ROLES OF CARBOHYDRATES AND PROTEINS IN THE STALING OF WHEAT FLOUR TORTILLA

A Dissertation

by

JUMA NOVIE AYAP ALVIOLA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Food Science and Technology

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ABSTRACT

Roles of Carbohydrates and Proteins in the Staling of
Wheat Flour Tortilla. (May 2007)
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Effects of enzymatic modification of starch, proteins and pentosans on dough and tortilla properties were determined to establish the role of these wheat components in tortilla staling. Starch, protein and pentosans were respectively modified with α-amylase, protease and transglutaminase (TG), and xylanase. Tortillas were stored at 22^{o}C and evaluated for at least three weeks.

Amylase improved shelf-stability of tortillas, produced a significant amount of dextrins and sugars, retarded decrease in amylose solubility, and weakened starch granules. However, control and treated tortillas had similar degrees of amylopectin crystallinity. Staling of tortillas appears to involve starch that reassociates into an amorphous structure.

Micrographs of control dough had thin protein strands forming a continuous matrix. Protease-treated dough had pieces of proteins in place of the continuous matrix, while TG-treated dough had thicker protein strands that were heterogeneously distributed. Both treatments resulted in shorter shelf-stability of tortillas. The organization of protein in dough is important for dough structure and appears to impact tortilla flexibility.

Protein solubility and SDS-PAGE results did not differentiate control and treated dough or tortillas. The fractions or molecular weight distribution are not significant determinants of protein functionality. Tertiary and quaternary protein structures of gluten may be more related to tortilla shelf-stability.

The 75 ppm xylanase treatment resulted in weaker tortilla structure and significantly higher amounts of low molecular weight saccharides and sugars. Control and the 25 ppm treatment sample had a similar shelf-stability and texture profile. Pentosans may affect staling indirectly through the effect on gluten development.

Fresh tortillas have amylopectin in an amorphous state, while amylose is mostly retrograded. The gluten matrix provides additional structure and flexibility to the tortilla. Pentosans may or may not be attached to the gluten network. Upon storage, amylopectin retrogrades and recrystallizes, firming the starch granules, resulting in firmer tortillas. Starch hydrolysis decreased the rigid structure and plasticized polymers during storage. It also reduced the restriction imposed by retrograded starch on gluten and allowed it more flexibility. Thus, the flexibility of tortillas results from the combined functionalities of amylose gel, amylopectin solidifying the starch granules during storage, and the changed functionality of gluten after baking.

DEDICATION

For Pete and Nathan

ACKNOWLEDGMENTS

I wish to thank my major adviser, Dr. Ralph Waniska, for patiently mentoring me through the rigors of research, from lab techniques to explaining results and looking at the big picture. I also thank Dr. Lloyd Rooney for giving me the opportunity to attend Texas A&M, and for regularly checking on my research progress and giving helpful advice. A big thank you to my other committee members, Drs. Jimmy Keeton and Luis Cisneros, for their valuable comments and suggestions to improve this work.

A special thank you to Dr. Norman White and Dr. Koushik Seetharaman for assistance in doing the X-ray diffraction and DSC analyses, respectively. Likewise, thanks to Dr. Stan Vitha for taking the confocal micrographs and for showing me how to use Image J and Olympus FV Viewer.

I am grateful to the companies that generously provided the flour, enzymes and other ingredients for my experiments.

I greatly appreciate the scholarships from the Intercollegiate Faculty of Food Science and Technology, and AACC International. Thanks, too, to the Philippine Rice Research Institute for letting me to pursue this program.

I thank the Cereal Quality Lab staff and fellow students for their assistance in processing the tortillas, and insightful discussions of my research results.

Thanks to Grace Bible Church, especially the Community class and friends from the Homebuilders class, for prayers and encouragement.

A big thank you to the Filipino families and friends in College Station, and the Tabiens in Beaumont for welcoming me and my family to their homes and lives, and giving us a break from the school routine.

Thanks to my parents, parents-in-law, and siblings for being an ever-present support system. My special thanks go to Pete for the love, patience and unwavering belief that I can do it, and to Nathan for being such a joy each day.

My utmost gratitude goes to the Almighty God who made this work possible. Thank you for seeing me through and providing everything I needed, as always.

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INTRODUCTION

Tortillas are now part of the American diet, aside from being a staple for Hispanics. Sales in the US in 2002 reached $5.2 billion (Anonymous 2003), and is expected to increase to $7 billion in 2006 (Sladky 2006). Versatility is one reason for the popularity of tortillas (Dally and Navarro 1999, Waniska 1999). Tortillas are currently not limited to burritos, tacos and enchiladas, but are also in soups, casseroles and desserts. They can also be low-fat, low-carb, high fiber, or whole grain, and they come in various flavors. Tortillas are so highly accepted that they are not confined to the grocery aisle, but are also served in fast food chains, restaurants and school cafeterias. With this increase in demand, the traditional processing of tortillas had to be modified. Tortillas are no longer prepared and served the same day, and yet consumers expect tortillas to retain "freshness" even after several days or weeks of storage.

Retention of flexibility during storage is a mark of good quality tortilla. However, despite the use of an optimized formulation and processing conditions, flour tortillas stale, i.e., become firm and break or crack when rolled. Relative to bread, tortillas generally stale slower, about 5 days for bread and 20 days for tortilla. This is thought to be due to differences in processing time and temperature, and dough moisture content (Seetharaman et al 2002). Several factors including ingredients, processing and post-processing conditions have been shown to significantly affect flour tortilla shelf-stability. Ingredients found to retard staling in flour tortillas include polyols, hydrocolloids and a leavening system with high acid-to-base ratio and bicarbonate of finer grade. Polyols (glycerol, propylene glycol, maltitol) prolonged shelf-stability only when used with flour with at least 11% protein content by acting as a plasticizer and interacting with gluten during processing to stabilize the structure (Suhendro et al 1995). Hydrocolloids, particularly carboxymethyl-cellulose (CMC) and cellulose-based commercial blends,

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This dissertation follows the style of Cereal Chemistry.

retained tortilla flexibility longer by allowing proper gluten development and retaining moisture in the baked product (Friend et al 1993, Waniska 1999). A high acid-to-base ratio (1.9) in the leavening system and finer grade of bicarbonate may have increased shelf-stability of tortillas by producing fewer air bubbles (Bejosano and Waniska 2004). A high level of cysteine (80 ppm), on the other hand, improves dough machinability but hastens staling by excessive depolymerization of the wheat proteins, which weakens the gluten matrix (Srinivasan et al 2000).

The hot-press pressure and time combination used during processing affects tortilla properties including shelf-stability (Adams and Waniska 2005). The mechanism involved is unclear, but it may lie in how the processing condition changes the protein and starch polymers during hot-pressing. Under- and over-mixing the dough also adversely affects tortilla shelf-stability (Srinivasan et al 2000). Under-mixing produces a heterogeneous dough while over-mixing disrupts the gluten network, and both conditions result in short shelf-stability.

Likewise, the temperature at which tortilla is stored has an effect on the rate of staling. Optimum storage temperature for flour tortillas was established at -12^{o}C or less where the product is in a "glassy state" and there is very minimal or no polymer mobility (Kelekci et al 2003). Tortillas firmed when they were stored at 0-35^{o}C, with the greatest firming observed at 22^{o}C. The reason for this observation is also unknown, but changes in protein hydration and associations with other components during storage may be involved.

The factors that affect shelf-stability of tortillas, as explained above, directly or indirectly affect the flour components, particularly starch and protein. Tortillas also stale because of the inherent changes in starch and proteins during storage. Thus, the flour components and the changes they undergo warrant a thorough study in relation to staling. Studies related tortilla shelf-stability with starch (Seetharaman et al 2002, Waniska et al 2002, Guo et al 2003) and protein (Suhendro et al 1993, Pascut et al 2004), but their specific involvement in staling needs further elucidation.

Research on the probable components that contribute to bread staling is extensive because of the desire to cut down the losses from unsold stale bread. Staling, specifically in bread, is defined as any change, other than microbial deterioration, which occurs after baking resulting in loss of freshness and quality of the baked product (Zobel and Kulp 1996). These changes may be in sensory (aroma, mouth feel) or physical (softness) attributes.

Studies on bread staling started as early as 1912 where the focus was on starch since bread and starch paste had the same changes with time, i.e., hardening, loss of swelling power and decrease in soluble starch (Katz 1928). Other researchers also suggested that the changes in starch alone can cause staling (Morgan 1997, Stauffer 2000, Kim and D'Appolonia 1977a-d). However, others showed that starch retrogradation and increase in firmness do not necessarily correlate, suggesting that there are other factors or components involved like wheat protein (Maleki et al 1980, Martin et al 1991), pentosans or non-starch polysaccharides (Michniewicz et al 1992), and moisture migration (Baik and Chinachoti 2000, Hallberg and Chinachoti 2002).

The purpose of this investigation is to understand the mechanism(s) of staling in wheat flour tortillas by developing a model based on the functionality of carbohydrates and proteins. Wheat flour starch, protein and pentosans have specific roles in tortilla processing. Starch in baked products (i.e., limited water) upon gelatinization becomes partly soluble and dispersed. Upon cooling, the reassociation of starch molecules contributes to the structure and texture of the final product. Gluten, on the other hand, gives the viscoelastic property of the dough, and makes holding of gases possible. The importance of pentosans lies in their ability to bind a large portion of water, competing with gluten and starch.

Knowing the roles and level of involvement of carbohydrates and proteins in tortilla staling will provide valuable information particularly to people in tortilla research and development both in academe and the food industry. The results of this study will increase the understanding of the staling phenomenon in flour tortillas. For practical

applications, the results can be used as a basis for improving formulations and/or modifying ingredient functionality to retard firming of tortillas.

RESEARCH OBJECTIVES

The overall goal of this research was to better understand the mechanism (or mechanisms) of staling in the wheat flour tortilla. Specifically, the objectives were:

(1) To determine the role of starch, protein and pentosans in the staling process using enzymes that modify these polymers, and
(2) To develop a model for tortilla staling based on the results of this study and from literature.

CHAPTER II
ROLE OF STARCH IN TORTILLA STALING

INTRODUCTION

Starch and Staling

Staling of wheat tortillas means loss of flexibility with time. Since starch comprises 63-72% of wheat flour on a 14% moisture basis (Atwell 2001), it is considered to be primarily involved in staling. Moreover, changes in starch properties including enzyme susceptibility, solubility, swelling power, iodine absorption and crystallinity have been related to changes in texture with time (Zobel and Kulp 1996).

Seetharaman et al (2002) studied the changes in wheat tortilla and buns during storage, particularly changes in the starch component. Stiffness of the tortilla was observed to correlate with storage time. Together with this, amylose solubility significantly decreased (about 33%) after baking and continued to decrease throughout storage. Amylopectin recrystallization in tortilla was almost immediate after baking while buns showed increasing recrystallization from 1 to 8 days of storage. The authors explained that this is because of the less harsh processing conditions of flour tortillas resulting in less dispersion of amylose and amylopectin, and consequently faster reassociation between polymers.

Amylose content of wheat flour also affects tortilla quality and shelf-stability. Flour with reduced or low amylose content produced thinner and less opaque tortillas because of the lack of retention of air bubbles (Waniska et al 2002, Guo et al 2003). Tortillas with waxy flour had lower tensile strength and higher rupture distance (i.e., more flexible) on the first day than the control. However, on the third day of storage, tortillas with waxy flour were less flexible than the control. Guo et al (2003) indicated that the addition of waxy flour delayed the initial starch recrystallization, but amylopectin retrogradation during storage adversely affected shelf-stability.

Damaged starch from dry-milling of wheat either positively or negatively affects the functionality of starch in baked products. It increases water absorption and

susceptibility to amylases. Damaged starch is another factor that has been reported to affect tortilla quality. High levels of damaged starch resulted in an increase in dough toughness, a decrease in tortilla diameter and opacity, but an improved shelf-stability (Arora 2003). The improved shelf-stability may be from better gluten formation and distribution. In contrast, Mao and Flores (2001) observed that as damaged starch increased, tortillas became less flexible and firmer. The authors explained that damaged starch increased the surface area resulting in inefficient covering of starch by gluten. Damaged starch has different effects on other baked products. A high level of damaged starch (14.1-16.5%) is desirable in making chapati, a staple flat bread in India, because it holds more water, which then provides steam to puff the product (Haridas Rao et al 1989). In contrast, cake flour has a low level of damaged starch for low and even hydration of starch granules.

Kim and D'Appolonia (1977a-d), in their kinetics study on bread staling, concluded that the primary staling mechanism is similar to starch crystallization. They stated that proteins and pentosans affect staling rate only by diluting starch, or by hindering starch crystallization. Stauffer (2000) also believes that staling is related to starch retrogradation because emulsifiers (e.g., monoglycerides) can be used as antistaling agents by complexing with gelatinized and solubilized amylose, thus hindering retrogradation.

Among the starch polymers, amylopectin is believed to play the most important role in staling. Reheating of bread above 50^{o}C (but below 90^{o}C) can bring back its freshness, and only retrograded amylopectin, not amylose, melts at this temperature (Gray and BeMiller 2003). Moreover, the decrease in solubility of amylopectin was observed to progress with staling, while decrease in amylose solubility was significant only during the first day of storage (Kim and D'Appolonia 1977c). On the other hand, Hug-Iten et al (1999) showed, through microscopy techniques, that amylose is more involved by enhancing the rigidity of starch granules during staling. They showed that amylose and amylopectin phase-separate, with amylose crystals in the inside cavity of the gelatinized starch granule and amylopectin crystals in the outer zone.

The functionality of starch is based mainly on its changes during heating in the presence of water. An increase in viscosity results from water uptake and swelling of the starch granules, which leads to the release of soluble starch (Hoseney 1994). Upon cooling, a starch solution again increases in viscosity, from loss of energy, which allows starch chains to associate through hydrogen bonding. In baked products, the outer branches of amylopectin and the dispersed amylose reassociate with other free chains after the baking process. This process of reassociation into ordered structures is called retrogradation. In this state, some crystalline regions may be formed (Atwell et al 1988), and likewise, starch becomes insoluble again (Zobel and Kulp 1996).

The extent of starch granule disruption and dispersion depends on several conditions including temperature, starch concentration, mechanical treatment, and the amount and availability of water (Zobel and Kulp 1996). A tortilla system has limited water; consequently, not all the starch becomes soluble and dispersed. A starch gelatinization gradient, which increases towards the center of the layers, develops in baked tortillas (McDonough et al 1996).

Amylase and Staling

Amylase was originally added to bread formulas to produce fermentable sugars. Formulas today have relatively higher levels of fermentable sugars, but amylase is still added to improve processing conditions and overall quality of the baked product (Kuracina et al 1987, Mathewson 2000). Amylases also slow down bread staling, thus these have been used extensively in trying to explain the staling mechanisms. In general, theories include the following: amylases produce soluble saccharides which exert antistaling effect, the enzymes attack long starch molecules which link crystalline regions, and these remove protruding amylopectin branches, thus, hinder cross-linking with amylose (Bowles 1996). The source and properties of the amylase affect the rate of bread staling. Bacterial amylase is generally more efficient in retarding staling than fungal amylase, and these are better than cereal amylase (Akers and Hoseney 1994, Martin and Hoseney 1991). Martin and Hoseney (1991) observed that bacterial and fungal α-amylase produce a larger amount of DP 3-9 dextrins than cereal amylase, so

these are said to lengthen shelf-stability. The authors further proposed that maltose diffuses from the starch and protein interface, thus giving no interference while the longer and larger dextrins hydrogen-bond to the proteins, increasing firmness of the bread crumb.

Duedahl-Olesen et al (1999), on the other hand, showed that linear maltodextrins (maltotriose, maltotetraose), glucose and maltose did not decrease enthalpy values of baked and aged flour-water dough, which means that they have no effect in amylopectin recrystallization. However, addition of 3% γ-cyclodextrin gave a significant decrease in enthalpy.

Arora (2003) studied the effect of different carbohydrases on tortilla quality. A bacterial α-amylase extended shelf-stability of flour tortillas from 12 to 28 days and improved tortilla diameter. A maltogenic enzyme and xylanase also improved shelf-stability but to a lesser extent. Two amyloglucosidases, a malted barley amylase and a fungal amylase did not contribute any improvement to tortilla quality.

This research was conducted to compare amylase-treated and control tortillas, and propose a mechanism on how starch is involved in the staling of this product.

MATERIALS AND METHODS

Flour Tortilla Preparation and Storage

The control tortilla formula had the following ingredients: 1000 g wheat flour (untreated, bleached, enriched; ADM Milling Co., Overland Park, KS), 60 g shortening (Sysco Corp., Houston, TX), 15 g salt (Morton International, Inc., Chicago, IL), 6 g sodium bicarbonate (Arm and Hammer, Church and Dwight Company, Inc, Princeton, NJ), 5.8 g sodium aluminum sulfate (Gallard-Schlesinger Industries, Plainview, NY), 5 g sodium steroyl lactylate (American Ingredients Company, Kansas City, MO), 4 g sodium propionate (ADM Arkady, Olathe, KS), 3.3 g encapsulated fumaric acid (Balchem Corp., New Hampton, NY), 4 g potassium sorbate (ADM Arkady, Olathe, KS), and 510 g of distilled water. The α-amylase-treated tortillas used the same ingredients except that it only had 480 g distilled water in which the 100 ppm enzyme (A7595,

Sigma-Aldrich, Inc., St. Louis, MO) was dissolved. The α-amylase (1,4- α-D-glucan glucano-hydrolase, EC 3.2.1.1) had an enzyme activity of 250 amylase units (AU) per gram. One AU is the amount of enzyme that dextrinizes 5.26 g dry starch per hour. The enzyme-treated tortilla had 25 AU per batch or about 0.69 AU per tortilla.

The dry ingredients were mixed for 2 min with a paddle at low speed (Model A-200, Hobart Corp., Troy, OH). Shortening was added and mixed for 5 min at low speed. Water, which was heated to about 35°C, was added and mixed with a hook for 1 min at low speed, and for 6 min at medium speed. The dough was proofed for 5 min (32-35°C, 70-75% RH, Model 57638, National Manufacturing Co., Lincoln, NE), then pressed into a stainless steel plate, divided and rounded into 36 dough balls (Dutchess Tool Co., Beacon, NY). Dough balls were held for 10 min in the proofing chamber before hot-pressing and baking.

