A QUANTITATIVE METHOD TO EVALUATE THE EFFECT OF XYLANASES IN BAKING

by

JESSE STINSON

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Approved by:
Major Professor
Dr. Fadi Aramouni

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Abstract

β-(1,4)-endoxylanases, commonly referred to as xylanases, have become integral to the industrial breadmaking process. This enzyme is known to cause improvement in dough rheology, loaf volume, and crumb grain. Significant research has been conducted regarding the structure, function, and inhibition of xylanases, but there is currently no quick and reproducible method to evaluate their effect in baking. The goal of this research was to develop a quantitative method for this purpose and to determine why the effect of xylanases varies with different wheat flours. The currently used methods of test baking, dough stickiness, and spectrophotometric analysis for reducing sugars were evaluated, and failed to provide reproducible results. Therefore, a new method was developed to measure the Flour Water Expression Rate (FWER) with the addition of xylanases. Commercially available enzymes from *Aspergillus niger* and *Bacillus subtilis* were evaluated in this study. The FWER method measures the amount of water released by the xylanase over a set period of time. This method consistently provided statistically significant data ($p < 0.05$), which was able to provide a comparison of xylanases from *A. niger* and *B. subtilis* in different flours. The results indicated that the xylanase from *A. niger* tends to release more water, have a higher FWER value, than the xylanase from *B. subtilis*. In one flour, *A. niger* xylanase resulted in an FWER of 15.18 compared to *B. subtilis* xylanase that resulted in an FWER of 9.57 at equivalent activities. However, inhibitors in the wheat appeared to cause an impact on the FWER, which was evaluated with an uninhibited xylanase from *B. subtilis*. This new method for the evaluation of xylanases in baking suggests varying levels of xylanase inhibitors in wheat may be the reason xylanases effect wheat flours differently.

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Chapter 1 - Literature Review

Bread products have been a staple in the diets of people from many different cultures for thousands of years. Traditionally, bread products are made with wheat flour and have evolved dramatically over the years (Cauvain and Young 1998). The current industrial breadmaking process utilizes new technologies to improve processing and product quality for high speed processing equipment. Enzymes in particular have become an integral ingredient in breadmaking. This work takes a closer look at one particular group of enzymes, the β-(1,4)-endoxylanases, further referred to as xylanases.

Basics of Breadmaking

The production of bread requires four basic ingredients: flour, water, yeast, and salt, although a variety of optional ingredients that can be added to improve processing, flavor, and overall product quality. Some of these optional ingredients include: sweeteners, lipids, oxidizing agents, reducing agents, emulsifiers, vital wheat gluten, antimicrobials, and enzymes (Cauvain and Young 1998, Pyler and Gorton 2009). The first step in breadmaking is the process of mixing. The mixing stage has three objectives: to combine the ingredients homogeneously, to develop the dough, and to incorporate air. Dough development is the creation of a 3-D gluten network that provides gas retention and expansion properties to the dough (Pyler and Gorton 2009). Water absorption plays an important role in the mixing process, and also affects dough handling properties and final bread quality.

In a sponge and dough process of breadmaking a portion of the ingredients are mixed briefly, and then fermented for 2-4 hours at 23-26°C. This fermentation process develops flavor compounds, decreases the pH of the dough, and improves dough processing. After sponge

fermentation, the remaining ingredients are added to the sponge (previously fermented material) and mixed to develop a dough (Pyler and Gorton 2009).

The fully developed and fermented dough is then divided into the appropriate weight and rounded into dough pieces. After resting, dough pieces are sheeted then moulded into the desired shape, and placed in a greased pan. Dough is then proofed for approximately 1 hour at 41-46°C at a relative humidity of 75-80%. The fermentation steps allow the yeast to produce carbon dioxide which causes the dough to expand. After proofing, the dough is baked at 204-238°C for 16-22 minutes for white pan bread. The baking process further expands and then sets the dough structure and will inactivate the yeast and enzymes. Bread is cooled to an internal temperature around 34-41°C before being sliced and packaged (Pyler and Gorton 2009).

Basics of Enzymology

Introduction

An enzyme is a protein that catalyzes a chemical reaction and can increase the rate of reaction by anywhere from 10^3-10^{11} fold. Enzymes are ubiquitous in living organisms and play important functions in many biological processes (Witaker 1996). In recent years exogenously added enzymes have come to play a major role in the breadmaking process. In bread production enzymes are used for such purposes as shelf life extension, improving dough handling, and improving finished product quality (Pyler and Gorton 2009).

Structure and Kinetics

Enzymes are proteins that have primary, secondary, tertiary, and sometimes quaternary structures. One very important aspect of an enzyme's structure is the active site, where the reaction is catalyzed. Enzymes have three different classes of active sites: pocket/crater, cleft/groove, and tunnel (Davies and Henrissat 1995). Enzymes are very specific to a particular

substrate. An enzymatic reaction involves the active site of an enzyme interacting with the substrate to form an intermediate. The intermediate is then converted into products, while the enzyme is unchanged. This process is captured in Equation 1.1 and includes the appropriate rate constants (K_1, K_{-1}, and K_2) for each step in the process.

Equation 1.1 Enzyme Kinetics Equation.

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$$
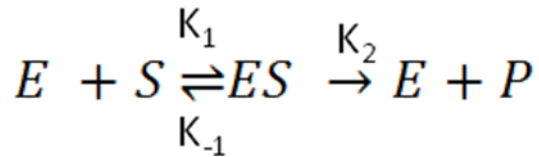
E=Enzyme, S=Substrate, P=Product

An important relationship in enzyme kinetics is the Mechaelis Menten Equation, Equation 1.2.

Equation 1.2 Mechaelis Menten Equation.

$$V_0 = \frac{V_{max}\,[S]_0}{K_m + [S]_0}$$

In this equation the reaction rate (v) is defined by the change of product concentration divided by change of time. Where V_o is initial velocity, V_{max} is maximum velocity, $[S]_o$ is initial substrate concentration, and Km is the Michaelis-Menten constant. Figure 1.1 is a visual representation of this model. Under basic assumptions, where k_1, k_{-1}, k_2 are the first order rate constants found in Equation 1.1 (Witaker 1996).
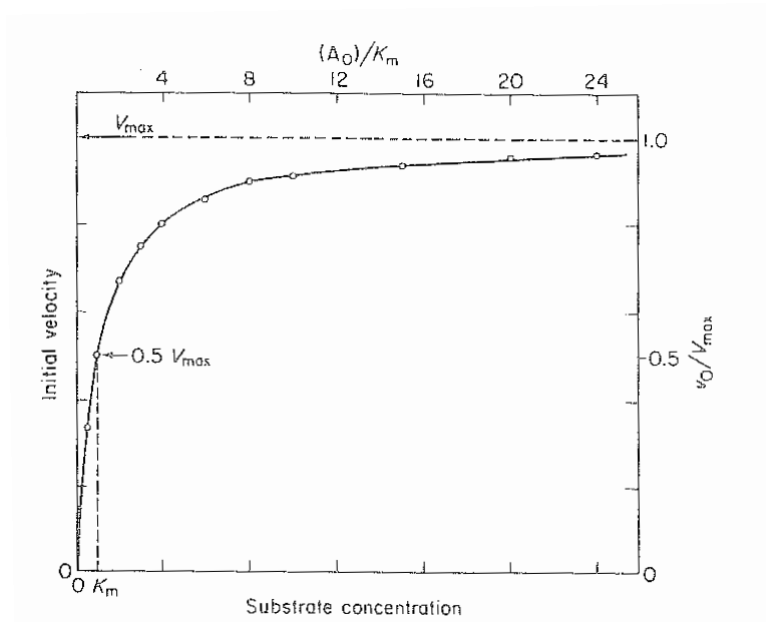
Figure 1.1 Michaelis-Menten kinetics, adapted from Witaker (1996).

There are six types of enzyme catalyzed reactions: oxidoreduction, transfer, hydrolysis, formation of double bonds without hydrolysis, isomerization, and ligation (Witaker 1996). Xylanases catalyze hydrolysis reactions. Many important factors affect enzyme reactions: temperature, pH, enzyme concentration, substrate concentration, and water activity are some of the most important. Enzymes can be inactivated by a number of different conditions including: temperature extremes, pH, pressure, radiation, solvents, and shearing (Witaker 1996).

Enzyme Inhibitors

Specific enzyme inhibitors also affect enzyme activity. These inhibitors are commonly found in many plants and food ingredients (Witaker 1996). There are two types of inhibitors, reversible and irreversible. Reversible inhibitors form noncovalent bonds with the enzyme and exist as four types: competitive, noncompetitive, uncompetitive, and allosteric. When both the inhibitor and the substrate compete to bind with the enzyme's active site, the inhibition is competitive. In noncompetitive inhibition, the substrate and inhibitor are able to bind to the

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enzyme at the same time. In uncompetitive inhibition, the inhibitor binds to the enzyme-substrate intermediate, rather than the enzyme. Allosteric inhibition behavior generally occurs when the inhibitor binds to multiple enzyme subunits within the quaternary structure of the enzyme. Irreversible inhibitors bond covalently to enzymes, which results in their inactivation (Witaker 1996).

Enzymes in Breadmaking

Amylases

Exogenously added enzymes play an important role in breadmaking systems, and are used mainly for shelf life extension, enhancement of dough properties, and improved loaf volume. One of the major contributors to the baking industry are amylases. Amylases hydrolyze starch polymers and exist as two types, α and β. α-amylases are members of the Family 13 glycosyl hydrolases, and cleave the interior α-(1,4) bond of amylopectin and amylose, producing dextrins (Goesaert et al 2009). Some amylases are used to decrease the rate of staling and improve loaf volume of bread (Pyler and Gorton 2009). Fungal sources of α-amylase do not have a large role in shelf life extension because they are largely inactivated before the onset of starch gelatinization (around 60°C). Bacterial α-amylases are generally more heat stable and, therefore, play a larger role in the baking process. They can also produce crumb gumminess if not completely deactivated during baking (Pyler and Gorton 2009). β-amylases are members of the Family 14 glycosyl hydrolases, and cleave alternate α-(1,4) bonds of the starch polymers from the non-reducing end, producing maltose. Maltogenic amylase is a member of the Family 13 glycosyl hydrolase family and is commonly used for shelf life extension of bread products by decreasing the rate of firming. It is an exo-acting bacterial amylase that produces maltose, and is considered to be inactivated during baking (Goesaert et al 2009, Pyler and Gorton 2009).

Glycoamylases are exo-acting and hydrolyze the α-(1,4) bonds in starch to produce glucose.

