ABSTRACT

GHARST, GREG. Biochemical and Rheological Characterization of Peanut Proteins Crosslinked with Microbial Transglutaminase. (Under the direction of Dr. Timothy H. Sanders.)

Initially, the rheological behaviors of aqueous peanut flour dispersions were characterized across a range of conditions, including controlled heating and cooling rates under both large and small-strain deformations. Fat content influenced the rheological patterns, as dispersions of higher fat flours were less viscous than lower fat flours. A roast effect was also displayed, especially for dispersions of the higher fat flours in which light roast flours were more viscous than dark roast flours. Dispersions of low fat peanut flours were more viscous than soy at higher temperatures (>75°C). Centrifugal separation revealed that the insoluble material of all dispersions primarily contributed to the observed rheological responses. SDS-PAGE was used to characterize the soluble and insoluble proteins making up the various dispersions.

In the second study, we investigated the polymerization of peanut proteins treated with microbial transglutaminase (TGase). TGase is an enzyme that promotes protein crosslinking reactions through an acyl transferase mechanism involving protein-bound glutaminyl residues and primary amines including the ε-amino group of lysine residues in soy, myosin, gluten, oat globulin, casein and whey. Herein, we report exogenous TGase catalysis of several peanut protein fractions, including purified Ara h 1. SDS-PAGE banding patterns revealed the formation of high molecular weight polymers while catalysis of Ara h 1 resulted in distinct dimer formation. Crosslinking effects were accomplished in the presence and absence of the reducing reagent, dithiothreitol. Ortho-

phthaldialdehyde assays (OPA), used to quantify the degree of polymerization, indicated ~21% and ~30% protein coupling using either cold hexane extracted peanut protein fractions or lightly roasted peanut flour dispersions, respectively. Rheological measurements established that TGase-modified light roast peanut flour-12% fat exhibited lowered viscosity readings compared to non-treated dispersions.

In the third study, light roast-12% fat peanut flour (PF), a high protein ingredient prepared after partial extraction of oil from roasted peanut seed, was polymerized by TGase resulting in modification of functional properties such as viscosity, gelation, solubility, and water holding capacity. These experiments were conducted with either TGase treated or untreated 20% w/w PF dispersions containing 0.5% or 1.0% w/w amidated pectin (AP). Gelation occurred at higher temperatures (~78°C) using PF dispersions treated with TGase compared to dispersions devoid of enzyme (~68°C). The addition of 0.5% AP to PF dispersions minimized shifts in gel point temperature and increased the apparent viscosity of all samples, especially those treated with TGase. High molecular weight polymers were formed in TGase treated PF dispersions both in the presence and absence of AP; however, polymer formation was more rapid in PF dispersions without AP. OPA data indicated ~40% protein coupling in PF dispersions treated with TGase compared to ~20% in those containing both AP and TGase.

In the final study, light roast-12% fat peanut flour (PF) is a high protein food ingredient. Recently, we observed that TGase crosslinked peanut proteins and changed the functional characteristics of the final product. Polymer formation was observed in TGase treated

PF-casein (CN) dispersions and occurred more rapidly with increasing concentrations of CN. Upon heating, the gelation temperature of all TGase-treated PF-CN dispersions increased. Moreover, the viscosity and yield stress of TGase treated PF-CN dispersions were less than in non-TGase treated controls. TGase treated PF + 5% CN dispersions increased in water holding capacity after 24 hr incubation at 37°C. Casein proved to be an effective co-substrate with PF, and enzymatic processing changed several rheological characteristics including gelation temperature, viscosity, yield stress, and WHC. Collectively, these data suggest the potential use of PF-PF, PF-AP, and PF-CN polymers in high protein peanut-based food products.

Biochemical and Rheological Characterization of Peanut Proteins Crosslinked with Microbial Transglutaminase

By

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Biography

Gregory Allen Gharst was born in Rota, Spain on February 1, 1968. He was raised, for the most part of his young life in Fredericksburg, VA with the guidance of his parents, Doug and Jane, older brother Mike, and older sister Linda. He graduated from Stafford High School in 1986 where he earned varsity letters in basketball and was the captain of the team. He then joined the United States Army and was an Airborne Medic while achieving the rank of E-4 before finishing his contractual obligation. At North Carolina State University, he studied Biological Life Sciences and received his B.S. in May, 1994. After graduating, Greg was hired as the microbiologist for McLane/MarketFare Foods in Fredericksburg, VA, then he was promoted to Quality Assurance Manager and finally to Technical Services Manager where he re-located to corporate headquarters in Austin, TX. In 2004, Greg received his M. S. in Food Science studying *Camplylobacter* in mature cattle at slaughter, under the direction of Dr. Sophia Kathariou. During the summer of 2004, Greg started working on his PhD in Food Science studying peanut protein chemistry, under the guidance of Dr. Debra A. Clare and Dr. Jack P. Davis and direction of Dr. Timothy H. Sanders.