Tortillas were pressed and baked in a three-tier gas-fired oven (Model 0P01004-02, Lawrence Equipment, El Monte, CA). The top and bottom platen temperature of the press was 400°F (204.4°C). The hot-pressing dwell time was 1.35 sec with a pressure of 1100 psi. The oven temperature was 350-365°F (177-185°C) and oven dwell time was 30 sec. The tortillas were cooled on a three-tier conveyor (Model 3106-INF, Superior Food Machinery Inc., Pico Rivera, CA) and individually placed on a sanitized table to cool further. These were then packed in polyethylene bags.

Tortillas were stored at ambient temperature (22°C), and were sampled at 0, 0.04, 0.21, 1, 3, 7, 14, 21 and 28 days after baking. Three batches of control and amylase-treated tortillas were prepared or separate days and evaluated.

Physico-chemical Properties

The dough was evaluated subjectively using a scale of 1 to 5 for smoothness, softness, extensibility, force to extend (elasticity) and press rating (force required to flatten dough). A score of 1 means the dough is very smooth, very soft, breaks immediately, needs less force to extend, and is easy to press, respectively. Conversely, a score of 5 means that the dough is very rough, very firm, excessively extensible, needs much force to extend, and is hard to press to the stainless steel round plate.

Ten tortillas from each batch were randomly selected and measured for weight, height, diameter and opacity after one day of storage. The stack of ten tortillas was weighed, and measured for height using a caliper (Chicago Brand 12" Electronic Digital Caliper, Chicago, IL). Diameter was measured from two points for each tortilla. Opacity was subjectively evaluated using a score of 100% for complete opacity and 0% for complete translucency. Color was measured from two tortillas with a colorimeter (CR-310 Chroma Meter, Minolta Corp., Ramsey, NJ). Measurements were taken from three points from both sides of the tortilla. Moisture content was determined using the AACC (2000) two-step method (Method 44-15A). About 5 g of air-dried (room temperature, 48 hr), ground tortilla was mixed with 50 g distilled water, and pH was immediately measured using a pH meter (Model IQ240, IQ Scientific Instruments Inc., San Diego, CA).

Shelf-Stability and Texture

Subjective Test (Rollability Test)

Tortillas were wrapped around a dowel (1 cm diameter) and evaluated based on a scale of 1 (breaks immediately; cannot be rolled) to 5 (no cracks; very flexible). Tortillas were considered unacceptable when the rollability scores were below 3.

Objective Test (2D Extensibility Test)

Textural changes were monitored using a texture analyzer (Model TA-XT2i, Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK). The TA-108 fixture and an acrylic probe of 7/16-inch diameter with a flat edge were used. The test was conducted using the return to start option with compression mode and trigger force of 0.05 N. Pre-test, test, and post- test speeds were 5.0 mm/s, 1.0 mm/s, and 5.0 mm/s, respectively, with a distance set at 25 mm. The modulus of deformation, work, maximum force and distance needed to rupture the tortilla were determined using the instrument's software program.

Carbohydrate Profile

A 0.4 g ground sample was suspended in 10 mL double distilled water, solubilized at 50°C for 10 min, sonicated for 15 sec at 20 KHz, centrifuged for 10 min at 6000 rpm, and filtered through a 5 μm nylon filter. The extract was fractionated with degassed double distilled-deionized water as the mobile phase at a 1mL/min flow rate, and through a series of columns (Showa Denko, Japan) held at 50°C. The refractive index detector (Waters Model 410, Millipore Co., Milford, MA) was held at 50°C. The total amount of dextrins and sugars was determined using the ChromPerfect LSi 5.5.4 software (Justice Laboratory Software, Justice Innovations Inc., Denville, NJ). Standard solutions of maltose (Sigma-Aldrich, Inc., St. Louis, MO) and DE 13 maltodextrin (Cargill Inc., Cedar Rapids, IA) were used to make a concentration vs. area standard curve for sugars and dextrins, respectively.

The molecular weight of the dextrins and sugars were estimated using pullulan standards (Showa Denko, Japan). The standards, with a molecular weight range of 5900 to 112000 Daltons, were used to make a molecular weight vs. retention time standard curve.

Stabilization of Tortillas

Two tortillas were mixed with 250 mL of methanol with 0.05% mercuric chloride in a blender for 2 min, and vacuum-filtered. The sample was rinsed again with 200 mL methanol and filtered before drying at 50°C for 3-4 hr in a forced-air oven, and ground in a cyclone mill. Stabilized samples were stored at –40°C and used for RVA, DSC, X-ray diffraction and amylose solubility analyses.

Amylose Solubility

The procedure of Seetharaman et al (2002) was followed, which used 0.1N NaOH (for processed starches). A 100 mg sample of stabilized tortilla was weighed into a 100 mL volumetric flask. Then 1 mL of 95% ethanol and 9 mL of 0.1N NaOH was added and mixed with the sample. The flasks were kept for 2 hr at room temperature, then filled to 100 mL with distilled water. A 5 mL aliquot was transferred to another 100 mL flask with about 50 mL distilled water, 1 mL of 1N acetic acid and 2 mL of iodine

solution. Distilled water was added to 100 mL, and the solution was thoroughly mixed. The blank contained 5 mL of 0.09N NaOH in place of the 5 mL sample and served as the reference. Absorbance of the samples was measured at 620 nm. Potato amylose (Sigma Chem. Co., St. Louis, MO) and normal corn starch (Argo, Bestfoods, Englewood Cliffs, NJ) were used as standards.

Pasting Properties (RVA Analysis)

Slurries of the stabilized samples (15% solids dry basis; total weight of 28 g) were prepared with 2% mercuric chloride solution and evaluated for pasting properties using a rapid viscoanalyzer (RVA Model 4, Newport Scientific PTY Ltd, Sydney, Australia). The temperature profile used started with heating from ambient temperature to 50^{o}C (0-1 min) followed by a linear temperature increase from 50-95^{o}C (1-4.45 min), then a holding step at 95^{o}C (4.45-7.15 min), cooling to 50^{o}C (7.15-11 min), and lastly another holding step at 50^{o}C (11-13 min). The peak, trough, breakdown, setback and final viscosities, and peak time were determined using the Thermocline software (Newport Scientific PTY Ltd, Sydney, Australia).

Amylopectin Crystallinity

X-ray Diffraction

The X-ray diffraction analysis was performed on a Philips defractometer (Philips Electronic Instruments, Mahwah, NJ) using 30 kV and 18 mA with Cu Kα radiation. The step scan mode was used with a step size of 0.05^{o} 2θ and a dwell time of 5 s at each step (Deng et al 2006). The x-ray patterns were interpreted using the method of Zobel (1964).

Differential Scanning Calorimetry

Stabilized tortilla samples (4 mg) were rehydrated with 8 mg of distilled water for 20 min in aluminum pans, and sealed for analysis. These were heated from 30 to 120^{o}C at a rate of 10^{o}C/min. Enthalpy of gelatinization/retrogradation were determined.

Statistical Analysis

The effects of amylase and storage time on dough and tortilla quality were evaluated using SPSS version 11.5 (SPSS Inc., Chicago, IL). Analysis of variance in a

completely randomized design was done to determine any significant contribution of the treatments and/or their interactions. LSD ($p<0.05$) was used to compare multiple means.

RESULTS

Dough Properties

The control and amylase-treated dough had the same softness, intermediate extensibility, and needed moderate force to extend, and were easy to press to the stainless steel plate for dividing and rounding (Table I).

The amylase-treated tortilla was prepared with less water (48% of flour weight) than the control (51%) to avoid having sticky dough which is difficult to process. Amylase hydrolyzes damaged starch in the dough, which decreases the water held by the damaged starch. This was observed in the initial stage of the mixograph test (Fig. 1) wherein the amylase-treated dough had a slightly lower viscosity. However, both treatments had similar viscosities upon optimum mixing.

Tortilla Physical and Texture Properties

Control tortillas were heavier, thicker, smaller in diameter and more opaque than amylase-treated tortillas (Table II). Both had similar specific volume, pH and color values.

Control tortillas had significantly higher moisture content than the amylase-treated tortillas (Table II), which is due to the higher amount of water used in the control. However, in both treatments, moisture content did not change significantly with storage (Table III). This means that, in this study, moisture content was not a factor that significantly affected loss of tortilla flexibility. This is in contrast to the study of Mao and Flores (2001) which included loss of moisture as a parameter that caused texture changes in tortilla.

TABLE I

Physical Properties of Control and Amylase-Treated Dough[a, b]

Dough properties[c]	**Control**	**Amylase-Treated**
Softness	2.0a	1.5a
Extensibility	3.1a	3.0a
Force to extend	3.5a	3.4a
Press rating	2.0a	2.0a

[a] Water absorption: control = 51%, amylase-treated = 48%

[b] Means from three trials; means in a row followed by the same letter are not significantly different ($p<0.05$)

[c] Softness: 1 – very soft, 5 – firm; Extensibility: 1 – not extensible, 5 – very extensible; Force to extend: 1 – less force, 5 – much force; Press rating: 1 – easy to press, 5 – hard to press

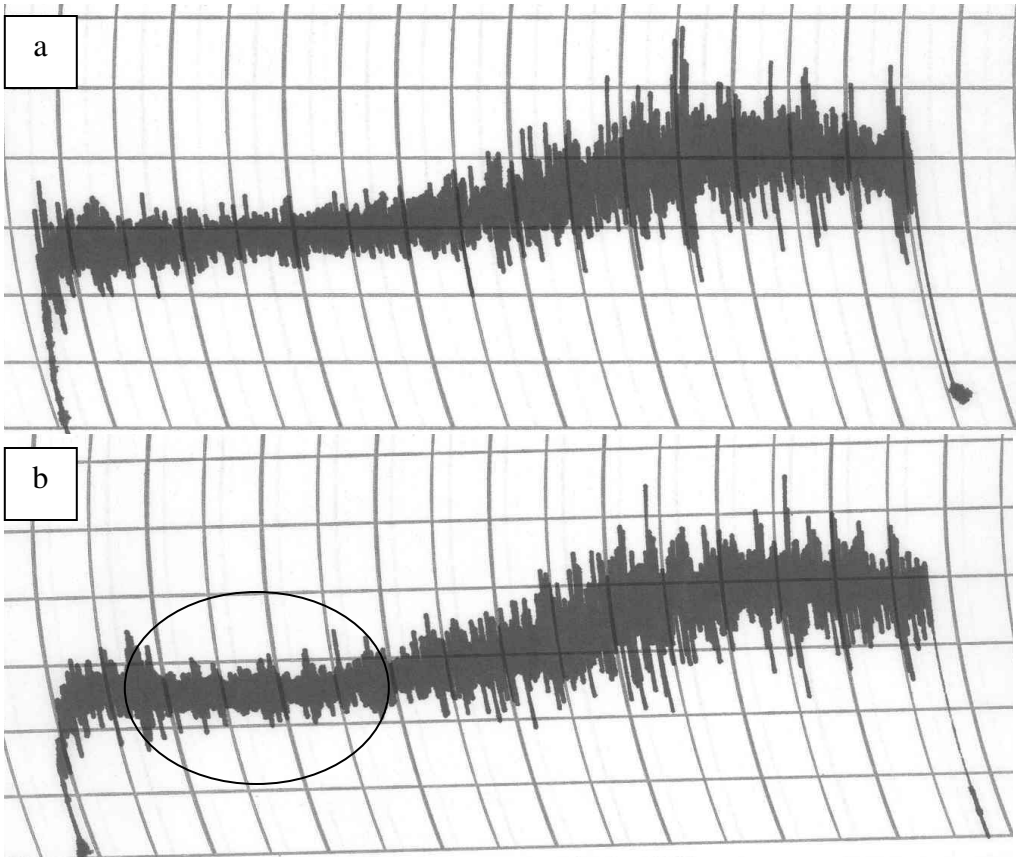

Fig. 1. Mixogram of (a) control and (b) amylase-treated dough. (Encircled part shows early decrease in viscosity of dough from amylase activity).

TABLE II

Physical Properties of Control and Amylase-Treated Tortillas[a]

Tortilla Properties	**Control**	**Amylase-treated**
Weight (g)	40.3a	38.8b
Height (mm)	2.78a	2.62b
Diameter (mm)	170.8b	175.6a
Opacity (%)	75.8a	73.5b
Specific volume (cm^3/g)	1.58a	1.64a
pH	5.4a	5.3a
Moisture (%)	33.2a	31.8b
Color		
L-value	82.38a	82.50a
a-value	0.16a	0.24a
b-value	17.76a	17.58a

[a] Means from three trials; means in a row followed by the same letter are not significantly different ($p<0.05$)

TABLE III

Moisture Content of Control and Amylase-Treated Tortillas Stored for Four Weeks[a]

Storage Time (day)	Control	Amylase-treated
0	33.2a	31.8a
7	32.9a	31.1a
14	32.9a	31.1a
21	32.8a	30.9a
28	32.8a	31.1a

[a] Means from three trials; means in a column followed by the same letter are not significantly different ($p<0.05$)

Control and amylase-treated tortillas had similar rollability scores after three days of storage (Fig. 2). This means that the enzyme did not significantly change the flexibility of the tortilla at this point, as determined by a subjective test.

After seven days of storage, the control tortillas were significantly less rollable than the amylase-treated tortillas. The control tortillas reached the unacceptable score of 3 (i.e., small breaks upon rolling) before two weeks while the amylase-treated tortillas were still flexible after 28 days of storage.

The significantly longer retention of flexibility in amylase-treated tortilla was likewise observed using an objective test (Fig. 3, Appendix A1). The deformation modulus, which is the ratio of force and distance before rupture, was significantly lower for treated tortillas. The treatment-storage time interaction was also significant, which means that the treatments differed in modulus response during storage. The deformation modulus for the amylase-treated tortilla was similar from 5 hr to 28 days of storage. For the control tortilla, the deformation modulus significantly increased after 1 day, leveled from 3 to 21 days, and increased on the 28^{th} day of storage.

The force required to rupture the control and the amylase-treated tortilla was similar (Fig. 3). Overall, force was greatest after 1 hr, 5 hr and 28 days of storage, but did not show any significant changes from 1 to 28 days.

The distance to which the tortilla was extended before rupture was significantly greater in the amylase-treated sample than the control. The treatment-storage time interaction was not significant, which means that both treatments had the same effect on the distance to rupture with storage. Significant decrease in distance was observed until 3 days of storage. No significant change in distance occurred from 3 to 28 days.

The work required to rupture the tortilla was significantly greater for amylase-treated tortilla than the control. Like force and distance to rupture, no significant interaction between treatment and storage time was observed for work. The 1 hr-old tortilla needed the greatest work, and this was significantly higher than the 5 hr and 1 day old tortilla. No significant change in work was observed after 1 day of storage.

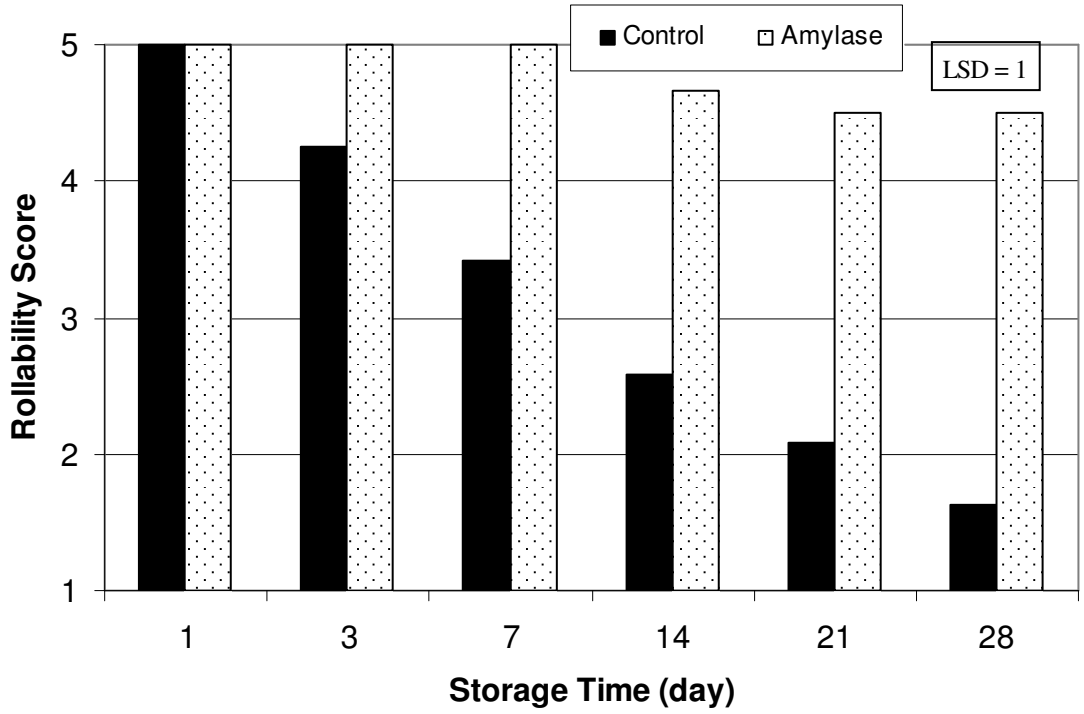

Fig. 2. Rollability scores of control and amylase-treated tortillas stored for 28 days.

