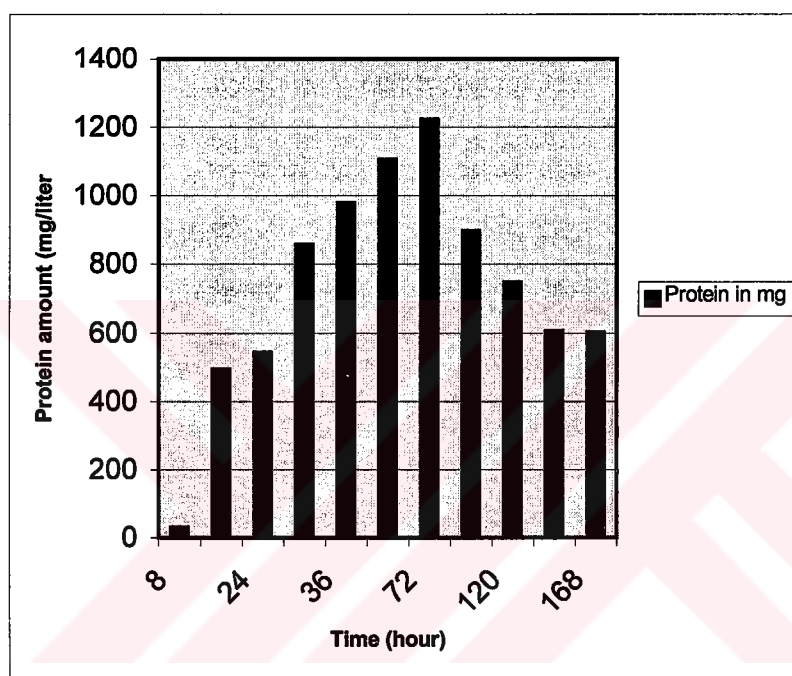


Figure 3.14. Time course analysis of secretory proteins during expression of transformed *P. pastoris*. Protein amount was determined by Bradford Protein Assay.

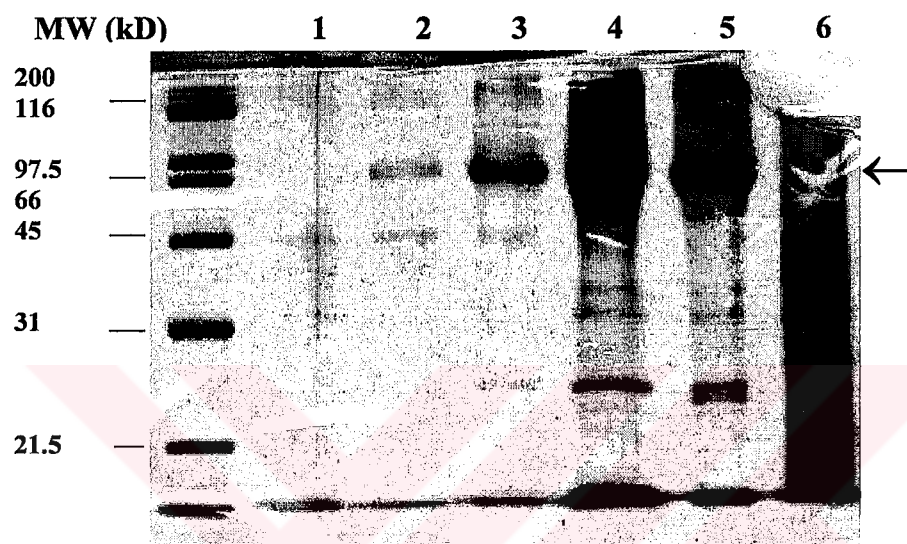


Figure 3.15. SDS-PAGE analysis of culture supernatants. Lane 1 is the aliquote from control culture after 3 days. Lane 2 is the aliquote from transformant culture after 12 hours, Lane 3 is after 24 hours, Lane 4 is after 72 hours, Lane 5 is after 96 hours, Lane 6 is the Ay protein as expressed in *E. coli* (Obtained from Lafiandra, University of Tuscia). The arrow indicates the location of Ay protein (66 kD).

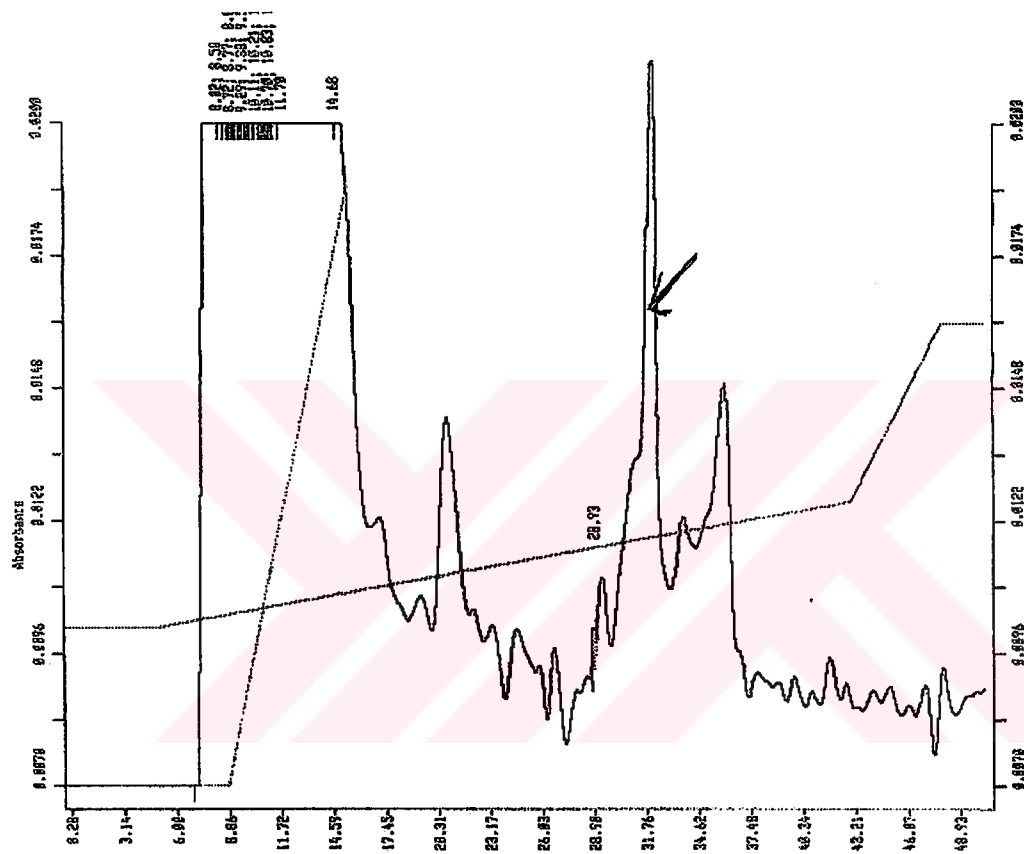


Figure 3.16. RP-HPLC of expression culture aliquote. The arrow indicates the Ay protein at 31.8 min elution as shown by an arrow.

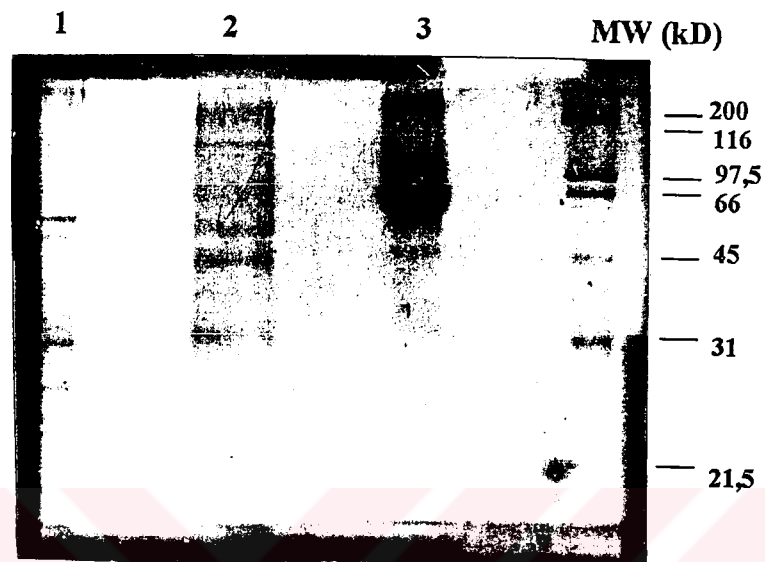


Figure 3.17. SDS-PAGE (11 %) of aliquots of culture medium 3 days after induction of expression of *Ay* gene in *Pichia pastoris*. Lane 1 is the purified *Ay* protein from expression in *E. coli* as supplied by Lafiandra, University of Tuscia., lane 2 is the parallel experiment with pPIC9 transformed cells, lane 3 is the precipitated sample of 1 ml 3-days grown culture of transformed cells.

transferred to PVDF membrane by electroelution and sequenced by N-terminal analysis.

3.9 N-Terminal Amino Acid Sequences

The N-terminal amino acid sequence of subunit *Ay* (16 residues) was identical to that of subunit from Lira (Figure 3.18). The durum cultivar Lira (*Triticum durum*) was previously used to clone *Ay* gene and to transform it into *E. coli*. by Buonocore (1996).

Lira	E-G-E-A-S-G-Q-C-E-R-Q-L-L-K-R-Q-L-E-A-Y-Q-Q-L-Q-D-V-S
Yayla	E-G-E-A-S-G-Q-C-E-R-Q-L-L-K-R-Q

Figure 3.18. Comparison of N-terminal amino acid sequences of *Ay* gene product from 'Lira' (Buonocore *et. al.* 1996) and 'Yayla'.

3.10 Effect of *Ay* Protein on Loaf Volume

The contribution of Yayla-*Ay* protein on loaf volume was determined by the microsedimentation test as described in section 3.2. The isolated *Ay* protein of 0.1 g were mixed with 0.6 g of flour (1/7 w/w ratio) from each genotype. The mixture were tested with the procedure to obtain its loaf volume and compared to

the value obtained from flour without *Ay* protein addition (Table 3.5). The replication were run by isolated *Ay* protein

Table 3.5. SDS-sedimentation volumes (ml) as determined by microsedimentation test. The values were the mean values of four replicates.

Genotypes	SDS Sedimentation Volume	SDS Sedimentation Volume (Protein extract from control added)	SDS Sedimentation Volume (<i>Ay</i> protein added)
Yayla	3.24±0.072	3.2±0.153	3.65±0.162
Dicle 95	3.15±0.136	3.20±0.327	3.12±0.78
Kıraç	2.8±0.041	2.950.1183	3.12±0.137
Sertak	2.54±0.054	2.53±0.178	2.45±0.194
Çakmak	2.32±0.270	2.32±0.235	2.34±0.102
Ak 702	1.27±0.148	1.26±0.472	1.39±0.132
Tosun	2.20±0.032	2.21±0.107	2.31±0.242
Bezostaja	2.12±0.050	2.15±0.176	2.31±0.231
Gün 91	2.1±0.040	2.13±0.231	2.22±0.102
Orso	1.9±0.043	1.88±0.194	2.38±0.98
Gerek	1.89±0.066	1.84±0.239	2.12±0.127
Atay 85	2.02±0.128	1.97±0.203	2.230±0.111
Bolal	1.43±0.078	1.53±0.182	1.56±0.128

Standard Error of LSD analysis: 0.201

from 4 different expression culture. Another set of experiment was designed to observe any contribution from the background proteins expressed during fermentation. *Ay* protein addition showed variable increases in the loaf volumes of corresponding flour on all genotypes. The loaf volume of Orso (from 1.9 to 2.38), Kırac, (from 2.8 to 3.12), Yayla (from 3.24 to 3.65), Gerek (from 1.89 to 2.12) increased significantly upon addition of *Ay* protein. The LSD analysis showed that SDS Sedimentation volume increases by *Ay* protein addition was statistically significant for 'Yayla' ($p=0.038$).