Pullulanases cleave the α-(1,6) bonds in starch, and are commonly called debranching enzymes

(Goesaert et al 2009). The mode of action for these amylases is portrayed in Figure 1.2.
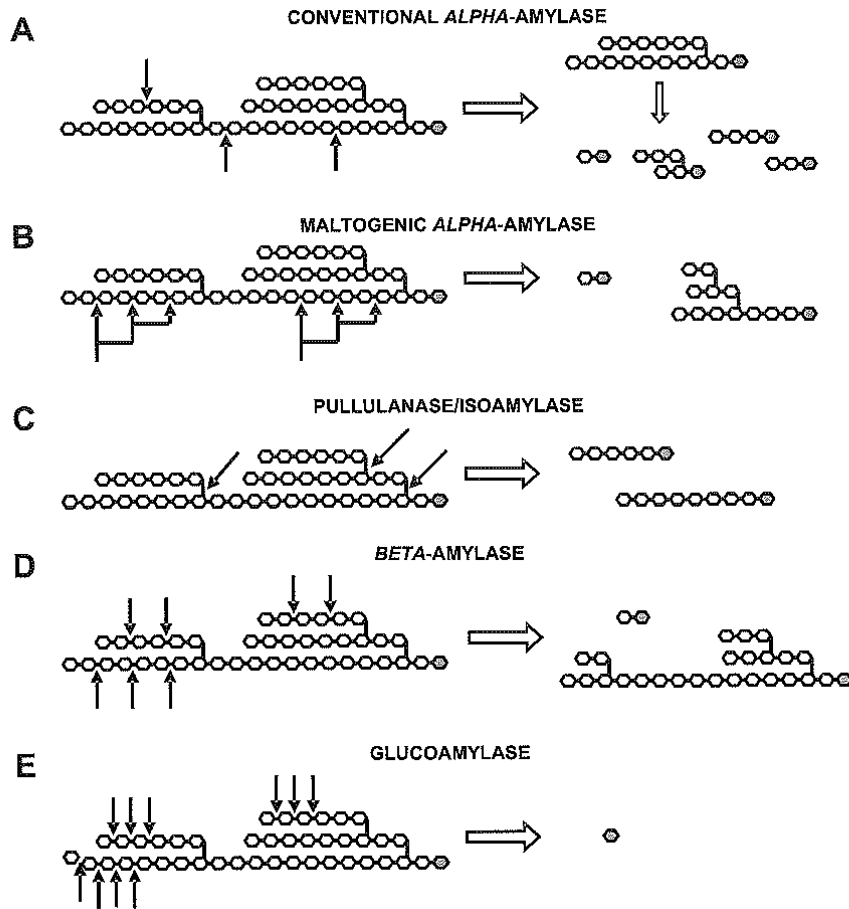
Figure 1.2 Mode of action for common amylases in baking adapted from Goesaert et al, 2009.

Lipases

Lipases are added in breadmaking to improve dough rheology and loaf volume. They

hydrolyze triglycerides to form mono-and-diglycerides, which are emulsifying agents, while

releasing free fatty acids (Pyler and Gorton 2009). In the last 20 years, three types of lipases

have been used in the baking industry. The first type of lipase hydrolyzes nonpolar triglycerides

at the 1 and 3 positions, resulting in the production of free fatty acids and mono-glycerides. The

removal of fatty acids from the glycerol backbone increases the polarity of the lipids. The second type of lipase hydrolyzes nonpolar and polar lipids, resulting in similar products as the first type, but with greater polarity. The newest lipases are claimed to be more consistent across different crops of flour, and improve expansion of the gluten network. In recent years lipases have been commonly used as clean label replacements for emulsifiers, because they perform similar functions (Moayedallaie et al 2010).

Proteases

Proteases hydrolyze the peptide bond between amino acids in proteins, which can modify or weaken the gluten network in dough. These enzymes are used to improve pan flow of the dough or act as a reducing agent to decrease mix time. A major challenge with the use of these enzymes is a lack of control of their activity. Too much enzyme action will degrade the gluten network excessively; this risk limits the use of proteases in breadmaking (Pyler and Gorton 2009). Research has also suggested proteases are capable of improving the shelf life of bread by delaying crumb firming (Barrett et al 2005).

Transglutaminase

Transglutaminase catalyzes three separate reactions: crosslinking of protein through glutamine and lysine amino acids, integrating proteins with free amines, and converting glutamine into glutamate. These processes have been shown to improve dough characteristics. It has been suggested that this enzyme crosslinks small globulin and albumin protein units into larger masses, which are beneficial in breadmaking (Gerrard et al 2001). In simple terms, some of the baking industry uses transglutaminase to improve dough strength (Pyler and Gorton 2009).

Glucose Oxidase

Glucose oxidase is an enzyme used commercially to replace chemical oxidants and dough strengthening emulsifiers such as azodicarbonamide (ADA) and diacetyl tartaric acid ester of monoglycerides (DATEM). This enzyme catalyzes the production of hydrogen peroxide and gluconic acid from glucose. The hydrogen peroxide produced in the reaction causes disulfide bonds to form in gluten, which improves dough strength. Glucose oxidase has been shown to increase loaf volume and decrease crumb firmness at optimum levels, but an overdosage causes a decrease in volume (Bonet et al 2006). The research of Rasiah et al (2005) suggests that glucose oxidase leads to the formation of dityrosine linkages as well as disulfide linkages.

Xylanases

Xylanases hydrolyze the non-starch polysaccharide, arabinoxylan. These enzymes are used to improve dough handling properties, pan flow, and increase bread volume (Pyler and Gorton 2009). This research focuses on the use of xylanases in bread. Therefore xylanases will be discussed in greater detail in the coming sections.

Arabinoxylan – Xylanase Substrate

Structure

The substrate for xylanases is arabinoxylan, a non-starch polysaccharide found in plant cell walls (Meuser and Suckow 1986, Prade 1995). The term pentosan was commonly used in the past when discussing non-starch polysaccharides composed of 5-carbon sugars. However, this terminology is vague and should be replaced with the specific compound of interest, generally arabinoxylan (AX) or arabinogalactan (AG) (Yeh et al 1980, Perlin 1951a). Non-starch polysaccharides as a whole make up 1.5-3% of wheat flour. They have extremely high water binding capacities, being able to absorb around 20-23% water (Izydorczyk et al 1990,

Meuser and Suckow 1986, Yeh et al 1980, Cleemput et al 1993, Pyler and Gorton 2009).

Arabinogalactan (Figure 1.3) are similar in structure to arabinoxylan (Figures 1.4 and 1.5); however their backbone is a linear chain of galactose rather than xylose. They also have significant protein content, contain no ferulic acid, and have a larger molecular weight (Fincher et al 1974, Izydorczyk et al 1990).

Proposed model of the arabinogalactan-peptide

Galactose (○); arabinose (●); hydroxyproline (■); other amino acids (□).
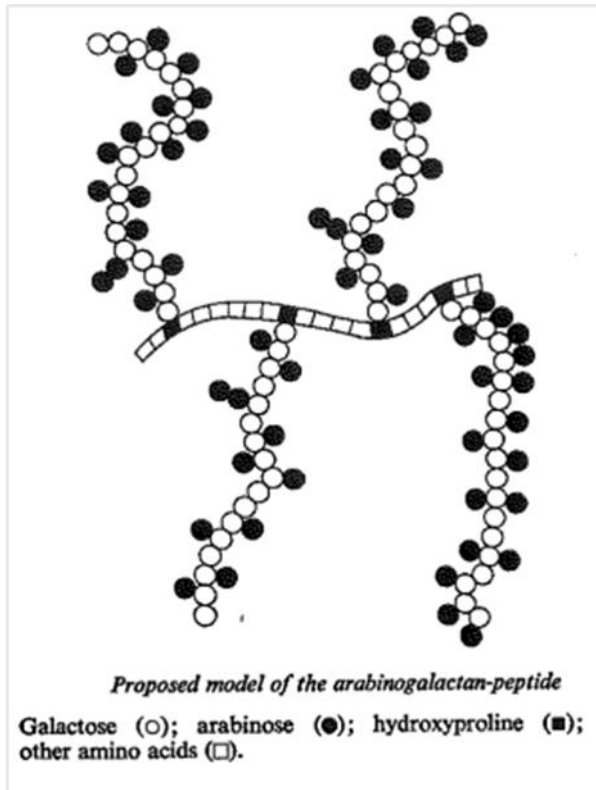
Figure 1.3 Arabinogalactan-peptide structure from Fincher et al (1974).

Arabinoxylan are composed of a linear backbone of (1,4) linked β-D-xylan with side chains of arabinose and ferulic acid (Perlin 1951b, Goldschmid and Perlin 1963, Prade 1995). The various side chain configurations are pictured in Figure 1.4. The arabinose branches are generally single unit side chains, but xylose can also be disubstituted with arbinose (Gruppen et al 1992). Ferulic acid may also be bound to the number 5 carbon of arbinose. The amount of branching in AX is reported as the arabinose/xylose ratio and is commonly used to characterize

AX. This ratio ranges from 0.45 to 0.63 in wheat AX (Izydorczyk et al 1990, Dervilly et al 2000, Loosveld et al 1997, and Cleemput et al 1993). It is known to differ in different structural regions of the caryopsis.

(A): non-substituted D-xylopyranosyl-residue, (B): D-xylopryanosyl residue substituted on C(O)-2 with a L-arabinofuranosyl residue, (C): D-xylopryanosyl residue substituted on C(O)-3 with a L-arabinofuranosyl residue, (D): D-xylopryanosyl residue substituted on C(O)-2 and C(O)-3 with a L-arabinofuranosyl residues. Structure C shows the link of ferulic acid to C(O)-5 of a L-arabinofuranosyl residue

Figure 1.4 Structural elements of arabinoxylan from Courtin and Delcour (2002).

Arabinoxylan have two distinct sequence regions as shown in Figure 1.5. The first region is highly branched, containing 1-3 xylose units with arabinose substitution followed by an unsubstituted xylose. The second region is "open", with many unsubstituted xylose units in a row separated by a pair or single substituted xylose. The ratio of the branched region to the open region relates to the arabinose/xylose (Ara/Xyl) ratio (Goldschmid and Perlin 1963, Gruppen et al 1993).
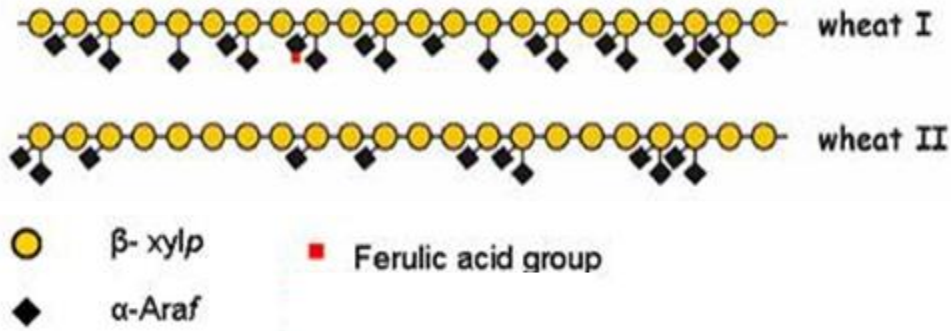
Figure 1.5 Wheat arabinoxylan structure, from Gruppen et al (1993).

Arabinoxylan can be grouped into two fractions; water extractable (WE-AX) and water unextractable (WU-AX). WE-AX make up 25-40% of wheat arabinoxylan (Meuser and Suckow 1986, Cleemput et al 1993, Geissmann and Neukom 1973). WU-AX make up 60-75% of wheat arabinoxylan, are more branched and, therefore, have larger molecular weights than WE-AX. Arabinoxylan are also able to form covalent bonds with other AX, proteins, lignin, and cellulose (Meuser and Suckow 1986, Cleemput et al 1993, Iiyama et al 1994, Gruppen et al 1992, Ordaz-Ortiz and Saulnier 2005). There is no difference between the xylan backbone of the two forms of AX, but the differences as described are found in the side chains (Meuser and Suckow 1986, Gruppen et al 1992). The arabinoxylan structure varies widely in different wheat varieties, and may play an important role in the baking process (Saulnier et al 2007).

