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Enzymes in Breadmaking

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INTRODUCTION

For several thousands of years, man has used wheat and other cereals to produce bread. The baking of leavened breads was already known by the ancient Egyptians and represents one of the oldest biotechnological processes. The use of enzymes in breadmaking dates from these earliest times as well. The first bakers unconsciously made use of the enzymes present in the cereals or produced by yeast and other microorganisms. Addition of (exogenous) enzymes started much later, but already more than a century ago, it became the practice to use malted barley to optimize the amylase levels in flour and improve its breadmaking properties. Later, fungal enzymes became available as a malt replacement. With increasing insight into the complex transformations occurring during dough mixing, proofing, and baking, new enzymes and enzyme activities were introduced. Today, a whole range of enzymes, representing different classes, such as hydrolases and oxidoreductases, are used in breadmaking to improve the breadmaking process and to maximize the functionality of flour and the quality of bread. Baking applications account for approximately 8% of enzyme sales in the grain-processing sector (Godfrey 2003). Amylolytic enzymes, and to a slightly lesser extent xylanases, have become commodity products, and the use of lipases and glucose oxidases has become established as well. Other enzymes, such as microbial lipoxygenases, so far remain highly specialized catalysts (Godfrey 2003).

In general, enzymes directly or indirectly affect the structure and physicochemical properties of

different flour constituents in a well-defined way. Therefore, prior to discussing the role of different enzymes in breadmaking, a concise overview of the structure and properties of the different flour constituents and their functionality in breadmaking is presented.

WHEAT FLOUR CONSTITUENTS IN BREADMAKING

The vast majority of bread is produced from wheat. In breadmaking, wheat flour, water, salt, yeast (and/or other microorganisms), and often also other, nonessential, ingredients, such as fat and sugar, are mixed into a viscoelastic dough, which is fermented and baked. Wheat flour mainly consists of starch (ca. 70–75%), water (ca. 14%), proteins (ca. 10–12%), nonstarch polysaccharides (ca. 2–3%), in particular arabinoxylans, and lipids (ca. 2%). These wheat flour constituents determine to a great extent dough processibility and bread quality. Indeed, during the different steps in the breadmaking process, complex chemical, biochemical, and physical transformations take place that affect and are affected by the various flour constituents (Goesaert et al. 2005).

STARCH

Structural Aspects

Starch is the most abundant constituent and most important reserve polysaccharide of cereals. On a molecular level, its major constituents are the glucose polymers amylose (25–28%) and amylopectin (72–75%). Amylose consists of some 500–6000 α-(1,4)-linked D-glucopyranosyl units. Although a fraction of the amylose molecules is slightly branched by α-(1,6)-linkages (Hizukuri et al. 1981, Shibanuma et al. 1994), it is for all practical purposes considered to be a linear molecule. In contrast, amylopectin consists of linear chains of 10–100 α-(1,4)-linked D-glucopyranosyl units which are connected by α-(1,6)-linkages, forming a very large and highly branched polysaccharide of up to 3 million glucose units (Manners 1979, Zobel 1988). Amylopectin is generally described as a cluster (French 1984, Robin et al. 1974) with polymodal chain length distribution (Hizukuri 1986) and a nonrandom nature of branching (Thompson 2000). In the cluster model, the short chains, that is, the unbranched outer A chains and the shortest inner branched chains (B1), form double helices and make up a single cluster, while the longer branched chains (B2–B4) extend into two to four clusters, respectively.

Starch occurs as intracellular, water-insoluble semicrystalline granules. In contrast to most plant starches, wheat, rye, and barley starches have a bimodal size distribution. They consist of large (10–35 μm) lenticular and small (1–8 μm) spherical granules (Karlsson et al. 1983, Stoddard 1999). When viewed in polarized light, native starch granules are birefringent, and a "Malteser cross" can be observed. This phenomenon results from a degree of order in the starch granule and a radial orientation of the macromolecules (Buléon et al. 1998). The partially crystalline nature of starch (20–40%) (Hizukuri 1996) is predominantly attributed to structural elements of amylopectin. Different packing of the amylopectin side chain double helices gives rise to different crystal types. Cereal starches have an A-type X-ray diffraction pattern, and retrograded starch a B type (Buléon et al. 1998).

Several extensive reviews provide a more detailed discussion of the structure and physicochemical properties of (wheat) starch (e.g., Buléon et al. 1998, Eliasson and Gudmundsson 1996, Hizukuri 1996, Parker and Ring 2001).

Starch in Breadmaking

During milling, a fraction (ca. 8%) of the starch granules is mechanically damaged. Damaged starch is no longer birefringent, is more susceptible to amylolysis, and has greater water absorption than its native counterpart (Hoseney 1994).

During dough preparation, starch retains its native state. It absorbs water and slightly and reversibly swells. Its role in dough is still not very clear (Bloksma 1990, Eliasson and Larsson 1993, Larsson and Eliasson 1997).

Due to the combination of heat and moisture during baking, the starch granules gelatinize when their gelatinization temperature is reached: their molecular order is irreversibly destroyed, and the partially crystalline granules are transformed into an amorphous state (Atwell et al. 1988). However, mainly due to the limited amount of water available in the dough, their granular identity is retained (Hug-Iten et al. 1999, Schoch 1965, Varriano-Marston et al. 1980). In addition to the loss of molecular order,

birefringence, and crystallinity, the gelatinization process is also associated with granule swelling and distortion (due to the increased water absorption) and limited starch solubilization (or amylose leaching), which increases the viscosity of the aqueous phase. Part of the (solubilized) amylose forms inclusion complexes with both added and endogenous wheat polar lipids, as evidenced by the V-type X-ray diffraction pattern of fresh bread crumb. Furthermore, due to phase separation, a nonhomogeneous distribution of amylose and amylopectin in the granules was observed, with amylose and amylopectin enrichment in the center and outer layers of the large granules, respectively.

Upon cooling and aging (storage) of the bread, the starch polysaccharides reassociate to a more ordered or even a new B-type crystalline state, differing from that of the native granules. This process is defined as retrogradation (Atwell et al. 1988). Initially, double helices are formed between amylose molecules, and a continuous network develops, in which the swollen and deformed starch granules are embedded and interlinked. Within 24 hours, these double helices form very stable crystalline structures. Amylose is hence an essential structural element of bread and a determining factor for initial crumb firmness (Eliasson and Larsson 1993). In contrast, amylopectin recrystallization is much slower and occurs in the swollen and deformed gelatinized starch granules. It determines the long-term development of structure and crystallinity in starch systems (Miles et al. 1985). Therefore, amylopectin retrogradation is an important process during storage and aging of bread.

During storage, bread gradually loses its appealing freshness and stales. The staling process is often evaluated by measuring crumb firmness, but includes other characteristics as well, such as loss of flavor and moisture. Bread staling is a complex process, which is not completely understood and involves multiple constituents and mechanisms (see Gray and BeMiller 2003). However, water migration and transformations in the starch fractions are widely accepted to be the most important factors in this process (Gray and BeMiller 2003). Most staling models attribute crumb firming during aging to amylopectin retrogradation, in particular the formation of double helices by the short amylopectin side chains and their recrystallization (Gray and BeMiller 2003, Kulp and Ponte 1981, Schoch and French 1947, Zobel and Kulp 1996), resulting in an increased rigidity of the swollen granules. Due to its rapid retrogradation upon bread cooling, amylose is believed to have little, if any, contribution to bread firming, although the formation of ordered amylose structures in the granule centers may also contribute to increasing granular rigidity (Hug-Iten et al. 1999, 2003). However, starch crystallinity often poorly correlates with crumb firmness, particularly in amylase-supplemented breads, suggesting that crumb firming and starch recrystallization are separate processes (Dragsdorf and Varriano-Marston 1980). This indicates that the formation of a structured network in which multiple crystalline and amorphous regions are linked by large starch polymers may contribute more to bread firmness than the extent and quality of crystallinity (Zobel and Kulp 1996). In this respect, a recent bread staling model proposed that both an increase in granular rigidity (due to molecular rearrangements in the amylopectin-rich and amylose-rich regions of the swollen starch granules) and the formation of a structured network (comprising interlinked crystalline regions) contribute to bread firming (Hug-Iten et al. 2003).

Proteins

Composition and Structural Aspects

Traditionally, cereal proteins have been classified according to a solubility-based fractionation subsequently using water, a dilute salt solution, aqueous alcohol, and dilute acid or alkali (Osborne 1924). This way, wheat proteins are classified as albumins, globulins, gliadins (or wheat prolamins), and glutenins (or wheat glutelins), respectively. However, due to their inextractability, some of the wheat proteins are not included in these fractions. In addition, the distinction between the groups is not always clear. In this respect, the Osborne fractionation does not provide a clear separation of proteins differing biochemically/genetically or in functionality during breadmaking (Goesaert et al. 2005, Veraverbeke and Delcour 2002).

Today, wheat proteins are preferentially classified from a functional point of view. Two groups of wheat proteins can be distinguished: the nongluten and the gluten proteins. The nongluten proteins (ca. one-fifth of the wheat proteins) are mostly found in the Osborne albumin and globulin fractions. Generally, they are monomeric metabolic or structural

proteins with either no role or only a minor role in breadmaking (Veraverbeke and Delcour 2002). The gluten proteins (ca. four-fifths of the wheat proteins) are largely insoluble in water or dilute salt solutions. They are the major storage proteins of wheat, and more, in particular, belong to the prolamin class of seed storage proteins (Shewry and Halford 2002). They play a crucial role in breadmaking. The gluten proteins can be classified as either gliadins or the functionally distinctly different glutenins.