CHAPTER 4

DISCUSSION

In the studies presented here, polymorphism of gluten proteins were investigated for 13 Turkish wheat genotypes. The genotype 'Yayla' was found to be polymorphic in the 1*Ay* locus fragment length compared to previous genotypes reported. This new allele of *Ay* protein was decided to be cloned by PCR amplification. The cloned fragment was expressed in a heterologous system and the product was characterized by SDS-PAGE and RP-HPLC. The isolated protein was further characterized by N-terminal amino acid sequencing. The influence of the Yayla-*Ay* protein on the loaf volume of flour was evaluated by a SDS sedimentation test.

4.1 Gluten Polymorphism at Protein and Genomic Level

The polymorphism of gluten proteins are useful in determining the quality of flour. Moreover, the variability in the glutenin protein composition can be used in improvement of flour quality of genotypes. The standard methods used for determination of polymorphism of gluten proteins are SE-HPLC and SDS-PAGE. Recently PCR based methods are considered as a more precise method for the same purpose. The analysis of 13 Turkish wheat genotypes with SE-HPLC, SDS-

PAGE and PCR amplification methods revealed a variety of polymorphism for the composition of gluten subunits.

4.1.1 HPLC Analysis of Gluten Proteins

SE-HPLC analysis of wheat seed proteins was performed by a TSK 4000-SW column which is a kind of column suitable for the fractionation of relatively large protein aggregates. It separates proteins in a large MW range (10 kD to 1000 kD). The application of SE-HPLC on selected flour samples revealed a diverse polymorphism in gluten protein subunits among genotypes. The F1/F2 ratio was highly polymorphic.

Previous studies showed clearly that the HPLC profile can be divided into three main fractions that are important in predicting the baking qualities as shown in the Figure 3.1. The F1 fraction elutes polypeptides with molecular weight more than 100 kD. These fraction represents the polymeric proteins. Polymeric fraction of gluten consist of a network of LMW and HMW glutenin subunits. The molecular weights of polymeric proteins are variable, however they can reach as high as one thousand kD.

To confirm the ability of HPLC column to separate predicted fractions, the elutions from each fraction were collected and prepared for SDS-PAGE analysis. The proteins in elutes were first precipitated by TCA and then dissociated into polypeptides by solubilizing in sample buffer containing reducing agent DDT and

by heating. This procedure was expected to give a variety of polypeptides ranging from 30 kD (from LMW Glutenin subunits) to as high as 80 kD (from HMW Glutenin subunits). This was shown to be true for the sample separated in the Figure 3.2. F2 fraction contains gliadins with molecular weight between 30 to 80 kD. The lane 2 in Figure 3.2 is the polypeptides forming this fraction and they are observed to be 20-50 kD. F3 fraction represents albumin/globulin proteins with molecular weight less than 30 kD. Lane 3 in figure 2.3 resolved polypeptides between 10-30 kD. The separation of HPLC fractions in SDS-PAGE showed that the column TSK 4000-SW separated the gluten proteins exactly as described in literature (MacRitchie, 1998).

Studies show that MW distribution of protein aggregates is a good predictor of baking qualities. The percentage of F1 peak and, especially F1/F2 ratio is highly variety dependent (MacRitchie *et. al.* 1990). The F3 peak is likely to consist of monomeric gliadin-like proteins and is known to be strongly influenced by the environmental effects and is a poor indicator of baking quality. F1/F2 ratio must be considered as a relative index. Environmental conditions result in the different molecular weight distributions (MacRitchie, 1998). The SE-HPLC analysis resulted in variable fractions among 13 cultivars studied. The analysis of fractions were tabulated in Table 3.1. The table also contained quality values for 8 of the genotypes used in this study as described by Kün (1988). These values categorize the flour into three groups according to their structural and functional characteristics. A value of 3 indicates a flour with high commercial

value and a value of 1 with low commercial value. F1 fractions were variable from 8.14 to 11.77 and correlated with quality values with a coefficient of -0.36.

The statistical analysis confirmed the significance of the differences of fractions among genotypes. Moreover, there was apparent correlation between quality values as given by Kün (1988) and F1 fraction or F1/F2 ratio values.

4.1.2 SDS Sedimentation Volume Determination

The loaf volume is a predictor test for the breadmaking quality of flour. The higher loaf volume is desirable since it gives a higher quality end-product. SDS Sedimentation test was used to evaluate the loaf volume potential of the flour of genotypes used in the HPLC studies. The method selected was a modified procedure of SDS sedimentation test. It is called the microsedimentation gel test of Sapirstein (1999). This method was preferred since 0.7 g of sample for analysis. Small amount of sample made the evaluation of *Ay* product in a heterologous system possible since the amount of protein obtained in such systems are always low compared to the amount required in the standard sedimentation tests.

Standard methods for rheological study of doughs give valuable information on the processing properties of wheat. Farinograph is used to determine the water absorption capacity of flour and the mixing behavior of dough. The extensograph and alveograph are used to determine strength and

extensibility of a dough at a constant kneading time. The mixograph was designed to test wheat dough strength, a particularly important determinant of breadmaking quality.

Eventhough there are many techniques for determination of dough values, the SDS Sedimentation volume was selected in this study since it was quick, simple to apply and reliable. The parameter were repeatedly shown to be correlated with the viscoelastic parameters such as farinograph, or mixograph results for a range of genotypes. It has been used in quality development programs.

4.1.3 Electrophoretic Analysis of Gluten Proteins

Electrophoretic patterns can separate subunit polypeptides and useful in fingerprinting of genotypes. SDS-PAGE was carried out following modified procedures of Laemmli (1970) as described by Khan *et. al.* (1989). This procedure revealed allelic differences in many subunits as shown in Figures 3.5 and 3.6. The bands were analyzed to study totally 19 polypeptides. The samples were sequentially extracted to obtain an unreduced and a reduced preparation for the electrophoretic analysis. These subunits were divided into 5 groups. HMW Glutenin subunits have molecular weight above 80 kD and four bands were evaluated in this region. The unreduced samples produced three different patterns in this group. Atay and Gün were distinguished by the absence of band two from other genotypes. Gerek had the absence of band three and Çakmak had the only

overexpressed band in this region. The rest of genotypes had all the subunits. ω -Gliadins gave a more variable subunit patterns compared to HMW glutenin subunit region. The molecular weight range for this group is between 51-80 kD. The unreduced fraction produced 9 different patterns. Atay, Bolal and Dicle had the same pattern by the absence of band three. Bezostaja and Gün had the same pattern by the presence of all the five bands. LMW Glutenin subunits with molecular weight 40-51 kD produced high polymorphism in the unreduced and reduced samples. Unreduced samples gave 7 band patterns. Bezostaja, Çakmak, Dicle, Gün and Kırac had the same pattern by having all the bands. α -, β -, δ -gliadin region is from 28 kD to 40 kD. Unreduced samples produced 5 different banding pattern. These banding patterns show that the SDS-PAGE analysis as applied in this study produced enough polymorphism only in the ω -gliadins and LMW glutenin regions to distinguish between the genotypes. Unreduced and reduced samples gave similar banding patterns.

4.1.4 DNA Polymorphism Analysis

The polymorphism of gluten proteins at the genomic level was investigated for three glutenin locus. The LMW glutenin subunits and HMW glutenin subunits were found to be nonpolymorphic for the 13 genotypes. The primer sets developed by Okita for LMW subunits were reported to give extensive variation among hexaploid varieties (Compenhout, 1995). This was attempted to be used for the detection of gluten subunit variance among Turkish wheat cultivars. The primer set that reported to be most polymorphic did not give any

variance among the six cultivars applied. The amplification with this primers produced a single 695 bp band for all the six cultivars studied. Similarly, another primer sets for LMW subunits did not produced any polymorphic bands. The third primer set for amplifying the whole HMW *Ay* gene were detected to be polymorphic (Figure 3.9). The *Ay* gene were reported to be a 1850 bp for Lira (D'Ovidio, 1989). The four cultivars resulted in the same molecular weight bands. On the other hand, Yayla and Bolal resulted in a different allelic form of the gene with a molecular weight approximately 1900 bp.

The use of PCR has made it possible to integrate information obtained with classical biochemical techniques. Determination of allelic forms by PCR amplification helped identification of cultivars with different qualitative characteristics. There are examples to distinguish between genotypes from their polymorphism in glutenin genes. Durum wheat cultivars with good and poor technological properties were distinguished by selective amplification of γ -gliadin genes or LMW Glutenin subunit genes (D'ovidio, 1990, 1993). Similar procedures were developed that make it possible to discriminate cultivars possessing subunit 5 from those possessing subunit 2 (D'Ovidio, 1994). Gene cloning and sequencing of glutenin subunits made it possible to detect discrepancies between subunits.

The current studies reports many polymorphic primer sets amplifying glutenin regions (D'Ovidio, 1996). The complete sequencing of glutenin genes results in application of many different markers with the new approaches. To further characterize the genetic forms of Turkish wheat cultivars, the number of

primers and genotypes must be increased in similar studies. This study continued in the following sections to clone the allelic form of *Ay* gene and express it in a heterologous system to evaluate its effect on breadmaking quality.

The polymorphism between genotypes should be further checked by using more genotypes and primer sets. The first phase of this study would be a complete screen of the primers previously developed and used in the hexaploid genomes. Later, the development of new primers can be studied. The identification of polymorphism by PCR amplification must be expected to be rewarding since it is more precise than the identification of polymorphism by biochemical methods.

4.2 Cloning and Expression Studies Concerning *Ay* gene

The amplified fragments by *Ay* locus primers contained a new allele. Bolal and Yayla genotypes produced a slightly higher molecular weight *Ay* gene fragment with an apparent molecular weight of 1.9 kb. The previously reported *Ay* gene fragments from hexaploid and tetraploid genotypes was around 1.8 kb (D'Ovidio, 1993). Thus, this new fragment were cloned from 'Yayla'. The *Ay* gene is an inactive gene in hexaploid genotypes. After characterization of the fragment, the cloned sequence was expressed in *Pichia* expression system for determining the properties of *Ay* product.

4.2.1 PCR Cloning of *Ay* Fragment from ‘Yayla’

The *Ay* gene sequence was already established by Forde *et. al.* (1985). The *Ay* gene product was never expressed in hexaploid wheat and the behavior of this subunit in the flour would be valuable as a new polymorphism increasing the quality parameters of flour. Therefore, the published sequence was used to clone the complete sequence from ‘Yayla’ aiming the heterologous expression of the protein. The ‘Yayla’ genotype was selected for cloning since it gave a slightly different molecular weight fragment after PCR amplification compared to previously cloned *Ay* gene parent cultivar ‘Lira’. Furthermore, ‘Lira’ was tetraploid and it is a possibility to have a different *Ay* gene by cloning it from a hexaploid cultivar, ‘Yayla’. The cloning strategy taken in this study was a direct ligation of PCR amplified fragment into a pGEMT vector. This vector uses the free Thymidine chain left after PCR reaction to insert in the multiple cloning site in a single step. The recombinant pGEMT-Yayla-*Ay* plasmid was transformed into *E. coli* for amplification and further use. The coding region of this construct was approximately 1850 bp and produced a polypeptide of 65.4 kD as determined by SDS-PAGE (Forde *et. al.* 1985).