Effect on Breadmaking

The effect of AX on the breadmaking process has been studied extensively and this relatively small component of wheat has been shown to affect mixing, dough absorption, dough processing, and overall bread quality (Courtin and Delcour 2002). The literature is inconsistent on the effect of AX on dough absorption (Cleemput et al 1993, Shogren et al 1987), which may be due to the different behaviors of the water soluble and insoluble portions. Yeh et al (1980) showed that water soluble AX decreased absorption, while the results of Michniewicz et al

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(1990) concluded that water insoluble AX increased absorption. Jelaca and Hylnka (1971)

concluded that the water binding capacity of AX is dependent upon mixing parameters, water

availability, other formula ingredients, and evaluation methods. The amount of WU-AX has

been shown to decrease during the mixing process (Yeh et al 1980). This was further supported

by Dornez et al (2007), who suggested WU-AX are solubilized by the mechanical forces during

mixing. Arabinogalactan have not been shown to have this same effect (Yeh et al 1980).

WE-AX increase the viscosity of the aqueous dough phase, which improves dough

properties by stabilizing gas cells (Courtin et al 1999). WE-AX have also been shown to

improve overall bread quality, which includes loaf volume and appearance (Courtin et al 1999,

Rouau et al 1994). On the other hand, WU-AX have a negative effect on bread quality and

produce bread with poor loaf volume (Courtin et al 1999). The proposed beneficial effects of

WE-AX and the negative effects of WU-AX are shown in Figure 1.6. Cleemput et al (1993)

suggested an increase in disubstituted xylose leads to improved bread quality. The literature

clearly suggests that protein is not the only factor in flour quality, but AX structure and

concentration also plays a role (Shogren et al 1987).

a) control dough b) xylanase addition that preferentially degrades WU-AX c) xylanase addition that preferentially degrades WE-AX
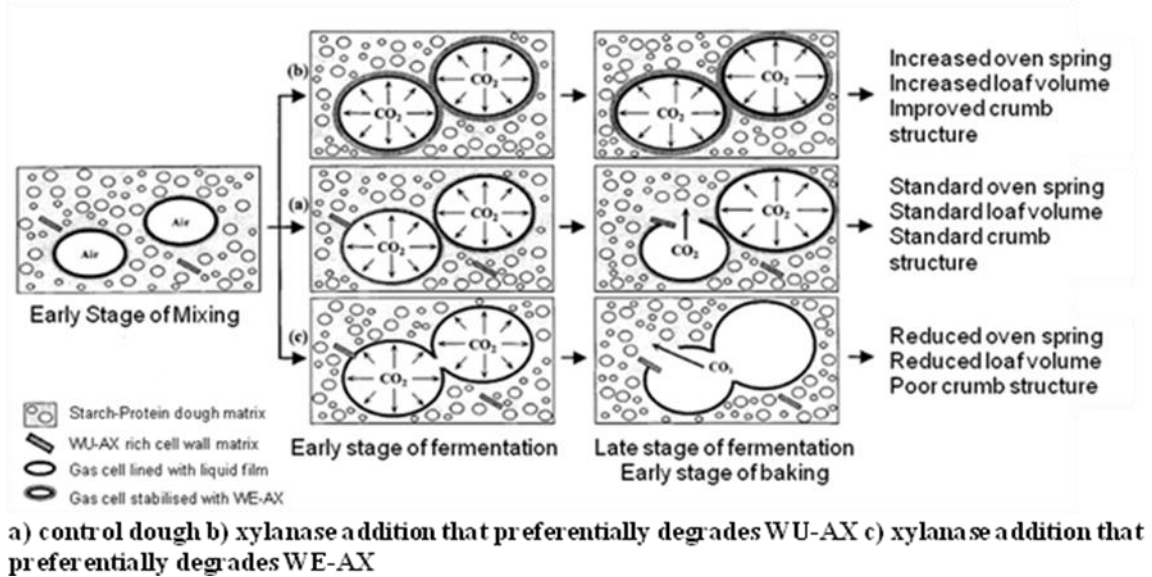
Figure 1.6 Arabinoxylan effect on gas cell stability, adapted from Courtin and Delcour (2002).

Enzymatic Attack

Arabinoxylan are susceptible to enzymatic attack at many points in their structure, and many different enzymes are required for complete degradation of this polymer. β-(1,4)-endoxylanases (xylanases) cleave the internal glycosidic bond between xylose units. This enzyme will be the subject of discussion in this work and will be described in detail in the next section. β-D-xylosidase is capable of cleaving one xylose unit from the non-reducing end (Collins et al 2005). The side chains of AX can also be cleaved by enzymes such as α-L-arabinofuranosidase and ferulic acid esterases (Van Laere et al 1997, Collins et al 2005). The sites for enzymatic attack are indicated in Figure 1.7 by the specified arrows.
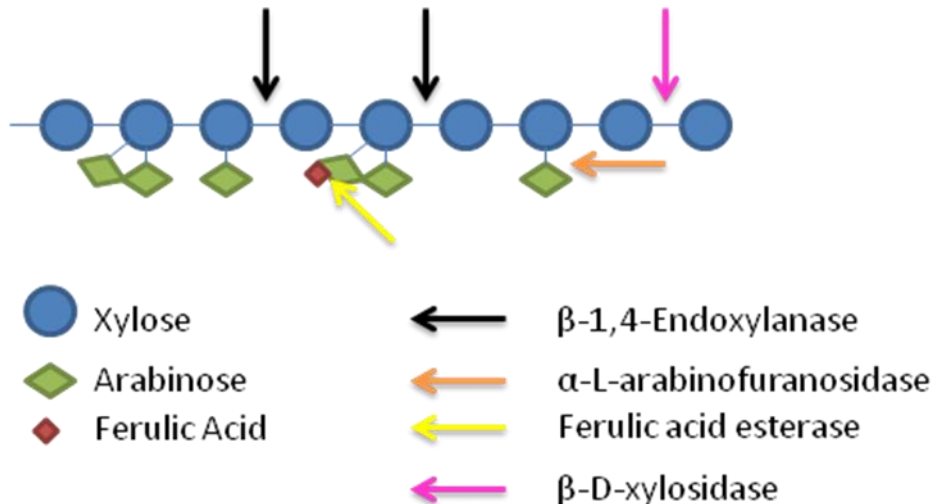
Figure 1.7 Arabinoxylan enzyme attack, adapted from Gruppen et al (1993).

β-1,4-Endoxylanases (Xylanases)

Sources

Xylanases are produced by many species of bacteria, fungi, protozoa, and algae (Torronen and Rouvinen 1997, Collins et al 2005, Sunna and Antranikian 1997, Prade 1995). These organisms use xylanases to produce xylose, which is their main source of carbon (Collins 2005). Two sources of xylanase activity exist in the wheat kernel. Endogenous wheat xylanases are found in the endosperm and are used by the plant for biological processes. Microbial xylanases contaminate the external portion of the wheat kernel (bran) and are used by microorganisms to attack the wheat kernel. The microbial xylanases make up around 80% of overall xylanase activity in wheat (Dornez et al 2006).

Classification

Xylanases fall into the glycosyl hydrolase category of enzymes. This group of enzymes also includes cellulases, amylases, lysozymes, glucanases, and chitinases (Henrissat and Bairoch 1993, Davies and Henrissat 1995). Glycosyl hydrolases are further categorized by family, which

is determined by commonalities in the sequence of amino acids (Henrissat and Bairoch 1993). β-(1,4)-endoxylanases (xylanases) are found in families 5,7,8,10,11 and 43 (Collins et al 2002, Collins et al 2005). Endogenous xylanases are members of Family 10 xylanases (Simpson et al 2003), while most xylanases used industrially are members of families 10 and 11 (Collins et al 2005). In the older classification system, families 10 and 11 were called F and G, respectively (Torronen and Rouvinen 1997). Basic Local Alignment Search Tool (BLAST) is able to search for commonalities in the sequence of amino acids. In 1996, this tool identified 77 Family 10 and 88 Family 11 xylanases (Jeffries 1996). The enzyme commission number is a classification tool utilizing the enzyme's catalytic reaction to classify enzymes. The β-(1,4)-endoxylanase reaction is classified as EC 3.2.1.8, regardless of the xylanase family (Cantarel et al 2009).

Structure

The xylanases of greatest importance to the food industry are Family 10 and 11 xylanases. These families have important structural differences, which affect their functionality and industrial applications. Family 10 xylanases generally have high molecular weights and low isoelectric points. Their structure is relatively common among other enzymes, and is classified as an 8-fold β/α-barrel. This family of enzymes has an open cleft active site, and the catalytic residues are the glutamates Glu-128 and Glu-236. Both of the catalytic residues are located on the carbonyl end of the β barrel. This structure is commonly referred to as a "salad bowl" configuration, which can be seen in Figure 1.8 (Collins et al 2005, Torronen and Rouvinen 1997, Derewenda et al 1994).

Family 11 xylanases generally have low molecular weight and high isoelectric points. This family of enzymes is very specific, and only includes xylanases. The "β jelly roll" structure consists of two β sheets (A and B) and one three turn α-helix. This configuration is commonly

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called a "partially closed right hand", which is portrayed in Figure 1.8 (Collins et al 2005,

Torronen and Rouvinen 1997). The active site is also an open cleft, with the catalytic residues

Glu-78 and Glu-172 (Davies and Henrissat 1995, Miao et al 1994, Wakarchuk et al 1993).
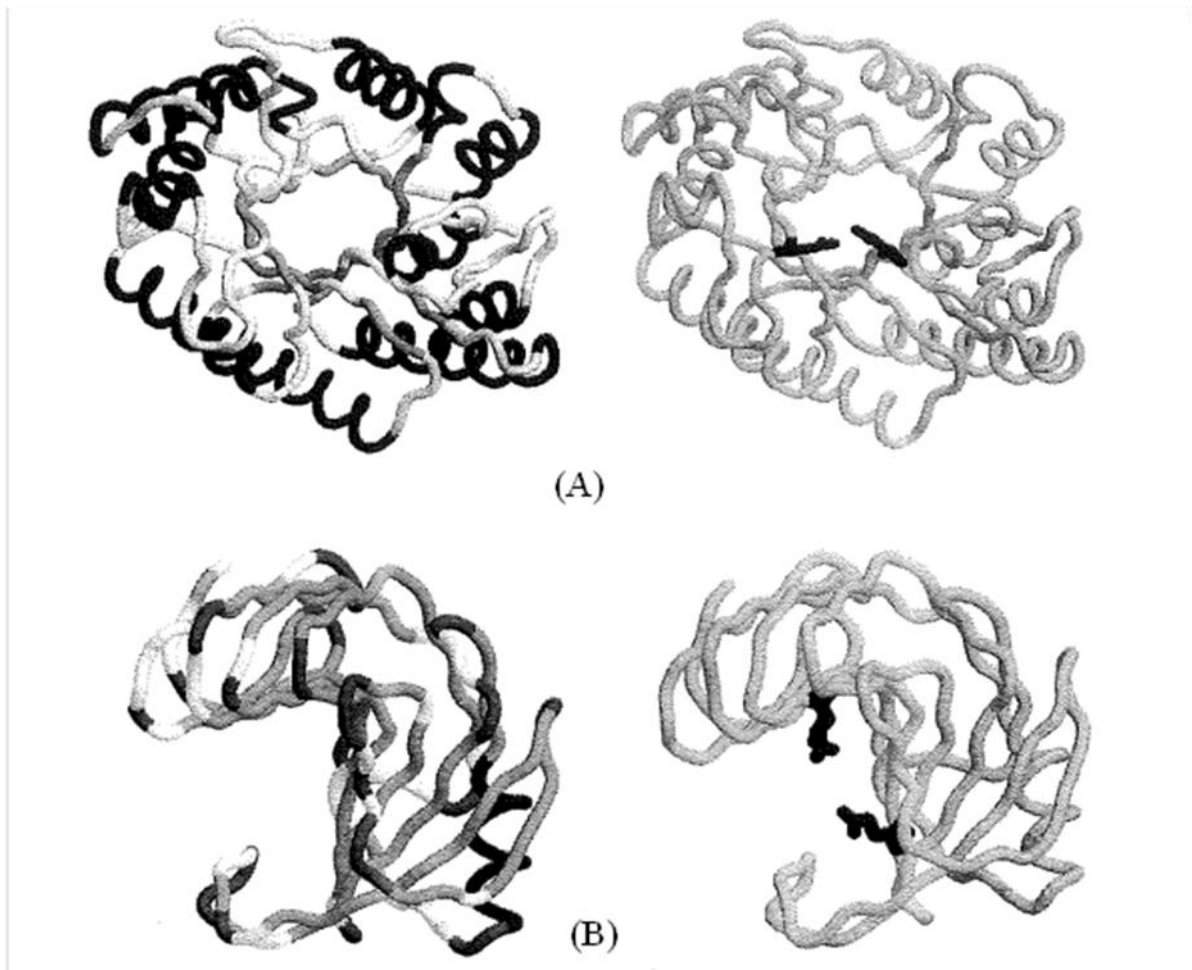
Figure 1.8 "Schematic representation of the molecular structure of endoxylanases member of (A) Family 10 and (B) Family 11. The left hand side representation shows structural information, while the right side picture shows the catalytic residues (drawn with RalMol v 2.6)" from Courtin and Delcour (2002).