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Chapter 1

Literature Review

INTRODUCTION - PEANUTS

History

Peanuts (*Arachis hypogaea* L.) originated in South America. Peanuts, beans, and peas are members of the Leguminosae family which are primarily defined by species that contain nitrogen-fixing bacteria on their roots (Hopkins and Huner, 2004), unlike tree nuts such as walnuts and almonds.

Peanuts can be grown in many parts of the world and the plant grows best in light, sandy loam soil. Plants require five months of warm weather, and an annual rainfall of 50 to 100 cm (20 to 40 in) or the equivalent in irrigation water (Woodruff, 1983). The top three peanut producing countries in the world as of 2002 were: China (14M tons), India (7.6M tons), and the United States (U.S.) (1.9M tons) (USDA, 2004). Although the overall production of the U.S. was third, productivity was first. In 2002, the U.S. produced 3.4 metric tons per hectare; while China only produced 2.9 metric tons per hectare (USDA, 2004). Moreover, in the U.S., 85% of the peanuts produced are consumed and the remainder (15%) used as peanut byproducts in products such as oil, flour, meal, etc. (Murugesu and Basha, 1989). Several factors, such as weather and technology, directly influence the productivity difference between China and the United States, although the U.S. has higher yields primarily because of a more efficient production process.

Production

Until approximately 1920, the harvesting and production of peanuts in the U.S. was extremely labor intensive and was concentrated on farms in the southern part of the United States (Woodruff, 1983). Planting and cultivating of peanuts was all done by hand, i.e., the peanut pod was shelled and then hand-planted. Cultivation by hand occurred as many as four times in one growing season which illustrates the intense labor that was involved in production, prior to development of modern mechanization (Woodruff, 1983).

By 1964, peanut production was greater than 90% mechanized in Alabama and Georgia (Woodruff, 1983). Mechanization of peanut production included but was not limited to, applying herbicides, deep tillage and smooth soil preparation, fertilizing, and mechanized drying (Woodruff, 1983). The advantages of mechanized peanut production were the increase in yield and the decrease in man-hours needed per acre which are directly related to increased profits.

Planting, Cultivation, and Harvesting

The two most important factors for determining the best time for planting peanuts are the soil conditions and the weather. Planting in the Southeast usually takes place in early spring (Sullivan, 2003). Yield increases can be achieved by planting early to allow sufficient time for the crop to mature before the weather turns cool, thus resulting in more mature peanut pods (Sullivan, 2003).

The harvesting of peanuts is a process consisting of digging, inverting into windrows, picking, and then drying. The timing of the peanut harvest is critical since it affects both yield and peanut quality (Woodruff, 1983). Harvesting the crop when the maximum amount of mature fruit are on the plants (Woodruff, 1983) results in the maximum yield possible.

The beginning of mechanical digging/inverting was accomplished in 1954 (Woodruff, 1983). This process entails cutting the taproot of the plant, lifting the plants from the soil, shaking any residual soil from the roots of the plant, and inverting the plants into windrows (defined as long row(s) of cut peanut plants left in a field before being bundled), so that the peanuts are exposed to the air for drying (Woodruff, 1983).

Picking is the production process between windrow and wagon drying. Picking by a peanut combine involves lifts the vine before the peanuts are pulled off with fingers or perforations (Sullivan, 2003). The picking action of the combine is adjusted to optimize peanut yield with minimal pod breakage (Sullivan, 2003).

The process of drying begins as soon as the plants are lifted from the soil and inverted into windrows. The process entails drying the peanuts in the windrows from 30-40% mean moisture at the time of digging to approximately 22-25% moisture at picking to approximately 10% mean moisture after heated air drying to ensure quality retention during storage (Pattee and Young, 1982).