4.2.2 Construction of Expression Vector

The Yayla-*Ay* fragment was isolated from pGEMT construct and inserted in the *Pichia* expression vector pPIC9. *Pichia* expression system presents four choices as expression vector. There were two vector for the intracellular

expression (pPIC3.5 and pHIL-D2) and two for section (pPIC9 and pHIL-S1). The pPIC9 vector was selected for this study since the multiple cloning site was appropriate for *Ay* gene fragment. The secretion strategy was a better choice because it eliminates the purification steps simpler compared to intracellular strategy.

The pPIC9-*Ay* construct contained the entire coding region of Glu-1y gene of A genome. Since one the cloning sites in pPIC9 was XhoI, the secretion sequence after XhoI site needed to be regenerated in the primers. The primer set for amplifying complete *Ay* region from Yayla were designed to have XhoI cut site in the 5'-end and EcoRI at the 3' end. The primers were highly specific with 37 and 33 bp length. The fragment obtained from Yayla was 1900 bp as observed in 1 % agarose gel electrophoresis (Figure 3.9). The vector is constructed so that the recombinant gene of High Molecular Weight Glutenin Subunit *Ay* of 'Yayla' is downstream from the *AOX1* promoter.

The α -factor secretion signal as supplied on the pPIC9 vector was decided to be used instead of native secretion signal of *Ay* gene. Therefore, 18 bp at the 3'-end of primer was complementary to the *Ay* gene after secretion signal cut site. The use of Xho I site removes the KEX2 site which is necessary for the proper removal of the α -factor secretion signal peptide during secretion. Thus, the cut site was regenerated in the 5'-primer. This was the region between XhoI and SnaBI corresponding to (Glu-Lys-Arg) and (Glu-Ala) repeat. The (Glu-Ala) repeat are removed by Ste13 after the cut by KEX2 enzyme. The sequence of *Ay*

gene after signal cut site is (Glu-Gly-Ala-Glu-Ala). The first four amino acid sequence resembles the (Glu-Ala) repeat except a glycine. This sequence would be enough for the recognition of KEX2, but at the same time not functional for Ste13. Therefore, the fragment amplified would produce a *Ay* fragment free of artificial amino acids added. The *Ay* protein expressed in *E. coli* (Buoconore, 1996) was containing an extra Methionine before the first Glutamine.

4.2.3 Expression of 'Yayla-Ay' in *Pichia*

The functional components of the *Pichia pastoris* yeast expression system are the transfer vector and the host. The *his4* host strain GS115 can metabolize methanol as its sole carbon source by the action of the gene product, alcohol oxidase I (*AOX1*). Since the plasmid to be integrated into yeast genome were linearized with Sal I restriction enzyme, it is expected to have His⁺-Mut⁺ recombinants. Selection on RDB plates confirmed His⁺ phenotype and selection on MM plates confirmed Mut⁺ phenotype. Recombination of the transfer vector into the yeast genome allows for auxotrophic selection as the transformed yeast clones are capable of growth in the absence of histidine. Figure 3.11 shows that transformed cells can grow in medium without histidine. The His⁺ presence was already confirmed by selecting them in a growing medium without histidine. Figure 3.12 shows that there is growth on methanol added plates indicating the Mut⁺ strains. The confirmation for the His⁺ and Mut⁺ phenotype of recombinants was important in the selection of expression conditions. Mut⁺ phenotype showed that methanol induced secretion of *Ay* protein would be successful.

The integration of pPIC9-*Ay* construct into yeast genome were confirmed by a direct PCR procedure by two primers amplifying two fragments in the recombinant genome. Moreover, the direction of integration was important in the expression of the gene. To check the correctness of integration in the recombinant yeast colonies, 5'-*AOX1* and 3'-*AOX1* primer pairs was used. If the integration were between homologous recombination of 5'-*AOX1* region, this primer set are expected to produce two bands, one corresponding to *AOX1* gene with 492 bp and the combination of *Ay* and *AOX1* gene with 2.392 bp (Figure 3.13).

Yeast cells harboring pPIC9-*Ay* construct in the correct direction were induced by adding methanol in the growth medium. Expression was achieved in 2 liters flasks with cheesecloth tappings and vigorous shaking for good oxygenation. The cultures were continued up to 168 hours in order to find the time for highest secretion of *Ay* protein. Methanol was added at an amount of 0.5 ml/day since it is repeatedly reported to be evaporated in the aeration. An aliquot of the yeast culture was taken after 8 hours of induction and analyzed by SDS-PAGE. The analysis of the protein pattern showed the presence of an additional band of 66 kD. This band as shown in Figure 3.14 was determined to be slightly higher molecular weight compared to the subunit cloned from 'Lira' and expressed in *E. coli* (D'Ovidio). The total amount of protein in each aliquot were analyzed by Bradford protein assay. The maximum expression was found after three days of expression and the yield was 1.2 g/liters.

The *Ay* protein is normally secreted, non-glycosylated, thus secretory expression is convenient. The sequence amplified from published sequence do not contain the native signal sequence. We used the α -factor signal sequence (in pPIC9) in order to secrete the *Ay* protein. This signal sequence include a KEX2-like processing signal (Laroche, 1994). The processing of the α -factor signal sequence in pPIC9 occurs in two steps; the preliminary cleavage of the signal sequence by KEX2 gene product, with final KEX2 cleavage between arginine and glutamine in the sequence Glu-Lys-Arg*Glu-Ala-Glu-Ala, where * is the site of cleavage. The Glu-Ala repeats are further cleaved by the STE13 gene product. In order to confirm the identity of the expressed protein, the band corresponding to expressed protein was transferred from SDS-PAGE separation to PVDF membrane and N-terminal amino acid sequence was determined. This is identical to that determined from the protein expressed in *E. coli* as reported by Rooke (1999). The N-terminal amino acid sequence showed that the signal peptide was removed successfully during secretion.

4.3 Isolation and Characterization of Yayla-*Ay* Gene Product

The expressed *Ay* protein was collected under the optimized conditions (72 hours). The identity of protein was first verified by SDS-PAGE analysis. There was a secreted protein corresponding to the *Ay* protein as expressed in *E. coli* (Supplied by D'Ovidio) and this band was absent in the control experiment with the parent plasmid (without *Ay* gene) transformed *P. pastoris* expression (Figure 3.14). The identity of the purified protein was further confirmed by analyzing the

expression medium by RP-HPLC. The retention time was 31.8 min as shown in Figure 3.15 and reported as the characteristics of *Ay* protein (Forde *et. al.* 1985). Partial N-terminal amino acid analysis also confirmed the identity of the protein to be similar to the previously reported *Ay* protein sequence (Buonocore *et. al.* 1996).

In order to determine the impact of the expressed subunit *Ay* on the functional properties of the grain, the flour samples of 13 wheat cultivars were combined with *Ay* protein and analyzed by measuring the loaf volumes by SDS-microsedimentation test of Sapirstein and Suchy (1999). A number of parameters could be measured by using mixograph, farinograph etc., however, loaf volume is an easy and reliable technique to be used in determining quality of flour. The mixed *Ay* protein was at 1/7 (w/w) ratio meaning that it simulated overexpression of the *Ay* gene for each genotype. This analysis showed that *Ay* protein increased the loaf volume of the flour (Table 3.3). This increase was variable among the genotypes. Although the *Ay* subunit was the major protein in the isolates, it was not the only protein. The effect of the additional proteins on the loaf volume was determined by mixing an isolate from control experiment which is designed to express the parental plasmid containing *Pichia*. This experiment showed that the increase observed by the above experiment was not related to the background secretion by parental plasmid.

4.4 Contribution of *Ay* Protein on Dough Volume

The influence of *Ay* protein on the flour quality was evaluated by mixing the isolated Yayla-*Ay* product with the flour of 13 genotypes at 1/7 (w/w) ratio. The amount of *Ay* subunit in the background of other subunits was significantly higher, simulating an overexpression of *Ay* product. The loaf volume of mixtures was found by SDS sedimentation method. The contribution of a single subunit on the end-product has been studied in several ways. The rough extraction of gluten fractions was the first approach to be studied. This straightforward approach was limited by the fractionation ability of solvents and did not give extensive information on each subunit, but group of subunits. Recently transgenic technology were started to be used for the purpose of overexpression of a single subunit to observe the influence on the end-product. The *Ay* gene is inactive in the wheat genome. The inactivation was thought to be caused by transcriptional regulation and the mechanism is not clear (D'Ovidio, 1995)

Studies of the structure of glutenin subunits and their interaction have been limited by the difficulty of isolating single subunit in large amounts from the complex gluten proteins present in wheat endosperm. One way to overcome this problem is the expression of individual wild-type and modified glutenin subunit in heterologous systems. These systems have additional advantage that specific gene modifications can be made and the new gene constructs could be quickly expressed. Recent studies have shown that *E. coli* system can express sufficient amount of individual subunits (D'Ovidio *et. al.* 1997, Galili, 1989; Shani, 1992).

However, the yield and the purification steps makes their use still limited. Comparison of expression systems of glutenin *Ay* gene has led to the development of an effective expression purification system. The *E. coli* expression system had success producing 0.3 g/l of protein (D'Ovidio, 1997). Expression in *Pichia* as described here achieved levels of up to 1.2 g/l in shake-flask culture which were improved 4 fold through the use of fermentation.

The evaluation of Yayla-*Ay* protein on loaf volume showed that the expression of the protein might be a new source of variation contributing on the breadmaking quality of flour. This result would urge the studies on the mechanism of silencing of *Ay* gene expression in wheat and would result in a transgenic wheat with a higher quality flour by overexpression of *Ay* protein.

4.5 *Pichia* Expression System as an Alternative to Overexpression Studies

The expression of *Ay* gene in *Pichia* expression system and studies on the effect of the protein on the baking quality of flour can be proposed as a model system to screen the potential effect of overexpression of glutenin subunits on the flour quality.

Genetic and biochemical analysis have shown that bread-making quality of wheat glutenin is particularly associated with allelic variation in High Molecular Weight subunits encoded by genes at the *Glu-D1* and *Glu-A1* loci (Payne, 1987). However, the effect of each glutenin subunits on flour quality has not been

studied. Studies of the structure of Glutenin subunits and their interaction have been limited by the difficulty of isolating single subunits in large amounts from the complex mixture of gluten proteins present in wheat endosperm. One way to overcome this problem is to transform a chimeric gene having a strong promoter into wheat and to evaluate the technological property of flour. This approach have been used to overexpress HMW Glutenin subunit 1D5 (Rooke *et. al.* 1999). This is the straightforward and decisive approach, however it needs a lot of time and investment. In total 30 different alleles at the Glu-A1 locus coding for (HMW-GS), 25 alleles at the Gli-A1 locus coding for ω - and γ -gliadins and 45 alleles at the Gli-A2 locus controlling the synthesis of α/β -gliadins were detected. (Saponaro *et. al.* 1995). Another way to overcome this problem is the expression of individual wild-type and modified high molecular weight glutenin subunits in heterologous systems. These systems have the additional advantage that specific gene modifications can be made and the new gene constructs quickly expressed. *E. coli* have previously been used expressing glutenin subunits (D'Ovidio *et. al.* 1997).