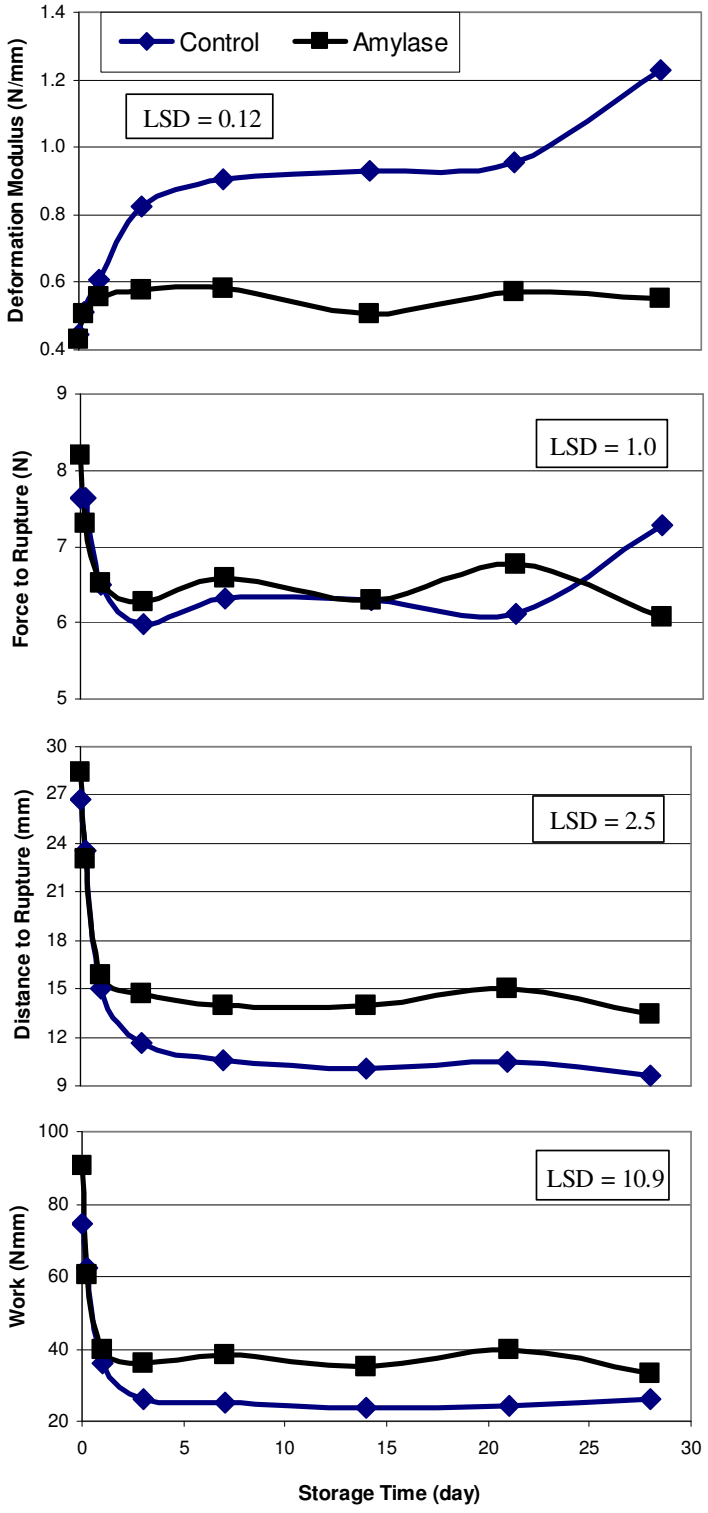

Fig. 3. Texture profile of control and amylase-treated tortillas stored for 28 days.

Carbohydrate Profile

In this study, dextrins refer to saccharides with molecular weight (MW) from 661 to 131,862 Daltons (i.e., eluted between 20-26 min) while sugars are those with MW less than 661 Daltons. The MW of the dextrins in the amylase-treated tortilla ranged from 1862 to 28184 Daltons. This corresponds to a degree of polymerization (DP) of about 10.9 to 164.8 glucose units. The molecular weight of sugars averaged at 447 or a DP of about 2.6.

Amylase-treated tortilla had significantly higher amount of dextrin than the control tortilla (Fig. 4, Appendix A2). No significant increase in dextrins was observed in the control dough and tortillas. These dextrins in the control samples may be from the flour used. In the amylase-treated tortillas, the least amount of dextrin was in the dough, since amylase activity is optimum during gelatinization of starch. The highest amount of dextrin was observed in the freshly baked tortilla until 5 hr of storage. This was followed by a decrease after the tortillas were stored for one day, which may be due to further hydrolysis of the dextrins. Palacios et al (2004) suggested that degradation of high molecular weight dextrins, specifically the external branches of amylopectin, during storage explains why thermostable amylases effectively retard firming.

The amount of sugars in the control dough significantly increased during pressing and baking, but did not change significantly during storage (Fig. 4, Appendix A2). The sugars may be from β-amylase activity inherent in the wheat flour (Mathewson 2000). The absence of change in the amount of sugar during storage indicates that β-amylase was inactivated during baking. The amylase-treated tortillas had a significantly higher amount of sugars than the control. Among the amylase-treated samples, the dough had the least sugars, but this greatly increased after pressing and baking. The increase in sugars continued until the third week of storage.

Martin and Hoseney (1991) reported that bread supplemented with bacterial amylase had dextrins with DP 3-7 while bread with malted barley flour had dextrins larger than DP 9. They concluded that dextrins with DP3-9 delay bread staling since

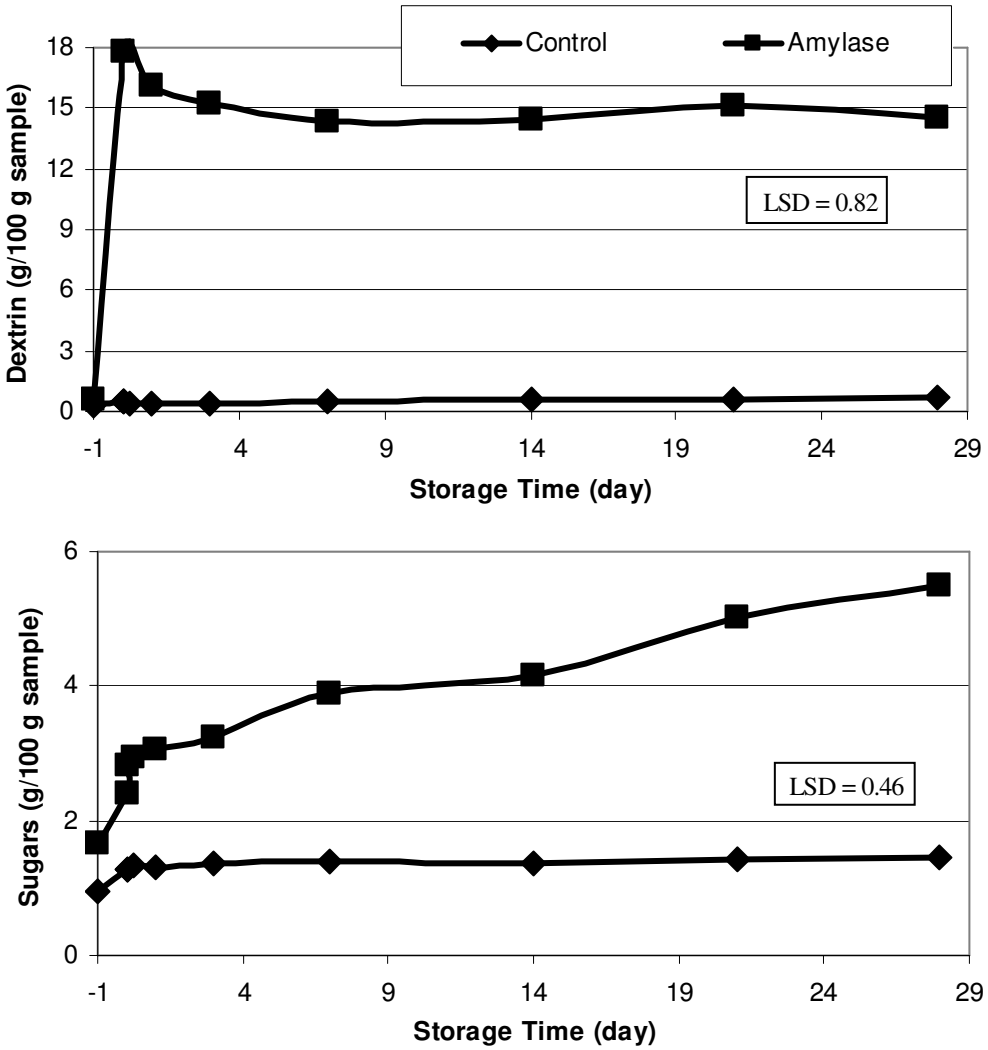

Fig. 4. Dextrin and sugar profile (g/100 g sample, dry basis) of control and amylase-treated dough (-1 day) and tortilla.

bread with bacterial amylase did not firm in 5 days of storage while bread with malted barley flour firmed faster than the control.

Amylose Solubility

Amylase-treated tortillas had significantly higher amylose solubility than the control after one day of storage (Fig. 5, Appendix A3). The amylose solubility in the amylase-treated samples did not significantly change immediately after baking until three weeks of storage. An increase was observed on the fourth week, but this was not significantly higher than the 2 or 3-week old tortilla. Kuracina et al (1987) also observed the decrease in loss of solubility when wheat starch was treated with bacterial, fungal and maltogenic amylases.

The freshly-baked control tortilla had the highest amylose solubility, which decreased drastically (about 28%) after 1 hr of storage. Amylose solubility was similar from 1 hr to 3 days, and from 3 to 28 days. The control dough had similar amylose solubility with the tortilla stored for 1 to 28 days. Amylose becomes soluble during baking and rapidly reassociates making it insoluble again (Zobel and Kulp 1996).

Pasting Profile

Amylase significantly affected the pasting characteristics of the dough and tortilla (Fig. 6, Table IV). When the slurries were heated, the amylase-treated tortillas had significantly lower peak, trough and breakdown viscosities, and a significantly earlier peak time than the control. The peak viscosity of the control and treated dough were similar. However, the breakdown viscosity of the amylase-treated dough was much lower. The hydrolytic action of the enzyme adversely affected the swelling capacity and integrity of the starch granule. Upon cooling of the mixture, the amylase-treated dough and tortilla had significantly lower final viscosities, and a corresponding lower setback, than the control. These low final and setback viscosities indicate minimal reassociation of starch, particularly amylose, when the slurry was cooled. Other studies also reported reduction in pasting viscosities of wheat starch treated with amylases (Kuracina et al 1987, Leman et al 2005).

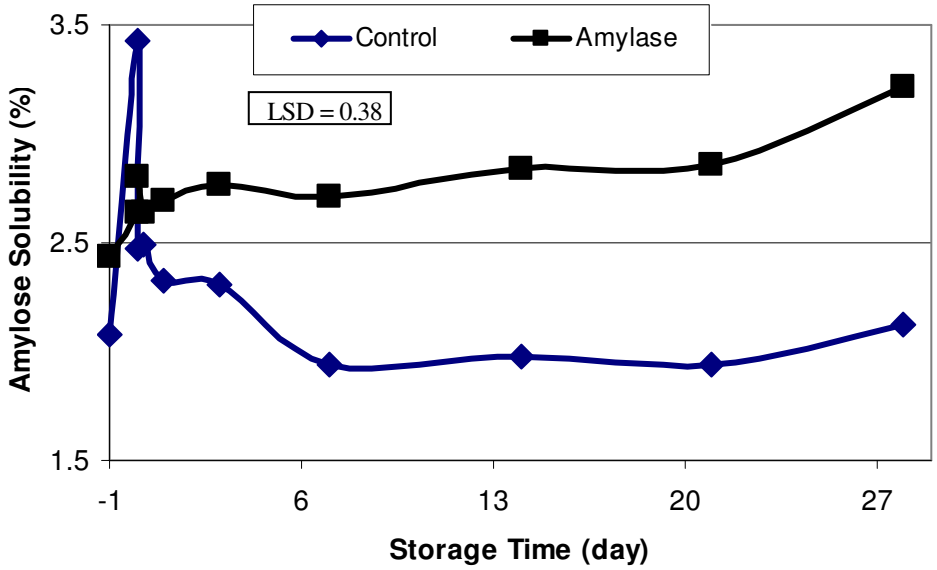

Fig. 5. Amylose solubility (% of flour weight) of control and amylase-treated dough (-1 day) and tortillas stored for 28 days.

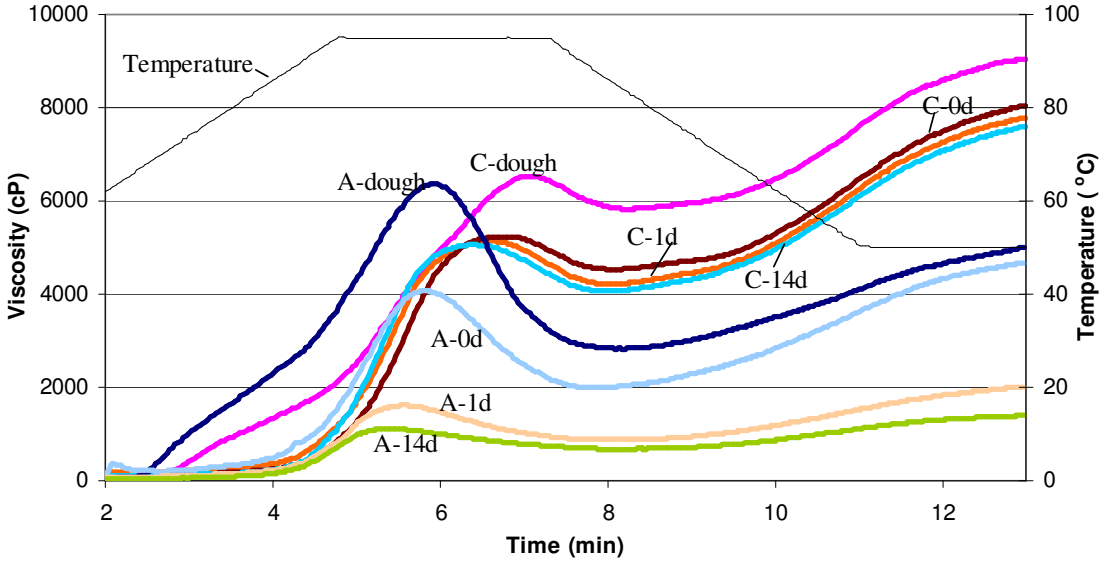

Fig. 6. Pasting profile of control (C) and amylase-treated (A) dough and tortillas stored for 0, 1 and 14 days.

TABLE IV

Viscosity Profile of Control and Amylase-Treated Tortillas[a]

Storage Time (day)	Peak (cP)	Trough (cP)	Breakdown (cP)	Final (cP)	Setback (cP)	Peak time (min)
Control						
Dough	6600	5829	771	8983	3154	7.0
0	5223	4207	1016	7793	3586	6.6
0.04	5160	4188	973	7809	3621	6.6
1	5238	4523	714	8050	3527	6.8
7	5280	4257	1022	7913	3656	6.5
14	5072	4054	1018	7598	3543	6.4
28	4838	3974	864	7342	3368	6.3
Amylase-treated						
Dough	6469	2815	3654	4990	2175	5.9
0	4161	1973	2188	4660	2686	5.8
0.04	3212	1656	1556	3555	1899	5.8
1	1619	871	749	2009	1138	5.6
7	1345	770	575	1674	904	5.4
14	1118	675	442	1395	720	5.4
28	811	510	302	1028	518	5.4
LSD[b]	185	236	182	243	130	0.1

[a] Means from two trials

[b] Least Significant Difference ($p<0.05$)

A significant decrease in the viscosity parameters and peak time was likewise observed with storage in both control and amylase-treated tortilla. This means that changes in the swelling capacity of the starch granules occur during storage. Since granule swelling is associated with amylopectin, amylopectin-amylopectin and amylopectin-amylose associations during storage may be a factor that affected the starch pasting properties.

Amylopectin Crystallinity

X-ray Diffraction

Control and amylase-treated tortillas increased in amylopectin crystallinity with time as indicated by the increase in the relative intensity and number of discernible peaks in the x-ray diffraction patterns (Fig. 7). Tortillas stored for 1 hr had a typical diffuse pattern of an amorphous system with a sharp peak (Lionetto et al 2005), which was centered at around 20.3^{o}. Tortillas stored for 3 and 14 days showed an A-type crystalline structure with peaks around $2\theta = 15.3$, 18.2 and 23.5^{o} (Zobel 1964). This increase in crystallinity is attributed to retrogradation of amylopectin during storage. However, control and amylase-treated tortillas had similar crystallinity patterns throughout storage (Fig. 8). This is in contrast to studies in bread where the amylase-treated samples had greater crystallinity than the control (Zobel and Senti 1959, Dragsdorf and Varriano-Marston 1980). However, results from these earlier studies and this study agree that increase in amylopectin crystallinity does not necessarily correlate with firming or loss of flexibility.

Differential Scanning Calorimetry

Control and amylase-treated tortillas had significant increase in enthalpies from 1 hr to 14 day-old tortillas (Table V). However, both had similar enthalpies for each storage time. This indicates that there was an increase in amylopectin crystallinity with time, but there was no difference in extent of amylopectin crystallinity between the control and amylase-treated tortilla. This agrees with the x-ray diffraction data. This, however, is not consistent with the observations of Defloor and Delcour (1999) wherein

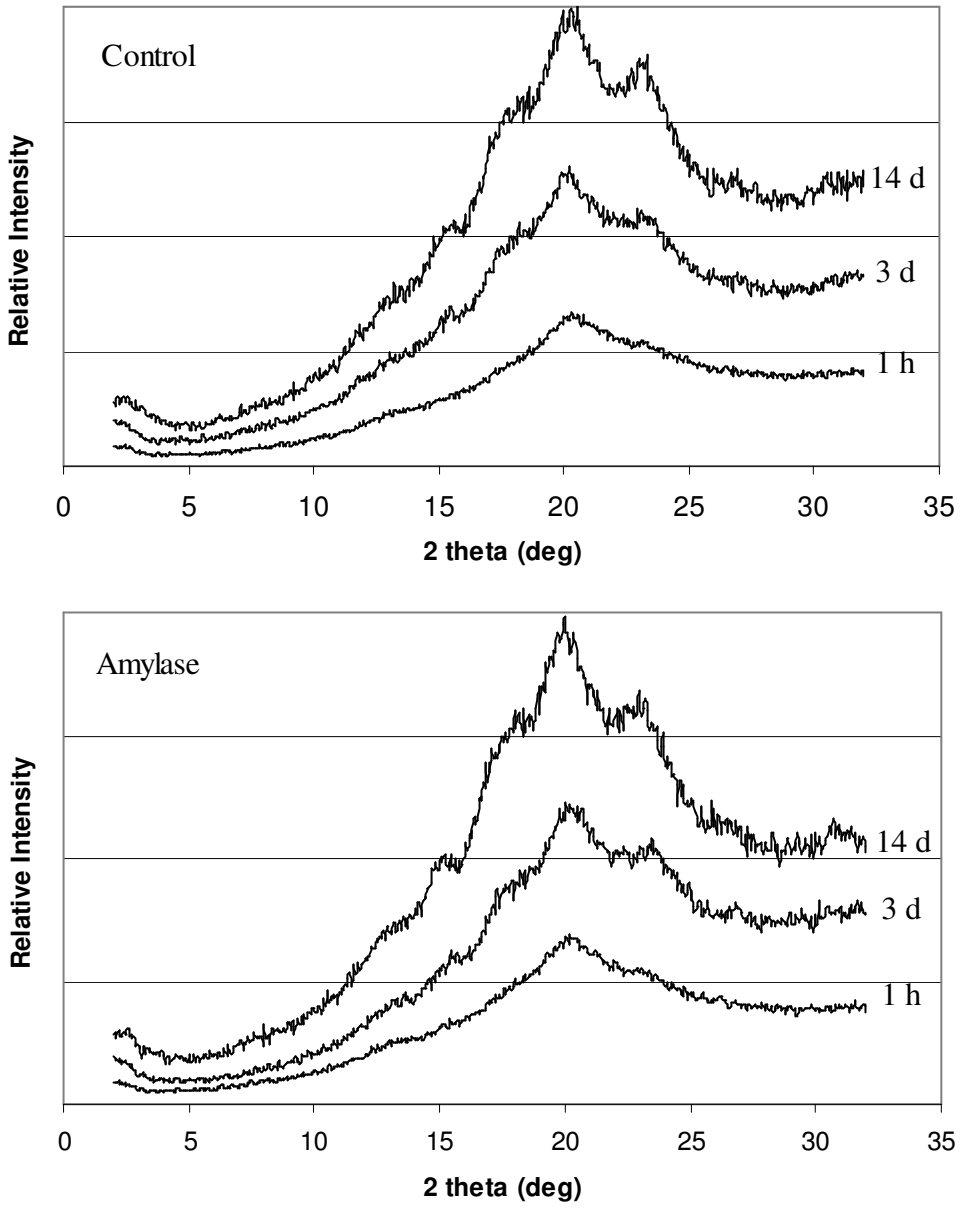

Fig. 7. X-ray diffraction patterns of control and amylase-treated tortillas after 0.04, 3 and 14 days of storage.