Mechanisms

The general mechanism followed by xylanases is acid catalysis, which requires a proton donor and a base (Davies and Henrissat 1995). There are two mechanistic types of hydrolysis, retention and inversion. The inversion mechanism (Figure 1.9) occurs in a single step, is acid/base catalyzed, and utilizes a water molecule to displace the glycosidic leaving group. The retention mechanism (Figure 1.10), utilized by both Family 10 and 11 xylanases, is a double displacement mechanism (Sinnot 1990). In this two step process, an intermediate is formed and then hydrolyzed with the enzyme and substrate. This method utilizes the carboxylic side chain of glutamic acid in the mechanism (Withers and Aebersold 1995).

Xylanases cleave the internal glycosidic bond between xylose molecules in arabinoxylan. The hydrolysis pattern is not random and is dependent upon length of substrate and degree of branching (Sunna and Antranikian 1997). Many glycosyl hydrolases have 5 catalytic subsites, which are labeled -2, -1, +1, +2, and +3. The positive numbers designate the direction of the reducing end, and the hydrolysis occurs between -1 and +1 (Torronen and Rouvinen 1997).
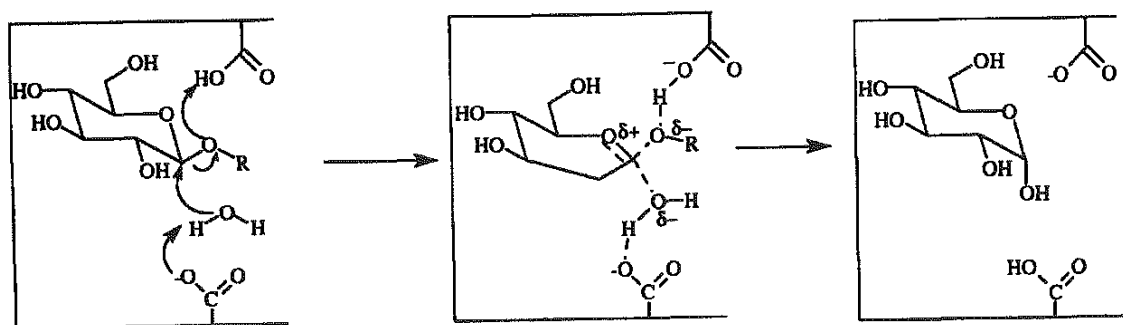
Figure 1.9 "Catalytic mechanism for inverting glycosidases" Withers and Aebersold (1995).

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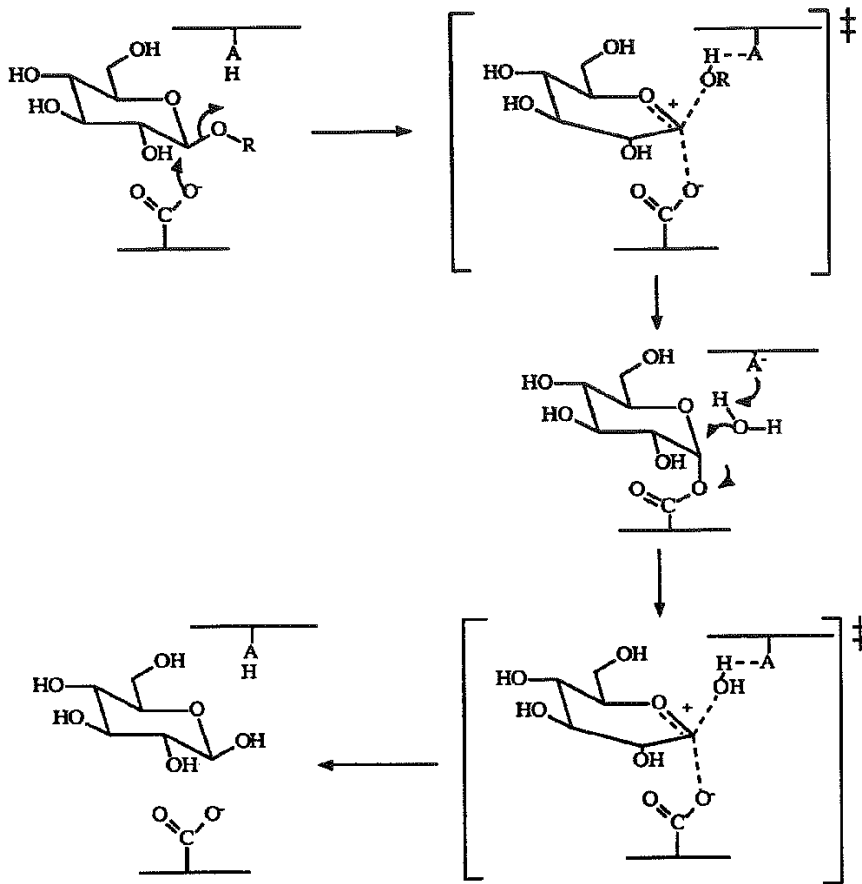
Figure 1.10 "Catalytic mechanism for retaining glycosidases" Withers and Aebersold (1995).

Family 10 xylanases contain 4-5 binding sites, with the subsites at the non-reducing (-1, -2) end more specific than the reducing end (+2). These xylanases are able to hydrolyze relatively small xylose chains (Collins et al 2005). Family 10 xylanases tend to have a specificity toward WE-AX over WU-AX (Moers et al 2003). Research by Bonnin et al (2006), suggests arabinose substitution inhibits functionality of this enzyme family to some degree.

Family 11 xylanases have more binding sites than Family 10; up to 7 sites have been identified for some species. This family is unable to hydrolyze xylan into as small of chains as is Family 10 (Collins et al 2005). Family 11 xylanases tend to have specificity toward Water Unextractable Arabinoxylan over Water Extractable Arabinoxylan (Moers et al 2003). Research

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by Bonnin et al (2006) suggests this family is strongly inhibited by arabinose substitution. Table 1.1 provides a summary of the differences between Family 10 and Family 11 xylanases.

Table 1.1 Xylanase Family Characteristics

Characteristics	Family 10	Family 11
Molecular Weight	High	Low
pI	Low	High
Structure	8-fold β/α-barrel	2 β sheets, 1 α-helix
Configuration	"Salad Bowl"	"Right Hand"
Active Site	Open Cleft	Open Cleft
Mechanism	Retention	Retention
Product Size	Small	Larger
Selectivity	WE-AX	WU-AX
Baking Performance	Poor	Excellent

Industrial Uses of Xylanases

Xylanases are used industrially in many different applications. Each application may require different types and/or sources of xylanases. Industries utilizing xylanases include: baking, fruit juice manufacturing, extraction of olive oil, beer brewing, wine making, animal feed, paper, textiles, and plant growth (Collins et al 2005, Bhat 2000). Family 10 xylanases are used in applications such as animal feed production and starch/gluten separation because the desired effect is the breakdown of WE-AX. Family 11 xylanases are used in the industrial breadmaking industry because they preferentially breakdown WU-AX (Courtin and Delcour, 2002).

General Functions of Xylanases in Breadmaking

Courtin et al (1999) outlined two possible functions for xylanases in a wheat dough system. The xylanase can either solubilize the WU-AX (desirable), or decrease the size of the

WE-AX (undesirable). The maximum observed solubilization of WU-AX in breadmaking is 70-80% (Courtin et al 2001, Moers et al 2005).

Xylanases have been shown to have significant effects on wheat dough properties. The role of xylanases in dough is to solubilize WU-AX. This increases the viscosity of the aqueous phase, which in turn improves gas retention. Water is also released from the AX, which may improve gluten development (Rouau et al 1994, and Courtin and Delcour 2002). If too much xylanase is added to a dough, stickiness can become a problem due to water release. Studies suggest the addition of peroxidase and/or glucose oxidase can minimize dough stickiness caused by xylanase addition (Hilhorst et al 1999, and Hilhorst et al 2002).

Xylanases have also been shown to increase bread volume (Martinez-Anaya and Jimenez 1997, Rouau et al 1994, Courtin et al 1999). Bread volume is improved for two main reasons, the first and most important of which is the reduction of the amount of WU-AX. WU-AX rupture gas cells, causing their coalescence and poor gas retention (Courtin and Delcour 2002). The second beneficial effect of xylanase is an increase in solubilized WE-AX, which improves gas retention by increasing the viscosity of the aqueous phase (Rouau et al 1994, Courtin and Delcour 2002). Courtin et al (1999) demonstrated that xylanase addition decreases loaf volume if the WE-AX is hydrolyzed.

There is some debate about the effect of xylanases on crumb firmness. Some research indicates that xylanases decrease the rate of firming (Martinez-Anaya and Jimenez 1997, Jiang et al 2005, Basinskiene et al 2007). Other studies suggest xylanases decrease crumb firmness, but do not effect the rate of firming. However, it is agreed that the effect of xylanase on crumb firmness is due to more than the effect an increase in volume has on firmness (Courtin et al 1999).

Overall, research clearly shows xylanases can have beneficial effects in breadmaking. The preferred xylanases for breadmaking hydrolyze the detrimental water-unextractable arabinoxylan but do not alter the beneficial water-extractable arabinoxylan found in flour (Courtin et al 1999).