Figure 4.1 shows estimated time required for the two approaches. The use of *Pichia* expression system resulted in a gain of at least 4 months. The comparison of time investment on the two approach shows clearly that the use of heterologous systems would be a valuable alternative for evaluating the influence of glutenin subunits on flour quality as a screening procedure for transgenic studies.

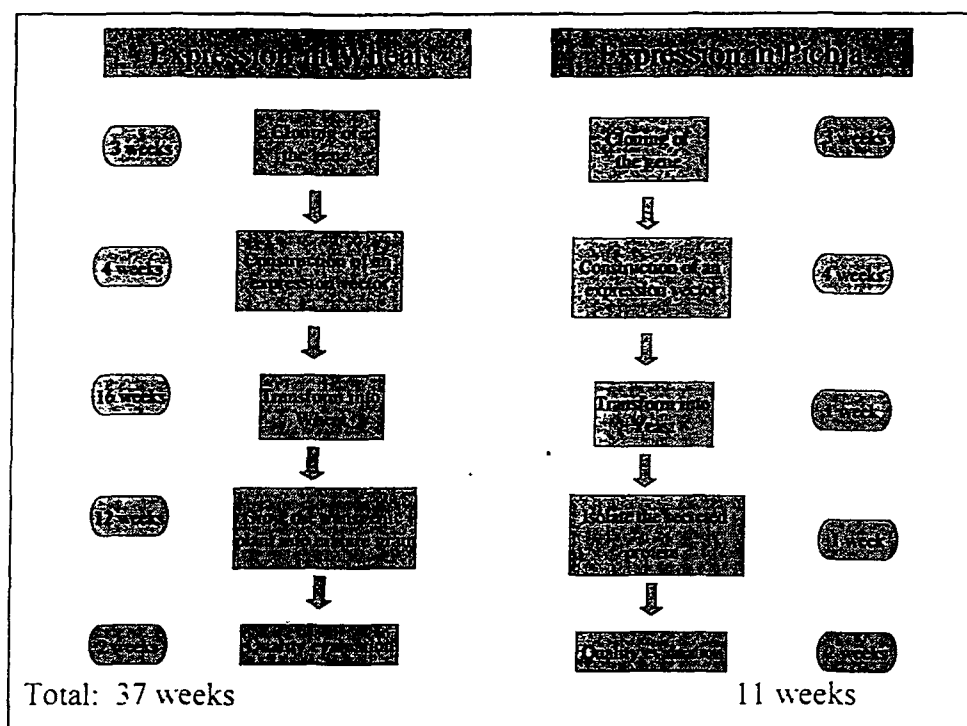


Figure 4.1. Estimated time required to complete the evaluation of a glutenin subunit for the transgenic approach and the proposed approach.

This research aimed at investigating the development of a system to study the role of Glutenin subunits on the bread-making quality. It is proposed that expression of glutenin subunits in yeast heterologous expression system may be used in the overexpression studies. The proposed system produce the gene product to be studied in *P. pastoris* expression system, isolated and included externally in the flour which is evaluated for the quality parameters. The information provided in this way will give a valuable insight for further study in a short time and with less investment compared to direct expression in transformed wheat. Therefore, the proposed system would be a pretransgenic tool to plan the final transformation studies.

CHAPTER 5

CONCLUSION

5.1 Gluten Protein Polymorphism

The main goal of this study was to investigate the gluten protein polymorphism of 13 Turkish wheat cultivars (*Triticum aestivum* and *Triticum durum*) with a view of improvement of the flour quality. The results of this study, showed that there was polymorphism as revealed by SE-HPLC, SDS-PAGE and PCR amplification to distinguish between the genotypes as to subunit composition. SE-HPLC determined the amount of polymeric proteins of flour, SDS-PAGE revealed various subunit patterns and PCR amplification showed polymorphism in HMW 1*Ay* locus.

5.2 Cloning and Expression of Yayla-*Ay* Protein

The polymorphism between cultivars was shown by *Ay* gene specific primers. The second aim was to test the potential effect of the product of the silent *Ay* gene on flour quality. So, the rest of the study was devoted to the cloning and expressing of this new allele of the *Ay* gene from 'Yayla'. The cloned *Ay* gene was expressed in a heterologous *Pichia pastoris* expression system. The

contribution of *Ay* gene product on the loaf volume of 13 different cultivars was determined by SDS microsedimentation measurement. To the best of our knowledge, this study reported the heterologous expression of a glutenin protein in *Pichia* Expression System for the first time. The amount of *Ay* protein isolated from the culture media was 1.2 mg /l and four fold higher than previously reported amount in *E. coli* expression system. SDS-PAGE, RP-HPLC and N-terminal amino acid analysis confirmed the identity of the overexpression protein to be *Ay* protein.

5.3 Influence of Yayla-*Ay* Protein on Loaf Volume

The Yayla-*Ay* protein caused significant increases in SDS-sedimentation volume of flour when added in a 1/7 (w/w) ratio. The contribution of silent *Ay* gene product on the loaf volume properties of flour were determined by mixing *Ay* product from yeast system with the flour of 13 genotypes and determining the SDS-sedimentation volume of mixtures. The amount of protein mixed corresponded to an overexpression study. The *Ay* protein added flours gave higher loaf volumes indicating a possible improvement of flour technological property.

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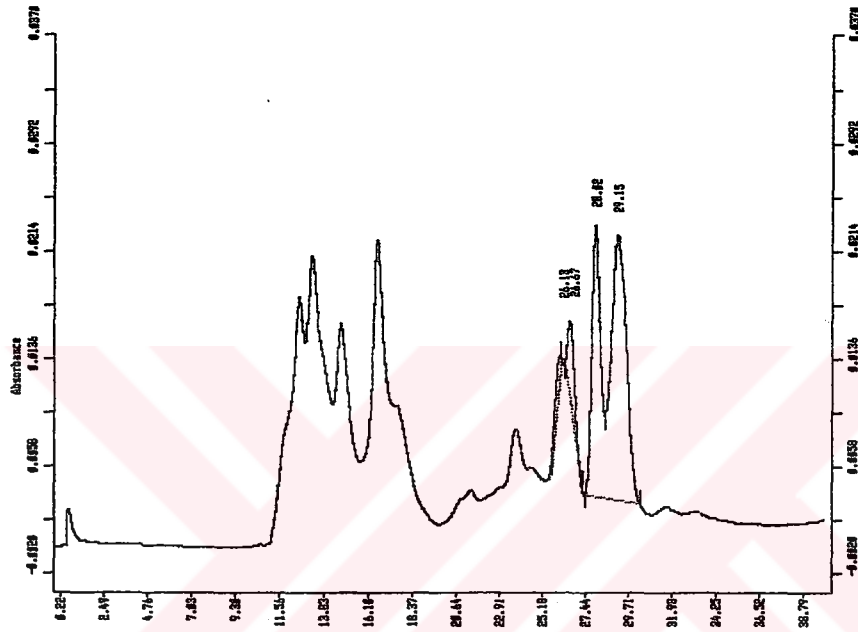
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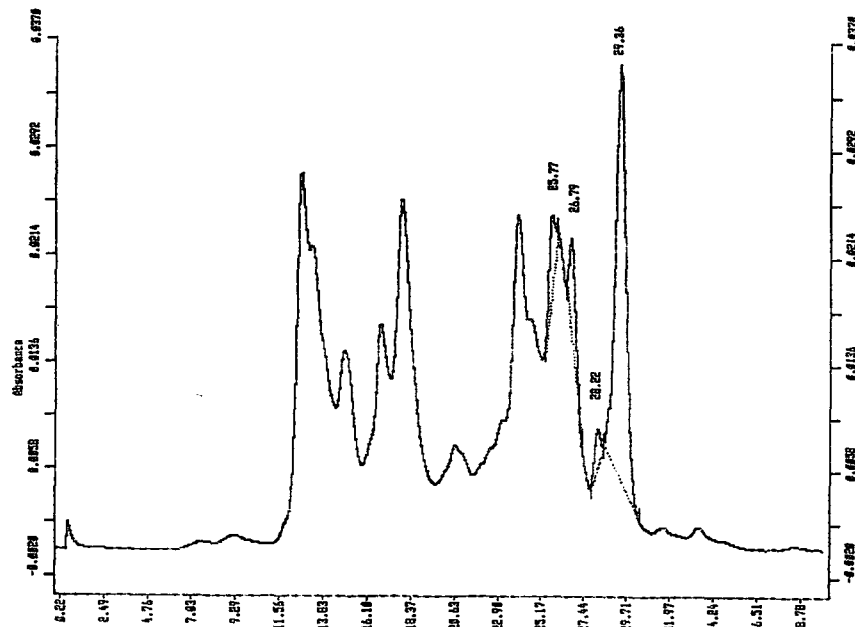


APPENDIX A

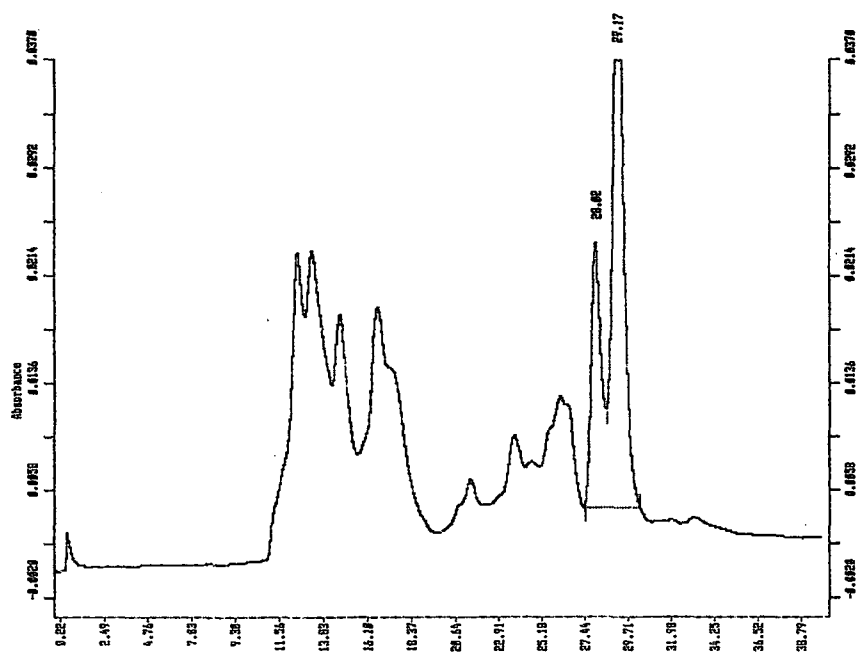
SE-HPLC chromatography profiles of reduced and unreduced fractions of 13 wheat genotypes .