PEANUT PROTEINS

Introduction

Proteins are compromised of many simple repeating units called amino acids, of which there are 20 different types commonly occurring in nature. In proteins, the levels of structure are defined as: 1. primary structure, linear sequence of amino acids based upon amino acid analysis or nucleic acid sequence. 2. secondary structure, an organized arrangement of simple amino acid sequences common to most proteins, with the two primary examples being α-helices and β-sheets. In an α-helix the amino acids are arranged in a helical structure as shown in Figure 1. β-sheets consist of two or more amino acid sequences within the same protein. The amino acids sequences are arranged adjacently and in parallel, but with alternating orientation such that hydrogen bonds can form between the two strands as displayed in Figure 2. 3. Tertiary structure is the three-dimensional arrangement of a protein. 4. Quaternary structure is the interaction of distinct polypeptides (defined as chains of amino acids linked together by covalent bonds) by specific forces such as hydrogen bonding, hydrophobic interactions, or electrostatic interactions (Creighton, 1984).

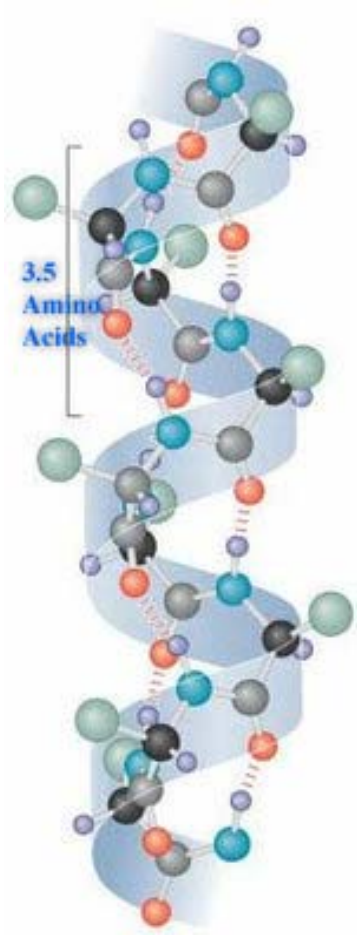


Figure 1 α-helix (Creighton, 1984)

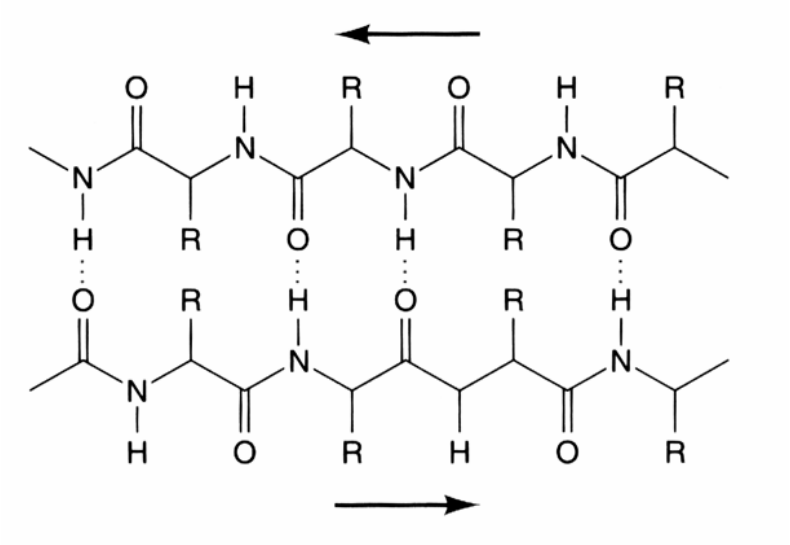


Figure 2 β-sheet (Creighton, 1984)

Proteins are often used in the food industry as functional ingredients, i.e. imparting some desirable trait to a food product. One potential application within the food industry includes gelation, or the forming of a three-dimensional network by making a fluid into a solid or semi-solid. This attribute contributes to the textural properties of food, flavor release, moisture holding, and water holding capacity. Other potential applications include foam formation and stability, which influences texture in products such as angel food cake. Proteins also are used in emulsions (defined as a dispersion of one liquid into another liquid or solid), as stabilizers due to interfacial properties with polar groups; thus allowing for interaction within a liquid such as water and a non-polar group that allows for interaction within a liquid such as oil (Fennema, 1996).