The gliadins form a highly heterogeneous group of monomeric gluten proteins, soluble in aqueous alcohol and ranging in molecular weight (MW) from 30,000 to 80,000. Biochemically, they can be further classified into three groups, that is, α-, γ-, and ω-gliadins (Shewry et al. 1986, Veraverbeke and Delcour 2002). Glutenins consist of a heterogeneous mixture of polymers with a broad MW range from approximately 80,000 up into the millions. A large part is soluble in dilute acid conditions and make up the Osborne glutenin fraction. The glutenin polymers are composed of a variety of glutenin subunits (GSs), which are cross-linked by disulphide bonds. GSs can be obtained upon reduction of the disulphide bonds and are biochemically related to the gliadins. Four different types of GSs have been identified: the high molecular weight GSs (HMW-GSs: MW 65,000–90,000) and the B-, C- and D-type low molecular weight GSs (LMW-GSs: MW 30,000–60,000). Different views on the structure of the glutenin polymer exist and are reviewed elsewhere (Shewry et al. 1992, Veraverbeke and Delcour 2002). In general, gluten proteins contain high levels of glutamine and proline. In addition, the α- and γ-gliadins and the B- and C-type LMW-GSs are characterized by relatively high levels of cysteine and methionine, while the ω-gliadins, which lack cysteine residues, and the D-type LMW-GSs are poor in sulphur-containing amino acids. Furthermore, the disulphide bonds in gliadins are intramolecular, while the sulfhydryl groups of the GSs can form either intra- or intermolecular bonds.

Gluten Proteins in Breadmaking

Proteins, in particular the gluten proteins, determine to a large extent the breadmaking performance of wheat flour. Although some nongluten proteins, such as certain enzymes and enzyme inhibitors, affect breadmaking as well, the unique property of wheat flour to form a viscoelastic dough, which retains the carbon dioxide produced by the fermenting yeast, is primarily due to the gluten proteins. During breadmaking, the gluten proteins undergo a number of complex changes, which are often poorly understood.

When wheat flour is mixed with water, the flour is hydrated and the discrete masses of gluten proteins are disrupted and transformed into a continuous cohesive viscoelastic gluten protein network (Amend et al. 1991, Singh and MacRitchie 2001). At the same time, protein extractability increases (Graveland et al. 1980, Veraverbeke et al. 1999). Furthermore, resistance to dough mixing, as evaluated by, for example, Farinograph and Mixograph, initially increases until an optimum is reached. Longer mixing times result in overmixing and decreased dough resistance. During fermentation, the protein network is essential for the gas-retaining capacity of the dough and the dimensions of the final loaf. Furthermore, the proteins become less extractable, indicating further changes and repolymerization.

Mixing and rheological properties of the dough and its sensitivity to overmixing are largely determined by the quantity and quality of gluten proteins. Gluten protein quality depends largely on the gliadin: glutenin ratio of the proteins as well as on the composition, structure and/or size distribution of the glutenin polymers, as reviewed elsewhere (Goesaert et al. 2005, MacRitchie 1992, Veraverbeke and Delcour 2002). Due to their large size, the glutenins form a network that is mainly responsible for the elasticity and cohesive strength of the dough (Belton 1999, Ewart 1972), whereas the gliadins act as plasticizers and hence mainly contribute to the viscosity, plasticity, and extensibility of the wheat flour dough (Schofield 1985, Veraverbeke and Delcour 2002). Furthermore, in the dough structure, both covalent and noncovalent bonds are involved (Bushuk 1998, Wrigley et al. 1998). The importance of disulphide cross-links is well established, and oxidative processes are very important during dough development (Wieser 2003). Reduction of the disulphide bonds reduces glutenin polymer molecular weight and weakens the dough, while oxidizing agents have a strengthening effect. Furthermore, hydrogen bonds, although much weaker than covalent linkages, can contribute significantly to dough structure because of their large number. Other noncovalent interactions include hydrophobic and ionic interactions.

In the baking phase, heat induces drastic transformations in the starch (cf. *supra*) and gluten fractions, which result in the typical foam structure of baked bread. The changes in the gluten proteins are probably a combination of changes in protein surface hydrophobicity, sulfhydryl-disulphide interchanges, and formation of new disulphide cross-links (Jeanjean et al. 1980, Morel et al. 2002, Schofield et al. 1983, Weegels et al. 1994). During bread storage, gluten proteins may be involved in the staling process as well, although their role is still not clear (Gray and BeMiller 2003). It is widely accepted that gluten plays an important role in water redistribution in the staling bread. In addition, hydrogen bonding between starch and gluten may contribute to bread firming by forming a structured, more rigid protein-starch network. These interactions may be more important than (Martin et al. 1991) or as important as (Every et al. 1998) starch-starch interactions. Maleki et al. (1980) proposed that differences in staling rate of bread are due to the gluten. In contrast, several studies found no significant correlation between protein concentration and quality and crumb firmness (Gray and BeMiller 2003).

Nonstarch Polysaccharides

Composition and Structural Aspects

Cereal nonstarch polysaccharides (NSP) consist mainly of arabinoxylans (AXs), β-glucan, cellulose, and arabinogalactan-peptides. They can all be classified as dietary fiber constituents and health-promoting effects have been ascribed to some of them (Lanza et al. 1987). AXs constitute the largest fraction, that is, 85%, of the wheat endosperm cell wall NSP, which in turn make up 75% of wheat endosperm cell wall dry matter weight (Mares and Stone 1973). One-fourth to one-third of the 1.5–2.5% AX found in wheat flour endosperm is water extractable (Meuser and Suckow 1986). The remaining, water-unextractable, AXs (WU-AXs), are strongly cross-linked into the cell wall (Iiyama et al. 1994, Lam et al. 1992).

Both WU-AX composing molecules and water-extractable AXs (WE-AXs) comprise a polydisperse population of polysaccharides with one general structure. AXs contain a backbone of β-1,4-linked D-xylopyranosyl residues, either unsubstituted or substituted at the *C(O)-3* and/or the *C(O)-2* position with monomeric α-L-arabinofuranoside (Perlin 1951a,b). The *C(O)-5* of some of the arabinose residues are ester linked to ferulic acid (Fausch et al. 1963). The degree of substitution of AX is expressed by the arabinose to xylose ratio (A/X), with a typical average value of 0.5–0.6 for the general wheat WE-AX population (Cleemput et al. 1993), but extreme values of 0.31–1.06 for WE-AX subfractions (Dervilly et al. 2000). In AX structure models, the arabinose substituents are not evenly distributed along the AX chain. Highly branched regions are interlinked with lowly substituted, more open regions (Goldschmid and Perlin 1963, Gruppen et al. 1993). Different proportions of these differently substituted areas could explain the above mentioned variation in A/X values. Compared with WE-AX, only small differences in molecular weight (Meuser and Suckow 1986) and A/X ratios (Gruppen et al. 1993) were reported for alkaline-solubilized WU-AX (AS-AX).

Arabinoxylans in Breadmaking

Arabinoxylans contribute significantly to breadmaking quality. It is generally accepted that WE-AXs, particularly the HMW fraction, positively affect breadmaking properties, while high levels of WU-AX are generally considered as having a negative effect on bread properties (Courtin et al. 1999, Maat et al. 1992). AX functionality in cereal processing in general and breadmaking in particular is based on their unique physicochemical properties. Generally, these properties are determined by the water-extractable or -unextractable nature and molecular structure of these polysaccharides.

The solubility of WE-AXs, AS-AXs, and enzymically solubilized AXs (ES-AXs) in water or water-ethanol mixtures increases with decreasing molecular weight (Courtin and Delcour 1998), increasing degree of substitution (Andrewartha et al. 1979, Neukom et al. 1967), and increasing proportions of highly substituted regions (Andrewartha et al. 1979, Rybka et al. 1993). AXs in solution can give high viscosities that are mainly dependent on AX chain length (Dervilly-Pinel et al. 2001, Izydorczyk and Biliaderis 1995). In this respect, approximately two-thirds of the intrinsic viscosity of flour extracts is attributed to WE-AXs (Udy 1956). Furthermore, under oxidizing conditions, covalent coupling of WE-AX–bound ferulic acid residues may occur,

resulting in WE-AX cross-linking (Figueroa-Espinoza and Rouau 1998, Vinkx et al. 1991). This can result in a strong increase of viscosity of AX solutions or the formation of a gel at high AX concentrations (Izydorczyk et al. 1990). Another important property of WE-AXs is their ability to stabilize protein films against thermal disruption. This is presumably due to their viscosity-increasing effect (Izydorczyk et al. 1991, Sarker et al. 1998) and interactions with proteins in the liquid films (Sarker et al. 1998). Alternatively, some proteins associated with WE-AXs may have surface-active properties (Eliasson and Larsson 1993). WU-AXs have high water-holding capacity, a property that is equally attributed to WE-AXs when considered in dough systems. In freshly prepared dough, AXs are estimated to hold up to one-quarter of the water (Atwell 1998).

In breadmaking, upon addition of WE-AXs, dough consistency and stiffness are increased, while mixing time is decreased (Jelaca and Hlynca 1972). At a constant dough consistency the addition of WE-AXs increases baking absorption (Biliaderis et al. 1995; Jelaca and Hlynca 1971, 1972; Kulp 1968), does not affect (Kulp 1968) or increases mixing time (Biliaderis et al. 1995, Jelaca and Hlynca 1971, Michniewicz et al. 1991), lowers the energy input to achieve optimal mixing (Jelaca and Hlynca 1971), enhances dough resistance to extension, and decreases dough extensibility (Jelaca and Hlynca 1972). HMW WE-AXs (MW 201,000–555,000) have a more pronounced impact on baking absorption and development time than LMW WE-AXs (MW 50,000–134,000) (Biliaderis et al. 1995, Courtin and Delcour 1998).

In general, WU-AX addition has effects similar to those of WE-AXs (Jelaca and Hlynca 1971, Kulp 1968, Michniewicz et al. 1991). Fractionation-reconstitution breadmaking experiments equally showed a positive correlation between flour WU-AX level and baking absorption for endogenous WU-AX (Courtin et al. 1999). These experiments also demonstrated that higher flour WU-AX content resulted in decreased extensibility and increased resistance to extension (Courtin et al. 1999). This is in agreement with the hypothesis that WU-AX rich cell wall fragments impair optimal gluten development during dough preparation. However, other researchers observed no effect of WU-AX addition on dough extensibility properties (Jelaca and Hlynca 1971, Kulp and Bechtel 1963).