Specific Xylanases and Breadmaking

The two most commonly used xylanases for breadmaking are Family 11 xylanases derived from *Bacillus subtilis* and *Aspergillus niger*. As mentioned previously, the preferred xylanase for this application preferentially degrades WU-AX over WE-AX. Family 11 xylanases have been shown to have this characteristic, with varying sources of xylanase having different levels of specificity. Moers et al (2003) developed a method to evaluate the specificity of xylanases for WU-AX and WE-AX, using a substrate selectivity factor (SSF). SSF was defined as xylanase activity on WU-AX divided by xylanase activity on WE-AX. Figure 1.11 demonstrates why *B. subtilis* and *A. niger* are the preferred xylanases for baking. They have the highest SSF values, and hence the greatest selectivity for WU-AX. It is currently unknown why xylanases have selectivity toward either WU-AX or WE-AX.
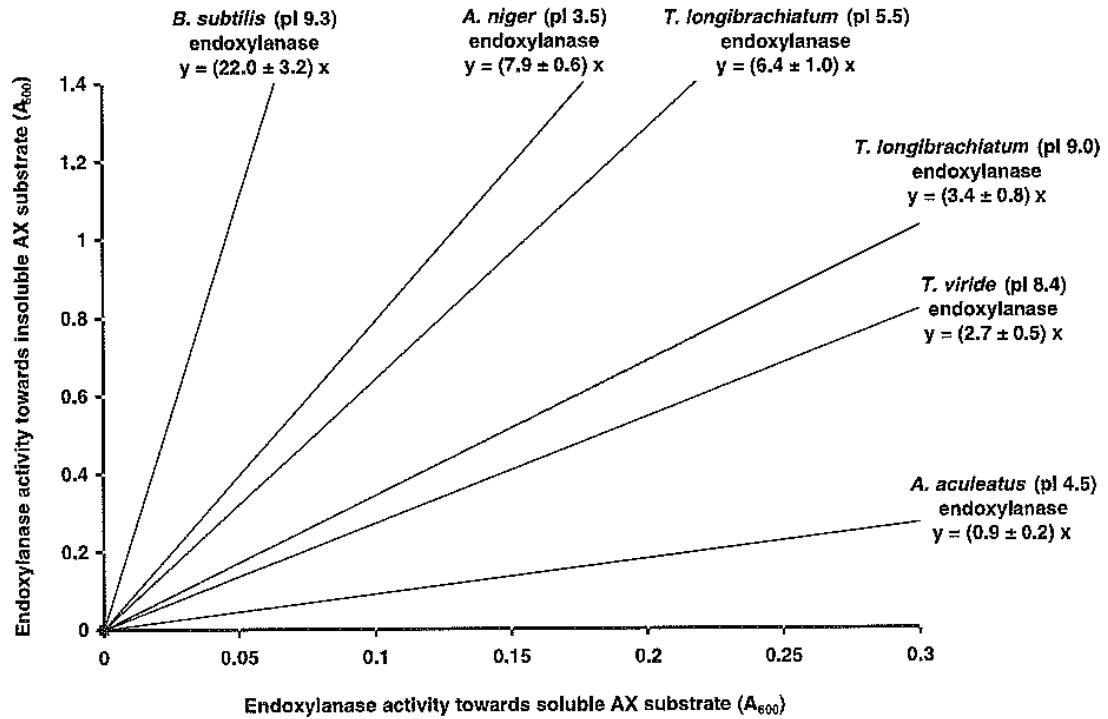
Figure 1.11 "Endoxylanase activities toward soluble and insoluble AX substrates on the X and Y axes, respectively" from Moers et al (2003).

Courtin et al (2001) studied the Family 11 xylanase from *B. subtilis*, and found that in its presence bread showed an improvement in crumb structure, loaf symmetry, and break and shred. Increased loaf volume was observed even with overdosed levels of the enzyme. Decreased crumb firmness was observed, but not a change in the rate of firming. The work also showed that the enzyme was not functional in the oven. In fact, during baking the solubilized WU-AX crosslinked to return to the original water unextractable state. In this study, the maximum solubilization of WU-AX using this enzyme was 80%. Out of 5 Family 11 xylanases tested by Moers et al (2003) *B. subtilis* had the greatest specificity for WU-AX.

The Family 11 xylanase from *Aspergillus niger* had the second greatest specificity toward WU-AX (Moers et al 2003). A maximum of 60% WU-AX was solubilized by this enzyme (Petit-Benvegnen et al 1998).

In comparison to these two effective Family 11 xylanases, the Family 10 xylanase from *Aspergillus aculeatus* is commonly used to demonstrate the negative effects a xylanase can have on bread. Courtin et al (2001) showed that this enzyme improved crumb structure and loaf symmetry as the *B. subtilis* xylanase did. However, the loaf volume decreased at low levels, and increased only slightly at higher levels. The maximum solublization from this experiment was 70%. The work of Moers et al (2003) demonstrates the specificity of this enzyme for WE-AX over WU-AX. Although this enzyme has no applications for breadmaking it is commonly used in the starch-gluten separation in milling due to its selectivity toward WE-AX (Frederix et al 2003).

Table 1.2 from Dekker and Richards (1976) outlines various attributes of xylanases from different sources. This same work also describes inhibitors for different xylanases. The *B. subtilis* xylanase is inhibited by some metal ions, while *A. niger* xylanases are inhibited by glycerol and ethanediol.

Table 1.2 pH and Temperature Characteristics for Xylanases, adapted from Dekker and Richards (1976).

Source of Enzyme	pH optimum	pH stability	Temperature Optimum (°C)	Temperature of Complete Thermal Inactiviation (°C)
Bacillus subtilis	6.0-6.2	5.0-7.0	37-45	70
Aspergillus niger I	4.5	NA	50	60
Aspergillus niger II	5.5	NA	50	60
Aspergillus niger III	4.5-5.0	2.0-9.0	50	80

Xylanase Inhibitors in Wheat

Data suggesting xylanase inhibitors exist in wheat was published for the first time by Debyser et al (1997). Since this time a total of three types of proteinaceous xylanase inhibitors have been identified. Croes et al (2009) studied the location and distribution of the three

inhibitors in the wheat kernel. They determined that all three inhibitors had similar distributions with the greatest concentration in the aleurone layer. The aleurone layer is the innermost part of the bran, and is highlighted in Figure 1.12. The study did not find a correlation between the levels of the three inhibitors. However, different varieties of wheat have been shown to have different inhibition effects on xylanases (Rouau and Surget 1998).
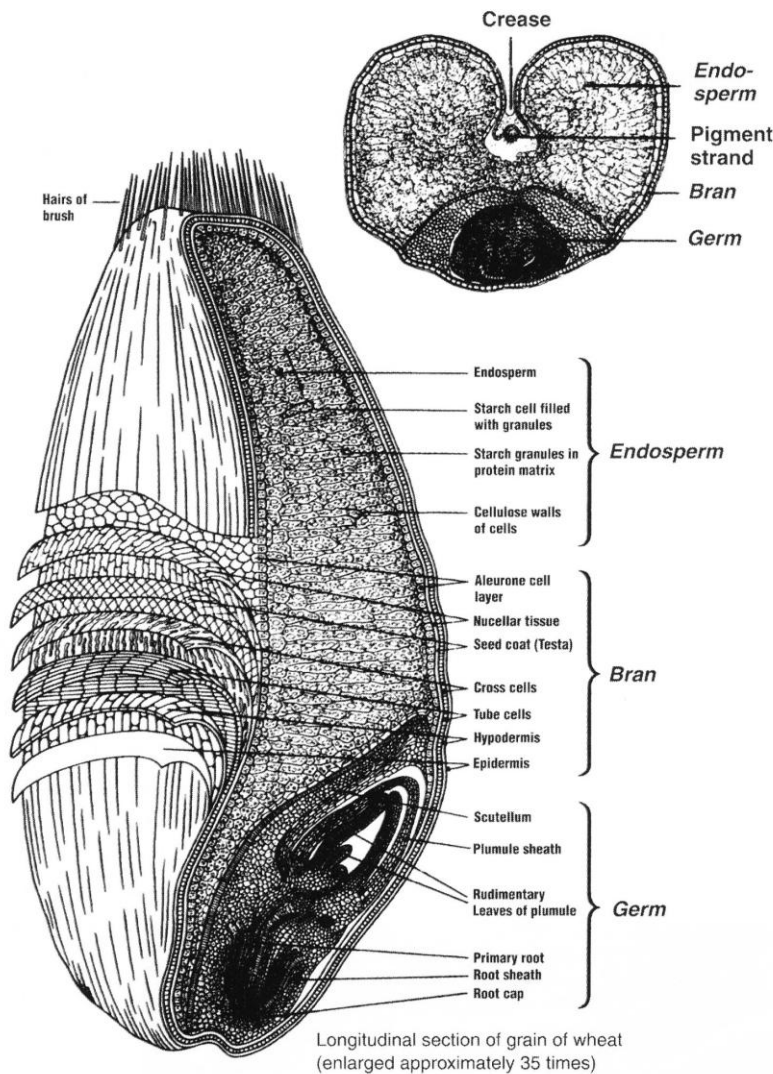
Figure 1.12 Illustration of wheat kernel components, adapted from Pyler and Gorton (2009).

The first type of xylanase inhibitor to be isolated was named TAXI (Triticum aestivum Xylanase Inhibitor) and has two forms. This type of inhibitor protects the plant at various stages of development. It is able to inhibit the endogenous wheat xylanases (Payan et al 2004, Debyser et al 1999). Via the competitive inhibition mechanism (Fierens et al 2007) this inhibitor can eliminate the effect of *A. niger* xylanase on loaf volume (Debyser et al 1999). TAXI exists as two forms, TAXI I and TAXI II. TAXI I inhibits all Family 11 xylanases, both from fungal and bacterial sources (Gebruers et al 2002). It was more effective against xylanases from *A. niger* than *B. subtilis* (Gebruers et al 2001, Sansen et al 2004). TAXI I is 40 kD with a pI of 8.8 (Gebruers et al 2001). TAXI II is unable to inhibit xylanases from *A. niger*, but does inhibit *B. subtilis* and *A. aculeatus* xylanases (Gebruers et al 2001, Gebruers et al 2002). Its molecular weight is 40 kD with a pI of 9.3 (Gebruers et al 2001).

The second of the three inhibitors to be isolated was named XIP (Xylanase Inhibiting Protein). Research indicates this protein inhibits only Family 11 xylanases from fungal sources. It is unable to affect Family 10 xylanases or bacterial sources of Family 11 xylanases. Of specific interest is the fact that the industrially used xylanase from *Aspergillus niger* is inhibited by this protein (McLauchlan et al 1999, Flatman et al 2002, Goesaert et al 2003). XIP is a small glycosylated protein with a basic pI. Its K_m against WE-AX is 20 ± 2 mg/ml and K_{cat} is 103 ± 6 sec^{-1} (McLauchlan et al 1999). This protein exhibits competitive inhibition and binds near the enzyme's active site. Many glycosylated inhibitors are slow binding, however XIP is not. It's K_i against fungal Family 11 xylanases ranges from 3.4-610 nM (Flatman et al 2002). XIP does not inhibit endogenous wheat xylanases, which indicates it is used by the plant to protect the grain from pathogens (Payan et al 2004). Pathogens on the exterior of the grain will release xylanases in order to attack the kernel. The xylanase inhibitors in the grain will inhibit the attacking

xylanases and therefore protect it from the pathogens. Research suggests the difference in inhibition against different sources of xylanase may be due to insertions in specific loops in the tertiary structure of the enzyme (Payan et al 2004, Gusakov and Ustinov 2009). XIP is deactivated relatively early in the baking process (Gebruers et al 2005) and has also been identified in rye, durum wheat, barley, and maize (Goesaert et al 2003).