Peanuts contain approximately 25-30% crude protein content (Basha et al., 1976; Hoffpauir 1953). The composition of crude peanut protein is broadly classified into three groups: albumins (water-soluble), globulins (~96% of total protein) (salt-soluble), and glutens (acid or alkaline soluble) (Basha and Cherry, 1976). The globulins are made up of two major fractions. The first fraction is arachin (Ara h 3) which constitutes about 63% of the total protein content. The second fraction is conarachin (Ara h 1) which constitutes about 33% of the total peanut protein (Mills et al., 2002). The molecular weight of arachin was reported at 350 kDa and composed of 6 subunits (Cherry et al., 1973).

Extraction

The extraction of peanut seed proteins for use as a functional food ingredient has potential to the peanut industry and the food industry as a food ingredient. For instance,

peanut proteins may be used to control the texture of products containing peanut flour dispersions, with examples being nutrition bars, confections, baked goods and peanut spreads. (McWatters and Cherry, 1982).

pH

Rustom and co-workers (1991) examined various parameters such as temperature, pH, time, solids-to-water ratio, soaking, and partial hydolysis with respect to total protein recovery. Within this study, the data determined that pH, solids-to-water ratio, temperature, hydrolysis (defined as chemical decomposition in which a compound is split into other compounds by reacting with water), and soaking had a significant impact on protein extraction.

Extraction buffers have been evaluated with respect to improving protein recovery from raw peanuts. Poms and co-workers (2004) found that high pH (8-11) buffers prepared from a range of salts resulted in the highest protein yield. They also reported that using water (pH 7.5) heated to 60°C was not significantly different than from a variety of commercially bought buffers when used to extract protein from raw peanuts.

Salt Effects

The use of salt (NaCl) to extract peanut proteins from the seed has been the subject of many investigations. Basha and Cherry (1976) demonstrated that maximum recovery of the arachin and nonarachin peanut seed proteins was achieved with a high salt buffer (1 M NaCl-20 mM sodium phosphate buffer pH 7.0). In the same study, other

extraction media were found to be less efficient, such as phosphate buffer (pH 7.9), deionized water, and 10% NaCl.

Kholief (1987) studied extraction of protein from peanut seed and demonstrated that both mono (defined as an atom combining with one other atom) and divalent (defined as an atom combining with two other different atoms) salts increased protein extraction at pH 4-5.5, and that increased salt concentrations improved protein extraction from peanut seed. Murugesu and Basha (1989) examined the effects of protein extraction using boiled peanuts in the presence and absence of 0-5% salt. Within this study, they established that the total protein content of the peanut seed was not significantly affected by either the salt or the boiling treatment and denaturation of the proteins via boiling decreased solubility.

Roasting Effects

In the presence of increasing moisture content (3.2-23.2%), peanut splits roasted at a temperature of 140°C were observed to decrease in the amount of extracted protein. Moreover, heat treatment, extent of heating, and moisture content displayed variations under gradient polyacrylamide gel electrophoresis (PAGE) from both arachins and non-arachins (Chiou and Tsai, 1989). Beyer and others (2001) conducted research, with regards to heating peanuts, and observed using sodium dodecyl sulfate (SDS) PAGE that the amount of the conarachin protein (Ara h 1) was reduced when peanuts were boiled or fried as compared to roasting. Dry roasting of peanuts was investigated and the findings observed indicated that the effects of the heat treatment (145°C/60 minutes) reduced the

solubility of peanut protein preparations by 50% (Ory et al., 1970). Poms et al. (2004) extended the findings of Ory et al. and investigated roasting as it affects protein extraction from peanut seed, dry-roasting temperatures and times were compared to oil-roasting, which generally involves lower temperatures and shorter times due to a more efficient heat transfer. The results indicated that increased roasting temperatures resulted in a significant decrease in the protein yield. For example, oil-roasting decreased protein recovery 50-75% and dry-roasting decreased protein recovery 75-80% as compared to raw peanuts.

Fractionation

The arachin fraction is stable in treatments such as heat and pH; however, the conarachin fraction was found to be more sensitive to these treatments (Natarajan et al., 1975). Two methodologies have been primarily utilized by investigators to fractionate these components. The first method was cryoprecipitation. This method involved mixing 0.2 M phosphate buffer (pH 7.9) with defatted flour. The mixture was then stirred for 4 hr, dialyzed against 4°C tap water and arachin precipitated while conarachin was solubilized (Owusu-Apenten, 2002). The second method involves ammonium sulfate fractionation. Within this method, arachin and conarachin were precipitated from solution using 0-40% and 60-80% ammonium sulfate, respectively (Owusu-Apenten, 2002). The fractionation of peanut seed protein by different methods has been investigated with an attempt to identify the remaining 4% peanut protein.