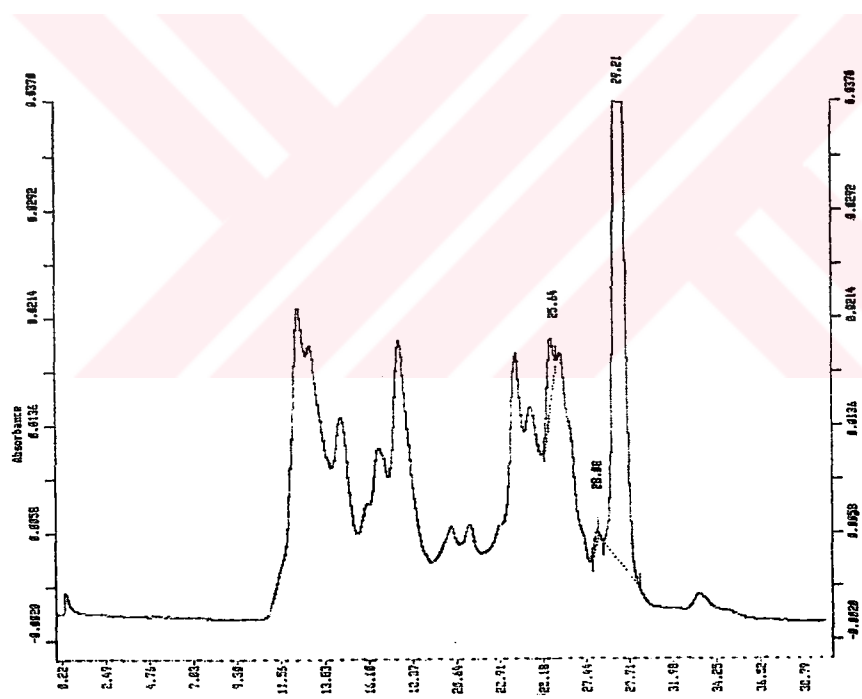
1. SE-HPLC profile of total insoluble fraction of Ak-702 grain.



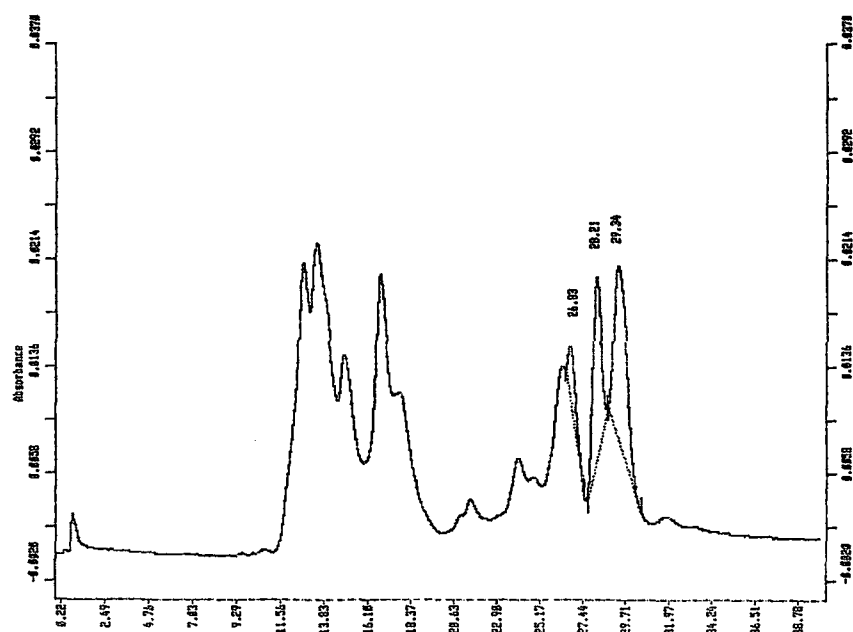
2. SE-HPLC profile of total soluble fraction of Ak-702 grain.



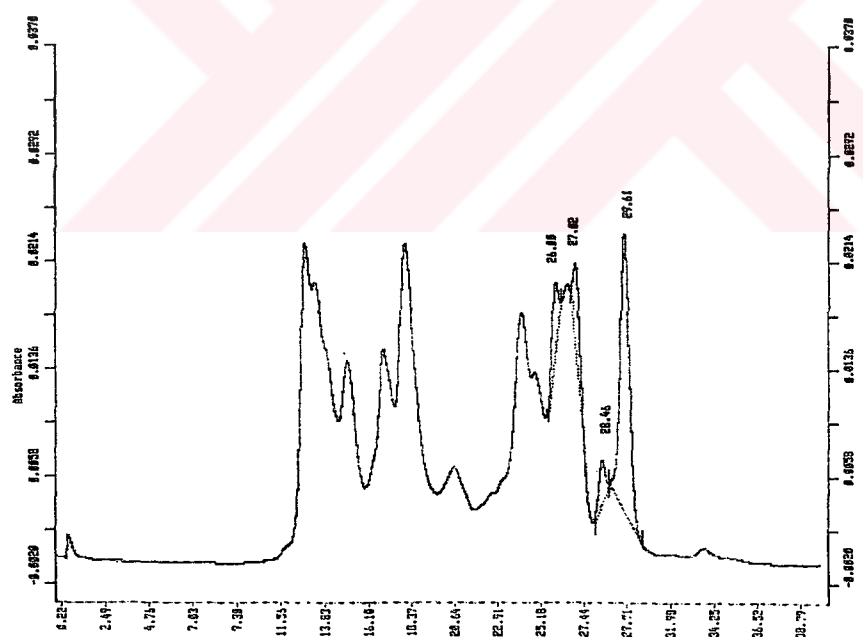
3. SE-HPLC profile of total insoluble fraction of Atay85 grain.



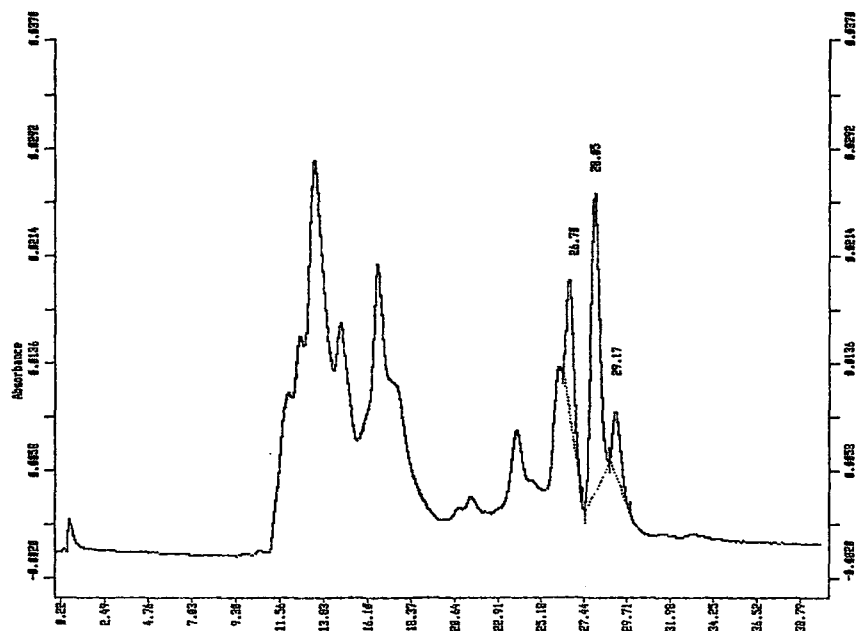
4. SE-HPLC profile of total soluble fraction of Atay85 grain.



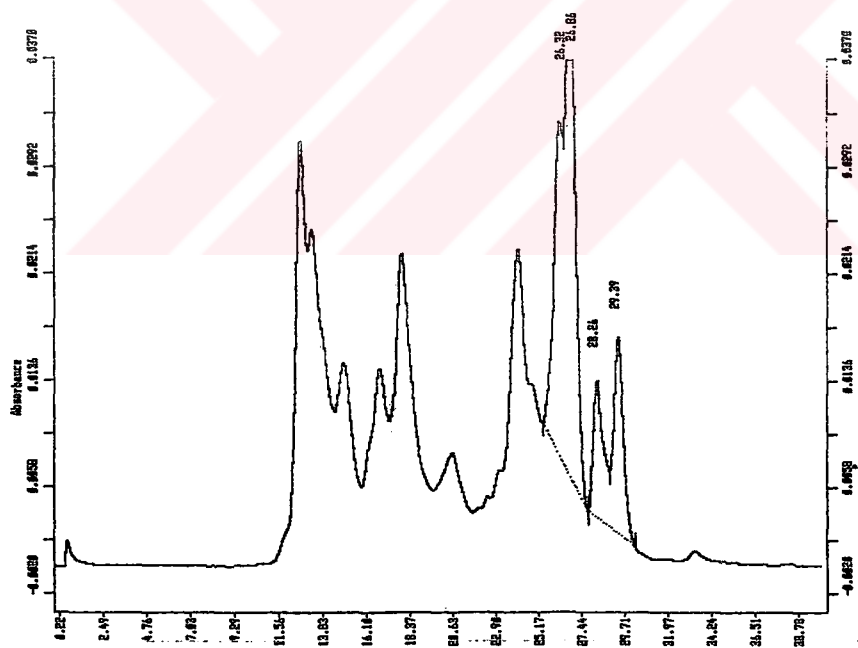
5. SE-HPLC profile of total insoluble fraction of Bezostaja grain.



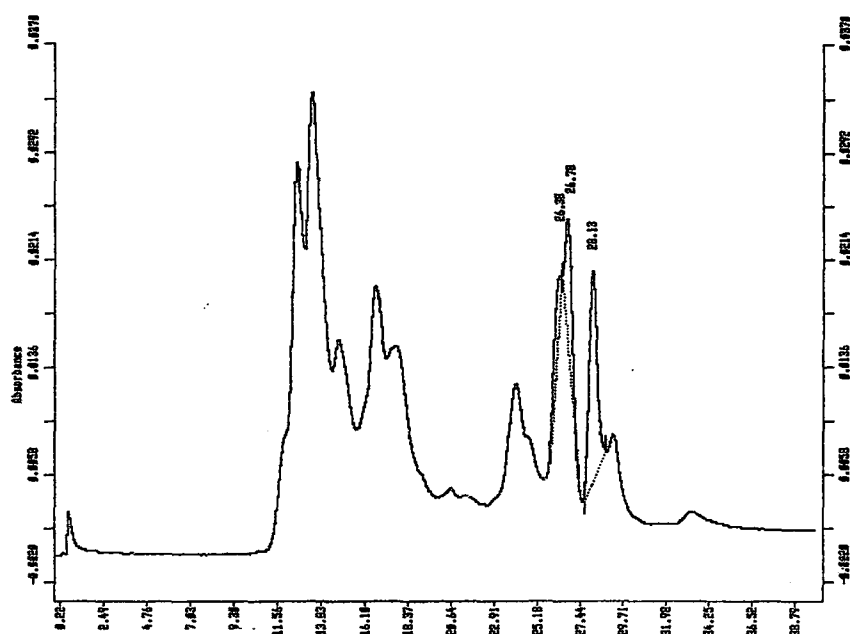
6. SE-HPLC profile of total soluble fraction of Bezostaja grain.



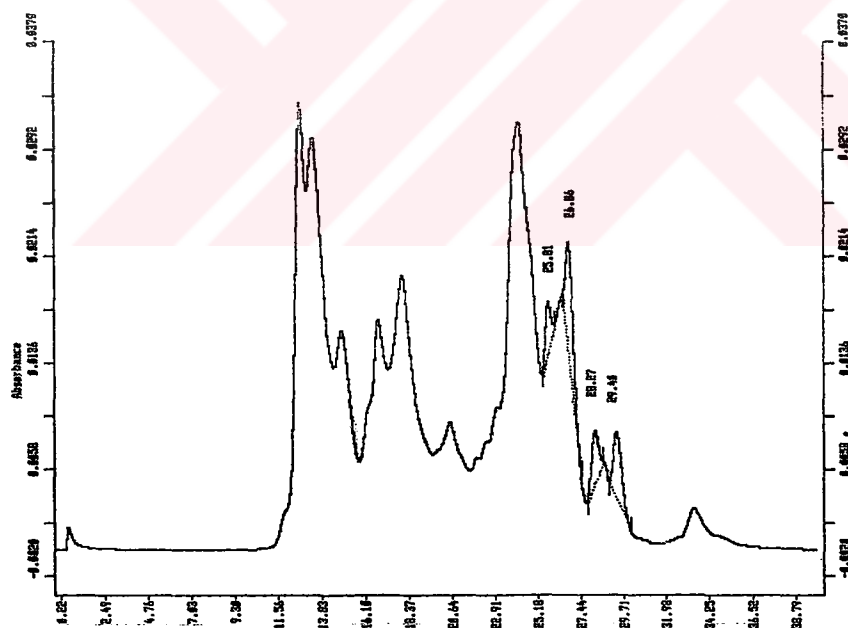
7. SE-HPLC profile of total insoluble fraction of Bolal grain.