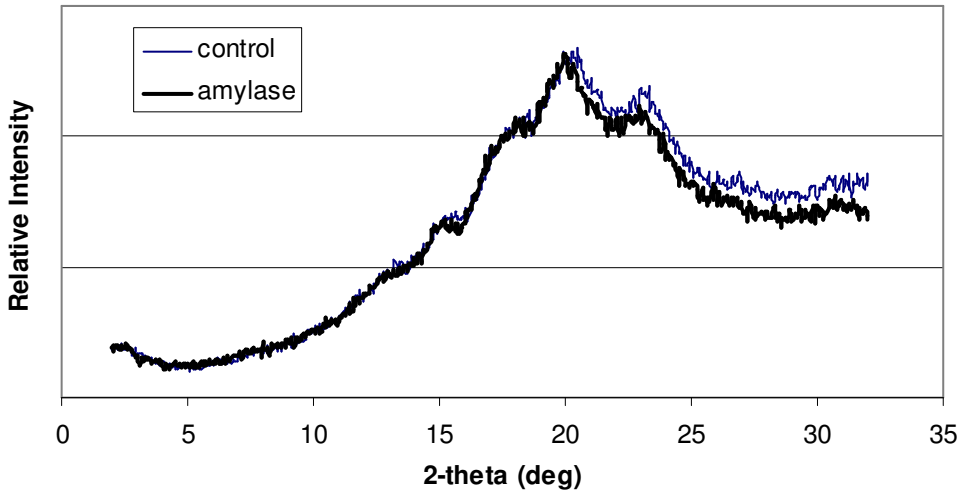

Fig. 8. X-ray diffraction pattern of control and amylase-treated tortilla stored for 14 days.

TABLE V

Enthalpy of Control and Amylase-Treated Tortillas After 1 Hr and 14 Days of Storage[a]

Treatment	Enthalpy (J/g)
Control	
1 hr	2.1b
14 day	3.2a
Amylase-treated	
1 hr	2.1b
14 day	3.0a

[a] Means of two trials; means in a column followed by the same letter are not significantly different ($p<0.05$)

enthalpies of breads supplemented with commercial antistaling amylases significantly reduced with time.

DISCUSSION

This study evaluated the effects of amylase and storage time on the shelf-stability, texture and starch properties of tortillas, with the end goal of explaining the role of starch in staling, i.e., loss of flexibility upon storage.

The texture of a food product is influenced by its structural organization, thus any change in structure has a corresponding change in texture (Aguilera and Stanley 1999). Starch, together with gluten, is primarily involved in tortilla structure. Starch granules comprise the discontinuous phase in the dough. Upon pressing and baking, starch granules gelatinize allowing part of amylose to disperse and be part of the continuous phase. The dispersed amylose reassociates to form a gel, thus contributing to the structure of the tortilla (Seetharaman et al 2002). Because of low moisture and shear during processing, amylopectin mainly remains inside the gelatinized starch granules, which also contribute to structure.

Theoretically, any treatment that will affect the structure functionality of starch will affect texture. The hydrolytic action of amylase changed starch properties, which significantly affected the texture and shelf-stability of the product (Figs. 2 and 3). Addition of amylase to the tortilla formulation resulted in a longer retention of flexibility, which was observed through an objective extensibility test and a subjective rollability test. This corroborates the results of Arora (2003). Amylase is also used commercially as anti-staling agent in bread.

Specific changes in starch properties caused by the addition of amylase that were documented in this study were the production of a significantly higher amount of dextrin and sugars, slower decrease in amylose solubility, and higher rates of viscosity development and reduction of pasting viscosities (Figs. 4-6). The production of dextrins and sugars in the amylase-treated dough and tortillas proved the amylolytic activity of the enzyme in the product. The enzyme had substantial activity during processing

because there was higher dextrin and sugars in the tortilla than in the dough. Moreover, the enzyme was not inactivated by the processing conditions used, because there was continued increase in sugars throughout the storage period.

The hydrolysis of starch into lower molecular weight components resulted in higher amylose solubility and reduction of pasting viscosities. Amylose molecules start reassociating once the temperature cools below 125^{o}C (amylose Tm), which means that they start to retrograde immediately once outside of the oven. The gel formed through retrogradation is insoluble. In the amylase-treated tortilla, the hydrolysis of the dispersed amylose meant less available amylose molecules to reassociate, and consequently not much insoluble gel is formed. The weaker gel formed after cooling was also observed in the RVA analysis of the amylase-treated samples. Leman et al (2005) reported that wheat starch treated with endo-amylases from *Bacillus* spp did not form gels upon cooling.

The lower pasting viscosities of the amylase-treated tortillas showed that the enzyme not only acted on dispersed amylose, but on the starch granule as well. The hydrolytic action of amylase resulted in a less rigid granule structure, a higher swelling rate, and greater susceptibility to disintegration, which was also observed by Leman et al (2005).

Wheat starch granules are semi-crystalline, which is attributed mainly to amylopectin (Atwell 2001). After tortilla processing, when temperature drops below 50^{o}C (amylopectin Tm), amylopectin molecules start to retrograde and crystalline regions are again formed. Aside from difference in Tm, amylopectin retrogradation is slower than amylose because the former is bulkier and needs more time to reach equilibrium lattice positions (Dragsdorf and Varriano-Marston 1980). Since amylopectin molecules retrograde during storage, amylopectin crystallinity has been implicated to cause staling (Kim and D'Appolonia 1977c). In this study, amylase retarded staling but the amylase-treated tortilla had the same amylopectin crystallinity as the control as evaluated by x-ray diffraction and DSC (Fig. 8 and Table 4). This means that the starch hydrolyzed by the α-amylase in the tortillas did not substantially interfere with the

crystallization of amylopectin. This occurred even though greater than 20% of the starch was hydrolyzed to dextrins and sugars. It is assumed that amylopectin crystallization occurs primarily inside the remaining gelatinized starch granule. It is proposed that amylopectin crystallinity does not significantly impact tortilla flexibility. Instead, starch between the gelatinized granules, and starch that retrograded to form the amorphous region, impacted retention of flexibility of tortilla during storage.

It is suggested that the anti-staling mechanism of amylase is through hydrolysis of the dispersed starch, starch molecules bridging the crystalline region, and protruding amylopectin branches. This action reduced retrogradation and also prevented formation of intra- and intergranular linkages. In addition, the hydrolysates acted as plasticizer for the protein and starch polymers, resulting in more flexibility. It has to be noted, though, that a high amount of amylase does not correspond to longer shelf-stability. Excessive hydrolysis of the starch will result in dough that is sticky and difficult to process. Moreover, this will weaken the tortilla structure, since dispersed amylose contributes to this structure.

CHAPTER III
ROLE OF PROTEINS IN TORTILLA STALING

INTRODUCTION

Protein and Staling

Flour with higher protein content and addition of vital wheat gluten to the tortilla formulation both improve shelf-stability. Suhendro et al (1995) observed that flours with 10.2% protein produced tortillas that were less shelf-stable than flours with $\geq$11.0% protein, and an evaluation of 61 commercial tortilla flours also showed that those with 11.0-11.5% protein content yielded more shelf-stable tortillas (Waniska et al 2004). In another study, addition of 2-3% wheat gluten improved elasticity and strength of dough, and shelf-stability of wheat tortillas (Suhendro et al 1993). The authors explained that this might be from the viscoelastic properties of gluten, which were partially retained after baking. They further proposed that the improvement of gluten functionality might be from having more gliadin and glutenin interactions through hydrogen bonding, and ionic and hydrophobic associations.

Pascut et al (2004) reported that tortillas with commercial wheat protein fractions (non-hydrolyzed and slightly hydrolyzed vital wheat gluten-based fractions, gliadin and glutenin, and vital wheat gluten) had similar or longer shelf-stabilities compared to the control tortillas. Addition of vital wheat gluten and glutenin resulted in tortillas with smaller diameter, while tortillas with gliadin had comparable diameters with the control. All three treatments significantly improved shelf-stability, but the authors suggested that tortilla quality would benefit more from an increase in extensibility, which is a gliadin functionality.

Contradictory reports are given on the involvement of proteins in bread staling. A review on bread staling by Zobel and Kulp (1996) states that there is no significant evidence that protein is a major contributor to firming of bread. Gray and BeMiller (2003), on the other had, say that evidence has accumulated that gluten proteins are important in explaining staling.

Approaches used to study the involvement of protein in bread staling include the comparison of aged gluten and starch gels, use of flours with different protein content and quality, and observation of water transfer between gluten and starch (Zobel and Kulp 1996). Gluten gels firm very little during storage while starch gels firm rapidly, thus starch and not protein is said to be involved in staling (Katz 1928).

Breads from high protein flours are generally more shelf-stable, but these also have higher loaf volumes, which translate into a softer crumb. It is not clear whether the longer stability is an effect of higher protein content or larger loaf volume. To eliminate this problem, Bechtel and Meisner (1954) used reconstituted flour with different protein contents, and made breads with comparable loaf volumes and moisture content. They proved that higher protein flours yield breads that keep longer.

Maleki et al (1980) used fractionated starch, gluten and water solubles from weak and strong flours, and showed that the staling rate of bread from reconstituted strong flour was similar to that of bread with starch and water solubles from the weak flour and gluten from the strong flour. From this, the authors proposed that gluten is the primary contributor to staling rate. Callejo et al (1999) supported this conclusion by showing that the addition of 1-2% vital wheat gluten increased flexibility and reduced firmness in bread stored for 48 and 72 hours, especially when water was also increased by 3-6%. Martin et al (1991) suggested that the role of protein, specifically gluten, is through its formation of hydrogen bonds with gelatinized starch, which strengthens during storage through loss of kinetic energy. Morgan et al (1997) disagree with the starch-gluten interaction theory because starch bread staled at a comparable rate to standard bread. They concluded that starch-gluten interactions are not important to bread firmness, and that starch-starch interactions cause most of the observed changes during staling. Likewise, an evaluation of starch bread with different gluten levels (0, 10 and 15%) did not have significant differences in firmness and firming rate (Every et al 1998).

Protein-Modifying Enzymes

Proteases are added to bread formulations to make the dough more extensible and consequently reduce mixing time, and improve flow characteristics, machinability and gas retention (Mathewson 2000). Unlike amylase, the effect of protease on bread or tortilla staling is not yet well established. However, since protein is believed to be involved in bread staling, hydrolysis by protease may have an effect on staling. Barrett et al (2005) studied the effect of two amylase-based and a protease-based enzyme system on the stability of bread. They observed that all three enzyme-systems retarded firming but the protease had the most significant effect. The authors explained that the protease might have cleaved the protein-starch structure formed during storage**,** while the amylases produced dextrins that inhibited the formation of the said structure. However, this assumes that the protease was not inactivated during baking.

Transglutaminases (TG) are enzymes that catalyze acyl-transfer reactions resulting in covalent cross-links between lysine and glutamine residues, and they also hydrolyze glutamine to glutamate, and incorporate amine groups via the amide moiety of glutamine (Gerrard et al 2001). The use of TG in the baking industry is relatively recent compared to other enzymes like amylase. Gerrard et al (1998) observed that pan bread formulations with TG yielded dough that held about 6% more water, required less work input and were less sticky, and breads with better texture and crumb strength (i.e., ability to withstand handling) compared to a control. Bauer et al (2003a) also reported that addition of TG resulted in doughs that developed faster, with lower stickiness, increased resistance, decreased extensibility and slight increase in work input. A problem associated with TG in pan bread is the reduction of extensibility, which consequently leads to lower loaf volume (Bauer et al 2003a, Autio et al 2005). In contrast, croissants improved in volume, flakiness and texture with the addition of TG, including croissants with wholemeal flour (Gerrard et al 2000). This improved quality was attributed to the cross-linking of albumins and globulins, making them part of the dough system, and the cross-linking of high molecular weight glutenin sub-units into more functional protein aggregates (Gerrard et al 2001). Other potential uses of TG include improvement of the

baking quality of weak flours (Autio et al 2005), and formation of a protein network in gluten-free breads (Moore et al 2006).

The studies, which showed that protein could extend shelf-stability, did not describe the mechanism involved in the process. Thus, this study was conducted to determine the role of protein in flour tortilla staling through evaluation of the effect of protease and TG on tortilla texture and microstructure, and determination of changes in protein during tortilla storage. The hypotheses were that the use of protease will disrupt the gluten matrix and thus shorten shelf-stability, while TG will form cross-links to strengthen the matrix and extend shelf-stability. Comparison of these contrasting treatments with a control in terms of protein profile will then be a basis to infer the involvement of protein in staling.

MATERIALS AND METHODS

Flour Tortilla Preparation and Storage

The control tortilla formulation is similar to that in Chapter II. The protease-treated samples were prepared like the control but with 40 ppm enzyme (P1236, Sigma-Aldrich, Inc., St. Louis, MO) dissolved in 470 g distilled water. The protease enzyme was an endo-protease from *Bacillus amyloliquefaciens* with an activity of at least 0.8 U/g.

The transglutaminase (TG)-treated samples were prepared with 0.5% enzyme (Activa TI, Ajinomoto, Japan) dissolved in about 50 g distilled water. This was added to the dough (prepared with 465 g water) after 2 min mixing in medium speed. The enzyme had an activity of 100 U/g.

Tortillas were stored at ambient temperature (22^{o}C), and were sampled at 0.04, 0.21, 1, 3, 7, 14 and 21 days after baking. Two batches of control and enzyme-treated tortillas were prepared on separate days and evaluated.

Microstructure

Dough and tortillas for microscopy study were prepared by mixing 0.002% Safranin-O dye to the distilled water as described by Moore et al (2006).

Dough, hot-pressed dough, partially baked and fully baked tortilla samples were evaluated for microstructure under a confocal laser scanning microscope (Olympus FV1000, Olympus Corp., Tokyo, Japan). Samples (about 1 mm thick) were taken with a scalpel, placed on a welled-glass slide, added with a drop of oil and covered with a glass cover slip. These were viewed with a 20x oil immersion objective, and optical slices were taken at 1 μm increments.

Physico-chemical and Texture Properties

The control and treated doughs were evaluated using the subjective test for smoothness, softness, extensibility, force to extend and press rating. Weight, height (thickness), diameter, opacity, pH and moisture content of the tortillas were also determined. Shelf-stability and texture were evaluated with the subjective rollability test and objective 2D extensibility test. Details of methods are described in Chapter II.

Protein Solubility

Protein fractions were sequentially extracted from 1.5 g of freeze-dried control and enzyme-treated dough and tortillas (Vivas-Rodriguez 1988). Fraction I (albumins and globulins) was extracted with 0.05 M NaCl, fraction II (prolamins) with 60% tert-butanol, and fraction III (cross-linked prolamins) with 60% tert-butanol with 2% β-mercaptoethanol (BME) with shaking at room temperature. Fraction IV (glutelins) was extracted with 2% SDS, 5% BME and 0.065M Tris buffer (pH 6.8) at 50^{o}C. All fractions were extracted twice, first for 2 hr and the second for 1 hr, using 9 ml of solvent. All extracts were centrifuged at 10,000 rpm for 10 min and the supernatants were collected and analyzed. Protein content of the extracts was determined using the *RC DC* Protein Assay Kit II with bovine serum albumin as standard (Bio-Rad 500-0121, Hercules, CA).

SDS-PAGE

Dough and tortilla samples were mixed with an aqueous solution containing 1.5% SDS (1:10 w/v) with shaking for 1 hr. Protein content of the extracts was determined using the *RC DC* Protein Assay Kit II. SDS-PAGE was done under reducing conditions. Supernatants of the extract were mixed with Laemmli sample buffer (Bio-Rad 161-0737,) in a 1:1 ratio, and incubated in boiling water for 5 min. Samples with

approximately 40 µg protein were loaded into a 10-well single-stacking Tris-HCl Ready Gel (Bio-Rad 161-1102, Hercules, CA) that had a 12% resolving gel and 4% stacking gel. The gel was stained with 0.02% Coomassie R350 and destained with methanol-trichloroacetic acid-water solution (3:1:6 v/w/v).

RESULTS

Dough and Tortilla Physical Properties

The control, protease-treated and TG-treated doughs were prepared with 51%, 48% and 51.5% water absorption, respectively, to have soft, non-sticky and machinable doughs. The doughs from all three treatments had similar softness, extensibility and force to extend ratings (Table VI). However, control and protease-treated doughs were easier to press into the stainless steel plate for dividing. This means that TG significantly increased cross-links during proofing, making the dough tougher and less extensible.

Tortillas from all treatments had similar weight, height and pH. Control and TG-treated tortillas were not significantly different in diameter, opacity, specific volume and moisture content (Table VII). In bread with TG (Autio et al 2005), the decrease in extensibility of the dough resulted in a significantly smaller loaf volume, but a significant decrease in tortilla diameter was not observed in this study. Protease-treated tortillas, in contrast, had significantly larger diameters. The enzyme hydrolyzed part of the gluten matrix, thus the dough had less resistance to hot-pressing. These tortillas were also more opaque, resembling tortillas from cake or pastry flour (i.e., low protein content). Moisture content of the protease-treated tortillas was significantly lower because of the lower amount of water used. It may also be from greater water loss during baking because the disrupted gluten held less water.

Dough and Tortilla Microstructure

Confocal microscopy was used to study the effect of protease and TG on dough and tortilla microstructure. All samples were viewed with an oil immersion objective at 20x magnification.