Native arachin is classified as an 11-12S globulin, which is found extensively in plant seeds. Arachin is insoluble in water and 50% ammonium sulfate, soluble in dilute salt solution, and it is coagulable by heat. A treatment of 2-mercaptoethanol (ME) and then SDS-PAGE displayed seven distinct bands (72.4, 60.3, 39.8, 33.1, 29.0 and 21 kDa) and the molecular weight was reported as 350 kDa (Owusu-Apenten, 2002). However, a previous investigation regarding the 11S or legume-like globulins in dicotyledonous (defined as a flowering plant with two embryonic seed leaves that usually appear at germination) seed plants found that the 11S consists of only six subunits (Plietz et al., 1987).

Native conarachin, a 7S globulin, has a molecular weight of 180 kDa (Owusu-Apenten, 2002) and is composed of a trimer of 60 kDa subunits. 2-ME and SDS-PAGE analysis of conarachin (Ara h 1) displayed eight bands (72.4, 39.8, 33.1, 26.9, 18.6, and 16 kDa) (Owusu-Apenten, 2002).

Natarajan and others (1975) investigated the use of sodium hypochlorite, a potent oxidizing agent that induces protein conformational changes and affects fractionation. Use of SDS-PAGE indicated that peanut protein isolate treated with 0.15–0.25% sodium hypochlorite at pH 8–10 displayed several minor bands. These minor bands were thought to be derived from the major peanut seed protein conarachin.

Allergenicity

Peanuts are part of a group of foods that cause allergic reactions. True food allergies happen in about 1–2% of the adult population and in about 3–8% of children (Magni et al., 2005). Peanuts, specifically, affect 0.6–1% of the adult population and they frequently have severe or fatal reactions (Magni et al., 2005). Minor amounts of ingested peanut (1/50 of a peanut or approximately 3 mg) are typically sufficient to induce allergic reactions and threshold levels as low as 100 μg have been reported (Wichers et al., 2004). Ara h 1 – Ara h 8 are a group of peanut proteins that have been implicated in peanut allergic reactions. Of these eight proteins, Ara h 1 and Ara h 3 account for 70 and 90%, respectively of all allergic responses and are considered to be the major allergens (Magni et al., 2005). Ara h 3, producing allergic reactions mostly in children, has been investigated most extensively (Magni et al., 2005).

Ara h 2, a peanut protein allergen, is a doublet (defined as consisting of two similar parts) with a molecular weight of 17-20 kDa as determined by SDS-PAGE and is rich in sulfur containing amino acids such as cysteine and methionine. Results have shown that Ara h 2 is present in all peanuts that are grown in various parts of the world (Koppelman et al., 2001).

Ara h 3 was identified as a particular subunit (14 kDa) of arachin by Eigenmann and co-workers (1996). However, molecular biology techniques indicated that the arachin gene expressed a protein that was approximately 60 kDA, referred to as peanut glycinin.

The quaternary structure of peanut proteins was shown to be similar to the subunit organization of soy glycinin (Koppelman et al., 2003).

Characterization

Shokraii and Esen (1992) investigated the differences in peanut proteins before and after reduction with 2-mercaptoethanol (2-ME). The results indicated that a number of new bands, all of which were lower molecular weights, do appear after the disulfide bonds were reduced.

Alam and co-workers (2000) investigated the proteins separated from other peanut proteins by solubility in methanol which were suggested to specifically contribute to flavor of the roasted peanut. These proteins were either methanol-soluble (MS) or methanol-insoluble (MIS) at a concentration of 85% methanol. Characterization of these proteins, as determined with SDS-PAGE, indicated that the MS fraction was less prevalent (9-10%) than the MIS fraction (15-33%). Also, seed maturation increased protein content more in the MIS fraction than the MS fraction.

Kella and Poola (1985) investigated the effects of sugar on the thermal denaturation and aggregation of arachin. This study demonstrated that at pH 3.6 and in the presence of sugars (D-xylose, D-glucose, D-sucrose) arachin became more compact thus decreasing its thermal unfolding compared to native arachin (pH 7.5). Accordingly, sugars decreased heat-induced aggregation of arachin. These attributes were attributed to protein-carbohydrate effects on intra-protein hydrogen and hydrophobic bonding.