Although WE-AXs do not have elastic properties, they are believed to function somewhat as gluten during fermentation and the initial baking phase (Hoseney 1984): they slow down the release of carbon dioxide from the dough, thus improving gas retention properties. In this respect, fractionation-reconstitution experiments showed that an increased loaf volume goes hand in hand with an increased level of medium and high MW WE-AXs (Courtin et al. 1999). According to Gan et al. (1995), WE-AXs increase dough foam stability by increasing the viscosity of the dough liquid phase and, consequently, the stability of the gas cell liquid films. In this view, WE-AX mediated gas cell stabilization extends the oven rise and positively affects bread properties, such as crumb firmness, structure, and texture and loaf volume (Gan et al. 1995). Other researchers ascribe the positive influence of WE-AXs to the formation of a weak secondary network, which enforces the gluten network (Jelaca and Hlynca 1972).

Kulp and Bechtel (1963) observed that the addition of WU-AXs did not significantly affect gas retention and dough evolution compared with the control dough. In contrast, according to fractionation-reconstitution experiments, loaf volume and bread quality increase when the WU-AX content in the dough decreases (Courtin et al. 1999). The postulated negative impact of WU-AX can be attributed to (1) the formation of physical barriers for gluten during dough development, (2) the absorption of a large amount of water, making it unavailable for gluten development and film formation, and (3) the perforation of the gas cells causing them to coalesce (Courtin et al. 1999, Courtin and Delcour 2002).

As discussed earlier, bread stales during storage, a process that is generally attributed to starch retrogradation. Some authors suggest that AXs may sterically interfere with the intermolecular associations of starch and, in this way, may lower the rate of retrogradation (Kim and D'Appolonia 1977a,b). Other authors relate the impact of AXs on bread staling to their pronounced effect on the dough water distribution. After all, the rate at which starch retrogrades depends to a large extent on the amount of available water (Biliaderis et al. 1995, Eliasson and Larsson 1993, Gudmundsson et al. 1991, Meuser and Suckow 1986). In addition, the available water may act as a plasticizer in the gluten-starch matrix (Levine and Slade 1990).

LIPIDS

Classification

The wheat flour lipids form a highly heterogeneous group with different chemical structures and different compositions. The fatty acid pattern is dominated by the unsaturated linoleic acid (C18:2), with lower levels of palmitic (C16:0) and oleic acid (C18:1) (Eliasson and Larsson 1993, Hoseney 1994). Flour lipids occur both free and bound to various other cereal constituents, particularly starch and proteins. These different fractions can be distinguished based on their solubility under selective extraction conditions (Eliasson and Larsson 1993, Hoseney 1994).

Starch lipids (ca. one-fourth to one-third of total wheat flour lipids) comprise mostly lysophospholipids and are important minor constituents typical of cereal starches. They form inclusion complexes with amylose during starch gelatinization, although such complexes may already exist in native cereal starches as well (Morrison et al. 1993). Because of their tight association in the starch granules, the starch lipids are effectively unavailable until the gelatinization temperature is reached in the baking phase, and hence have little if any effect on breadmaking.

The nonstarch lipids (NSLs) (ca. two-thirds to three-fourths of total wheat flour lipids) consist of similar levels of polar and nonpolar lipids. Part of the NSL fraction, the so-called bound NSLs, cannot be extracted with nonpolar solvents, presumably because of their association with the flour proteins (Chung 1986). The free and bound NSL fractions have different compositions. The bound NSL fraction consists predominantly (ca. two-thirds) of nonpolar lipids, particularly triacylglycerols, while the free NSL fraction is dominated by the polar glyco- and phospholipids (e.g., digalactosyl diacylglycerols) (Chung 1986, Eliasson and Larsson 1993, Hoseney 1994).

Lipids in Breadmaking

Although the NSLs are a minor component of flour, they contribute significantly to breadmaking quality. It is generally accepted that the nonpolar flour lipids negatively influence baking performance, particularly loaf volume, which is presumably due to the presence of fatty acids (MacRitchie 1981). Polar lipids have a positive effect (Eliasson and Larsson 1993). Indeed, loaf volume is strongly correlated with the polar lipid content, the ratio of polar to nonpolar lipids, and the galactolipid content of the free NSLs (Békés et al. 1986, Chung et al. 1982, Matsoukas and Morrison 1991). Furthermore, Graybosch et al. (1993) reported a substantial positive contribution of the polar lipids to dough handling properties.

Lipid functionality in breadmaking strongly depends on their physical state (Eliasson and Larsson 1993, Gan et al. 1995) and is mainly attributed to their surface-active properties and their effect on the gas cell stability (Gan et al. 1995). Presumably, the polar lipids can form lipid monolayers at the gas-liquid interphase of the gas cells, thereby increasing the gas retention capacity of the dough. Furthermore, during mixing, dough lipids are affected by two specific processes. During dough development, a large fraction of the free NSLs become "bound," and their extractability is reduced. This is mainly attributed to specific interactions between lipids and proteins during gluten network formation (Addo and Pomeranz 1991, Chung and Tsen 1975). In addition, mixing under air induces oxidation of the polyunsaturated fatty acids, due to the endogenous or added lipoxygenases (cf. *infra*).

FUNCTIONALITY OF ENZYMES IN BREADMAKING

The application of enzymes in breadmaking to improve the processing and/or quality of flour, dough, and bread is well established. A whole range of enzymes, representing different enzyme classes, specificities and functionalities, is available (Table 19.1), and development of new enzymes continues. Whereas in the past hydrolytic enzymes were used almost exclusively, today oxidizing and/or cross-linking enzymes receive increasing attention.

Enzyme application has a number of advantages from both the producer's and the consumer's point of view (van Oort et al. 1997). From a technological perspective, several enzymes improve dough machinability and stability, which are important requirements in modern day breadmaking. Indeed, industrial automated processes subject the dough to mechanical vibrations during transport. In addition, decreased processing time and reduced energy input cut down production costs, and new technologies, related to refrigerated and frozen dough production

Table 19.1. Application of Different Enzymes in Breadmaking

Enzyme	Substrate	Action	Function in Breadmaking
Amylolytic enzyme (e.g., α-amylase; maltogenic α-amylase)	Starch	Hydrolysis of glycoside bonds of damaged and/or gelatinized starch Generation of LMW dextrins	Flour standardization Increased loaf volume Improved crumb texture and crust color Antistaling
Endoprotease	Gluten proteins	Hydrolysis of peptide bonds	Improved dough handling and machinability
Endoxylanase	Arabinoxylans	Solubilization of WU-AXs Degradation of WE-AXs, preferably to a limited extent	Improved dough handling and machinability Increased loaf volume Improved crumb texture Possibly antistaling
Lipase	Flour lipids and glycolipids	Reduction of level of triglycerides Generation of mono- and diglycerides Liberation of polyunsaturated fatty acids	Increased dough strength and stability Increased loaf volume Improved crumb texture
Glucose (hexose) oxidase	Glucose (and other mono- and oligosaccharides, e.g., maltose)	Generation of hydrogen peroxide (Formation of lactones)	Increased dough strength and stability Increased loaf volume Improved crumb texture
Lipoxygenase	Polyunsaturated fatty acids	Formation of hydroxyperoxides Generation of radicals Cooxidation of carotenoids and proteins	Improved dough handling Crumb bleaching
Transglutaminase	Proteins	Cross-linking of proteins by formation of covalent isopeptide bonds	Increased dough strength Increased loaf volume

and new baking methods, have been introduced. These developments rely heavily on the stability and manageability of the dough. Furthermore, enzymes can correct for the variable quality of wheat, which mainly originates from different climatological and environmental conditions, and lead to a more standardized flour quality. Many enzymes also improve several aspects of bread quality, including an increased bread volume, a finer and more even crumb structure, and a reduced staling (firming) rate. This meets consumer demands in terms of a large variety of healthy, high quality breads, which stay fresh for a longer period of time and are prepared without chemical additives. In this respect, the use of enzyme technology has been suggested as an alternative for emulsifiers and oxidizing agents, the most important chemical aids in breadmaking.

Amylolytic Enzymes

Classification

In the classification of glycoside hydrolases (GHs), based on structural and amino acid sequence similarities (Coutinho and Henrissat 1999, Henrissat 1991), a range of amylolytic enzymes are found in GH families 13, 14, and 15.

GH family 13, the so-called α-amylase family, consists of a variety of amylolytic enzymes hydrolyzing the α-(1,4)- and/or α-(1,6)-linkages in the starch polymers, such as α-amylases and debranch-

ing enzymes (Coutinho and Henrissat 1999, MacGregor et al. 2001, Svensson et al. 2002). α-Amylases (EC 3.2.1.1) are typical endo-enzymes, which more or less randomly hydrolyze the α-(1,4)-linkages in the starch polymers, generating LMW α-dextrins (Fig. 19.1) (Bowles 1996, Hoseney 1994). Maltogenic (EC.3.2.1.133) and other malto-oligosaccharide producing α-amylases also act on the α-(1,4)-bonds of starch. In many cases their mode of action is still much debated (MacGregor et al. 2001). However, they are supposedly mainly exo-acting enzymes that mainly release maltose or other malto-oligosaccharides from starch (Fig. 19.1) (van der Maarel et al. 2002). Pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), the most important debranching enzymes, hydrolyze the α-(1,6)-bonds, thereby removing the side chains (Fig. 19.1).