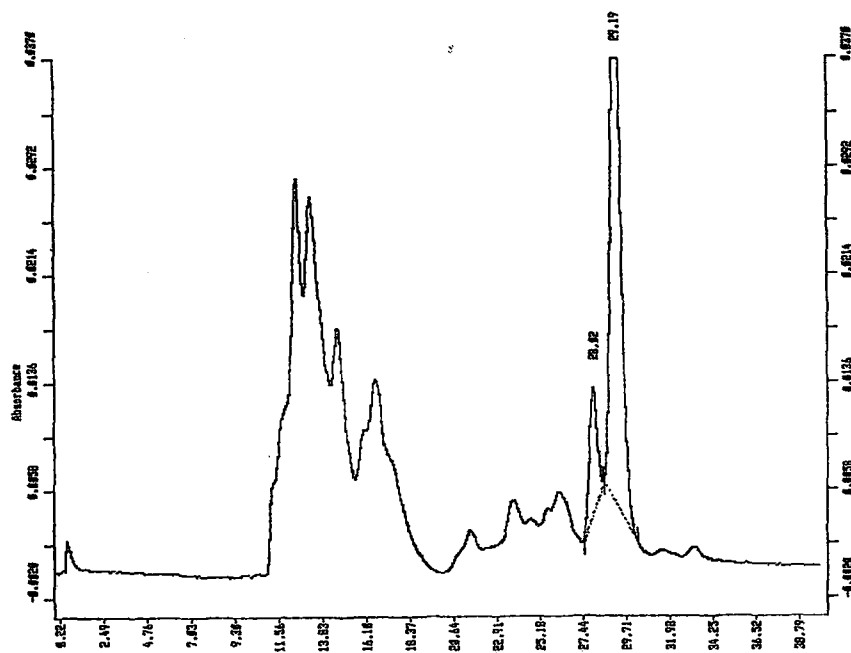
8. SE-HPLC profile of total soluble fraction of Bolal grain.



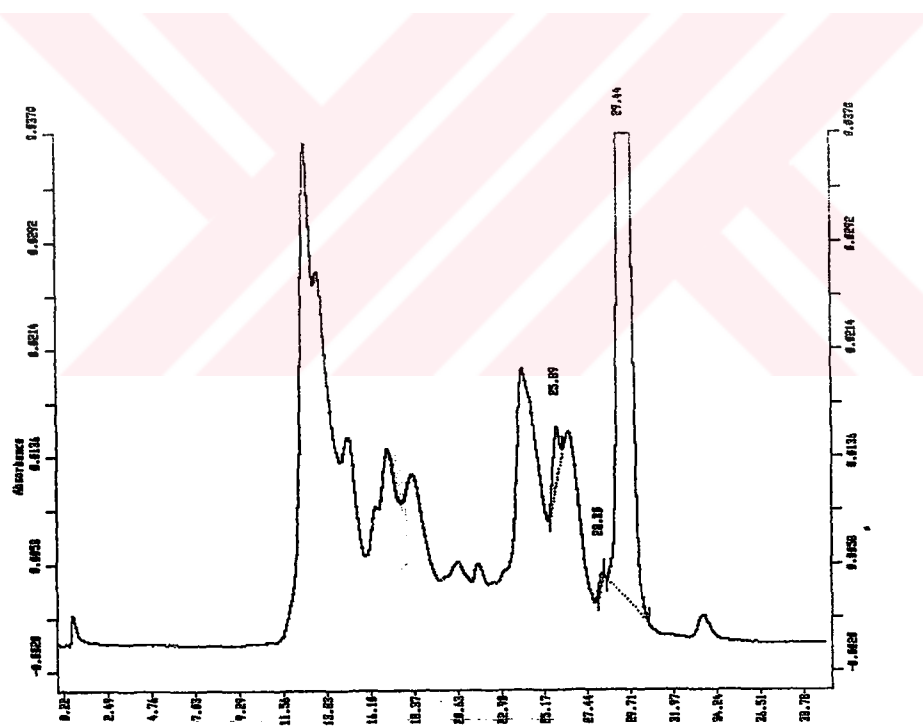
9. SE-HPLC profile of total insoluble fraction of Çakmak grain.



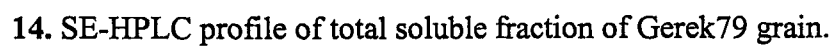
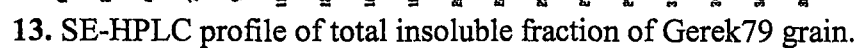
10. SE-HPLC profile of total soluble fraction of Çakmak grain.

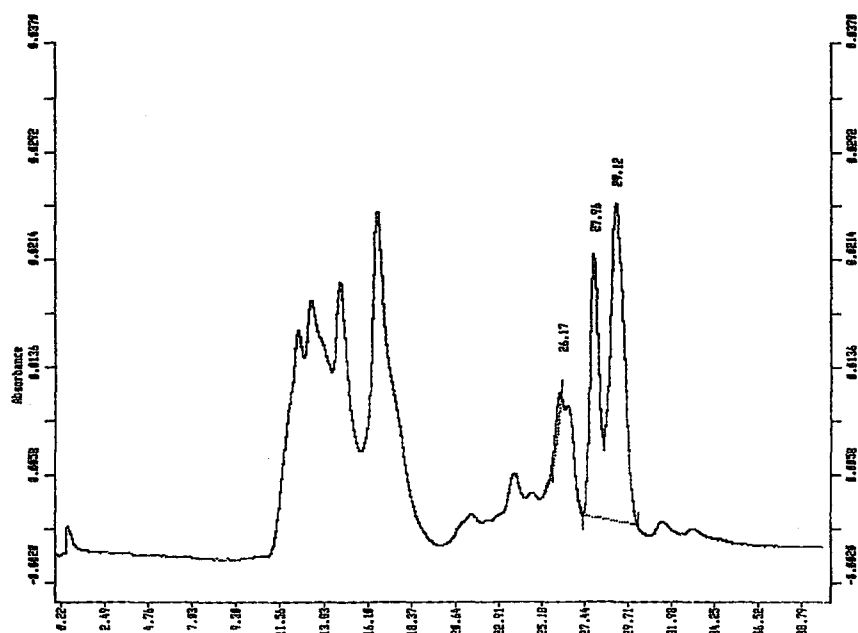


11. SE-HPLC profile of total insoluble fraction of Dicle95 grain.

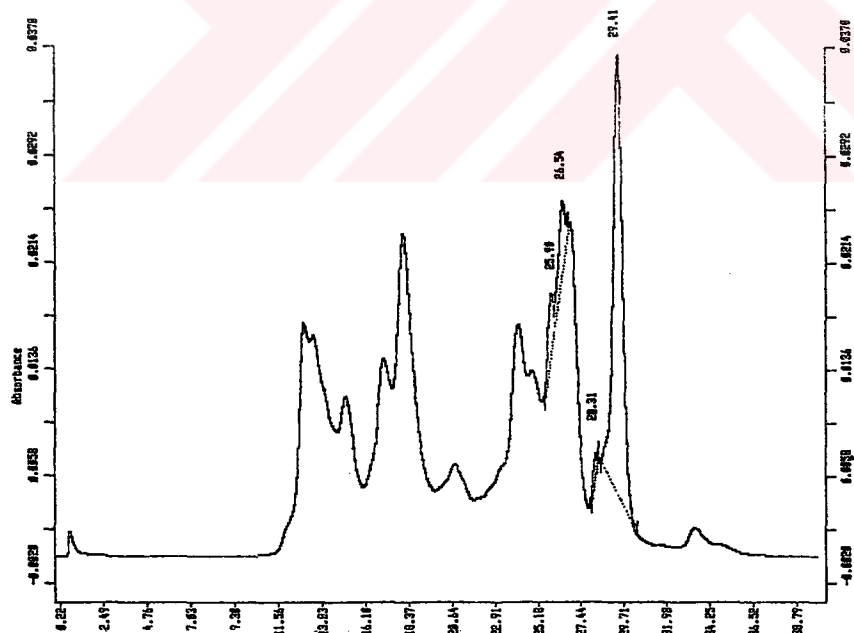


12. SE-HPLC profile of total soluble fraction of Dicle95 grain.

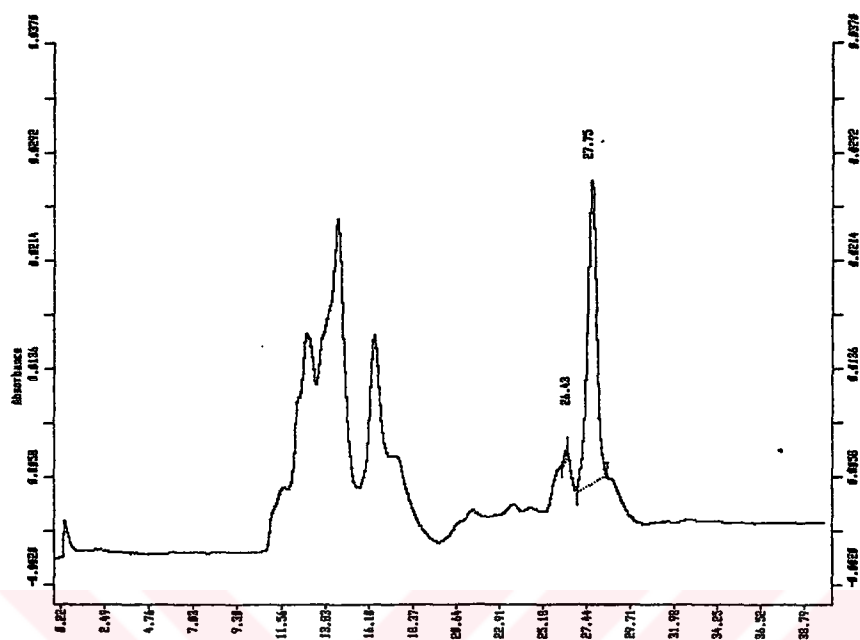




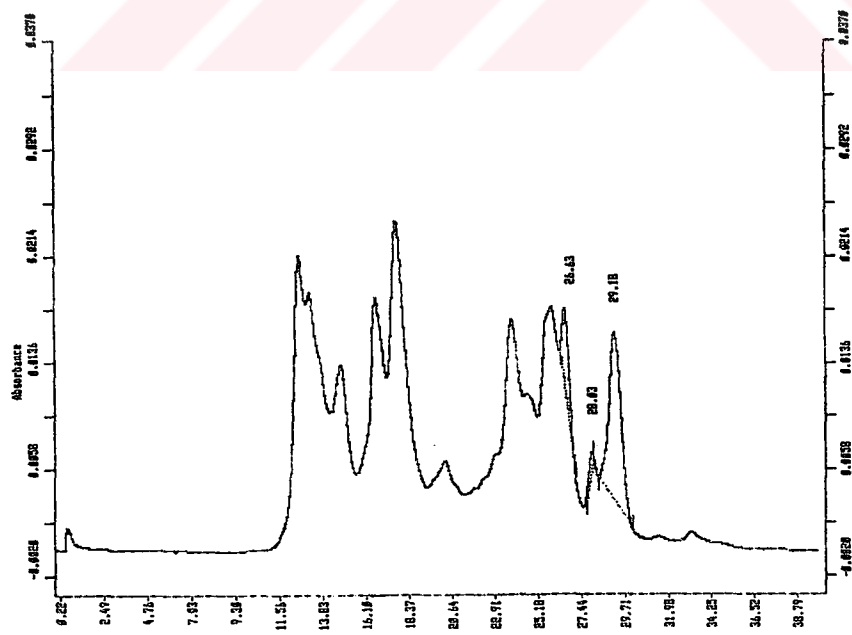
15. SE-HPLC profile of total insoluble fraction of Gün95 grain.