TABLE VI

Physical Properties of Control and Protease- and TG-Treated Dough[a, b]

Dough properties[c]	Control	Protease-Treated	TG-Treated
Softness	2.0a	1.8a	2.2a
Extensibility	3.0a	2.8a	3.3a
Force to extend	3.5a	3.0a	3.5a
Press rating	2.0b	1.8b	2.4a

[a] Water absorption: control = 51%, protease-treated = 48%, transglutaminase (TG) = 51.5%

[b] Means from two trials; means in a row followed by the same letter are not significantly different ($p<0.05$)

[c] Softness: 1 – very soft, 5 – firm; Extensibility: 1 – not extensible, 5 – very extensible; Force to extend: 1 – less force, 5 – much force; Press rating: 1 – easy to press, 5 – hard to press

TABLE VII

Physical Properties of Control and Protease- and TG-Treated Tortillas[a]

Tortilla Properties	Control	Protease-Treated	TG-Treated
Weight (g)	40.6a	38.1a	39.2a
Height (mm)	2.75a	2.49a	2.88a
Diameter (mm)	172.1b	191.8a	161.1b
Opacity (%)	75.5b	90.5a	79.5b
Specific volume (cm^3/g)	1.6b	1.9a	1.5b
pH	5.4a	5.4a	5.3a
Moisture (%)	32.9a	30.2b	32.7a

[a] Means from two trials; means in a row followed by the same letter are not significantly different ($p<0.05$)

The gluten matrix and starch granules can be clearly distinguished in the dough micrographs (Fig. 9a-c). Control dough had a thin protein film that formed a continuous matrix (Fig. 9a). McDonough et al (1996), using environmental scanning electron microscopy (ESEM), also observed hydrated starch granules held together by a film of gluten. Addition of protease hydrolyzed the protein resulting in dough that had dispersed discontinuous protein pieces (Fig. 9b). Transglutaminase (TG), an enzyme that cross-links lysine and glutamine residues, gave a dough with clumps of protein (Fig. 9c). Autio et al (2005) also observed thicker fibers or interaction of fibers, and an uneven distribution of the protein network in bread dough with added TG.

Tortilla micrographs show only the continuous matrix (Fig. 9d-f), which is composed of protein and dispersed starch. Voids can either be gelatinized starch granules or air bubbles. Control tortilla had a well-developed and well-distributed continuous structure (Fig. 9d). Protease-treated tortilla had intact structure despite the hydrolyzed gluten matrix observed in the dough (Fig. 9e). This structure held the tortilla together and prevented crumbling in the oven. The starch molecules that leached out of the starch granules may have contributed to the formation of this structure. The TG-treated tortilla retained clumps of proteins, which may be from excessive cross-linking (Fig. 9f). TG-cross-linked gluten is relatively heat-stable (Larre et al 2000), thus the thick protein strands in the dough was still observed after processing.

It was interesting to observe that a protein matrix was still formed in the protease-treated tortilla. Thus, more microscopy work was done to determine in what step of the pressing and baking process did the tortilla structure form. Micrographs were taken from control and protease-treated samples after hot-pressing and after they came out of the first tier of the oven.

The platens that press the dough balls are heated to 400^{o}F and press at a pressure of 1100 psi for 1.35 sec. These conditions flatten the dough, dehydrate the surfaces and create a “seal” to limit the escape of gas and moisture during baking (McDonough et al 1996). The hot-pressed, unbaked control disc had gluten fibrils stretched in one direction in both the inner and surface samples (Fig. 10a, c). The hot-pressed protease-treated disc

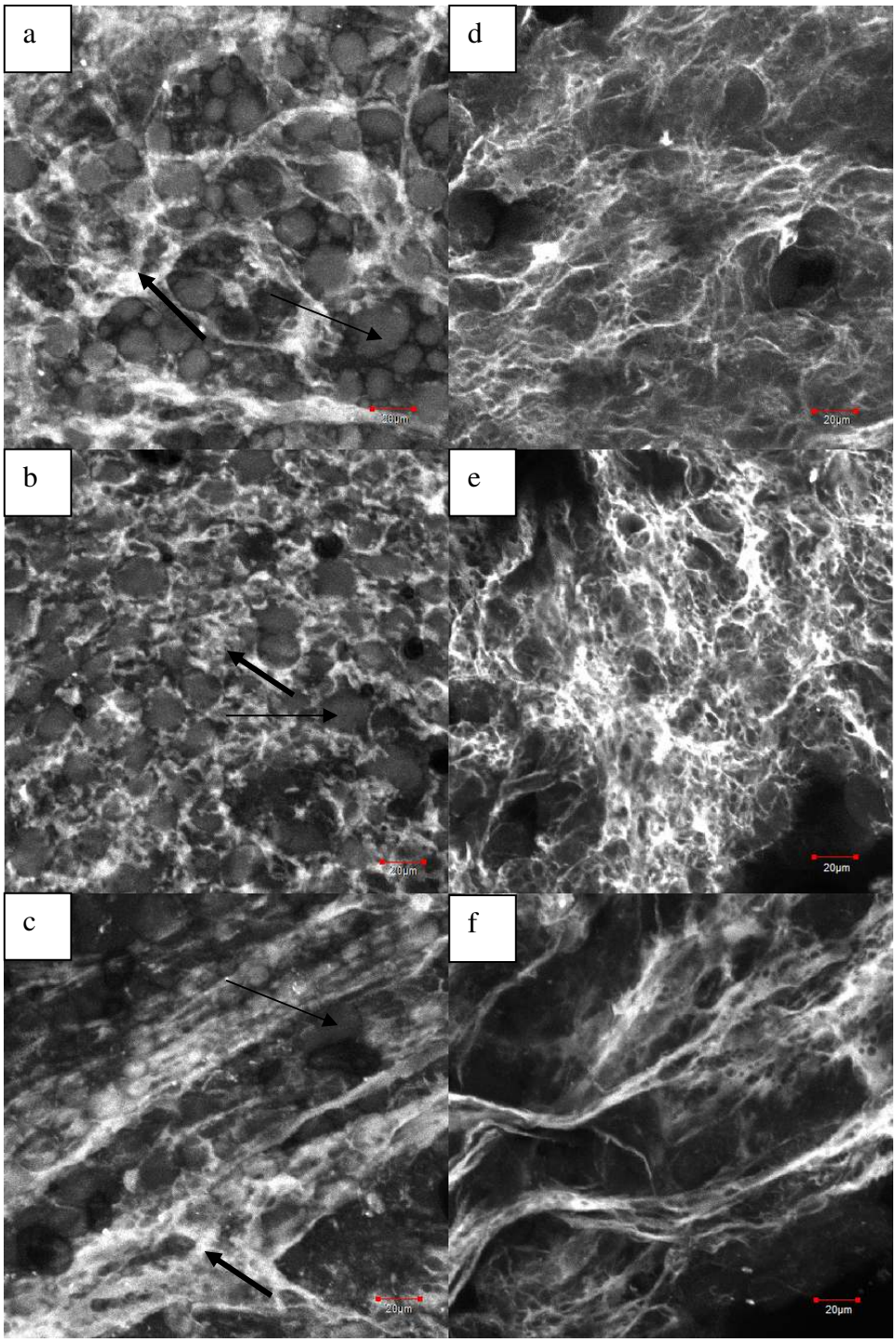

Fig. 9. Confocal micrographs of control (a, d), protease-treated (b, e) and TG-treated (c, f) dough and tortilla, respectively, viewed at 20x magnification. Thick arrows point at gluten matrix, thin arrows point at starch granules.

did not have the stretched fibrils, but pressure and temperature conditions may have allowed the protein pieces that were observed in the dough to associate and form a continuous matrix (Fig. 10b, d). The hot-pressing conditions have increased mobility of the molecules, including the dispersed proteins, allowing covalent and non-covalent (e.g., ionic and hydrogen bonds) bonds to be formed, and entanglements to occur.

The starch granules are not very distinct at the hot-pressing stage, and may be from less uptake of the dye relative to the protein. McDonough et al (1996) reported that at this stage, the starch granules at the surface are dehydrated and flat while those at the middle or inside the disc are still hydrated, showing that heat penetration is not that extensive yet.

The tortilla is baked on one side for about 10 sec after going through the first tier of the oven. The control and protease-treated tortillas had the same well-developed web-like structure especially on the baked surface (Fig. 11a-d). This compact continuous structure on the surface may have been formed by dehydration upon heating, which led to smaller air bubbles and compressed gelatinized starch granules (i.e., based on size of voids). The dispersed starch, specifically amylose molecules, may have strengthened the structure of the tortilla with protease. The samples from the inside of the tortilla had more voids than that from the surface, which may be gas bubbles and/or gelatinized starch granules. Observations with ESEM showed stretched gluten matrix and distended concave starch granules from expansion of tortilla from steam and leavening gases (McDonough et al 1996).

Tortilla Shelf-Stability and Texture

The effects of protease and TG on shelf-stability and texture of tortilla samples were determined with a subjective rollability evaluation, which uses a 1 to 5 scale, and an objective extensibility test using a texture analyzer.

All treatments significantly decreased in rollability scores with time, which means loss of flexibility during storage (Fig. 12). Tortillas with protease had cracks at 1 day of storage. These tortillas were unacceptable (i.e., score below 3) on the third day.

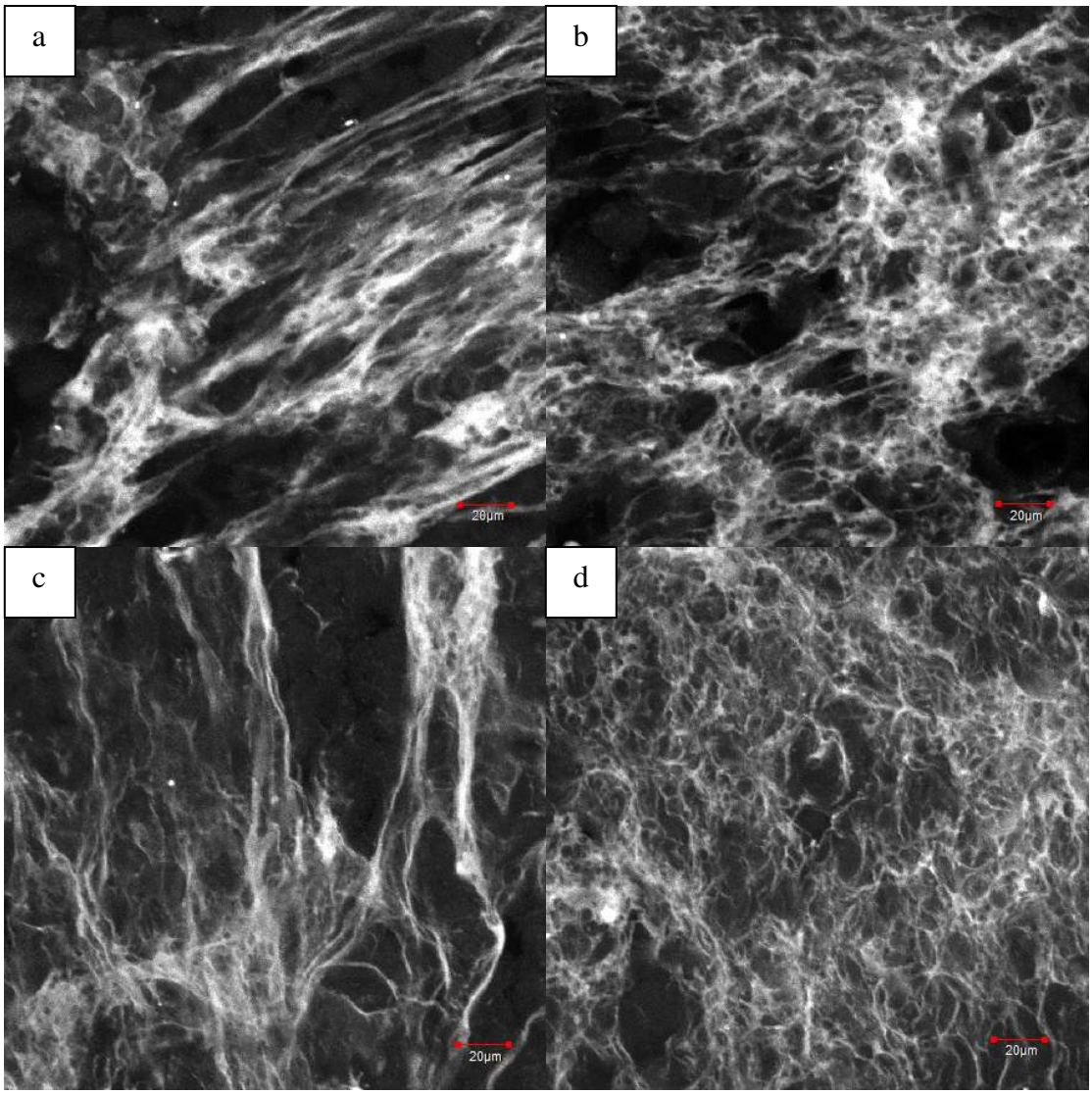

Fig. 10. Confocal micrographs of hot-pressed control (a, c) and protease-treated (b, d) tortillas viewed at 20x magnification; sampled from inside (a, b) and outside/surface (c, d) of tortilla.

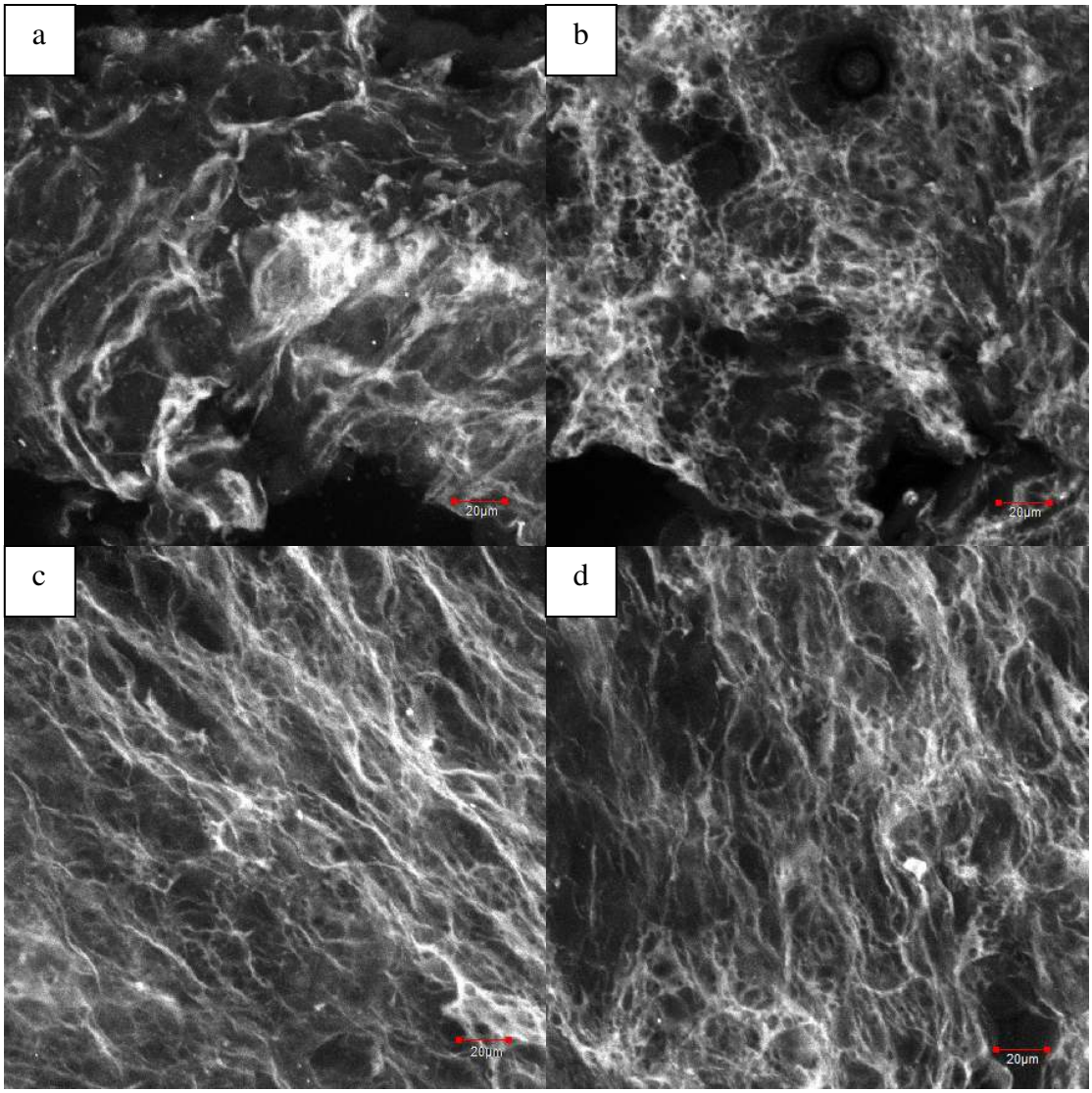

Fig 11. Confocal micrographs of control (a, c) and protease-treated (b, d) tortillas from tier 1 of oven viewed at 20x magnification; sampled from inside (a, b) and outside/surface (c, d) of tortilla.

Control and TG-treated tortillas were both rollable after 1 day, but control tortillas retained flexibility longer.

Deformation modulus, the ratio of force and rupture distance, was significantly affected by enzyme treatment and storage time (Fig. 13, Appendix A4). This was consistently highest in the control tortillas throughout storage, and lowest in protease-treated tortillas. TG-treated tortillas had similar deformation modulus with protease-treated tortillas after 1 and 5 hr of storage, and with the control on the third day.

Rupture force was likewise affected by treatment and storage time (Fig. 13), but the interaction of these variables was not significant. Control tortillas required the greatest force to rupture, while the protease-treated tortillas required the least force. TG-treated tortillas had the same force values with the control until 3 days of storage, but had significantly lower values thereafter. For all three treatments, rupture force was highest on the day of baking, and this decreased with time. The change in rupture force was not significant from 3 to 21 days of storage.

TG-treated tortillas generally extended more before breaking (i.e., greater rupture distance) than the two other treatments (Fig. 13). Protease-treated tortillas had the least rupture distance values. Rupture distance did not change significantly after 1 day in TG-treated tortillas, and after 7 days in control and protease-treated tortillas.

Work to rupture tortillas was greatest in the control (Fig. 13). However, this was not significantly different from TG-treated tortillas from 3 to 21 days of storage. Protease-treated tortillas required the least work to rupture. Fresh tortillas had the highest work values, and this decreased with time. All three treatments did not have significant change in work needed to rupture tortillas from 3 days onwards.