FUNCTIONAL PROPERTIES OF PEANUT PROTEINS

Introduction

The effective use of peanut seed proteins as an ingredient in food processing is highly dependent upon the functional properties of the proteins (Ahmed and Schmidt, 1979). Functional properties that determine whether or not a protein is successful as an ingredient(s) within a manufactured food are solubility, foaming ability, viscosity, and gelation. Accordingly, the processing conditions of peanut seed proteins are important with regards to their functional properties because of the general sensitivity of protein structure to changes in environment (Ahmed and Schmidt, 1979).

Solubility

Ahmed and Schmidt (1979) initially investigated the effects of processing and storage on the solubility of peanut proteins. The results indicated that centrifugation to solubilize peanut proteins was not effective. Spray and freeze-drying solubilized more protein than drum-drying. Solubility was not affected by steaming of peanuts for 20 minutes at 107°C as compared to non-heating. Storage at 24°C for 36 months decreased solubility of spray-dried peanut proteins.

The capacity of proteins to dissolve or disperse into a solvent strongly relates to their functionality within a food system. Protein solubility is influenced by factors such as pH, temperature, protein source, processing parameters, and protein concentration (Shen 1981). pH is an important factor concerning the solubility of peanut proteins as pH 4.0

represented the least soluble conditions whereas pH 9.0 the best (Ahmed and Schmidt, 1979). However, this contradicts earlier findings that indicated pH 8.0 was optimal for solubilization and it was not improved at pH levels higher than 8.0 (Rhee et al., 1973). Neutral pH (7.0) had 5-11% lower amounts of soluble protein compared to pH 9.0.

Rhee and co-workers (1972) determined that divalent salts such as $CaCl_2$ and $MgCl_2$ at pH 7.0 or greater suppressed peanut protein solubility at low concentration of the salt (i.e. 0.01 M $CaCl_2$). However, monovalent salts such as NaCl and KCl had the opposite effects on peanut protein solubility. A 1.0 M NaCl solution increased the amount of soluble protein at pH 7.0 or greater. Additionally, low concentrations of (< 0.1 M – > 0.25 M) monovalent and divalent salts present at a pH range of 3.5-5.5 increased the solubility of peanut protein. This is important because water is least effective at solubilizing peanut proteins within this pH range (Rhee et al., 1972). At pH 3.0 there was a decrease in protein extraction with an increase in monovalent and divalent salt concentration (0.25 M or greater). Very low pH (< 2.0) in combination with the salts suppressed solubility (Rhee et al., 1972).

However, Basha and Cherry (1976), who were among the first to investigate peanut protein solubility, determined that the maximum recovery of arachin and conarachin was achieved with the use of 1 M NaCl-20 mM sodium phosphate buffer (pH 7.0) compared to water, 10% NaCl, and sodium phosphate buffer (pH 7.9).

Neucere (1972) investigated the effect of heat on the solubility of peanut proteins. The findings from this investigation indicated that solubility decreased linearly with use of dry-heat (110-155ºC). However, when samples absorbed distilled water for 16 hours at 25°C (40% final moisture content) heat for one hour at temperatures ranging from 110-155°C) resulted in a sigmoidal solubility curve when plotted against temperature. α-arachin, a peanut protein characterized by immunochemical techniques using specific antisera, exhibited maximum solubility using wet-heat at 120°C. Disc electrophoresis demonstrated qualitatively that wet-heated peanut proteins were similar in solubility to native proteins regardless of the heat treatment level used.

Cherry and co-workers (1975) found that heating raw peanuts from 50 and 75°C for 15-90 minutes did not alter solubility characteristics of proteins dispersed in water. Only minor solubility increases were found after treatment at 50-75°C for 15-90 minutes. However, an increase in temperature above 100°C to a maximum of 120°C induced a decrease in protein solubility. Another study also confirmed that heating peanut protein dispersions to 80°C or higher decreased protein solubility (Schmidt and Mendelsohn, 1977).

The effect of moist heat (100°C) on peanut protein solubility was also studied using peanuts grown at different locations and then stored for different lengths of time. Increasing the heating time to 210 minutes or greater decreased protein solubility; however, the rate of decline was slower for recently harvested peanuts compared to peanuts that had been harvested from the previous year (Cherry and McWatters, 1975).