The third type of xylanase inhibitor identified is TLXI (Thaumatin-like Xylanase Inhibitor). It acts by noncompetitive inhibition and does not inhibit Family 10 xylanases or Family 11 xylanases with high pI values (e.g. *B. subtilis*). TLXI is able to inhibit most other Family 11 xylanases, such as those from *A. niger* (Fierens et al 2007). The structures of all three xylanase inhibitors have been identified and are included in Figure 1.13. Table 1.3 summarizes properties of all three xylanase inhibitors.
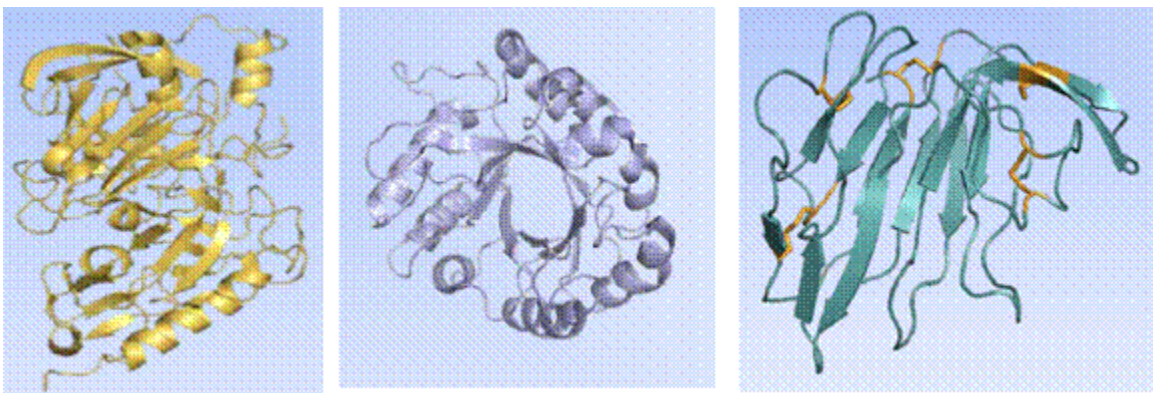
Figure 1.13 Xylanase inhibitor structures A) Triticum aestivum Xylanase Inhibitor (TAXI) B) Xylanase Inhibiting Protein (XIP) C) Thaumatin-like Xylanase Inhibitor (TLXI), adapted from Fierens et al (2008).

Table 1.3 Xylanase Inhibitor Characteristics, adapted from Fierens et al (2008).

Attributes	TAXI I	TAXI II	XIP	TLXI
Molecular Mass	40 kDa	30 + 10 kDa	30 kDa	
Molecular Form	Monomer	Monomer	Monomer	Monomer
pI	> 8.8	> 8.8	> 8.0	> 9.3
Specificity	*A. niger, B. subtilis*	*B. subtilis*	*A. niger*	*A. niger*
Mechanism	Competitive	Competitive	Competitive	Noncompetitive
Kinetics	Fast-binding	Fast-binding	Fast/Slow-binding	Slow-binding
K_i	1-20 nM	1-20 nM	2-600 nM	60 nM
Optimal pH	4.8-5.5	4.8-5.6	4.5-6.5	5.0-5.5
Optimal Temperature	20°C-40°C	20°C-40°C	30°C	40°C

TAXI: Triticum aestivum Xylanase Inhibitor; XIP: Xylanase Inhibiting Protein; TLXI: Thaumatin-like Xylanase Inhibitor

Effect of Xylanase Source in Different Wheat Flours

A significant amount of research has been conducted to understand both the mechanisms and applications of xylanases in improving the breadmaking process. However, important areas of interest are yet to be understood. One area yet to be explored is the effect that xylanase source plays in different wheat flours. It has been observed anecdotally that the xylanase from *A. niger* performs differently in American and European wheat flours than does the xylanase from *B. subtilis*. Specifically, the *A. niger* enzyme appears to cause greater dough stickiness with American flours, while the *B. subtilis* enzyme is observed to cause more dough stickiness with European wheat flours (at equivalent usage levels).

An objective and reproducible method needs to be developed to test this observation. Courtin and Delcour (2002) discussed the difference in effect of xylanases on strong and weak wheat flours. Because American flours are generally stronger than European flours, this may play a role in the observed effects of different xylanase sources. Xylanase inhibitors also differ

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between different wheat varieties (Rouau and Surget 1998) and different inhibitors have varying levels of effectiveness against *A. niger* and *B. subtilis* xylanases. This may also explain the observed differences between the two xylanases. The first goal of this work was to find a method to objectively and consistently measure the effect of xylanases on wheat flour dough stickiness.   The second objective was to use this method to determine if the previously described anecdotal observation is real. The desired outcome was to develop a new hypothesis as to why xylanases have varying effects on different wheat flours.

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Chapter 2 - Materials and Methods

List of Abbreviations

The following abbreviations will be used throughout the following two chapters.

TA…………………….. Texture Analysis
SSL………………….Sodium Stearoyl Lactylate
ADA……………….. Azodicarbonamide
FWER……………… Flour Water Expression Rate
DNSA……………….3,5-dinitrosalicylic acid
IWLR………………. Initial Water Loss Rate
WU-AX……………..Water Unextractable Arabinoxylan
WE-AX……………... Water Extractable Arabinoxylan
HMW WE-AX………High Molecular Weight Water Extractable Arabinoxylan
LMW WE-AX………Low Molecular Weight Water Extractable Arabinoxylan
AX…………………. Arabinoxylan
TAXI………………..Triticum Aestivum Xylanase Inhibitor
XIP…………………Xylanase Inhibiting Protein
TLXI……………….. Thaumatin-like Xylanase Inhibitor

Dough Stickiness

The Texture Analyzer (TAXT+, Stable Micro Systems, Surrey, UK) based Chen-Hoseney Dough Stickiness method (Stable Micro Systems, 2007) was used to evaluate dough stickiness. Using the formula in Table 2.1 bread dough was mixed in a 100 gram micro pin mixer (National Manufacturing Co, Lincoln, NE) for 7 minutes (or optimum development). The test settings for the TA method are shown in Table 2.2.

Table 2.1 Formulation for Dough Stickiness Method

Ingredient	grams
Flour	100
SSL*	0.5
Salt	2
Sugar	8
Ice Water	57
Soy Oil	2

*Sodium Stearoyl Lactylate

Table 2.2 Texture Analysis Method Settings

Option	Adhesive Test
Pre-Test Speed	0.5 mm/s
Test Speed	0.5 mm/s
Post-Test Speed	10.0 mm/s
Distance	4 mm
Force	40 g
Time	0.1 sec
Trigger Type	Auto - 5g
Tare Mode	Auto
Data Acquisition Rate	500 pps

Figure 2.1 is a sample curve produced with this method. The area of interest is the portion of the graph in the positive region (shaded). Dough stickiness and dough rheology can be characterized by three measurements on the graph: peak force (stickiness), area under the

curve (work of adhesion), and distance between anchor 1 and anchor 2 (dough strength/cohesiveness).
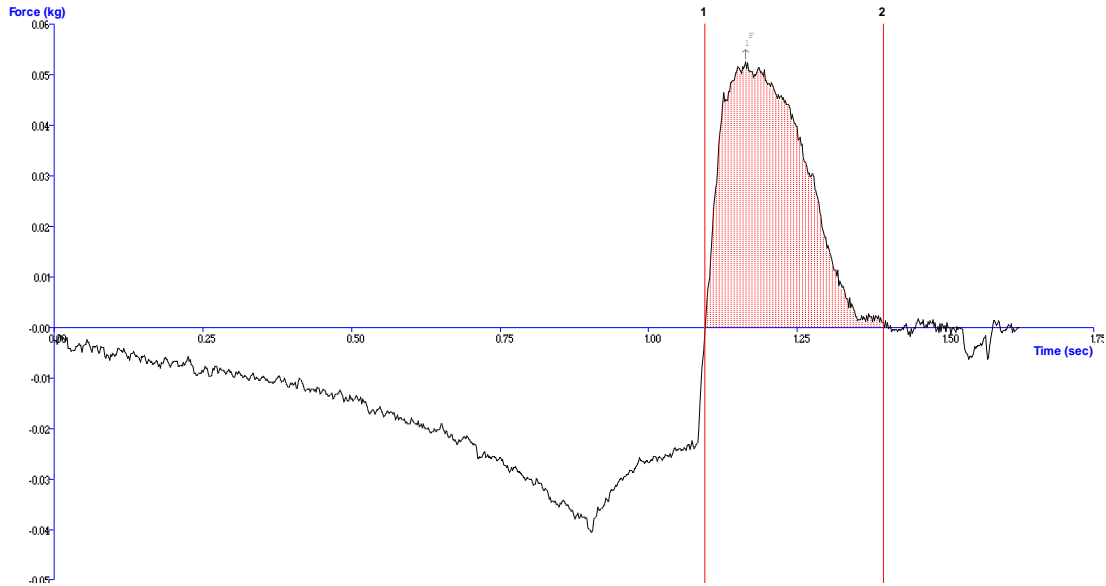
Figure 2.1 Sample dough stickiness curve from Texture Analyzer (TAXT+).

Using this method, a dose response of Veron® 191 from *Aspergillus niger* (AB Enzymes, Darmstadt, Germany) was conducted for the U.S. ADM Chattanooga flour (ADM Milling Company, Decatur, IL) shown in Table 2.6. The test was conducted in triplicate with 10 repeated measures for each individual dough. The experimental design was completely randomized. The data was analyzed using the Tukey HSD paired comparison test with $\alpha = .05$.

Bread Baking

Pup loaf baking tests, adapted from AACC Method 10-10B, were conducted incorporating one of three xylanases, Veron® 191 from *Aspergillus niger* (AB Enzymes, Darmstadt, Germany), Bakezyme® BXP 25001 from *Bacillus subtilis* (DSM Food Specialties

USA, Inc, Parsippany, NJ), and an uninhibited xylanase from *Bacillus subtilis* HPI BS3

(Dansico, Copenhagen, Denmark). The adaptations from the AACC Method include:

- Mix time held at 7 minutes

- Straight dough process replaced with no time dough procedure

All tests were conducted in the U.S. ADM Chattanooga flour (ADM Milling Company, Decatur,

IL) with the formula in Table 2.3. All ingredients were mixed for 7 minutes in a 100 gram micro

pin mixer (National Manufacturing Co, Lincoln, NE) to optimum development. The dough was

allowed to rest for 10 minutes before dividing into 168 gram pieces. The dough was allowed to

rest for an additional 5 minutes before shaping in a straight grain moulder (Gemini Bakery

Equipment Company, Philadelphia, PA). The dough was placed in greased pup loaf pans before

proofing for approximately 60 minutes (proofed to template height) and then baked at 216°C for

13 minutes. Bread volumes were measured with a Volscan Profiler (Stable Micro Systems,

Surrey, UK).

Table 2.3 Bread Formula

Ingredient	Grams
Flour	100
Instant Active Dry Yeast	2
SSL*	0.25
Salt	2
Sugar	8
Calcium Propionate	0.2
Fungal Amylase	10 ppm
ADA**	25 ppm
Ascorbic Acid	40 ppm
Ice Water	55
Soy Oil	2

*Sodium Stearoyl Lactylate
**Azodicarbonamide

Dose responses for each enzyme were conducted in triplicate with a completely randomized design. The enzyme levels tested are shown in Table 2.4. The bread volume data was analyzed using the Tukey HSD paired comparison analysis with $\alpha = 0.05$.

Table 2.4 Enzyme Levels.

Enzyme	Levels Tested	Suggested Usage Level from Supplier
Veron® 191	0 ppm, 50 ppm, 100 ppm, 150 ppm	100 ppm
Bakezyme® BXP 25001	0 ppm, 5 ppm, 10 ppm, 20 ppm	10 ppm
HPI BS3	0 ppm, 10 ppm, 20 ppm, 40 ppm	20 ppm

Xylanase Activity Assay

A spectroscopic method (adapted from Miller, 1959) was used to determine the xylanase activity using birchwood xylan (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) as substrate and 3,5-dinitrosalicylic acid (DNSA) to measure the amount of the reducing sugar (xylose) produced by the xylanase. The substrate, in a buffer solution, was reacted with the xylanase for 30 minutes at 37°C before the reaction was stopped with the DNSA solution and the reducing sugar content was measured by absorbance at 540 nm. A standard curve of xylose was developed in order to calculate the amount of xylose in the experimental samples. This experiment was set up in a completely randomized design and was conducted in triplicate.

Table 2.5 Xylanase Activity.