GH families 14 and 15 comprise β-amylases (EC 3.2.1.2) and amyloglucosidases (EC 3.2.1.3), respectively. These enzymes are typical inverting exo-amylases, which act on the α-(1,4)-linkages at the nonreducing ends of the starch molecules. β-amylase releases β-maltose until a branching point is encountered (Fig. 19.1). The end products of β-amylase action on starch are β-maltose and β-limit dextrins. In contrast, amyloglucosidase, also referred to as glucoamylase, has a limited activity on the α-(1,6)-bonds and can hence bypass the side chains. Theoretically, it can completely convert starch to β-glucose (Fig. 19.1) (Bowles 1996, Hoseney 1994).

Application of Amylases in Breadmaking

Amylases were among the first enzymes to be used in breadmaking and are today routinely added to flour. Amylase functionality in breadmaking comprises several aspects, including their action on native starch granules, their specificity, the formed degradation products, and the presence of enzyme inhibitors. In this respect, it is well known that proteinaceous α-amylase inhibitors are present in wheat. They have been extensively reviewed (e.g., Franco et al. 2002, Garcia-Olmedo et al. 1987, Silano 1987, Svensson et al. 2004). The cereal α-amylase inhibiting proteins are mainly found in two major families, that is, the "Kunitz"-type and the "cereal trypsin/α-amylase" or "chloroform/methanol extractable proteins" inhibitor family. The wheat "Kunitz"-type inhibitor (wheat α-amylase/subtilisin inhibitor or WASI) inhibits the germination specific, high pI cereal α-amylase 2, which is present in malted barley and wheat (Mundy et al. 1984), whereas the cereal-type α-amylase inhibitors target insect, mammalian, and/or bacterial α-amylases (Franco et al. 2002, Silano

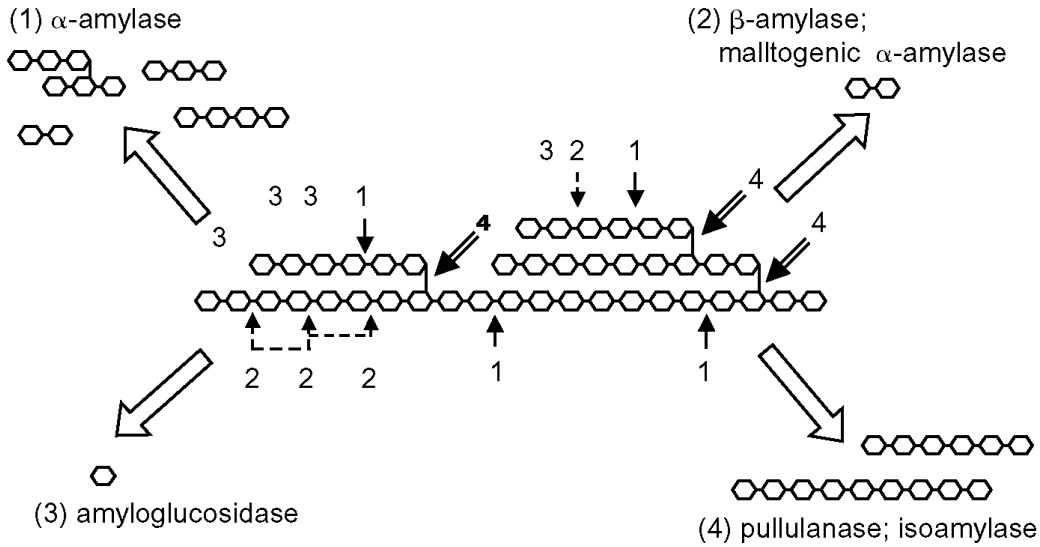

Figure 19.1. Action of different enzymes in the enzymic hydrolysis of starch; the gray ring structure represents a reducing glucose residue. (**1**) α-amylase action yielding branched and linear LMW dextrins. (**2**) β-amylase and maltogenic α-amylase yielding mainly maltose. (**3**) Amyloglucosidase action yielding glucose. (**4**) Debranching enzyme action yielding linear dextrins.

1987, Svensson et al. 2004). It is still unclear to what extent the wheat α-amylase inhibitors affect amylase functionality in breadmaking.

Rosell et al. (2001) found that commercial α-amylases from different sources (wheat flour, malted barley, fungi, bacteria) are affected to different degrees by process conditions and the presence of other dough ingredients. In addition, the thermal stability of the enzymes is an important parameter determining amylase functionality in breadmaking. In particular, fungal, cereal, and bacterial enzymes differ in their resistance to heat denaturation (Bowles 1996). In general, fungal enzymes are to a large extent inactivated at temperatures below that of starch gelatinization. Their main substrate is hence the easily degradable damaged starch fraction, because these enzymes are unable to degrade the native starch granules to a significant extent in breadmaking. In addition, their low thermostability reduces the risk of overdosing. In contrast, several bacterial amylases may survive the baking process. Their action during baking and storage is hence difficult to control, and upon overdosing, crumb structure properties can be negatively affected, resulting in, for example, gumminess. Enzymes with an intermediate thermostability are highly active at temperatures above the gelatinization temperature, when starch is easily degradable, but are inactivated by the baking process. They are hence less susceptible to overdosing (Hebeda et al. 1991).

In general, amylases are applied in breadmaking to optimize the amylase activity of the flour (i.e., flour standardization) and to retard bread staling.

Flour Standardization Amylolytic enzymes, in particular α- and β-amylases, are present in ungerminated wheat and hence in flour as well. However, amylase activity can vary considerably. Mature, ungerminated kernels of wheat and the other *Triticeae*, such as barley and rye, have high β-amylase levels. However, β-amylase exerts little if any activity on undamaged, native starch granules (Ziegler 1999). α-Amylase activity in ungerminated wheat is low, which results in high falling numbers. Amylolysis in the dough phase is thus limited, resulting in low bread volume and quality (Drapron and Godon 1987). Flour is routinely supplemented with amylases in order to optimize the amylase activity. It is widely accepted that the added amylases primarily function by increasing the level of fermentable and reducing sugars in flour and dough, thereby maximizing the fermentation (Bowles 1996). Indeed, the degradation of the more susceptible damaged starch granules by the supplemented α-amylase in the dough generates LMW dextrins. These are converted by the endogenous β-amylase to maltose (Kragh 2003, Linko et al. 1997), which can then be fermented by the yeast. Furthermore, an increased level of reducing sugars promotes the formation of Maillard reaction products, which intensify bread flavor and crust color (Bowles 1996, Drapron and Godon 1987). In addition, the degradation of damaged starch, which has a high hydration capacity, affects dough rheology (Drapron and Godon 1987). In this respect, several α-amylases have a dough softening effect (Dogan 2002, Harada et al. 2000, Si 1997). An alternative hypothesis relates amylase functionality to the reduction of dough viscosity during the initial stage of starch gelatinization. In this view, amylose leaching during gelatinization leads to a sudden viscosity rise, which terminates the oven spring. Amylases decrease the viscosity of the gelatinizing starch, thereby allowing for a prolonged oven spring and hence, increased loaf volume (Kragh 2003).

Amyloglucosidase can also be used to increase the levels of fermentable sugar and hence to support yeast fermentation. Although the high level of wheat β-amylase in flour generates sufficient levels of fermentable maltose (Bowles 1996), amyloglucosidase application may reduce fermentation time because glucose is fermented at higher rates than maltose. Furthermore, amyloglucosidase is used to reduce the level of added sugar and to improve crust color. Because this exo-amylase remains active after yeast inactivation, the generated glucose cannot be consumed and remains in the bread, thereby increasing sweetness and enhancing crust browning.

α-Amylase supplementation of flour has become standard practice. Most flour is supplemented at the mill or at the bakery by addition of a fungal α-amylase from *Aspergillus oryzae*, the so-called TAKA-amylase, or barley malt. Fungal amylase has a low thermal stability, making it less prone to overdosing. In addition, the use of fungal enzyme preparations has other advantages as well, such as more standardized protease levels compared with malt (Bowles 1996).

Antistaling Certain amylases can delay crumb firming and hence function as antistaling agents. Like the staling mechanism itself (cf. *supra*), the

mechanism or mode of action whereby these enzymes retard the staling/firming process, is still not completely understood. Obviously, (limited) amylolytic hydrolysis of starch yields enzyme specific LMW dextrins as well as a residual starch fraction. Several models, which generally focus on one of these products of amylolysis, propose different explanations for the antistaling action of these amylases.

Several authors suggested that the antifirming effect is due to production of dextrins of a specific size, which interfere with starch-starch interactions, in particular the reassociation and retrogradation of amylopectin (Defloor and Delcour 1999, León et al. 1997, Min et al. 1998), and/or with other interactions responsible for crumb firming (Akers and Hoseney 1994, Lin and Lineback 1990). In this respect, Martin and Hoseney (1991) attributed the antistaling effect to maltodextrins, which inhibit formation of starch-protein cross-links in aging bread. Several dextrin populations have been suggested as the agents responsible for the antifirming action of amylases, including branched LMW oligosaccharides originating from limited amylolysis of amylopectin (Lin and Lineback 1990), maltotriose and/or maltotetraose (Min et al. 1998), and maltodextrins of DP 3–10 (Martin and Hoseney 1991). However, the hypothesis that maltodextrins interfere with amylopectin retrogradation is somewhat in contradiction to the observed higher crystallinity in amylase-supplemented breads. In addition, other studies did not find a positive correlation between the presence of dextrins and an antistaling effect (Gerrard et al. 1997).