Additionally, McWatters and Holmes (1979) studied the effects of moist heat upon the solubility of peanut flour (8% suspensions w/v, pH 8.0), and reported that peanut flour solubility was reduced under moist heat (steam at 10, 20, and 30 minutes).

Mazhar and Basha (2002) examined the effect of plant moisture stress on peanut proteins. They found that peanut pod maturity affected the solubility of peanut proteins. Immature peanuts had higher moisture content and less soluble protein. Mature peanuts had lower moisture contend and more soluble protein; however, no major qualitative differences were evident using SDS-PAGE (Mazhar and Basha, 2002).

Viscosity/Gelation

Viscosity is an important attribute of proteins. Many factors such as shape, size, charge, and solubility influence viscosity or flow properties and these factors are influenced by pH, temperature, ionic strength, and environmental conditions (Shen, 1981). Viscosity of dispersions prepared with defatted peanut meal in water, 0.1 M NaCl, and 1.0 M NaCl (8% w/v) at pH 1.5, 4.0, 6.7, and 8.2 were investigated by McWatters et al. (1976) and indicated that all the dispersions at different pH's and salt concentrations had low viscosities and were similar.

The key properties of food gels are texture, appearance, water-holding capacity, spreadability, and structural rigidity (van Vliet et al., 2002). Gelation involves the transformation of a solution into a highly deformable solid without the solution losing its fluid component (Kella, 1989).

Proteins usually form gels after a heat treatment followed by a cooling period. There are three general steps with respect to protein gelation: 1) native molecular state (no interaction) 2) activation in which proteins are heated, causing them to unfold and 3) activated interaction of molecules resulting in network formation. This continuous network (3-D) of protein is critical for gel formation in order to become solid or solid-like thus a self-supporting system (van Vliet et al., 2002).

Gelation is attributed to two main types of chemical interactions. The first is covalent bonds (permanent bonds), and involves peptide or disulfide bond formation. The second and weaker type of interaction is non-covalent bond formation such as hydrogen bonding, hydrophobic interactions and electrostatic interactions (van Vliet et al., 2002).

Research conducted with, arachin, the primary protein of peanut seed, indicated that at concentrations above 7.5% w/v heat-reversible gel are formed at pH <3.8 using empirical measurements (Kumar et al., 1980); however no fundamental rheological measurements were performed. Schmidt and others (1978), employed empirical methods as well, and reported that solutions composed of peanut flour (≥25%) with whey protein concentrate (WPC) (10% protein), diminished the gelling characteristics of the WPC. Again, fundamental rheological tests were not conducted. The addition of salt to commercial peanut flour decreased the apparent gel strength but not in blended formulations where the levels of the peanut flour were low. These differences may be due to the complex nature of peanut proteins. The heat and salt combination was suggested to cause a decrease in gelation due to the dissociation (defined as the process by which the

action of a solvent causes a molecule to split into simpler groups of atoms, single atoms, or ions) in the quaternary structure. Furthermore, the use of calcium (concentrations of 5, 10, and 30 mM) decreased peanut flour gelation and caused proteins to precipitate in the presence of heat at the 30 mM $CaCl_2$ level. A slight increase in gel strength was noted because blended formulations were used and there was no precipitation (Schmidt et al., 1978).

Kella and Poola (1985) indicated that arachin (15% w/v) forms a gel when heated at 90°C for 15 minutes and then cooled to 5°C for 24 hours. Another study using purified arachin at pH 3.6 demonstrated that low levels of SDS ($< 1 \times 10^{-2}$ M) enhance heat-induced gelation by decreasing the net positive charge and thus freeing nonpolar groups during thermal denaturation allowing for protein-protein interactions which increase gel strength (Kella and Rao, 1985).

Foaming

Foams are defined as a gas (discontinuous phase) dispersed into a liquid or solid (continuous phase) and are similar to emulsions in that both have hydrophobic fluid dispersed into a hydrophilic liquid (Fennema, 1996). Foams are either bubbly or polyhedral, determined by the amount of air incorporated into the structure. Champagne and soda are examples of liquid continuous phase foams. Solid continuous phase foams are products like bread and cake. Foams are formed by three processes: whipping, bubble formation at the orifice, and bubble generation at the site of origin such as a chemical reaction, normally caused by bacteria or yeast, or a pressure change with beer (Fennema,

1996). Four factors, drainage, coalescence, creaming, and disproportionation are responsible for foam destabilization

Proteins function during foam formation by acting as surfactants (a surface active substance). During foam formation tremendous amounts of interfacial area are created. The interface is the air (hydrophobic)/aqueous (hydrophilic) boundary. Interfacial tension is defined as the surface tension at the interface between the dispersed and continuous phases (Fennema, 1996). Proteins are amphipathic and lower interfacial tension by rearrangement of their amino acids, i.e. putting hydrophobic amino acids in air and hydrophilics in water. At the interface of air and water two-dimensional networks (protein-protein interactions) may be formed. Properties that influence the creation of a protein solution into foam include pH, temperature, lipids, and solubility (Fennema, 1996).