Enzyme	Activity (nkat/mg)	ppm for equivalent activity
Veron® 191	10.94	100.0
Bakezyme® BXP 25001	80.32	13.70
HPI BS3	1.610	N/A*

*The usage level of HPI BS3 for equivalent activity to the other xylanases is not applicable based on this analysis due to the uninhibited nature of the enzyme.

Flour Water Expression Rate

The Flour Water Expression Rate (FWER) is a method developed as part of this work to evaluate the activity of xylanase on wheat flour doughs by measuring the amount of water released over time. Two white bread flours from America (ADM Milling Company, Decatur, IL) and two from Europe (Grands Moulins de Strasbourg, Strasbourg, France) were evaluated and their flour characteristics are shown in Table 2.6. Three xylanases were also tested with this method, Veron® 191 from *Aspergillus niger* (AB Enzymes, Darmstadt, Germany), Bakezyme® BXP 25001 from *Bacillus subtilis* (DSM Food Specialties USA, Inc, Parsippany, NJ), and an uninhibited xylanase from *Bacillus subtilis* HPI BS3 (Danisco, Copenhagen, Denmark).   The experiments were completely randomized with the treatments completed in triplicate with four repeated measures for each individual dough. Statistical analysis was completed in JMP® 9 (SAS Institute Inc, Cary, NC) using the Tukey HSD paired comparison analysis with $\alpha = 0.05$.

Table 2.6 Flour Characteristics.

Flour	Moisture	Protein	Ash Content	Falling Number
U.S. ADM Chattanooga	13.58	11.0	0.53	263
U.S. ADM Camp Hill	13.90	14.1	0.54	272
EU Manitoba	13.90	15.1	0.56	418
EU Mirebelle	13.90	11.8	0.58	386

All powdered enzymes were dissolved into a buffer/glycerol solution because the enzymes were more accurately measured in liquid form with an automatic pipette. The enzymes were stored in the freezer to maintain activity and glycerol was added to the solutions to keep them from freezing. A 10x concentrated citric acid buffer solution of pH 5.3 was prepared by combining the materials shown in Table 2.7.

Table 2.7 Citric Acid Buffer.

Ingredients	Grams	Source
Sodium Phosphate Dibasic Anhydrous	14.8	Avantor Performance Materials, Phillipsburg, NJ
Citric Acid Monohydrate	10.3	Thermo Fisher Scientific, Geel, Belgium
Water	Add to 1 liter	

A 4% (w/v) Veron® 191 solution was prepared by mixing 4 g Veron® 191, 9.6 g 10x Citric Acid Buffer, and 86.4 g distilled water. One ml of the resulting solution was transferred to 1.5 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) and centrifuged for 3 minutes at 8,000 rpm (Eppendorf Centrifuge 5418, Hamburg, Germany) to remove the enzyme diluents. A 500 μl portion of supernatant was transferred to a 1.5 ml microcentrifuge tube with an automatic pipette (VWR International, Radnor, PA). Then 500 μl of an 80% glycerol (ADM Company, Decatur, IL) solution was added to the tube and vortexed. The final enzyme solution contained 2% Veron® 191 and was stored at 0°C. Enzyme solutions of 1% Bakezyme® BXP 25001 and 1% HPI BS3 were prepared in the same manner.

The water absorption of each flour was determined using a 35g Mixograph (National Manufacturing Co, Lincoln, NE), based on AACC Method 54-40A. The FWER absorption was the absorption used in making the dough for the FWER test. The FWER absorptions were 12% higher than flour absorptions to ensure the doughs were saturated with water; the final FWER absorptions used for testing are shown in Table 2.8.

Table 2.8 Water Absorption.

Flour	Mixograph Absorption	FWER Absorption
U.S. ADM Chattanooga	56%	68%
U.S. ADM Camp Hill	62%	74%
EU Manitoba	60%	72%
EU Mirebelle	56%	68%

To prepare the FWER dough, 35 g of flour was mixed with the FWER absorption in

Table 2.8 using the 10x citric acid buffer and distilled water. Enzymes at varying levels were

also added at the beginning of mixing. Enzyme addition levels were determined based on DNSA

activity and standard usage levels in baking. The activity for each enzyme is shown in Table 2.5.

Each enzyme was added at the optimum usage level, found in Table 2.9, and 5 times this level.

Based on 35 g of flour, the enzyme solutions were added at the levels shown in Table 2.9. If

enzyme was added to the dough, the same amount of buffer/water was removed to maintain the

same absorption.

Table 2.9 Xylanase Addition.

Enzyme	No Add		Optimum		5x Optimum	
2% Veron® 191 Soln	0 ppm	0 µl	100.0 ppm	175.0 µl	500.0 ppm	875.0 µl
1% BXP 25001 Soln	0 ppm	0 µl	13.70 ppm	48.00 µl	68.50 ppm	253.5 µl
1% HPI BS3 Soln	0 ppm	0 µl	20.00 ppm	70.00 µl	100.0 ppm	350.0 µl

The doughs were mixed for 4.5 minutes in a Mixograph (National Manufacturing Co,

Lincoln, NE). Approximately 1.5 g of dough was packed into each of four pre-weighed 1.5 ml

microcentrifuge tubes (Eppendorf, Hamburg, Germany). The weight difference of all four dough

filled tubes was less than 0.1 g. The weight of the tube plus sample was recorded as "+ Sample".

The tubes were then loaded into a microcentrifuge (Eppendorf Centrifuge 5418, Hamburg,

Germany) and spun for 1 min at 13,500 rpm.

If the dough contained no enzyme or the recommended level of enzyme, the tubes were then incubated in a laboratory oven (VWR International, Radnor, PA) set to 30°C for 40 minutes. The tubes were then centrifuged for 10 minutes at 13,500 rpm. Supernatant was removed from the tube with a pipette, and the weight recorded as "1st Spin". The Initial Water Loss Rate (IWLR) was calculated based on Equation 2.1.

Equation 2.1 IWLR (Long Method).

$$IWLR = \frac{(+sample - 1stSpin) \times 100}{(+sample - Tubewt) \times 0.66667} \ (\text{g/hr})$$

The remaining dough was then incubated at 30°C for three hours before centrifuging the tubes for 10 minutes at 13,500 rpm again. The supernatant was removed and the weight of the tube recorded as "2nd Spin". FWER was calculated as the rate by Equation 2.2.

Equation 2.2 FWER (Long Method).

$$FWSR = \frac{(1stSpin - 2ndSpin) \times 100}{(1stSpin - Tubewt) \times 3} \ (\text{g/hr})$$

If the dough contained enzyme at 5x the optimum level, the initial incubation of the tubes was 15 minutes. The tubes were then centrifuged for 10 minutes at 13,500 rpm. Supernatant was removed from the tube, and the weight recorded as "1st Spin". IWLR was calculated using Equation 2.3.

Equation 2.3 IWLR (Short Method).

$$IWLR = \frac{(+sample - 1stSpin) \times 100}{(+sample - Tubewt) \times 0.25} \text{ (g/hr)}$$

An additional 30 minute incubation was completed before centrifuging the tubes for 10 minutes at 13,500 rpm. The supernatant was removed and the weight recorded as "2nd Spin". FWER was calculated as the rate using Equation 2.4.

Equation 2.4 FWER (Short Method).

$$FWSR = \frac{(1stSpin - 2ndSpin) \times 100}{(1stSpin - Tubewt) \times 0.5} \text{ (g/hr)}$$

Flow Chart for Experimentation

Dough Stickiness	
Texture Analysis Method	Not Reproducible

Bake Test	
Bread Baking Method Adapted from AACC Method 10-10B	Not Reproducible

Enzyme Activity Assay	
Spectroscopic Method to Measure Reducing Sugars	Substrate is not from wheat and has different properties

FWSR Method Development	
Measured the amount of water released from a dough over time	Reproducible, objective, and significant results

FWSR Test for Initial Hypothesis	
Fungal vs Bacterial xylanases would function differently in US and EU Flours	Hypothesis was false

FWSR Test for New Hypothesis	
Xylanase inhibitors are responsible for variations in xylanase activity across wheat flours	Tests support this theory, but additional work is recommended

Chapter 3 - Results and Discussion

Method Development

Dough Stickiness

A dose response curve was generated for the Veron®191 *Aspergillus niger* xylanase. Those data are presented in Table 3.1. Differences in dough stickiness could be observed subjectively. However, the Chen-Hoseney Dough Stickiness method did not give clear trends for stickiness, work of adhesion, or dough strength/cohesiveness. Stickiness, which is the most relevant output from this method, did not show statistically significant differences. This is due partly to the high variability within the same sample set. Grausgruber et al (2003) found similar difficulties with this method due to variation within the same sample. Hoseney and Smewing (1999) addressed the many challenges involved in measuring dough stickiness. The variation in stickiness is compounded by other changes in dough rheology caused by xylanase activity. Xylanases not only release water, which could increase dough stickiness, but also produce a more relaxed dough with different rheological properties. These rheological changes make this method even more difficult to measure an increase in dough stickiness. The conclusion is that this method was not effective in measuring the effect of xylanases in breadmaking.

Table 3.1 Dough Stickiness Texture Analysis Data with Varying Levels of Veron® 191 Xylanase Addition.

Treatment	Stickiness		Work of Adhesion		Dough Strength/Cohesiveness	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
0 ppm Veron® 191	54.49^A	2.43	6.15^{BC}	0.31	2.33^{BC}	0.24
100 ppm Veron® 191	52.93^A	1.66	5.49^C	0.93	1.96^C	0.40
400 ppm Veron® 191	56.13^A	2.29	7.79^A	0.44	3.06^A	0.37
700 ppm Veron® 191	54.43^A	4.45	6.65^B	0.70	2.76^{AB}	0.35
1000 ppm Veron® 191	54.91^A	5.55	6.67^{AB}	1.08	2.73^{AB}	0.39
^{ABC}For each column, mean values with the same superscript are not significantly different (p>.05)						

Bread Baking

The volume data (Table 3.2) did not show significantly improved loaf volume. However, there is a clear trend that loaf volume increases with xylanase addition (Table 3.2). A significant amount of research suggests xylanases increase bread volume (Martinez-Anaya and Jimenez 1997, Rouau et al 1994, Courtin et al 1999). However, similar to the dough stickiness results it was difficult to show statistical significance in the response. This lack of statistical significance of the baking tests further demonstrated the need for an objective and reproducible method to evaluate the effect of xylanases in bread dough systems.

Table 3.2 Pup Loaf Bread Volume with Varying Levels of Three Commercially Available Xylanases.

Treatment	Volume	Treatment	Volume	Treatment	Volume
0 ppm Veron® 191	660^A	0 ppm HPI BS3	604^B	0 ppm Bakezyme® BXP 25001	665^A
50 ppm Veron® 191	670^A	10 ppm HPI BS3	643^{AB}	5 ppm Bakezyme® BXP 25001	710^A
100 ppm Veron® 191	720^A	20 ppm HPI BS3	639^{AB}	10 ppm Bakezyme® BXP 25001	710^A
150 ppm Veron® 191	700^A	40 ppm HPI BS3	676^A	20 ppm Bakezyme® BXP 25001	734^A
^{AB}For each column, mean values with the same superscript are not significantly different (p>.05)					

Xylanase Activity Assay

Activity measurements as assessed by the DNSA method are shown in Table 3.3. It is important to note that the method used to generate this data used birchwood xylan as the substrate. There are structural differences between birchwood xylan and wheat arabinoxylan, mainly the presence of arabinose side chains, which can cause discrepancies between the activity assays and baking results. Therefore, this method is a first step when comparing xylanases and their functions, but cannot be used solely to predict baking functionality.