Other researchers believe that the presence of generated dextrins merely reflects the amylolytic modification of the starch, rather than inhibiting bread firming (Duedahl-Olesen et al. 1999, Gerrard et al. 1997). They attribute the antistaling effect of the amylases to the modified starch structure, which has different retrogradation properties. Zobel and Senti (1959) proposed that (endo-)amylolytic cleavage of long starch chains, which are the interconnecting links in the network of starch crystallites, results in a weakened and less rigid starch structure, and hence, a softer bread. Alternatively, the amylolytic removal, in particular by exo-acting amylases, of the easily accessible outer amylopectin branches of gelatinized starch, which protrude into the intergranular space, hinders double helix and crystal formation and/or amylose-amylopectin interaction (Bowles 1996, Zobel and Kulp 1996). In this respect, β-amylase was reported to reduce bread firmness (Martin and Hoseney 1991). Furthermore, the efficient antistaling effect of a maltogenic amylase from *Bacillus stearothermophilus* has been related to its action on the starch polymers as well (Hug-Iten et al. 2001, 2003). Presumably, this enzyme modifies both the amylopectin and the amylose structure. More particularly, the degradation of the amylopectin side chains, possibly by an exo-type action, hampers their reorganization (Hug-Iten et al. 2001, 2003). In addition, a limited degradation by an endo-mechanism presumably increases amylose mobility and facilitates its association, resulting in enhanced amylose crystallization and increased initial bread firmness. It is possible that the enhanced amylose aggregation contributes to the reduced bread firming rate. Hug-Iten et al. (2001, 2003) suggest that, because of rapid amylose aggregation, a weaker network is formed, which rearranges less on aging, or that further interaction between the starch polymers is hindered.

Many amylase-containing antistaling products are commercially available. Typically, they consist of bacterial or fungal α-amylases with intermediate thermostability (Bowles 1996, Hebeda et al. 1991). The above mentioned *B. stearothermophilus* maltogenic α-amylase is among the most effective antistaling agents available to date. This enzyme of intermediate thermostability, classified in GH family 13, shows significant sequence homology to cycloglycosyl transferases. In addition, compared with endo-α-amylases, it has some unusual structural (Dauter et al. 1999) and starch-degrading (Christophersen et al. 1998) properties. The mode of action of this enzyme is not completely understood. It produces mainly α-maltose from starch, suggesting an exo-type action. However, it has some endo-activity as well, as evidenced by the hydrolysis of amylose and β-limit dextrins (Christophersen et al. 1998, Outtrup and Norman 1984). Structurally, the position of the active site in an open gully is also consistent with an endo-action (Dauter et al. 1999).

Considerable research is still performed on the use of starch-converting enzymes as antistaling agents (van der Maarel et al. 2002), as evidenced by the large number of patents and publications. Research efforts focus on finding new enzymes, such as maltogenic and malto-oligosaccharide-producing amylases (Ben Ammar et al. 2002, Cherry et al. 1999, Kragh 2003, Kragh et al. 1999, Nielsen and Schäfer

2000) or modifing the properties of the enzymes, in particular their thermostability (Cherry et al. 1999, Maeda et al. 2003).

Proteases

Proteases catalyze the hydrolysis of peptide bonds in proteins. Endoproteases, also referred to as proteinases, cleave the internal bonds in the polypeptide chains, while exoproteases release amino acids from the chain ends. Most proteolytic enzymes can be classified according to the chemistry of their catalytic mechanism in serine, thiol or cysteine, metallo, and aspartic proteases, which require a hydroxyl group (serine residue), a sulfhydryl group (cysteine residue), a metal ion (e.g., zinc), and a carboxylic function (aspartic acid residue), respectively, at the active site to function properly (Mathewson 1998). In addition, many enzymes also prefer to cleave peptide bonds involving specific amino acids.

Proteolytic enzymes have been used for many years in some types of baked products, mainly to modify dough handling properties and machinability (Mathewson 2000). In breadmaking, proteinase application decreases mixing time by increasing the speed of water absorption (Lindahl and Eliasson 1992, Stauffer 1987). Furthermore, these enzymes increase dough extensibility, reduce dough consistency, and are used to control gluten strength (Kruger 1971). When used in suitable concentrations, proteinases improve the plastic properties of the dough, resulting in easier dough handling (Drapron and Godon 1987) and pan filling for rolls and buns (Bombara et al. 1997, Mathewson 2000). In biscuit manufacturing, where a weak gluten matrix is required, proteinases can increase dough elasticity and improve texture (Gaines and Finney 1989). They can be used to replace chemical dough softeners, such as glutathione or sodium metabisulphite (van Wakeren and Popper 2004). Clearly, proteinase functionality is due to the degradation of the gluten proteins, and thus affects the covalent interactions in the gluten network. Already very low protease levels have large effects on gluten physicochemical properties. In contrast to the impact of chemical reducing agents, the hydrolytic action is not reversible. In addition, proteinase action can be influenced by the presence of protease-inhibiting proteins in flour, such as WASI, a wheat "Kunitz"-type inhibitor which strongly inhibits subtilisin, and several members of the "cereal trypsin/α-amylase" inhibitor family, active against trypsin and/or chymotrypsin (Garcia-Olmedo et al. 1987, Stauffer 1987).

Exoproteolytic enzymes only have a limited impact on dough rheological properties. However, the generated free amino acids readily undergo Maillard-type reactions with reducing sugars and therefore contribute to color and flavor (Goesaert et al. 2005).

Endoxylanases

Whatever the functionality of different AX-fractions in breadmaking, on a commercial scale it is at present not possible to change dough properties through AX addition, due to the lack of industrially feasible AX isolation procedures and, therefore, the lack of commercial AX products. Optimization of AX functionality in breadmaking, however, is today obtained through the use of selected bacterial and fungal endoxylanases (EC 3.2.1.8). They are the key enzymes in the degradation of AXs as they are able to hydrolyze the AX xylan backbone internally. They are assisted by three types of exo-enzymes in achieving complete hydrolysis of AX, that is, α-L-arabinofuranosidases, β-D-xylosidases, and feruloyl esterases. From a breadmaking point of view, the latter enzymes are nowadays regarded as of minor importance, and as a consequence, they are not included in the present discussion.

Classification

Endoxylanases are mainly classified in GH families 10 and 11 (Coutinho and Henrissat 1999, Henrissat 1991), representing enzymes with different structures and catalytic properties (Biely et al. 1997, Jeffries 1996, Törrönen and Rouvinen 1997). Although endoxylanases of both families have similar catalytic residues and mechanisms, GH family 10 endoxylanases are regarded as less specific and more catalytically versatile, releasing shorter fragments than GH family 11 endoxylanases. The latter enzymes are more easily hindered by the arabinose substituents of AXs (Biely et al. 1997, Jeffries 1996). Some endoxylanases belonging to GH families 5, 8, and 43 have also been identified (Coutinho and Henrissat 1999), but so far few of them have been studied in detail, and little or nothing is known of their breadmaking functionality.

While each of the above cited GH families contains microbial endoxylanases, all plant endoxylanases thus far identified, including those of cere-

als, have been classified in GH family 10 (Simpson et al. 2002).

Application of Endoxylanases in Breadmaking

Specific microbial endoxylanases are routinely used in breadmaking to improve dough properties, such as softness, stability, elasticity and extensibility (Courtin et al. 2001, Rouau et al. 1994), oven spring (Sprössler 1997), and loaf volume (Courtin et al. 2001, Rouau et al. 1994, Sprössler 1997). The impact of endoxylanases on bread staling remains to be substantiated. Published data on their potential anti-staling properties are still controversial (Courtin et al. 2001, Martínez-Anaya and Jiménez 1997).

The performance of endoxylanases in breadmaking strongly depends on their activity towards WE-AX and WU-AX substrates (Fig. 19.2) (Courtin et al. 2001). In line with the above discussed WE-AX and WU-AX breadmaking functionalities, it can easily be apprehended that endoxylanases that have an (apparent) preference for WU-AX hydrolysis, that is, causing a reduction in the level of WU-AXs and an increase in the level of solubilized (HMW) AXs, improve dough and bread properties (Courtin et al. 1999, 2001). The use of a suitable endoxylanase in the first place increases dough stability, which means that throughout the fermentation stage the dough retains its optimal volume for a longer time and is more resistant to mechanical stress. This also implies that the oven rise, occurring during the initial stage of baking, is prolonged, leading to an increased loaf volume and a finer, softer and more homogeneous bread crumb (Courtin et al. 1999, 2001). In contrast to the above, endoxylanases that have a(n) (apparent) preference for WE-AX or solubilized AX hydrolysis, that is, reducing their molecular weight, result in little if any improvement (Fig. 19.2) (Courtin et al. 1999, 2001). Irrespective of their action towards either AX fraction, excessive endoxylanase levels result in slack and sticky doughs and loaves with poor crumb structure and color, poor gas cell distribution, and poor crust color. This effect is related to the extensive degradation of the general AX population and, therewith, the drastic loss in water-holding capacity of the dough

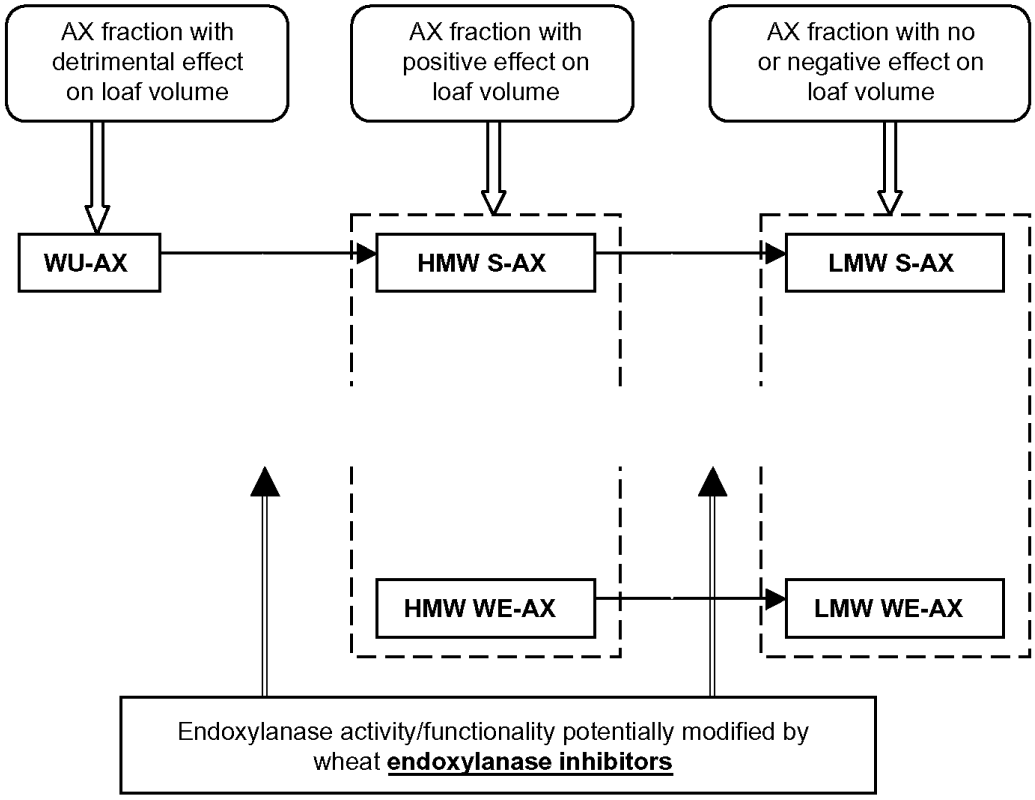

Figure 19.2. Schematic overview of arabinoxylan (AX), endoxylanase, and endoxylanase inhibitor functionality in relation to bread loaf volume.