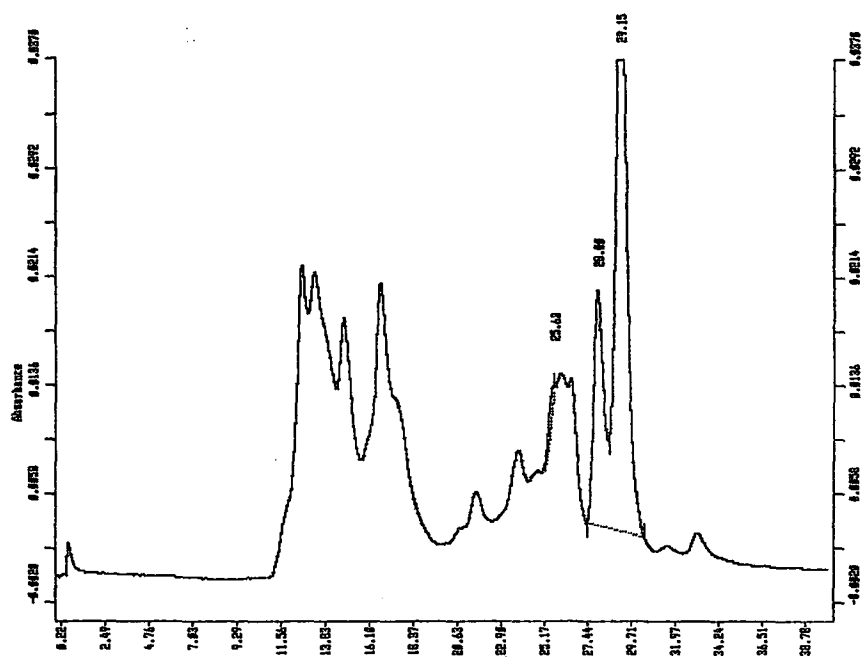
16. SE-HPLC profile of total soluble fraction of Gün95 grain.



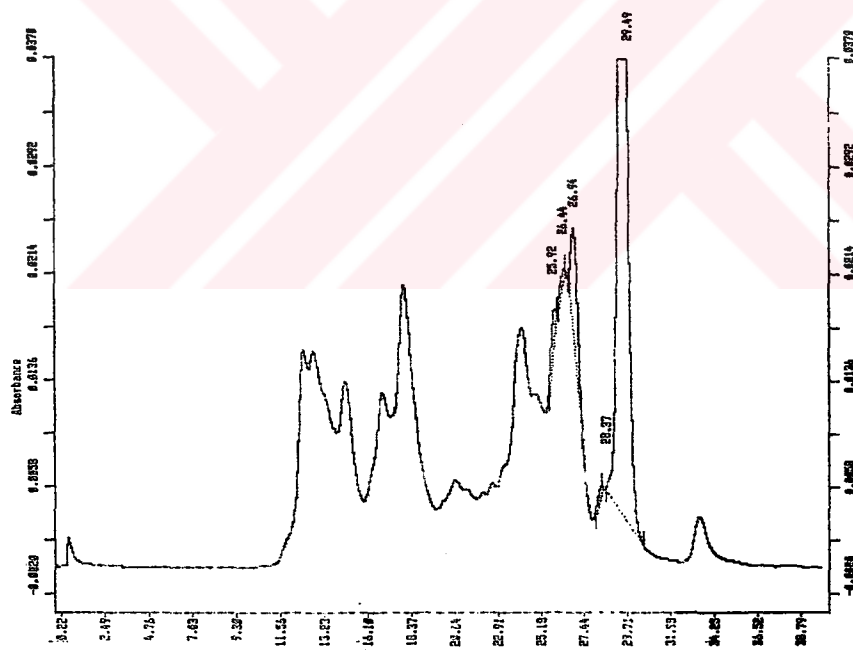
17. SE-HPLC profile of total insoluble fraction of Kırac grain.



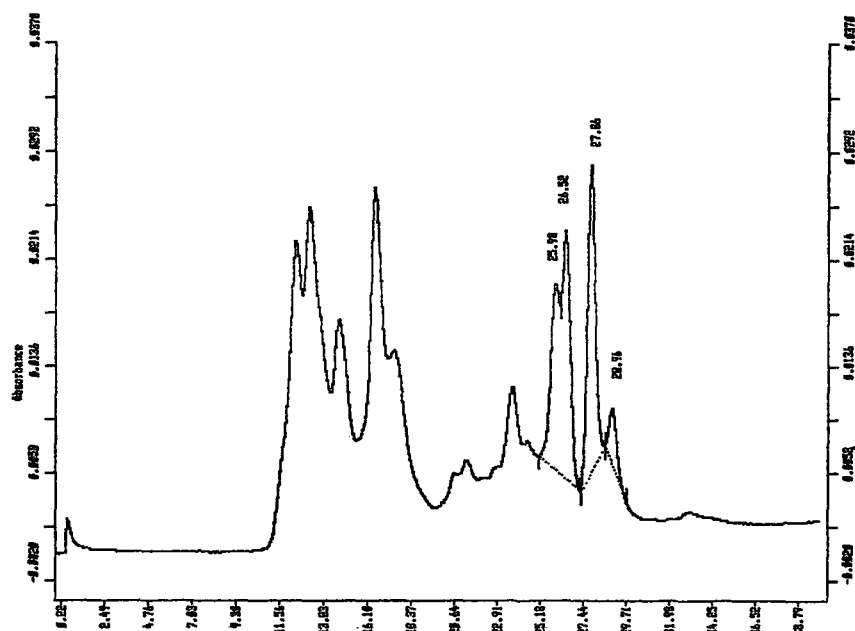
18. SE-HPLC profile of total soluble fraction of Kırac grain.



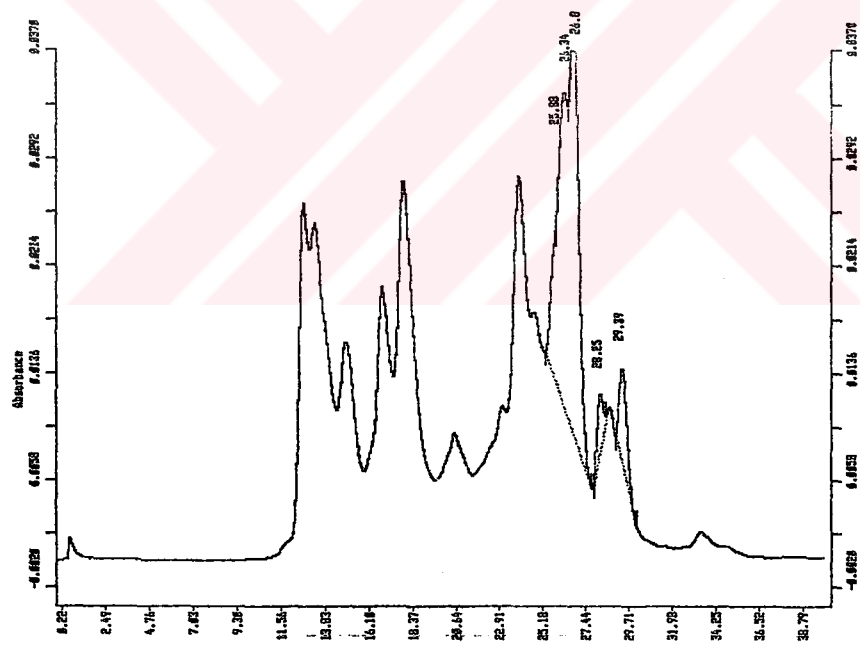
19. SE-HPLC profile of total insoluble fraction of Orso grain.



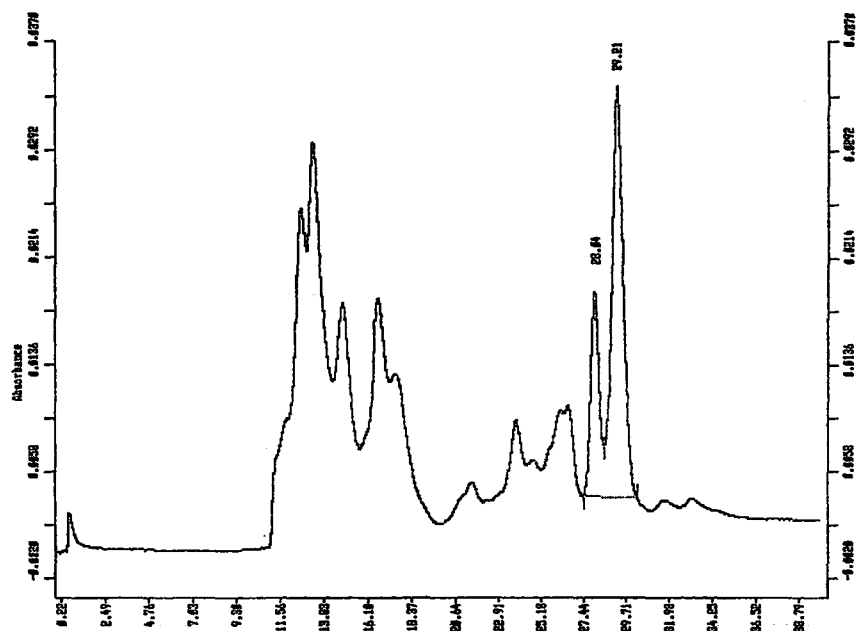
20. SE-HPLC profile of total soluble fraction of Orso grain.



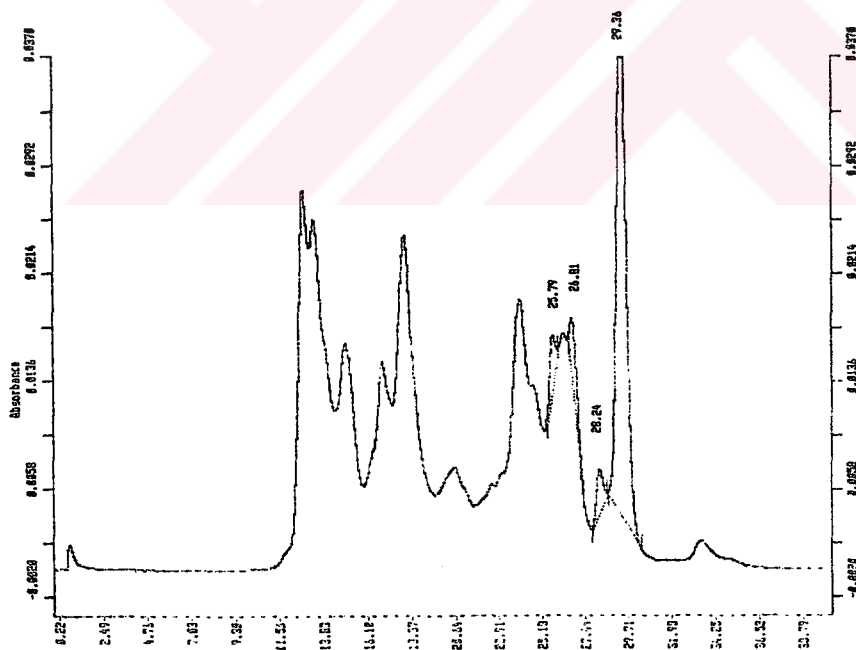
21. SE-HPLC profile of total insoluble fraction of Sertak grain.