Table 3.3 Xylanase Activity Assay Data for Three Commercially Available Xylanases.

	Activity (nkat/mg)	
Xylanases	Mean	Std Dev
Veron®191	10.94^B	0.61
Bakezyme® BXP 25001	80.32^A	5.18
HPI BS3	1.580^C	0.01
[ABC]Values with the same superscript are not significantly different (p>.05)		

Flour Water Expression Rate (FWER)

A statistically significant difference among all three treatments ($p<0.05$) was observed, and both enzymes increased the FWER, the Veron® 191 to a much greater degree than the Bakezyme® BXP 25001 (Table 3.4). The results of this method provide objective and reproducible data regarding the action of xylanases in a bread dough, whereas both the dough stickiness method and baking tests failed to show reproducible differences in the effect of xylanases in this system.

Table 3.4 FWER Data for the EU Mirebelle Flour.

Enzyme Treatment	FWER	
	Mean	Std Dev
No Enzyme	2.40^C	0.04
500 ppm Veron® 191	16.4^A	0.61
68.5 ppm Bakezyme® BXP 25001	5.66^B	0.28
^{ABC}Values with the same superscript are not significantly different (p>.05)		

Effect of Xylanases on American and European Flours

The initial hypothesis for this study was that the Veron®191 xylanase from *A. niger* would release more water, resulting in a higher FWER, in American flours than the Bakezyme® BXP 25001 xylanase from *B. subtilis*. The opposite observation was predicted to occur in European flours. Different wheat varieties are grown in American than in Europe due to different growing conditions. These genetic differences could be the contributing factor to varying xylanase functionality in American and European flours. The four flours used in this study were commercial blends of wheat varieties, rather than one singular variety, but they represent the varieties grown in American and Europe, respectively. This first designed experiment in this study was sufficient to show that this hypothesis was not supported. As demonstrated in Table 3.5, the treatments with Veron® 191 produced the highest FWER in three out of four flours. These findings support the research of Moers et al (2003), who's method for evaluating xylanase substrate selectivity suggests the xylanase from *B. subtilis* has a greater selectivity toward WU-AX than does the xylanase from *A. niger*. Substrate selectivity is an important factor because it is directly related to water release by the arabinoxylan. Courtin and Delcour (2002) describe three types of wheat arabinoxylan, Water-Unextractable Arabinoxylan (WU-AX), high molecular weight Water-Extractable Arabinoxylan (HMW WE-AX), and low molecular weight Water-Extractable Arabinoxylan (LWM WE-AX). HMW WE-AX are

desirable in breadmaking and have a high capacity to hold water. WU-AX and LMW WE-AX

are less desirable in breadmaking and do not hold as much water. The desired effect of

xylanases in breadmaking is to breakdown the WU-AX into HMW WE-AX. However, the

breakdown of HMW WE-AX into LMW WE-AX will also occur, which will cause water to be

released from the AX, causing dough stickiness. Moers et al (2003) show that the xylanase from

A. niger will more preferentially breakdown WE-AX than will the xylanase from *B. subtilis*.

This would suggest the xylanase from *A. niger* (Veron®191) would produce a higher FWER

than the xylanase from *B. subtilis* (Bakezyme® BXP 25001).

The FWER data in Table 3.5 supports these theories in three out of four flours (U.S.

ADM Chattanooga, EU Manitoba, and EU Mirebelle). However, the fourth flour (U.S. ADM

Camp Hill) shows the opposite effect. In this flour, the treatment with Bakezyme® BXP 25001

resulted in a higher FWER than the Veron®191. The original hypothesis would have predicted

this result for a European flour, but in this experiment it was observed for an American flour.

These results do not support the original hypothesis, and pose a new question. If xylanase

functionality is not related to a difference between American and European flours, what is the

reason? It may be due to differences in the types and levels of xylanase inhibitors found in those

wheats.

Table 3.5 Comparison of IWLR and FWER Data for Flour Wheat Flours.

	Initial Water Loss Rate (IWLR)			Flour Water Expression Rate (FWER)		
Flour	No Enzyme	500 ppm Veron® 191	68.5 ppm Bakezyme BXP® 25001	No Enzyme	500 ppm Veron® 191	68.5 ppm Bakezyme BXP® 25001
US ADM Chattanooga	2.178^B	10.99^A	3.732^B	2.075^C	15.18^A	9.673^B
EU Manitoba	2.415^B	9.745^A	3.397^B	1.318^C	11.34^A	8.570^B
US ADM Camp Hill	2.395^A	2.491^A	4.621^B	1.949^A	7.126^B	9.352^C
EU Mirebelle	2.185^A	6.354^B	$.9273^A$	2.399^C	16.40^A	5.656^B
[ABC]For each row, average values with the same superscript are not significantly different (p>.05)						

Adapted from Fierens et al (2008)

The FWER method was intended to produce one critical type of result, the FWER value. The IWLR is measured to remove any excess water from the dough and to improve the accuracy of the FWER value. However, an unexpected observation was made when lower levels of Bakezyme® BXP 25001 were added to the dough, as the IWLR decreased with the addition of xylanase (p=.0008) as shown in Table 3.6. One hypothesis to explain this observation is the breakdown of WU-AX by the xylanase to produce HMW WE-AX, which can hold large amounts of water. If the production of HMW WE-AX is optimized and not further broken down into LMW WE-AX, then the AX could absorb rather than release water. Additional work in this area would be required to test this hypothesis.

Table 3.6 IWLR Data for the U.S. ADM Chattanooga Flour with the Addition of Bakezyme® BXP 25001.

	Initial Water Loss Rate (IWLR)	
Enzyme Treatment	Mean	Std Dev
No Enzyme	2.030	0.30
13.7 ppm Bakezyme® BXP 25001	0.4654	0.03
p=.0008, treatments are significantly different		

New Hypothesis: Effect of Xylanase Inhibitors

Three different proteinaceous xylanase inhibitors have been discovered in wheat: TAXI, XIP, and TLXI (Debyser et al 1999, Fierens et al 2007). As Table 3.7 shows, they vary in their ability to inhibit different types of xylanases, specifically *A. niger* and *B. subtilis*. Research has shown that wheat variety has an effect on xylanase inhibition (Rouau and Surget 1998). Based on this information a new hypothesis was formulated. The unique behavior of the U.S. ADM Camp Hill flour and the currently unexplained variation in xylanase functionality across different flours may be due to differences in their xylanase inhibitors. This suggests the U.S. ADM Camp Hill flour had higher levels of XIP and TLXI inhibitors than did the other flours, and this caused

inhibition of the *A. niger* xylanase to a much greater degree. There were no commercially

available uninhibited versions of the xylanase from *A. niger* to directly test this theory.

However, there was a commercially available uninhibited xylanase from *B. subtilis* (HPI BS3),

which was used to evaluate this hypothesis.

Table 3.7 Comparison of the Attributes of the Xylanase Inhibitors Found in Wheat.

Attributes	TAXI I	TAXI II	XIP	TLXI
Molecular Mass	40 kDa	30 + 10 kDa	30 kDa	
Molecular Form	Monomer	Monomer	Monomer	Monomer
pI	> 8.8	> 8.8	> 8.0	> 9.3
Specificity	*A. niger, B. subtilis*	*B. subtilis*	*A. niger*	*A. niger*
Mechanism	Competitive	Competitive	Competitive	Noncompetitive
Kinetics	Fast-binding	Fast-binding	Fast/Slow-binding	Slow-binding
K_i	1-20 nM	1-20 nM	2-600 nM	60 nM
Optimal pH	4.8-5.5	4.8-5.6	4.5-6.5	5.0-5.5
Optimal Temperature	20˚C-40˚C	20˚C-40˚C	30˚C	40˚C

TAXI: Triticum aestivum Xylanase Inhibitor; XIP: Xylanase Inhibiting Protein; TLXI: Thaumatin-like Xylanase Inhibitor

The FWER method was used to evaluate the *B. subtilis* xylanase in its standard form and

the uninhibited forms each in the EU Mirebelle flour. The data in Table 3.8 shows interesting

results. The activities shown in Table 3.8 were generated using the DNSA method and clearly

show that this method is not applicable for uninhibited xylanases. The uninhibited xylanase

(HPI BS3) produced high FWER values at a very small addition level based on active units when

compared to the other xylanase tested. The standard enzyme at 192.6 nkat resulted in an FWER

of 5.831, while the uninhibited version at 5.520 nkat was 19.91. This data suggests the EU

Mirebelle flour has enough TAXI inhibitors for inhibition of the *B. subtilis* xylanase.

Interestingly, the observation from previous tests that the addition of xylanase can decrease the

IWLR was also confirmed with this experiment.

Table 3.8 IWLR and FWER Data for the EU Mirebelle Flour Comparing the Standard Xylanase from *Bacillus subtilis* to the Uninhibited Xylanase from *B. subtilis*.

Treatment	Active Units (nkat)	IWLR	FWER
No Enzyme	0	2.329^C	2.390^D
13.7 ppm Bakezyme® BXP 25001	38.55	0.4297^D	2.463^D
20 ppm HPI BS3	1.106	3.977^B	3.897^C
68.5 ppm Bakezyme® BXP 25001	192.6	.9698^{CD}	5.831^B
100 ppm HPI BS3	5.530	9.400^A	19.91^A
^{ABCD}For each column, average values with the same superscript are not significantly different (p>.05)			

A similar test was conducted in the U.S. ADM Chattanooga flour. Similar results were found in this flour as shown in Table 3.9. The consistency with the HPI BS3 was also very interesting with FWER values of 19.91 for the Mirebelle flour and 19.89 for the Chattanooga flour.

Table 3.9 IWLR and FWER Data for the U.S. ADM Chattanooga Flour Comparing the Standard Xylanase from *B. subtilis* to the Uninhibited Xylanase from *B. subtilis*.

Treatment	Active Units (nkat)	IWLR	FWER
No Enzyme	0	1.861^B	2.079^C
68.5 ppm Bakezyme® BXP 25001	192.6	2.929^B	7.559^B
100 ppm HPI BS3	5.530	18.74^A	19.89^A
^{ABC}For each column, average values with the same superscript are not significantly different (p>.05)			

Conclusions

A new method for the evaluation of xylanases in baking has been developed and evaluated. This method improves upon other commonly used methods by using wheat flour as the substrate and providing consistent statistically significant results. The FWER method demonstrates that the xylanase from *A. niger* will release more water than the xylanase from *B. subtilis* in most wheat flours. This research also clearly shows the impact of proteinaceous xylanase inhibitors in wheat on the functionality of standard commercially available xylanases. These findings indicate the baking industry should use an uninhibited xylanase because they would achieve greater consistency across different crops of flour.

Suggested Future Work

Two interesting findings from this study would benefit from additional work. The first unique finding was that the IWLR decreased with the addition of optimized levels of the xylanase from *B. subtilis*. In theory, this can be explained by the xylanase breaking down the Water-Unextractable Arabinoxylan into High Molecular Weight Water-Extractable Arabinoxylan (HMW WE-AX). The HMW WE-AX have a much greater capacity to hold water than the WU-AX, which would explain the decrease in initial water loss. However, this hypothesis should be tested.

The second finding worthy of additional work was the clear effect of the flour's xylanase inhibitors on the functionality of standard xylanases. These findings would be validated by analyzing each of the four flours in this study for the levels of the three xylanases inhibitors. This data would be able the test the hypothesis that the U.S. ADM Camp Hill flour had higher levels of XIP and TLXI inhibitors than the other flours and the EU Mirebelle flour had high levels of TAXI inhibitors.

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