(Courtin et al. 1999, 2001; McCleary et al. 1986; Rouau et al. 1994).

In addition to the apparent preferential hydrolysis of either WE-AXs or WU-AXs (substrate selectivity), their sensitivity towards wheat endogenous endoxylanase inhibitors is another important factor influencing endoxylanase breadmaking functionality (Fig. 19.2). These cereal proteins have been recently reviewed (Gebruers et al. 2004, Goesaert et al. 2004, Juge et al. 2004). To date, two endoxylanase inhibitors with different structures and specificities have been identified in wheat, that is, *Triticum aestivum* endoxylanase inhibitor (TAXI) (Debyser et al. 1999, Gebruers et al. 2001) and endoxylanase inhibiting protein (XIP) (Flatman et al. 2002, McLauchlan et al. 1999). The endogenous inhibitor levels will almost invariably exceed the level of microbial endoxylanase added for bread-improving purposes, potentially resulting in significant reduction of the activity of the inhibition-sensitive endoxylanases frequently used in breadmaking (Gebruers et al. 2002a, 2002b). In this respect, inhibition kinetics determines to a large extent the impact of the inhibitors in the dough phase. Indeed, while one endoxylanase may be almost immediately inhibited, other xylanases are not inhibited or only gradually lose their activity in the process due to inhibition (Gebruers et al. 2005, Trogh et al. 2004). Furthermore, it has been postulated that the inhibitors may affect the substrate selectivity of the endoxylanase by inhibiting the enzymic degradation of WE- and WU-AX fractions to different degrees (Sibbesen and Sørensen 2001). Recently, a mutant *B. subtilis* XynA endoxylanase that is insensitive towards wheat endoxylanase inhibitors and has improved breadmaking functionality was developed by molecular engineering (Sørensen 2003, Trogh et al. 2004).

Lipases

Lipases (glycerol ester hydrolases EC 3.1.1.3) hydrolyze ester bonds of acylglycerols, yielding mono- and diacylglycerols and free fatty acids. Generally, they act more readily on tri- and diacylglycerols, and many preferentially remove the fatty acids from position 1 and 3 of the glycerol residue (Mathewson 1998). Lipases usually function at the interface between aqueous and nonaqueous phases.

Commercial lipase application in breadmaking is quite recent compared with that of other enzymes. Some lipases, particularly those enzymes with 1,3-specificity, increase dough strength and stability, thereby improving dough machinability and increasing oven spring (Olesen et al. 1994, Poulsen and Borch Søe 1997, Si 1997). Therefore, they may be an alternative to the use of chemical dough strengthening agents and emulsifiers. Furthermore, lipase addition increases loaf volume and improves crumb structure and softness (Poulsen and Borch Søe 1997, Si 1997). In addition, lipases may have antistaling properties (Johnson and Welch 1968, Poulsen and Borch Søe 1997, Si 1997).

While several theories have been proposed to explain their effects, there is no generally accepted view on the way lipases contribute to bread quality. Undoubtedly, lipase functionality comprises several aspects (Castello et al. 2000, Poulsen and Borch Søe 1997). Hydrolysis of triacylglycerols reduces the level of the negative nonpolar flour lipids. At the same time, the level of emulsifying molecules increases, which may result in an increased stability of the gas cells (Castello et al. 1998, 2000). Furthermore, lipase action can improve the gluten network. By analyzing the biochemical changes induced by the exogenous lipases, Castello et al. (1998, 2000) proposed a more indirect impact of lipases on the gluten. Lipase action supplies polyunsaturated fatty acids to the wheat lipoxygenases, thus increasing the intensity of oxidation reactions during mixing and proofing. Alternatively, it has been postulated that lipases modify the interaction between flour lipids and the gluten proteins (Olesen et al. 1994) and improve glutenin/gliadin interaction (Poulsen and Borch Søe 1997). Several authors attributed the positive effects, in particular the antistaling effect, to the increased level of monoacylglycerols and the formation of amylose-lipid complexes (Johnson and Welch 1968, Poulsen and Borch Søe 1997). However, the in situ production of monoacylglycerol is probably insufficient to explain all reported effects (Si 1997).

Most commercial lipase preparations are from fungal origin. Recently, a lipase with high activity towards the polar lipid fraction has become commercially available (Christiansen et al. 2003). It converts phospho- and galactolipids (e.g., digalactosyl diacylglycerols) into even more polar lipids, such as digalactosyl monoacylglycerols. Its dough stabilizing effect may be related to the selectivity and rate of hydrolysis (Christiansen et al. 2003). Possibly in

a similar way, other lipolytic enzymes may improve breadmaking as well. In this respect, phospholipase A (EC 3.1.1.4), which liberates a fatty acid from phospholipids, has been claimed to improve dough handling properties, to reduce dough stickiness and to increase loaf volume (Inoue and Ota 1986).

Oxidoreductases

Oxidoreductases comprise a family of enzymes that catalyze oxidoreduction reactions. When oxygen is used as an electron acceptor, these enzymes are referred to as oxidases. Oxidase classification is based on the molecule or functional group that functions as electron donor (http://www.chem.qmul.ac.uk/iubmb/enzyme). Several oxidases, such as glucose and hexose oxidases, lipoxygenases, and peroxidases, can have a significant impact in breadmaking. In general, due to the great number and complexity of oxidoreduction reactions occurring during dough preparation (Nicolas and Potus 2000, Wieser 2003), the effect of these enzymes is not fully understood. Usually, their functionality is ascribed to the enhancement of oxidative cross-linking reactions between gluten proteins and/or AXs or to the decrease in the level of small thiol compounds, such as glutathione or cysteine (Nicolas and Potus 2000). These molecules participate in thiol-disulphide exchange reactions, leading to a weakening of gluten (Wieser 2003). However, the availability of oxygen may be a limiting factor due to oxygen consumption by the different redox systems and the yeast (Nicolas and Potus 2000).

Glucose and Hexose Oxidase

Glucose oxidase (EC 1.1.3.4) catalyzes the conversion of glucose and oxygen into gluconolactone and hydrogen peroxide (H_2O_2) (Fig. 19.3A). The former reaction product can in its turn convert spontaneously into gluconic acid. Hexose oxidase (EC 1.1.3.5) catalyzes a similar reaction but can convert several mono- and oligosaccharides into the corresponding lactones.

Glucose oxidase functions as an effective, fast-acting oxidizing agent in breadmaking (Vemulapalli et al. 1998). It increases dough strength and improves loaf volume and crumb structure. In addition, dough drying and reduced dough stickiness have been reported (Si 1997, Vemulapalli et al. 1998). However, at higher concentrations loaf volume is reduced, probably because of too high dough stiffness (Mitani et al. 2003). In breadmaking trials, hexose oxidase (from the red algae *Chondrus crispus*) has similar effects. It has a higher affinity for glucose and can use different substrates of which, in the case of breadmaking, maltose needs to be cited (Poulsen and Høstrup 1998).

The mechanism whereby glucose and hexose oxidases affect breadmaking is probably the same (Poulsen and Høstrup 1998). Probably, glucose and hexose oxidase functionality is related to the production of hydrogen peroxide. In one view, oxidation of free thiol groups by hydrogen peroxide results in formation of disulphide bridges between gluten proteins (Poulsen and Høstrup 1998) or reduces the level of reduced glutathione, which can weaken the dough (Mitani et al. 2003). Indeed, glucose oxidase decreases the thiol content of the dough (Poulsen and Høstrup 1998) or its SDS-soluble fraction (Vemulapalli and Hoseney, 1998). In addition, Primo-Martin et al. (2004) reported an increased incorporation of HMW-GSs in the SDS-unextractable glutenin polymer fraction. In contrast, several researchers did not observe significant differences in the solubility (Vemulapalli and Hoseney, 1998) or the size and profiles (Rosell et al. 2003) of the gluten proteins upon glucose oxidase supplementation. Alternatively, the produced hydrogen peroxide may activate the endogenous peroxidase system (Vemulapalli et al. 1998). This way, free radicals are formed, which may lead to macromolecular cross-linking by phenolic linkages, in particular the oxidative gelation of WE-AXs. Gelation may limit water mobility, resulting in drier dough (Vemulapalli et al. 1998).