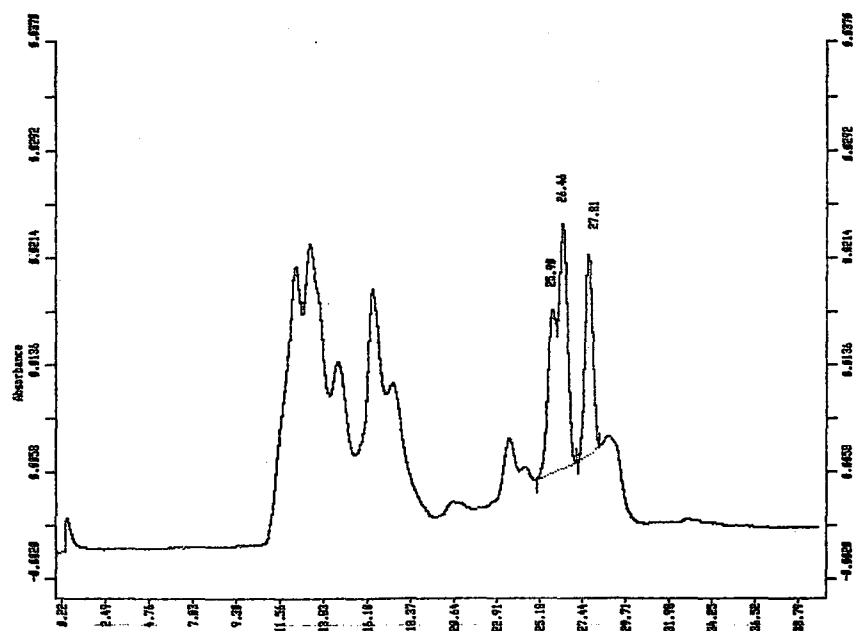
22. SE-HPLC profile of total soluble fraction of Sertak grain.



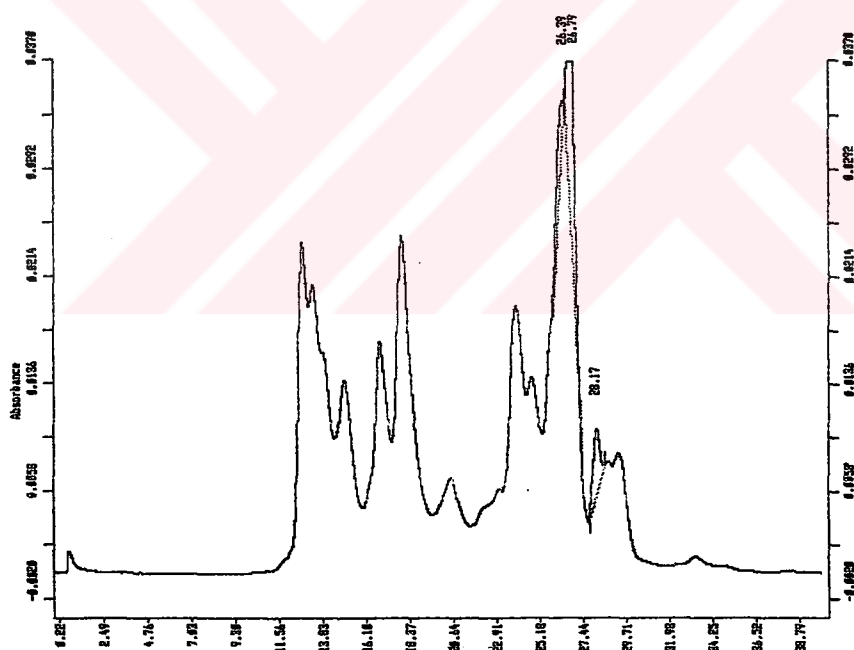
23. SE-HPLC profile of total insoluble fraction of Tosun144 grain.



24. SE-HPLC profile of total soluble fraction of Tosun144 grain.



25. SE-HPLC profile of total insoluble fraction of Yayla grain.



26. SE-HPLC profile of total soluble fraction of Yayla grain.

APPENDIX B

Data and ANOVA Analysis (with post-hoc test LSD) of SDS Sedimentation

Volume of Ay mixed Proteins (Table

ANOVA TABLE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	36.469	25	1.459	18.065	.000
Within Groups	6.298	78	8.075E-02		
Total	42.767	103			

LSD Analysis

Cultivars	Mean Diffrence (SDSSV-SDSSV-Ay)	St Error	Significance	95 % CI Lower	95 % CI Upper
Yayla	-0.425	0.201	0.038*	-0.825	-0.025
Dicle95	-0.225	0.201	0.266	-0.625	0.175
Kıraç	-0.370	0.201	0.069	-0.770	0.030
Sertak	0.273	0.201	0.179	-0.128	0.673
Çakmak	0.093	0.201	0.647	-0.308	0.493
Ak 702	-0.175	0.201	0.386	0.575	0.225
Tosun	-0.240	0.201	0.236	-0.640	0.160
Bezostaja	-0.260	0.201	0.199	-0.660	0.140
Gün 91	-0.140	0.201	0.488	-0.540	0.260
Orso	0.015	0.201	0.941	-0.385	0.415
Gerek	-0.325	0.201	0.110	-0.725	0.073
Atay 85	-0.215	0.201	0.288	-0.615	0.185
Bolal	-0.240	0.201	0.186	-1.640	-0.840

Genotypes	SDSSV	SDSSV (4y Protein added)	SDSSV(Protein extract from control added)
Yayla	3.30	3.38	3.63
	3.30	3.42	3.63
	3.37	3.34	4.08
	3.26	3.60	3.59
Dicle95	2.96	3.08	3.71
	3.24	3.68	2.74
	3.02	2.77	1.99
	2.96	3.20	4.64
Kıraç	2.70	3.50	2.81
	2.81	2.97	3.26
	2.78	3.45	3.07
	2.83	3.22	3.46
Sertak	2.46	2.60	2.14
	2.54	2.93	2.42
	2.59	2.74	2.36
	2.51	2.62	2.09
Çakmak	2.42	2.17	2.36
	2.41	1.91	2.33
	2.42	2.18	2.37
	2.50	2.62	2.32
Ak702	1.34	1.06	1.43
	1.13	1.26	1.37
	1.28	1.69	1.35
	1.19	0.86	1.49
Tosun144	2.25	2.39	2.54
	2.18	2.04	2.46
	2.19	2.15	2.49
	2.21	2.15	2.30
Bezostaja	2.12	1.99	2.51
	2.06	2.21	2.32
	2.06	2.37	2.35
	2.13	2.27	2.23
Gün91	2.13	2.26	2.28
	2.14	2.02	2.39
	2.13	1.95	2.20
	2.08	1.98	2.17
Orso	1.95	1.78	2.82
	1.92	1.90	1.09
	1.89	1.89	1.85
	1.88	1.89	1.82
Gerek79	1.85	1.69	2.31
	1.91	1.87	2.13
	1.92	1.53	2.15
	1.83	1.68	2.22
Atay85	1.93	1.81	2.20
	1.97	1.85	2.28
	2.07	2.36	2.15
	2.11	1.83	2.31
Bolal	1.34	1.89	2.60
	1.39	1.38	2.70
	1.32	1.36	2.50
	1.41	1.27	2.62

CURRICULUM VITAE

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March 12th, 1967, İskenderun Turkey

Turkish

Single

EDUCATION

1990

B.Sc. in Biology

Middle East Technical University, Ankara, Turkey

1993

M.Sc. in Biology

Middle East Technical University, Ankara, Turkey

2000

Ph.D. in Biology

Middle East Technical University, Ankara, Turkey

PROFESSIONAL EXPERIENCES AND EMPLOYMENT

1991-1998

Research Assistant in Department of Biology, METU.

1993

Visiting scientist, Biological Research Centre, H.A.S,
Szeged, Hungary (3 months) in the Plant Cell
Differentiation Lab of Dr. Denes Dudits.

1994-1995

Research Assistant, The Department of Plant and Soil
Sciences, Texas Tech University, Lubbock, Texas-USA (8
months) in the Plant Genetics Lab of Dr. Henry Nguyen.

1998 Visiting Scientist, The Department of Agrobiological and Agrochemistry, University of Tuscia, Viterbo, Italy (6 months) in the lab of Dr. Domenico Lafiandra.

LANGUAGES

English (excellent), German (fair), Italian (fair), Turkish (native)

PROFESSIONAL SKILLS

- ◆ Association of RFLP and RAPD markers with environmental stress tolerance traits.
- ◆ Evaluation techniques of drought and salt stress physiology of plants.
- ◆ Recombinant DNA techniques, cDNA and genomic library screening, vector construction, PCR related techniques, radioactive and dig-labelling detection systems.
- ◆ Heterologous gene expression systems in yeast.
- ◆ Protein purification systems with column chromatography and rotaphore.
- ◆ Size exclusion and reverse phase HPLC applications, electrophoresis of proteins, sequencing gels.
- ◆ Plant tissue culture techniques and transformation systems (direct DNA uptake, bombardment, Agrobacterium-mediated). Molecular analysis of transgenic plants.

FELLOWSHIPS

UNESCO Short-term Biotechnology fellowship 1994.

Texas Tech University Biotechnology Fellowship 1994.

NATO-A2 Short-term Ph.D. Fellowship 1998.

PUBLICATIONS

Özalp, V.C. (1993). Biochemical and Physiological Responses of Wheat under Salt Stress, Metu M.Sc. Thesis, Ankara, Turkey

Naqvi, SMS., **Özalp, VC.**, Oktem, HA, Yucel, M. 1994. Two-dimensional Electrophoresis with a Different Approach to Isoelectric Focusing, *Analyst*, 119:1341-1344.

Oktem, HA, Özkan, F., **Özalp, VC.**, Yucel, M. 1994. Agrobacterium Mediated Gene Transfer, *Turkish Journal of Botany*, 119:1341-1344.

Naqvi, SMS, **Özalp, VC**, Oktem, HA, Yucel, M. 1995. Salt-induced Synthesis of New Proteins in The Roots of Rice Varieties. *Journal of Plant Nutrition*, 18(6):1121-1137.

Gündoğuş, N., Sayılır, C., **Özalp, VC**, Ozkan, F, Yucel, M., Criss, EW. 1995. Effects of Polyamine Stimulated Casein Kinase II From Rat Brain on Cellular Oncogene Activities. *Turkish Journal of Medical Sciences*, 23:107-116.

Özalp, VC, Naqvi, SMS, Oktem, HA, Yucel, M. 2000. Photosystem II and Cellular Membrane Stability Evaluation in Hexaploid Wheat Seedlings under Salt Stress Conditions. *Journal of Plant Nutrition*, 23 (2): 275-283.