Both the subjective rollability evaluation and objective extensibility test showed that the protease-treated tortillas had weaker structure (i.e., shorter shelf-stability; less force, distance and work required to rupture). The addition of TG was expected to increase cross-linking in the protein fraction, and form a stronger and more flexible gluten matrix. However, TG-treated tortillas lost flexibility faster than the control based on the rollability test.

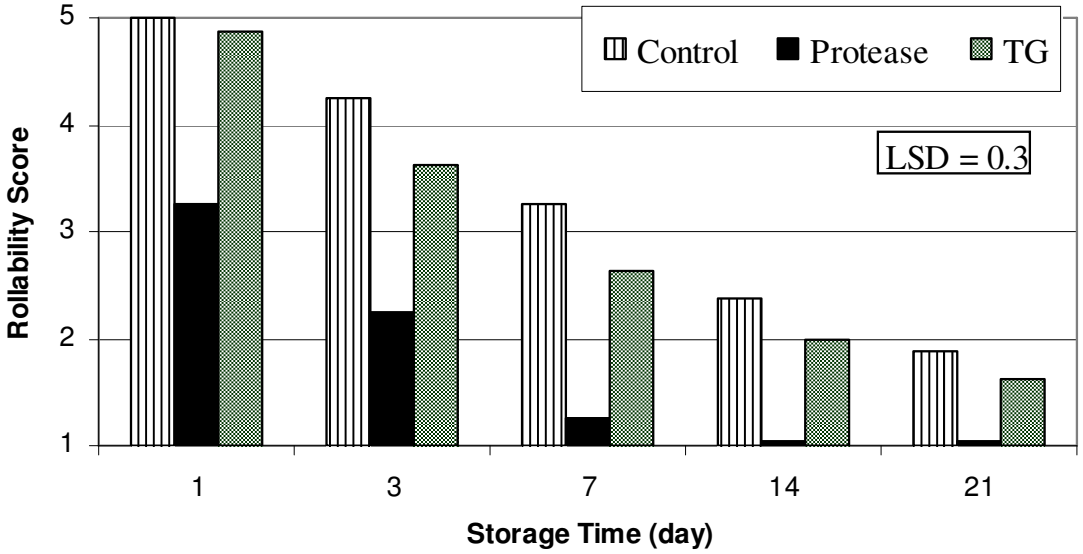

Fig. 12. Rollability scores of control and protease- and TG-treated tortillas stored for 21 days. LSD value is for both storage time and enzyme treatments.

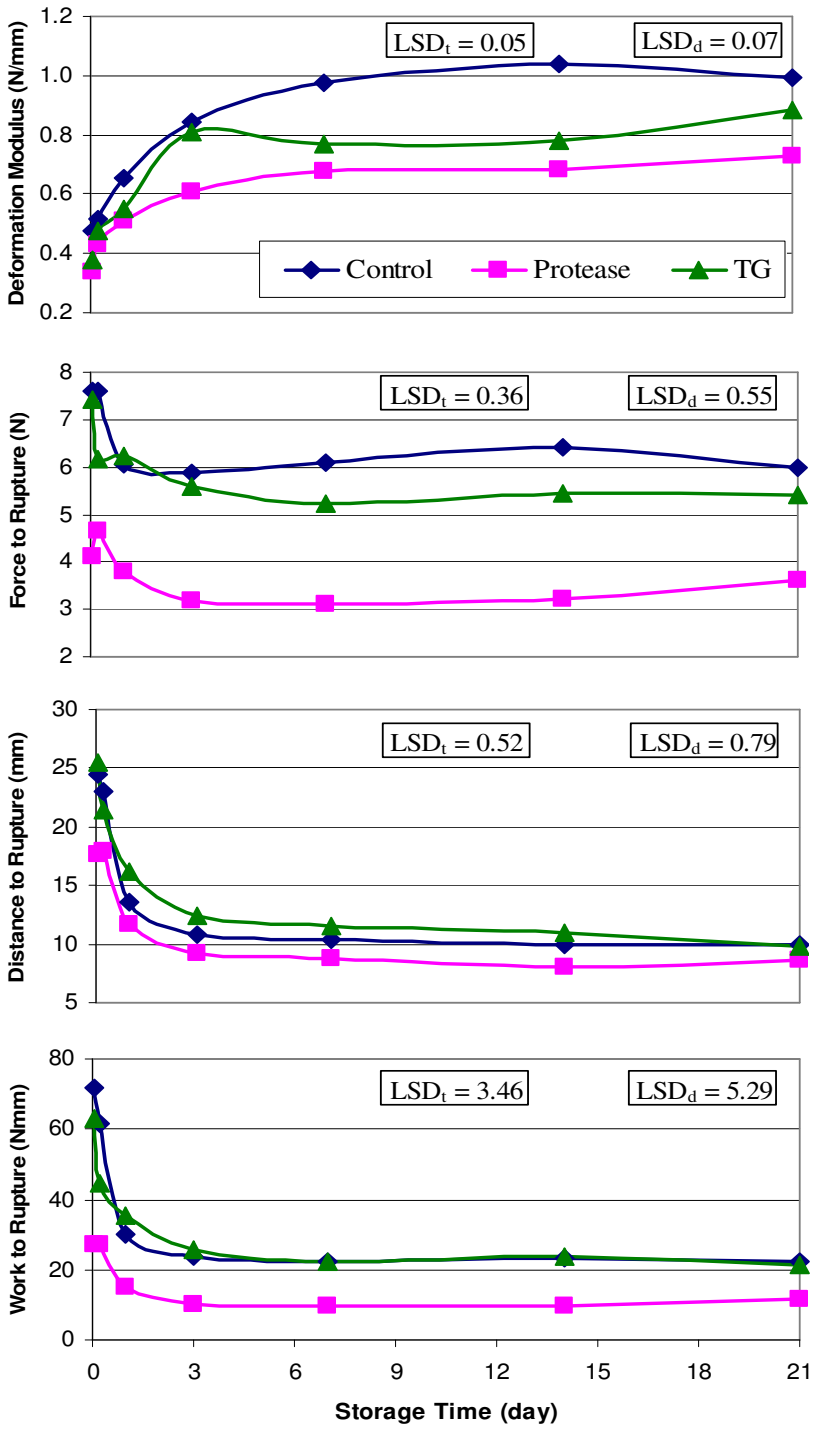

Fig. 13. Texture profile of control and protease- and TG-treated tortillas stored for 21 days. LSD_t is for treatment, LSD_d is for storage time.

Protein Solubility

Wheat proteins can be classified based on their solubility. Albumins and globulins are soluble in aqueous salt solutions, gliadins in concentrated aqueous alcohol solutions, and glutenins in dissociating solvents and in concentrated alcohol solutions with a reducing agent (Gerrard et al 2001, Schofield 1986).

Addition of protease significantly increased extraction of Fraction I (albumins and globulins) in both dough and tortilla samples (Table VIII). Control dough and 1-day tortillas had significantly higher Fraction I amounts than the TG-treated samples, but both treatments had similar amounts in 7-day tortillas. For all treatments, Fraction I significantly decreased from dough samples to 1- and 7-day tortillas.

Protease-treated tortillas, but not dough, had the highest Fraction II (alcohol soluble prolamins) extract (Table VIII). Control dough had significantly higher amounts than TG-treated dough, but both treatments had similar Fraction II amounts from tortilla samples. Fraction II amounts in all three treatments were significantly higher in dough than in tortilla samples. Storage of tortilla did not give any significant change in the amount of extract.

TG-treated dough had the highest Fraction III (cross-linked glutenins) amount among the three dough samples (Table VIII). However, TG-treated tortillas were similar with control tortillas. Protease-treated dough and tortillas had the least Fraction III values. In all treatments, Fraction III was significantly greater in the tortilla than in the dough.

Control and TG-treated dough and tortillas were not significantly different while protease-treated dough and tortillas had the least Fraction IV (SDS-glutenins) extract (Table VIII). Like Fraction III proteins, Fraction IV was significantly higher in tortilla than in the dough samples.

TABLE VIII

Protein Solubility Distribution (mg protein/g sample, dry basis) of Control and Protease- and TG-Treated Tortillas[a]

Storage Time (day)	Control	Protease-Treated	TG-Treated
Fraction I (albumins and globulins)			
dough	11.9Ab	12.5Aa	9.5Ac
1-day	5.1Bb	6.3Ba	4.7Bc
7-days	4.4Cb	5.7Ca	4.2Cb
Fraction II (prolamins)			
dough	37.2Aa	35.7Ab	34.1Ac
1-day	28.0Bb	30.8Ba	27.5Bb
7-days	27.8Bb	30.1Ba	27.3Bb
Fraction III (cross-linked glutenins)			
dough	19.6Cb	15.6Bc	21.2Ca
1-day	23.4Bab	22.8Ab	23.9Ba
7-days	24.3Aa	23.4Ab	25.03Aa
Fraction IV (SDS-glutenins)			
dough	37.6Ca	33.4Cb	39.5Ba
1-day	45.5Ba	41.4Bb	45.6Aa
7-days	47.8Aa	43.6Ab	44.8Ab

[a] Means from two trials; means in a row with the same small letter, and means in a column with the same capital letter are not significantly different ($p<0.05$)

Dough and tortillas with protease generally had greater solubility of Fractions I and II proteins, and conversely lower Fractions III and IV proteins. This may mean that the hydrolytic action of the enzyme made albumins, globulins and alcohol-soluble prolamins (which includes gliadins) more available by disrupting the gluten network. Addition of TG, on the other hand, created cross-links and interactions in the dough. Albumins and globulins are not involved in dough formation, but the lower Fraction I value in TG-treated dough may mean that cross-linking occurred to create dough-like insoluble polymers (Gerrard et al 2001). TG can also induce polymer formation in gliadins (Larre et al 2000, Bauer et al 2003b), which may explain the lower Fraction II value. The greater cross-linking in TG-treated dough is also evident in the significantly greater Fraction III proteins. In all three treatments, hot-pressing and baking reduced solubility of Fractions I and II and increased Fractions III and IV.

SDS-PAGE of Dough and Tortilla Proteins

SDS-PAGE was done to determine if the differences in shelf-stability and texture among the treatments would be reflected in their electrophoretic patterns or protein profiles.

The control, protease-treated and TG-treated dough and tortilla samples had similar protein profiles. One-day and 7-day tortilla also had the same electrophoretic patterns, which was expected since the enzymes were inactivated during processing. Primo-Martin et al (2006) did not observe any difference in degree of cross-linking among bread crust samples treated with protease and TG despite the differences in crust crispness. They suggested that the amount of proteins modified by the enzymes was not significant to be detected by SDS-PAGE, and this may be the case in this study as well. On the other hand, Larre et al (2000) showed through SDS-PAGE that treatments of gluten with TG reduces HMW-GS and forms new higher molecular weight molecules, and to a lesser extent reduces the concentration of gliadins and LMW-GS. Other studies agree that HMW-GS are primarily involved in TG-cross-linking (Gerrard et al 2001, Mujoo and Ng 2003, Bauer et al 2003b).

DISCUSSION

This study evaluated the effects of protease and TG, and storage time on the shelf-stability, texture, microstructure and protein profile of dough and/or tortilla, with the end goal of inferring the importance and involvement of proteins in tortilla staling.

Gluten is the primary flour component comprising the continuous matrix in the dough and tortilla, which means that it provides structure to the dough and tortilla. This makes gluten development important for it affects product quality, particularly structure and texture, and consequently, shelf-stability. As reported by Srinivasan et al (2000), over-mixing and under-mixing of dough produce tortillas with poor shelf-stability because the gluten matrix is disrupted and non-homogeneous, respectively. Protease and TG are ingredients that also alter gluten development. These are enzymes with opposite effects on gluten. Protease hydrolyzes protein while TG strengthens gluten through formation of crosslinks.

Addition of protease and TG to the tortilla formulation affected microstructure, shelf-stability and texture. Confocal microscopy visually showed that protease hydrolyzed the continuous film into pieces of proteins. A structure was still formed to hold the tortilla after baking, but the product had very poor shelf-stability (i.e., breaking at 3 days). The lower force, work and distance required to rupture the tortilla relative to the control was also evidence of this weak structure.

The clumps of proteins that were observed in the micrographs of the TG-treated dough means that cross-linking occurred. Cross-linking in dough is possible because wheat proteins are rich in glutamine, and the relatively low amount of lysine is not a hindrance (Larre et al 2000). These cross-links by TG, however, did not result in an improved shelf-stability of tortillas compared to the control. The same trend is reflected in the rupture force where the control and TG-treated tortilla had similar values for 3 days but the latter needed less force thereafter. This trend was not as obvious in the rupture distance and work parameters.

The above results showed that the organization or formation of the gluten network before hot-pressing and baking significantly affects the shelf-stability of the

product. Among the three treatments, the control, which had a well-formed gluten network, had the best shelf-stability. In contrast, the protease-treated dough with its continuous matrix hydrolyzed into pieces of protein had the poorest shelf-stability even if a structure was formed after hot-pressing. TG may have strengthened the dough with added cross-links, but this did not improve shelf-stability because the gluten matrix formed strands that were unevenly distributed within the dough.

The negative effect of TG on tortilla shelf-stability does not invalidate the hypothesis that a stronger gluten network through increased cross-linking improves shelf-stability. Addition of vital wheat gluten, which results in more disulfide linkages and stronger dough, yields tortilla that retains flexibility longer (Suhendro et al 1993, Pascut et al 2004). More studies have to be done on the effect of other oxidizing agents that strengthen, and at the same time maintain a homogeneous distribution, of the gluten network.

Protease and TG affected protein fraction extractability of the dough and tortilla samples. Protease-treated dough and tortilla had more albumins, globulins and prolamins, and less glutenins. In contrast, TG-treated dough and tortillas had less albumins, globulins and prolamins, and more glutenins. No consistent significant enzyme effect on protein solubility was observed with storage time. This was expected since it is assumed that the enzymes were inactivated with the processing conditions used.

Protein solubility and SDS-PAGE, methods that respectively characterize solubility and molecular weight of isolated proteins, did not clearly differentiate the control from the enzyme-treated samples. The effect of the enzymes on microstructure and shelf-stability was not reflected in these tests. This means that the protein fractions or composition are not determinants of staling. The tertiary and quaternary protein structures, their configuration and interactions, are important in gluten functionality. These may be the factors related to the staling of tortillas.

Theoretically, tortillas have less dispersed starch molecules (i.e., mostly amylose) than bread because of shorter baking time and lower moisture content. This means that dispersed starch limits the flexibility of protein to a lesser extent in tortillas. Aside from

a well-developed and homogeneously distributed gluten, retention of the viscoelastic property of protein after processing may improve shelf-stability.

CHAPTER IV
ROLE OF PENTOSANS IN TORTILLA STALING

INTRODUCTION

Pentosans and Staling

Early studies by Kim and D'Appolonia (1977a) on the effect of pentosans on starch gels revealed that both soluble and insoluble fractions retard firming of gels, with the latter being more effective. On the other hand, Devesa and Martinez-Anaya (2003) reported that commercial soluble arabinoxylan reduced the firming rate of starch gels while insoluble arabinoxylan gave a significant, but much lesser effect. The authors proposed that the soluble arabinoxylan interfered with starch swelling, and also weakened amylose gel formation between granules.

There is limited literature on the effect of pentosans and pentosanase on tortilla quality. Arora (2003) reported that the addition of 100 ppm xylanase improved tortilla shelf-stability. However, the mechanism involved was not elucidated. Addition of pentosans to bread formulations decreased staling and firming rate (Kim and D'Appolonia 1977d, Biliaderis et al 1995, Fessas and Schiraldi 1998), and the mechanisms suggested include inhibition of amylose inter-association in the starch-gluten matrix during storage (Michniewicz et al 1992), and creating a loose gluten network by limiting cross-linking, which leads to more expansion by fermentation gases (Fessas and Schiraldi 1998), dilution of the amount of starch for recrystallization (Kim and D'Appolonia 1977d, e), and higher moisture content of breads with pentosans that yields softer crumb because water acts as plasticizer (Michniewicz et al 1992, Biliaderis et al 1995).

Pentosanases and Bread Quality

Pentosanases in bread release pentosan-bound water making the dough more machinable and with better oven spring (Mathewson 2000, Si 1997). Endo-xylanases, which hydrolyze the xylopyranosyl backbone of pentosans, are the most effective pentosanase for bread making (van Oort et al 1995). The enzymes improve dough

tolerance and increase loaf volume. These positive effects have been attributed to improved gluten development through better water distribution, and increased viscosity from the increased solubility of pentosans (van Oort et al 1995).

Haros et al (2002) observed that xylanase, cellulase and β-glucanase significantly increased specific volume of bread, improved crumb texture and decreased firming rate during storage. They proposed that the hydrolysates interfered with the protein-starch interaction postulated by Martin et al (1991). However, since increase in loaf volume is related to crumb firmness, the slower firming rate may not be primarily an enzyme effect. Gil et al (1999) produced the same loaf volume by using lidded-pans and observed that addition of pentosanase (endo 1,4-β-xylanase) or a combination of pentosanase and lipase did not give a significant effect on staling of white bread. Differences in reported effects of xylanase on bread quality may be due to difference in source and type of xylanase used (Hilhorst et al 2002) and type or molecular structure of pentosans in the flour (van Oort et al 1995).

Pentosan Functionality

Pentosans are important wheat flour components because of their high water absorbing capacity. In a dough, about 23% of the water is bound by the pentosans (Neukom 1976). Pentosans, specifically the water-soluble ones, are important because they are involved in oxidative gelation, which occurs in the presence of small amounts of oxidizing agents (e.g., peroxidase, hydrogen peroxide). This is different from starch gelation because it does not need a heating and cooling cycle (Neukom 1976, Lineback and Rasper 1988). The mechanism for oxidative gelation was identified as formation of diferulic acid through linking of ferulic acid residues of adjacent arabinoxylan molecules (i.e., arabinogalactans do not have diferulic acid residues). Proteins may also be involved through formation of tyrosine-ferulic acid linkages (Neukom 1976).

The ability of pentosans to bind a significant amount of water and form viscous gels affects mixing, dough development and baking properties (van Oort et al 1995). However, studies on the specific effect of pentosans on baking quality are contradictory. Kim and D'Appolonia (1977d) reported that pentosans decreased mixing time and

yielded a more relaxed dough, and loaf volume slightly decreased with water-insoluble pentosans while soluble pentosans had no effect. This is in contrast to reports that water-soluble pentosans significantly increased loaf volume, and that the insoluble fraction had limited or no effect (Jelaca and Hlynka 1972, Michniewicz et al 1992). Biliaderis et al (1995) showed that there is an optimum concentration of water-soluble pentosans to get an increase in loaf volume. These contradicting results may be due to differences in purity and composition of pentosans used, amount of pentosans and method of mixing into dough, molecular size of polymers, and quality of base flour (Michniewicz et al 1992, Biliaderis et al 1995).