Lipoxygenase

Lipoxygenases (EC 1.13.11.12) are iron-containing enzymes catalyzing oxidation of polyunsaturated fatty acids, in particular linoleic (C18:2) and linolenic (C18:3) acid, into their corresponding hydroxyperoxides in the presence of oxygen with intermediate formation of (free) radicals (Fig. 19.3B) (Nicolas and Potus 2000, Belitz and Grosch 1999). Type I lipoxygenase only acts on free fatty acids, generates few, if any, free radicals, and is characterized by a high stereo- and regioselectivity, while type II lipoxygenase has a lower reaction

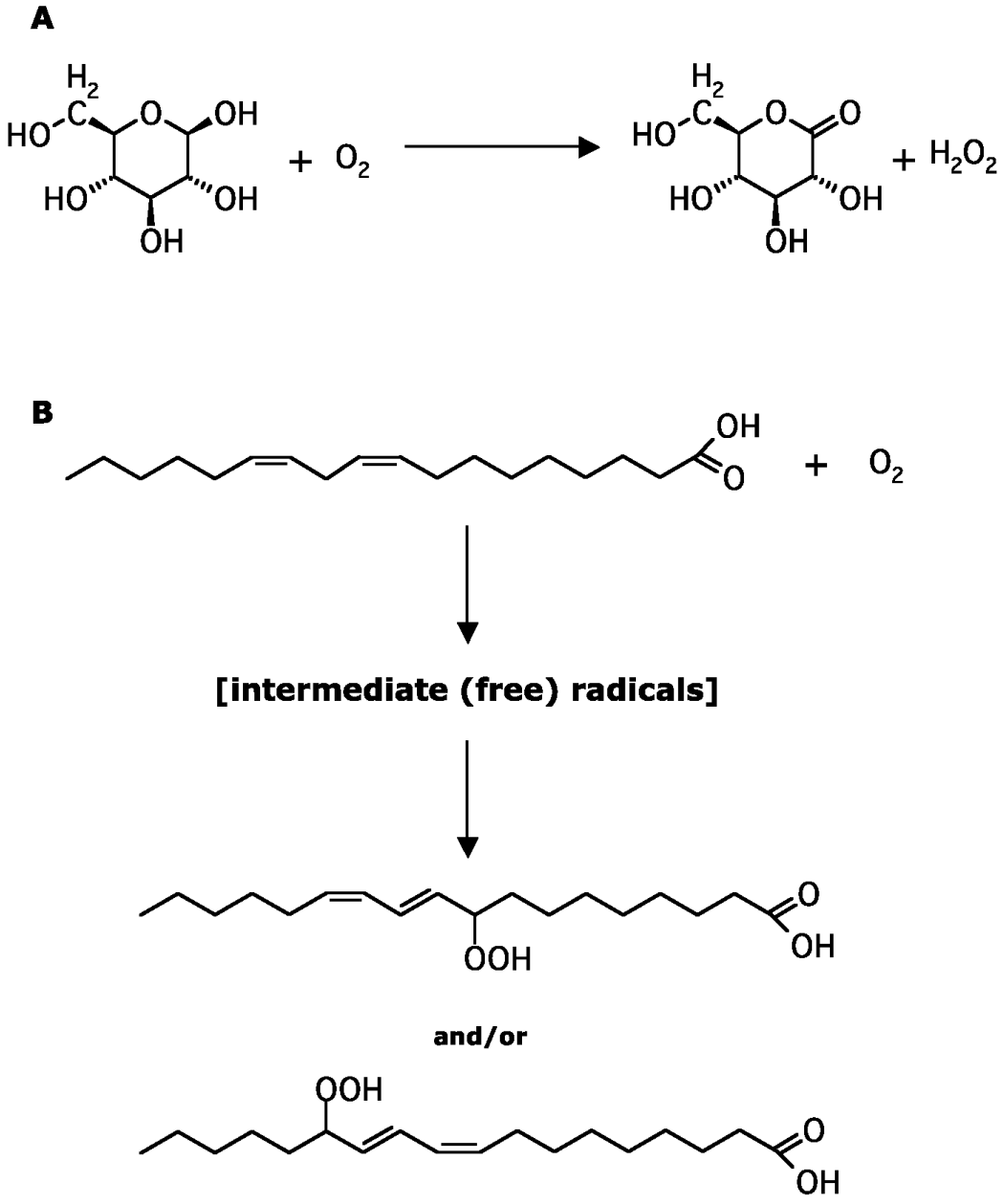

Figure 19.3. Primary reactions catalyzed by glucose oxidase (**A**) and lipoxygenase (**B**).

specificity and is able to react with esterified substrates, such as di- and triacylglycerols (Belitz and Grosch 1999, Mathewson 1998).

In breadmaking, lipoxygenase bleaches dough, resulting in a whiter bread crumb (Faubion and Hoseney 1981). Furthermore, lipoxygenase addition increases mixing tolerance and improves dough handling and rheological properties, resulting in an increased loaf volume (Hoseney et al. 1980, Faubion and Hoseney 1981). However, high lipoxygenase activities and the concomitant decomposition of the fatty acid hydroxyperoxides may lead to undesirable bread flavors.

Lipoxygenase functionality is related to the generated hydroxyperoxides and intermediate free radicals (Hoseney et al. 1980). These highly reactive substances can co-oxidize several substances, including lipophilic pigments and thiol groups (Nicolas and Potus 2000). In this respect, co-oxidation of carotenoids results in color loss. Furthermore, it has

been suggested that co-oxidation of accessible thiol groups leads to formation of intermolecular disulphide bridges between the gluten proteins, thus improving the gluten network (Casey 1997, Nicolas and Potus 2000). However, the cross-linking mechanism remains rather ill defined: the interaction between peroxidizing or peroxidized lipids and proteins or other flour constituents is complex (Matheis and Whitaker 1987). Whatever the case, in a tentative scheme for the enzyme-catalyzed redox reactions in dough, Nicolas and Potus (2000) proposed a central role for the lipoxygenase system.

Lipoxygenase supplementation generally comprises the addition of low levels of enzyme-active soybean or fava bean flour (Casey 1997, Mathewson 2000, Wieser 2003). Although wheat flour contains endogenous lipoxygenase, its role in breadmaking is unclear. During dough mixing, the wheat lipoxygenase may oxidize the polyunsaturated free fatty acids generated during the flour maturation period by the wheat endogenous lipase, thus affecting the oxidation reactions (Castello et al. 2000). On the other hand, the wheat lipoxygenase is a type I enzyme. Therefore, in contrast to the soy and fava bean type II lipoxygenases, its action is limited to free fatty acids, which may limit its functionality in breadmaking.

Other Oxidizing Enzymes

Many other oxidizing enzymes can promote cross-linking reactions of cereal proteins and/or phenolic carbohydrates and therefore have great potential in breadmaking applications (Reinikainen et al. 2003, Nicolas and Potus 2000).

Several oxidative enzymes, such as peroxidase and laccase, can oxidize phenolic compounds. Peroxidases (EC 1.11.1.7) comprise a family of oxidative enzymes that use hydrogen peroxide as an electron acceptor. A wide variety of substrates can be oxidized while generating radicals that can further react nonenzymically with other substrates (van Oort et al. 1997). Breadmaking trials with peroxidase addition showed a strengthening of the dough and improved loaf and crumb characteristics (van Oort et al. 1997). The generation of hydrogen peroxide may represent a limiting factor to the use of peroxidases as a dough-oxidizing agent. Laccases (EC 1.10.3.2) are copper-containing enzymes that catalyze the oxidation of different phenolic substrates to free radical products, which nonenzymically react with other compounds. Laccase is claimed to increase dough stability and dough strength and reduce dough stickiness (Si 1994). Primo-Martin et al. (2003) observed an increase in dough stiffness as well, but this effect was not statistically significant. Other researchers reported an accelerated dough formation and breakdown upon laccase addition (Labat et al. 2000).

The mode of action of these enzymes in breadmaking is less understood than that in model systems. The generated free radicals may have different fates in the dough. A first mechanism involves oxidation of ferulic acid esterified to AX (Hillhorst et al. 2000, Labat et al. 2000). Indeed, both peroxidases and laccases promote the oxidative gelation of AX in model systems (Fig. 19.4) (Figueroa-Espinoza and Rouau 1998). In wheat flour doughs, laccase decreases AX extractability by chain cross-linking through dimerization of the ferulic acid residues (Labat et al. 2000). Other researchers suggested that, in doughmaking, peroxidases only induce AX cross-linking (Hillhorst et al. 2000), thus forming a second network, independent of the gluten protein network (Dunnewind et al. 2002). Secondly, both cysteine and tyrosine residues may be involved in the oxidative covalent cross-linking of the gluten proteins (Matheis and Whitaker 1987). In this respect, laccase presumably promotes the formation of thiol radicals by a displacement reaction from phenoxyl radicals to thiol groups. Proteins or LMW thiol-containing molecules can participate in disulphide bonds, produced by dimerization of thiol radicals (Labat et al. 2000). In addition, gluten proteins may be linked to AXs by ferulic acid moieties and tyrosine or cysteine residues (Reinikainen et al. 2003, Nicolas and Potus 2000).

Because of the essential role of sulphide-disulphide interchange reactions in the development of the gluten protein network, enzymes acting on sulfhydryl groups and disulphide bonds can affect breadmaking as well (Reinikainen et al. 2003). Sulfhydryl oxidase catalyzes oxidation of sulfhydryl groups, resulting in the de novo formation of disulphide bridges and the formation of hydrogen peroxide (Matheis and Whitaker 1987). A mammalian sulfhydryl oxidase did not affect loaf volume, Mixograph measurements, or the level of free sulfhydryl groups in flour (Kaufman and Fennema 1987). However, several patents claim a dough improving

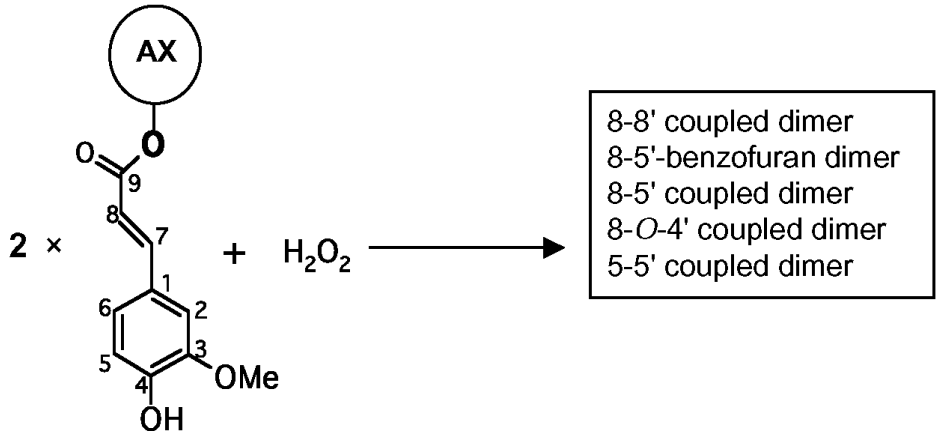

Figure 19.4. Schematic representation of the oxidative cross-linking of AX-esterified ferulic acid monomers (Based on Figueroa-Espinoza and Rouau 1998).

effect, with, for example, increased strength and resistance to overmixing when using a microbial sulfhydryl oxidase either alone (Verbakel et al. 1996) or in combination with glucose oxidase (Haarasilta and Väisänen 1989) or endoxylanase (Souppe 1996).

It is still unclear whether sulfhydryl oxidase action directly promotes the formation of intermolecular cross-links in proteins. Alternatively, the generated hydrogen peroxide, and the subsequent activation of the peroxidase system, may explain the observed effects (Nicolas and Potus 2000). Furthermore, oxidation of glutathione affects the participation of the glutathione thiol groups in the thiol-disulphide exchange reactions as well. In this respect, glutathione was very rapidly oxidized in model systems by an *Aspergillus niger* sulfhydryl oxidase (Louarme et al. 2003).

Transglutaminases

Transglutaminase (EC 2.3.2.13) is an acyl transferase catalyzing the transfer of the γ-carboxamide group of protein-bound L-glutamine to primary amines, such as the ε-amino group of protein-bound L-lysine, generating a so-called isopeptide bond (Fig. 19.5) (Matheis and Whitaker 1987). This way, new covalent nondisulphide cross-links are formed between peptide chains. Side reactions include deamination or amination of glutamine residues (Reinikainen et al. 2003).

In breadmaking trials, transglutaminase improved loaf volume and crumb texture and increased water absorption (Gerrard et al. 1998, Basman et al. 2002). Similarly, it improved the lift of puff pastry and the volume of yeasted croissants (Gerrard et al. 2000). Furthermore, it increased dough strength and stability and reduced extensibility (Larré et al. 2000, Basman et al. 2002, Bauer et al. 2003a). In addition, the required energy input for dough development in a high-speed mixing system was reduced (Gerrard et al. 1998). Weak flours were most affected by transglutaminase action (Larré et al. 2000), whereas high enzyme dosages, particularly in flour with high breadmaking quality, resulted in deteriorated dough handling properties and loss of dough structure, probably due to a too high dough strength (Basman et al. 2002, Bauer et al. 2003a).

In breadmaking, transglutaminase can modify the covalent linkages between gluten proteins. In contrast to oxidases, the formation of new inter- and intramolecular bonds is independent of the redox system in dough (Reinikainen et al. 2003). Due to their high glutamine content and despite their low lysine content, gluten proteins are a good substrate for this enzyme. Extractability studies and structural analysis confirmed the cross-linking of the gluten proteins in dough systems to larger, insoluble gluten polymers (Bauer et al. 2003b, Larré et al. 2000). HMW-GSs are affected to a large extent by transglutaminase action, but gliadins are incorporated as well (Bauer et al. 2003b, Larré et al. 2000, Rosell et al. 2003).

Today, the enzyme's use in the manufacture of baked goods is still relatively new. Transglutaminase-

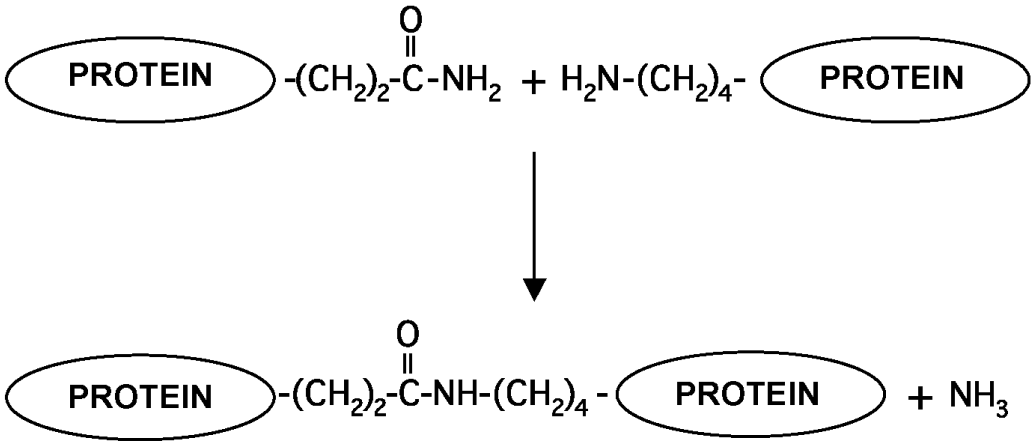

Figure 19.5. Cross-linking reaction between protein-bound glutaminyl and lysyl residues catalyzed by transglutaminase.

containing enzyme preparations are suitable for a multitude of baking applications (Diez Poza 2002), particularly those with weaker protein network structures. In the production of frozen doughs, transglutaminase action increases the freeze-thaw stability of the gluten network. In this respect, this enzyme is able to limit the deterioration of puff pastry and croissant doughs in frozen storage (Gerrard et al. 2000). Like the oxidizing enzymes, it can be used in enzyme formulations to replace the use of chemical emulsifiers and oxidizing agents (Diez Poza 2002). Other (potential) applications include the production of high-fiber and rye breads and gluten-free baked goods, where transglutaminase action can significantly improve the protein network (Reinikainen et al. 2003, Diez Poza 2002). Finally, Köksel et al. (2001) reported an improved baking performance of heavily bug-damaged wheat with transglutaminase addition. The enzyme restored the polymeric glutenin structure, which had been degraded by the grain pest proteases.

SYNERGETIC EFFECTS

Many commercially available enzyme preparations include multiple enzyme specificities. Several enzyme combinations have synergistic effects in breadmaking. Using enzyme combinations often permits lower dosages of the individual enzymes to obtain the same effect. In addition, the positive effects of the individual enzymes can be combined or the possible negative side effects of one enzyme can be nullified. However, the use of these enzyme combinations is even more empirically driven than the use of the individual enzymes because, as indicated before, in many cases the mechanism underlying the functionality of the individual enzymes in breadmaking is insufficiently understood. Furthermore, it is not clear at this time how well enzyme cocktails, which are claimed to have the potential to replace chemical oxidizing agents, function in a variety of baked goods and processes (Mathewson 2000).

An important example is the combination of endoxylanases and glucose oxidases, which can potentially replace certain emulsifiers and oxidizing agents, such as bromate and ascorbic acid. The dough strengthening and drying effect of glucose oxidase can remedy problems with dough handling associated with high endoxylanase dosages, such as soft and sticky doughs (Mutsaers 1997). This was confirmed by Martínez-Anaya and Jiménez (1998), who reported that the combination of glucose oxidase or specific lipases with endoxylanases reduced the endoxylanase-induced dough stickiness. It has been suggested that endoxylanase may remove interfering compounds, thereby promoting the oxidation and repolymerization of the glutenin polymers (van der Lugt et al. 1997). In addition, Primo-Martin et al. (2003) suggested that small ferulic acid-containing AX fragments, generated by endoxylanase action, affect AX gelation by the glucose oxidase.

Another type of synergy is seen with the combination of endoxylanases and cellulolytic enzymes (Hille and Schooneveld-Bergmans 2004). Probably, the enzymic removal of cellulose promotes the accessibility of the WU-AX polymers in the cell wall fragments to the endoxylanases. Further, the combined use of amylase and lipase was reported to improve bread quality and keeping properties (León et al. 2002). In the enzyme-supplemented breads, a more thermostable amylose-lipid complex was found, and amylopectin retrogradation was inhibited.

Synergetic effects have also been observed between enzymes with similar action. Hille and Schooneveld-Bergmans (2004) described the synergy between bacterial and fungal endoxylanases in their effect on dough characteristics, loaf volume, and crumb softness.

CONCLUSIONS

In modern day baking, the application of enzymes is well established. Both the amount and diversity of enzymes used is increasing. They are well suited to maximize the flour potential and meet the demands of industrial breadmaking, which is a dynamic, continuously changing process, and of the consumer, who desires high quality, nutritious, and "clean label" products. Selected enzymes can improve many aspects of processing (e.g., dough handling and stability properties) and product quality (e.g., crumb texture and shelf life), can minimize the effect of wheat flour variability, and can replace chemical additives.

However, enzyme applications in breadmaking remain to a large extent empirically driven. Indeed, during the transformation of flour to dough to bread, which then firms during storage, a series of complex biochemical, chemical, and physical processes, which are often poorly understood, occur. Therefore, it has in many instances not been possible to elucidate the mechanism underlying the enzyme-induced improving effect. However, the use of new, well-characterized enzymes will undoubtedly contribute to a better understanding of breadmaking and the role of the different flour constituents and enzymes in this process. In general, hydrolases act in a direct manner by generating modified wheat flour constituents with different properties, by removing components detrimental for breadmaking, and/or by liberating degradation products. Oxidoreductases act generally more in an indirect manner. Their action generates hydrogen peroxide or free radicals, which then react with and modify different flour constituents, often in a nonenzymic manner. In addition, enzyme application to obtain optimal, desirable effects depends to a large extent on enzyme specificity and properties (such as thermal stability). Processing time, the presence of inhibiting substances, and enzyme concentration are also important factors determining enzyme functionality.

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