This study was conducted to determine the functional role of pentosans in tortilla staling with the use of enzymatic hydrolysis.

MATERIALS AND METHODS

Flour Tortilla Preparation and Storage

The control tortilla formulation is similar to that in Chapter II. The xylanase-treated samples were prepared like the control but with 25, 50 and 75 ppm enzyme (Enzeco Xylanase S-200, Enzyme Development Corp, New York, NY) which was dissolved in water. The endo-xylanase activity is 190000-230000 BXU/g. One BXU is defined as the amount of enzyme that produces carbohydrates having a reducing power corresponding to 1 μmol xylose from birch xylan in one second at 50^{o}C and pH 5.3 (Technical data sheet, Enzyme Development Corp, New York, NY).

Tortillas were stored at ambient temperature (22^{o}C), and were sampled at 0.04, 0.21, 1, 3, 7, 14 and 21 days after baking. Two batches of control and enzyme-treated tortillas were prepared separately and evaluated.

Physico-chemical and Texture Properties

The control and treated doughs were evaluated using the subjective test for softness, extensibility, force to extend and press rating. Weight, height (thickness), diameter, opacity, pH and moisture content of the tortillas were also determined. Shelf-

stability and texture were evaluated with the subjective rollability test and objective 2D extensibility test. Details of methods are described in Chapter II.

Carbohydrate Profile

The carbohydrate profile of control and xylanase-treated (75 ppm) dough and tortillas stored for 0.04, 1 and 3 days was determined using SE-HPLC as detailed in Chapter II.

RESULTS

Dough and Tortilla Physical Properties

Control and xylanase-treated doughs were prepared with 51% water absorption. All doughs had similar softness, extensibility and press rating, and required the same force to extend (Table IX). Pentosans in the flour used in this study may not be associated in significant amounts with gluten since xylanase did not affect dough properties.

Control and treated tortillas were also similar in weight, thickness, opacity, specific volume, pH and moisture content. Treated tortillas with 50 and 75 ppm xylanase had significantly larger diameters than tortillas with 0 and 25 ppm xylanase (Table X). Overall, xylanase treatment at rates used had no significant effect on dough and tortilla physical properties.

Tortilla Shelf-Stability and Texture

Rollability scores of control and xylanase-treated tortillas were not significantly different (Figure 14, Appendix A5). Preliminary tests using higher rates of xylanase also did not improve tortilla shelf-stability. All treatments significantly decreased in rollability scores with time. Scores after 1 and 3 days of storage were similar, and these progressively decreased until third week of storage. In general, the treatments were unacceptable (i.e., rollability score less than 3) after being stored for two weeks.

Unlike the rollability evaluation, the objective test using the texture analyzer showed significant differences in the texture profile between treatments. In all parameters (deformation modulus, force, distance and work to rupture), control and 25

TABLE IX

Physical Properties of Control and Xylanase-Treated Dough[a]

Dough properties[b]	**Control**	**Xylanase (ppm)**		
		25	**50**	**75**
Softness	2.0a	2.2a	2.3a	2.2a
Extensibility	3.3a	3.3a	3.0a	3.3a
Force to extend	3.5a	3.5a	3.5a	3.5a
Press rating	2.0a	2.0a	2.0a	2.0a

[a] Means from two trials; means in a row followed by the same letter are not significantly different ($p<0.05$)

[b] Softness: 1 – very soft, 5 – firm; Extensibility: 1 – not extensible, 5 – very extensible; Force to extend: 1 – less force, 5 – much force; Press rating: 1 – easy to press, 5 – hard to press

TABLE X

Physical Properties of Control and Xylanase-Treated Tortillas[a]

Tortilla Properties	Control	Xylanase (ppm)		
		25	**50**	**75**
Weight (g)	40.3a	40.4a	40.2a	40.2a
Height (mm)	2.83 a	2.83a	2.76a	2.77a
Diameter (mm)	165.2b	164.4b	168.1a	168.1a
Opacity (%)	75.8a	73.0a	73.8a	76.0a
Specific volume (cm^3/g)	1.5a	1.5a	1.6a	1.6a
pH	5.2a	5.3a	5.2a	5.2a
Moisture (%)	33.1a	33.4a	32.8a	32.4a

[a] Means from two trials; means in a row followed by the same letter are not significantly different ($p<0.05$)

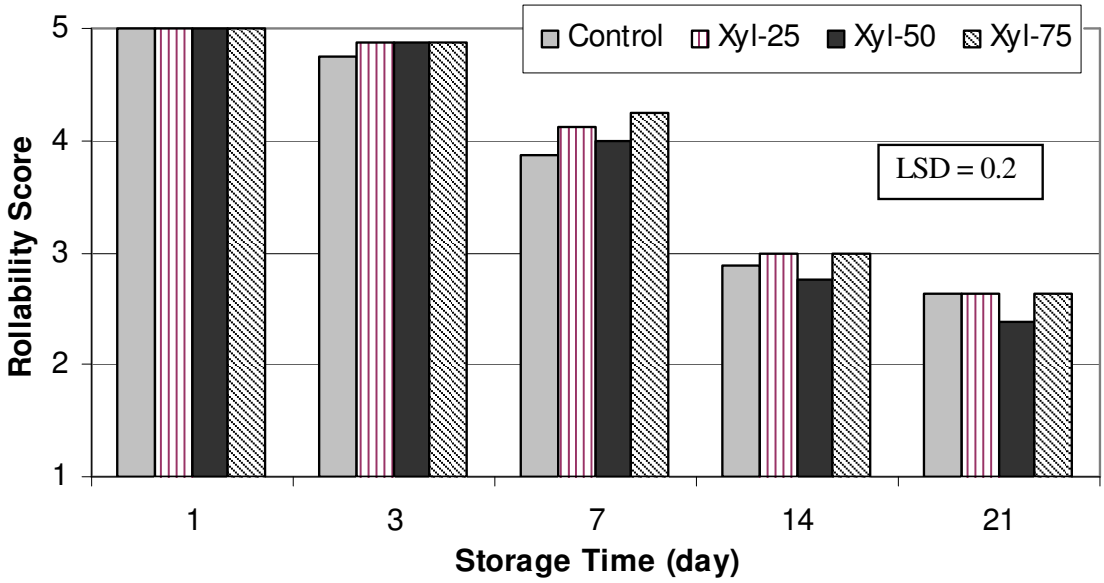

Fig. 14. Rollability score of control and xylanase-treated tortillas stored for 21 days. LSD value is for both storage time and enzyme treatment.

ppm xylanase-treated tortillas were not significantly different. These tortillas required significantly greater force and work to rupture than tortillas with 50 and 75 ppm xylanase (Fig. 15, Appendix A6).

Across storage times, the control tortillas had the highest deformation modulus value, while 50 and 75 ppm xylanase tortillas had significantly lower values, but were not different from tortillas with 25 ppm. Tortillas with 25 ppm xylanase were most extensible (i.e., greatest rupture distance value), followed by the control tortilla, which was not different from tortillas with 50 and 75 ppm. The lower values for rupture force, distance and work of tortillas with 50 and 75 ppm xylanase implies a weaker structure (i.e., less flexible; easier to break).

The loss of flexibility of the tortillas with time can be observed in the increase in deformation modulus and decrease in the rupture force, distance and work (Fig. 15). The lowest deformation modulus was at 1 hr after baking, and this significantly increased after 4 hr. Significant increase in deformation modulus occurred until one week and this stayed the same until three weeks of storage.

The rupture force was significantly higher for tortillas stored for 1 and 5 hr, and this decreased after 1 day. No significant change was observed thereafter. Distance to rupture decreased significantly until 3 days of storage, then stayed the same until 7 days. This was followed by another significant decrease at 14 days, which was similar to the value after 3 weeks of storage. Work to rupture was highest at 1 and 5 hr after baking. This decreased significantly until 3 days of storage, and did not change significantly thereafter.

Carbohydrate Profile

In this study, low molecular weight carbohydrates (LMW-C) refer to saccharides that eluted between 20.1-26 min with MW from 794 to 63096 Daltons, while sugars are those with MW less than 794 Daltons.

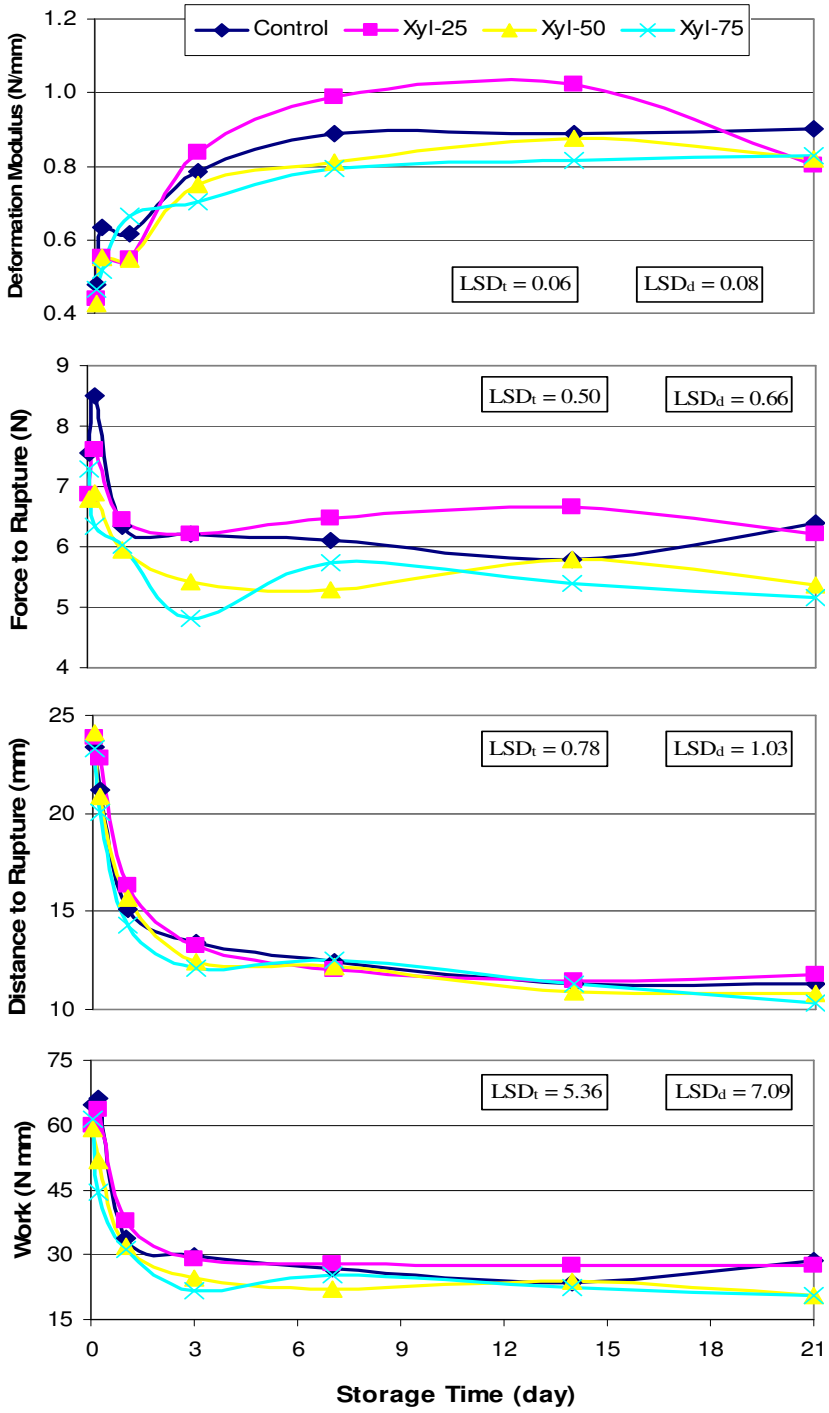

Fig. 15. Texture profile of control and xylanase-treated tortillas stored for 21 days. LSD_t is for treatment, LSD_d is for storage time.

Xylanase-treated dough and tortillas had significantly higher LMW-C ($p = 0.008$) and sugars ($p = 0.005$) than the control samples (Table XI). This is more evident in the amount of sugars than in LMW-C. This implies that the enzyme acted on the pentosans present in the flour. However, this hydrolytic activity and increase in sugars and LMW-C is not very substantial.

No significant change in LMW-C and sugars was observed after baking (i.e., dough to tortilla) and during tortilla storage in both control and xylanase-treated samples (Table XI). Xylanase hydrolyzed pentosans at the dough stage, and was inactivated during hot-pressing and baking. The LMW-C and sugars detected in the tortilla were the ones produced in the dough.

DISCUSSION

This study was done to determine the role of pentosans in tortilla staling through the use of an endo-xylanase. All studies on wheat pentosans state that these wheat components, despite their limited amount, affect dough and bread properties (Kim and D'Appolonia 1977d, Jelaca and Hlynka 1972, Michniewicz et al 1992, Biliaderis et al 1995). However, these studies differ in results on how pentosans affect dough and bread quality, the extent of these effects, and the explanation behind these effects.

Proper dough development, which involves hydration of gluten, is important in forming desirable tortilla structure, which in turn affects shelf-stability (Chapter III). Pentosans compete with gluten for water, with the water-insoluble pentosans absorbing more than the water-soluble type. Thus, partly hydrolyzing pentosans, specially the insoluble type, may improve dough development, and consequently improve shelf-stability. In this study, rollability scores of tortillas with xylanase were similar to the control. This is in contrast to the report of Arora (2003) where shelf-stability improved with xylanase. This may be due to the difference in xylanase used (Hilhorst et al 2002).

The objective test showed that tortillas with higher xylanase rates (50 and 75 ppm) needed less force and work to rupture, which means these had significantly weaker

TABLE XI

Carbohydrate Profile of Control and Xylanase-Treated Dough and Tortillas[a]

Sample	Control		Xylanase (75 ppm)	
	LMW-C[b] **(g/100g)**	**Sugars**[b] **(g/100g)**	**LMW-C (g/100g)**	**Sugars (g/100g)**
Dough	0.08a	0.66a	0.12a	0.71a
Tortilla				
1 hr	0.10a	0.67a	0.13a	0.74a
1 day	0.10a	0.67a	0.14a	0.71a
3 days	0.08a	0.65a	0.20a	0.75a

[a] Means from two trials; means in a column followed by the same letter are not significantly different ($p<0.05$)

[b] LMW-C – low molecular weight carbohydrates, eluted between 20.1-26 min (MW from 794 to 63096 Daltons); Sugars – eluted after 26 min., (MW less than 794 Daltons)

structures than the control and tortillas with 25 ppm xylanase. Hydrolysis of pentosans increases solubility (van Oort et al 1995), and the hydrolysates in the tortillas with 50 and 75 ppm may have acted like soluble fiber. Seetharaman et al (1997) reported that soluble fiber adversely affects dough development by combining with gluten to form a thick continuous matrix, resulting in poor quality tortillas.

Action of xylanase significantly increased LMW-C and sugars. However, the amount detected for both fractions in the dough was similar to that in fresh and stored tortillas. This means that xylanase was active only in the dough stage. This further shows that the weakening effect of the hydrolysates in tortilla with 75 ppm xylanase occurred during dough development.

Pentosans do not directly contribute to dough and tortilla structure, but they indirectly affect product quality through their interaction with protein (i.e., effect on dough development). It is proposed that pentosans have an indirect role in tortilla staling, which is by interfering with gluten development.

CHAPTER V
CONCLUSIONS

The mechanism of flour tortilla staling is not yet fully elucidated. Understanding the components involved in this phenomenon is important to have a basis for further studies to improve shelf-stability of flour tortillas. The results of this study reveal the role of starch, protein and pentosans in tortilla staling.

Staling of tortilla is defined as loss of flexibility or extensibility upon storage. As such, it is related to the texture of the product, which in turn is partly determined by the product's structure. Tortilla structure is primarily formed by starch and protein, with gluten and dispersed starch forming the continuous phase, and the gelatinized starch granules the discontinuous phase. Treatments (amylase, protease and transglutaminase) used in this study to modify starch and proteins affected texture and shelf-stability of tortilla.

Amylase improved shelf-stability of tortillas. The enzyme also produced dextrins and sugars, decreased loss of amylose solubility and decreased pasting viscosities. It hydrolyzed the dispersed starch and weakened granule integrity. Amylopectin crystallinity, as measured by DSC and X-ray diffraction, increased with time but was similar for the control and amylase-treated tortillas. Only the amorphous region of starch appears to be involved in staling. This means the regions with starch molecules that reassociate into a non-crystalline, disordered state.

Tortillas stale relatively slower than bread because less dispersed starch hinders the flexibility of the protein matrix. Hydrolysis of dispersed starch by amylase may have further freed the protein resulting in a more flexible tortilla. Moreover, the hydrolysates may have acted as plasticizer for the gluten matrix and gelatinized starch granules, thus giving more flexibility.

Modifying the gluten matrix through enzyme hydrolysis and increased cross-linking with protease and transglutaminase (TG), respectively, gave a corresponding change in texture and shelf-stability of tortillas. Microscopic observations of doughs

showed that protease hydrolyzed the continuous protein matrix into protein pieces, while TG created thick protein strands and a non-homogeneous continuous network. Both treatments resulted in tortillas with shorter shelf-stability than the control. This means that the formation and distribution of the gluten network in the dough affects the texture and shelf-stability of tortillas.

Protein solubility and SDS-PAGE tests did not give significant trends to differentiate the control and treated samples. It may not be the protein composition or fractions of the dough or the glutenin macropolymer (GMP) that is involved in staling, but its conformation or interactions. Further research is needed in this area, particularly on the GMP properties and any changes that occur after processing. This may further prove that retention of viscoelastic properties of protein after processing retards staling.

Pentosans are minor wheat components that have been shown to affect dough and bread quality. In this study, hydrolysis of pentosans with xylanase did not affect shelf-stability as measured by the rollability test. However, the objective test using a texture analyzer showed that higher amounts of xylanase (50 and 75 ppm) resulted in weaker structures, which was accompanied by significantly higher low molecular weight carbohydrates and sugars. It is proposed that the effect of pentosans on staling is indirect. It is not a major contributor to tortilla structure, but it can significantly affect it by modifying the gluten network or by affecting gluten development.

The involvement of starch, protein and pentosans in staling, as explained above, is illustrated in the proposed models for a fresh flour tortilla, stale control tortilla and stale amylase-treated tortilla (Figs. 18-20). Immediately after baking, amylopectin is in an amorphous state, while amylose (both dispersed and non-dispersed) is mostly retrograded. The protein or gluten matrix provides structure and flexibility to the tortilla. Pentosans may or may not be attached to the gluten network. Upon storage, amylopectin retrogrades and recrystallizes, forming intra- and inter-granular linkages. This firms the starch granules, resulting in a firmer tortilla structure. Addition of amylase retained flexibility longer by hydrolyzing starch, particularly those involved in the amorphous

region. Moreover, starch hydrolysis reduced retrograded starch surrounding the gluten matrix, making it more flexible.

Among the tools and methods used to evaluate staling, the subjective rollability test was advantageous in that it detected changes in tortilla flexibility beyond one week of storage, and approximated the actual rolling of tortilla. The objective texture analysis was advantageous in detecting differences between 0-3 days of storage, which is not measured with the rollability test, but it generally does not detect significant changes in texture after 3 days. The DSC and X-ray diffraction tests showed increase in amylopectin crystallinity with time, but the data cannot be related to the staling process as seen in the effect of amylase.

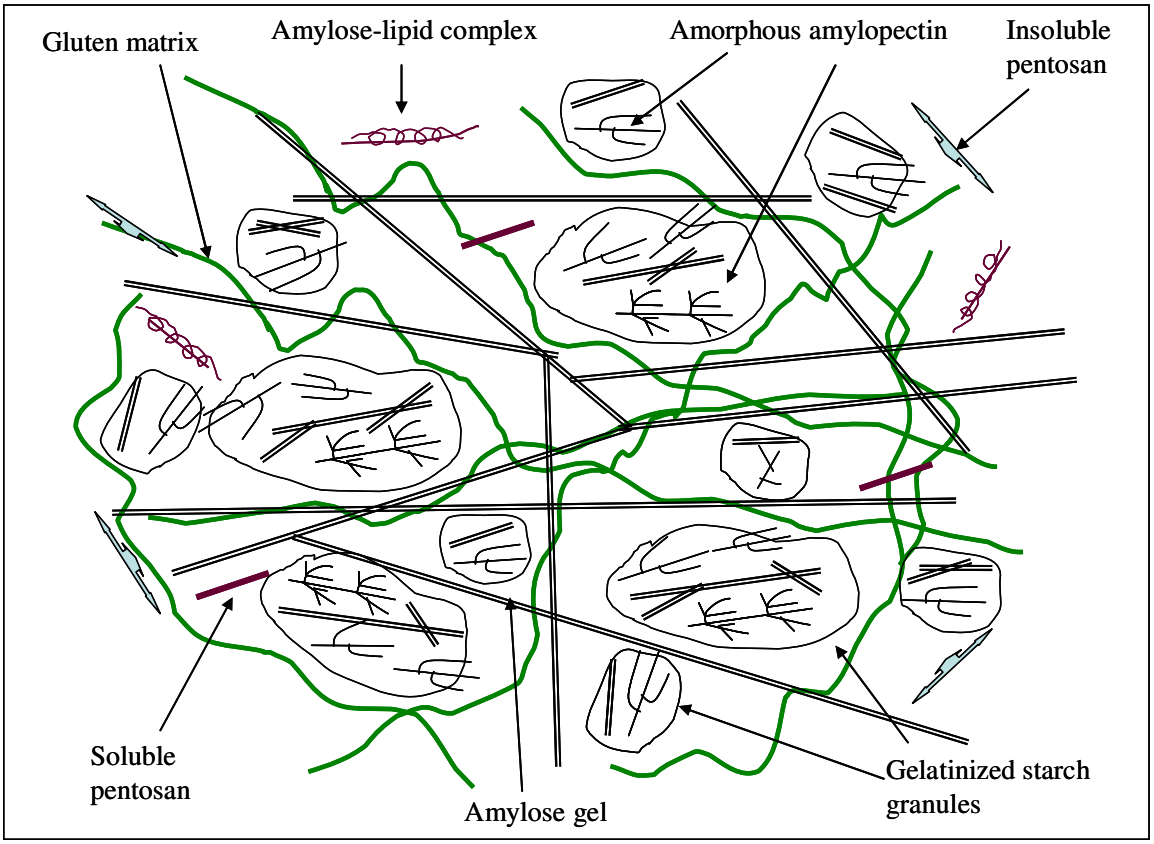

Fig. 16. Model for a fresh flour tortilla.

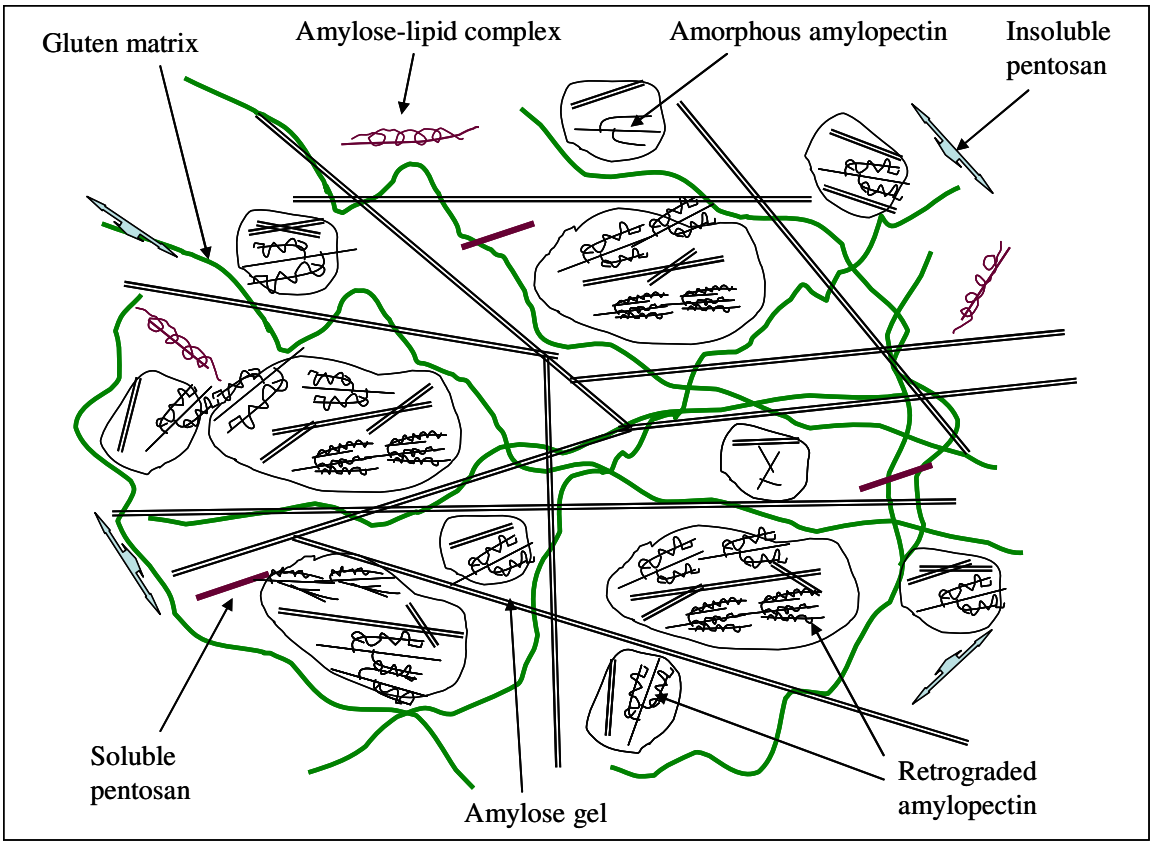

Fig. 17. Model for a stale control flour tortilla.

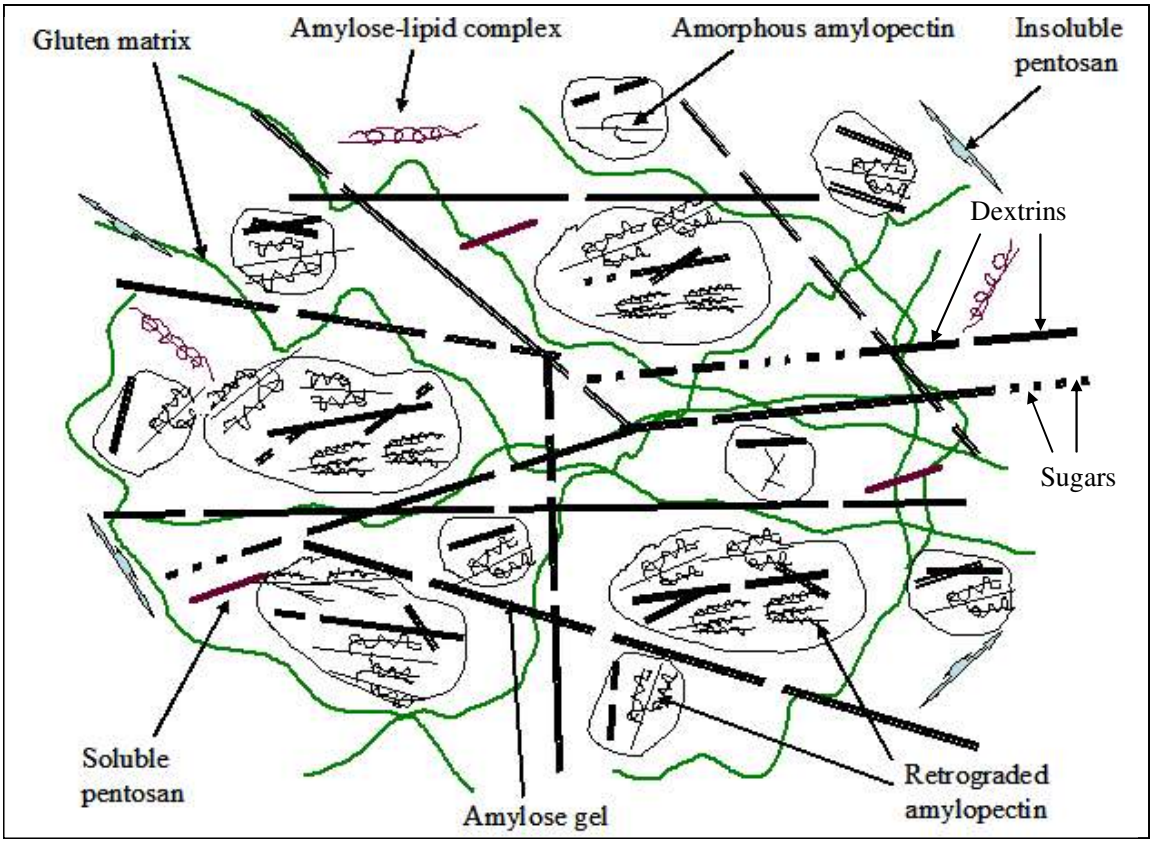

Fig. 18. Model for a stale amylase-treated flour tortilla.

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APPENDIX A

DATA TABLES

TABLE A1

Effect of Amylase and Storage Time on the Texture Profile of Tortillas

Storage Time (day)	Deformation Modulus (N/mm)	Force to Rupture (N)	Distance to Rupture (mm)	Work (N.mm)
Control				
0.04	0.44	7.63	26.76	74.49
0.21	0.51	7.64	23.57	62.19
1	0.61	6.50	15.06	35.91
3	0.83	5.99	11.67	26.03
7	0.91	6.31	10.63	25.04
14	0.93	6.29	10.10	23.66
21	0.96	6.11	10.45	24.01
28	1.23	7.28	9.61	26.14
Amylase-treated				
0.04	0.43	8.20	28.46	90.42
0.21	0.51	7.30	23.07	60.27
1	0.56	6.52	15.87	39.97
3	0.58	6.28	14.68	36.06
7	0.58	6.59	14.00	38.30
14	0.51	6.29	13.96	34.96
21	0.57	6.76	15.01	39.96
28	0.55	6.08	13.46	33.06
LSD	0.12	1.00	2.50	10.90

Means from three trials

TABLE A2

Effect of Amylase and Storage Time on the Carbohydrate Profile of Dough and Tortillas

Storage Time (day)	Dextrins (g/100 g sample, db)		Sugars (g/100 g sample, db)	
	Control	Amylase	Control	Amylase
dough	0.28a	0.57e	0.94b	1.66e
0	0.47a	17.81a	1.28a	2.41d
0.04	0.49a	18.05a	1.29a	2.83cd
0.21	0.42a	18.31a	1.34a	2.93c
1	0.36a	16.09b	1.29a	3.07c
3	0.44a	15.20c	1.35a	3.25c
7	0.49a	14.31d	1.38a	3.89b
14	0.60a	14.49cd	1.38a	4.16b
21	0.61a	15.16c	1.42a	5.03a
28	0.70a	14.55cd	1.45a	5.48a

Means from two trials; means in a column followed by the same letter are not significantly different ($p<0.05$)

TABLE A3

Effect of Amylase and Storage Time on Amylose Solubility of Dough and Tortillas

Storage Time (day)	Amylose Solubility (%)	
	Control	Amylase
dough	2.08cd	2.43c
0	3.43a	2.64bc
0.04	2.47b	2.80bc
0.21	2.49b	2.64bc
1	2.33bc	2.69bc
3	2.31bcd	2.77bc
7	1.94d	2.71bc
14	1.98cd	2.84ab
21	1.94d	2.86ab
28	2.12bcd	3.21a

Means from two trials; means in a column followed by the same letter are not significantly different ($p<0.05$)

TABLE A4

Texture Profile of Control and Protease- and TG-Treated Tortillas

Storage Time (day)	Control	Protease-Treated	TG-Treated
Deformation Modulus (N/mm)			
0.04	0.48Da	0.34Eb	0.38Eb
0.21	0.51Da	0.43Db	0.47Dab
1	0.65Ca	0.51Cb	0.55Cb
3	0.84Ba	0.61Bb	0.81ABa
7	0.97Aa	0.68ABc	0.77Bb
14	1.04Aa	0.68ABc	0.78Bb
21	0.99Aa	0.73Ac	0.88Ab
Force to Rupture (N)			
0.04	7.61Aa	4.13ABb	7.44Aa
0.21	7.62Aa	4.65Ac	6.16Bb
1	6.07Ba	3.79Bb	6.24Ba
3	5.90Ba	3.19Cb	5.58Ca
7	6.11Ba	3.12Cc	5.23Cb
14	6.42Ba	3.23Cc	5.45Cb
21	5.99Ba	3.62BCc	5.40Cb
Distance to Rupture (mm)			
0.04	24.49Ab	17.68Ac	25.45Aa
0.21	22.98Ba	17.96Ac	21.36Bb
1	13.56Cb	11.64Bc	16.14Ca
3	10.76Db	9.21Cc	12.47Da
7	10.31DEb	8.72CDc	11.61Da
14	9.89Eb	7.99Dc	10.96Da
21	9.96Ea	8.70CDb	9.79Da

Storage Time (day)	Control	Protease-Treated	TG-Treated
Work to Rupture (N.mm)			
0.04	71.79Aa	27.01Ac	62.86Ab
0.21	61.71Ba	27.25Ac	44.69Bb
1	30.22Cb	15.16Bc	35.36Ca
3	23.65Da	10.42BCb	25.78Da
7	22.14Da	9.82Cb	22.07Da
14	23.11Da	9.82Cb	23.69Da
21	22.20Da	11.55BCb	21.24Da

[a] Means from two trials; means in a row with the same small letter, and means in a column with the same capital letter are not significantly different ($p<0.05$)

TABLE A5

Effect of Xylanase and Storage Time on Rollability Scores of Tortillas

Storage Time (day)	Control	Xylanase (ppm)		
		25	50	75
1	5.0Aa	5.0Aa	5.0Aa	5.0Aa
3	4.8Aa	4.9Aa	4.9Aa	4.9Aa
7	3.9Bb	4.1Bab	4.0Bb	4.3Ba
14	2.9Ca	3.0Ca	2.8Ca	3.0Ca
21	2.6Da	2.6Da	2.4Da	2.6Da

Means from two trials; means in a row with the same small letter, and means in a column with the same capital letter are not significantly different ($p<0.05$)

TABLE A6

Effect of Xylanase and Storage Time on the Texture Profile of Tortillas

Storage Time (day)	Control	Xylanase (ppm)		
		25	50	75
Deformation Modulus (N/mm)				
0.04	0.48Da	0.44Da	0.42Da	0.47Ca
0.21	0.64Ca	0.55Cb	0.55Cb	0.52Cb
1	0.62Ca	0.55Cb	0.55Cb	0.67Ba
3	0.78Bab	0.84Ba	0.75Bbc	0.70Bc
7	0.89Ab	0.99Aa	0.81ABc	0.79Ac
14	0.89Ab	1.02Aa	0.88Abc	0.82Ac
21	0.90Aa	0.80Bb	0.82ABb	0.83Ab
Force to Rupture (N)				
0.04	7.56Ba	6.87Bb	6.80Ab	7.29Aab
0.21	8.50Aa	7.60Ab	6.89Ac	6.34Bd
1	6.34Cab	6.45BCa	5.94Bb	6.03BCab
3	6.21Ca	6.20Ca	5.42Bb	4.82Ec
7	6.10Cab	6.47BCa	5.30Bc	5.73BCDbc
14	5.79Cb	6.65BCa	5.78Bb	5.38CDEb
21	6.40Ca	6.22BCa	5.37Bb	5.16DEb
Distance to Rupture (mm)				
0.04	23.38Ab	23.85Aab	24.11Aa	23.26Ab
0.21	21.23Bb	22.78Ba	20.87Bb	20.01Bc
1	15.11Cb	16.35Ca	15.69Cab	14.31Cc
3	13.42Da	13.21Dab	12.43Dbc	12.11DEc
7	12.40Da	12.05Ea	12.1Da	12.48Da
14	11.28Ea	11.45Ea	10.89Ea	11.29EFa
21	11.32Eab	11.82Ea	10.78Ebc	10.36Fc

Storage Time (day)	Control	Xylanase (ppm)		
		25	50	75
Work to Rupture (N.mm)				
0.04	64.73Aa	59.76Aab	59.17Ab	61.56Aab
0.21	65.98Aa	63.46Aa	51.87Bb	44.56Bc
1	33.90Bab	37.98Ba	31.88Cb	31.32Cb
3	29.75BCa	29.17Ca	24.44Dbc	21.58Dc
7	26.90BCab	28.00Ca	22.07Db	25.40CDab
14	23.46Ca	27.61Ca	23.80Da	22.28Da
21	28.56BCa	27.34Ca	20.62Db	20.55Db

Means from two trials; means in a row with the same small letter, and means in a column with the same capital letter are not significantly different ($p<0.05$)

VITA

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