McWatters and Cherry (1975) investigated the foam properties of peanut paste prepared with moist heat treatments at 50, 75, and 100ºC for 15-90 minutes. Their results indicated that foaming capacity was highest at 100ºC compared to 50 and 75ºC. Results of their experiment were measured by milliliters of foam recorded due to the increase in moisture content during the heating process.

McWatters and co-workers (1976) conducted research concerning the effect of pH on defatted peanut meal suspensions and the resultant foaming capacity. The resulting data indicated that stability and foam formation are affected by pH adjustments before

whipping. At pH 1.5, defatted peanut meal produced foams that were stiff and had small air bubbles. In contrast, foams produced at neutral pH (6.7) lacked stiffness, consisted of large air bubbles, and were frothy.

In an extension of earlier work, the foaming characteristics of defatted peanut flour (8% w/v) in distilled water were investigated by McWatters and Cherry (1977). Their results indicated that peanut flour formed thick egg white type foams after undergoing a two-step pH adjustment using 1 N HCl or NaOH while stirring (6.7 → 4.0 → 8.2). A 120% increase in volume was observed as compared to a one step pH adjustment (4.0 → 6.7 or 6.7 → 8.2) that produced 100% increase in volume. Another result established that protein solubility was related more closely to the quality of the foam produced compared to the amount of oil or air bound by the flour. Finally, the investigators found that the pH of the mixed flour system was highly important.

Sekul and co-workers (1978) investigated the partial hydrolysis of defatted peanut proteins (8% dispersions w/v) with papain and the subsequent effect upon foaming capacity. The data indicated that partial hydrolysis of peanut proteins with papain did increase the foaming capacity due to modification of protein structure allowing for better interaction and foam formation. A two-step pH adjustment (6.7 → 4.0 → 8.2) was important for the formation of foam and resulted in a three fold increase in foaming capacity.

Kim and co-workers (1992) studied foam formation using nine different peanut cultivars. Their findings demonstrated that there were no significant differences among these cultivars in foams produced after whipping.

A more recent study of glycinin, an 11S globulin protein isolated from soy (with similarities to arachin) demonstrated that the improvement in foam performance at pH 3.0 was related to the interfacial measurements, i.e. increased adsorption (defined as the accumulation of gases, liquids, or solutes on the surface of a solid or liquid) rates and interfacial rheology (defined as the deformation and flow of matter using stress or strain) (Martin et al., 2002).

TRANSGLUTAMINASE

Transglutaminase (TGase) is an enzyme that catalyzes protein crosslinking through acyl-transfer reactions between glutaminyl residues (donor) and primary amines (acceptor) (Truong et al., 2004; Zhu et al., 1995). TGase activity is temperature and pH dependent with the optimal temperature being approximately 40°C at pH 7.0 (Schorsch et al., 2000[A]). The utilization of TGase within food products may modify important characteristics and impact chemical and physical properties such as protein size, stability, conformation, viscosity, formation of heat and water resistant films, water holding capacity, and solubility (Truong et al., 2004; Zhu et al., 1995).

Zhu and co-workers (1995) summarized some of the current food applications based on past and current usage of the enzyme. In one example, TGase was used to improve the elasticity and texture of meat proteins in hamburger and meatballs. Fish paste texture was enhanced after treatment with the enzyme. TGase improved the texture of wheat proteins, used in baking goods, providing increased dough volume. Soy based foods such as tofu, treated with TGase had improved shelf-life, whereas gelatin products were found to exhibit enhanced firmness and elasticity.

Previously, Tanimoto and Kinsella (1988) conducted research which indicated that β-lactoglobulin (β-lac). β-lac (whey proteins) treated with TGase in the presence of high heat (100°C) did not denature while β-lac, alone, did denature at 70°C. The importance of this work was that proteins and heat treatments cause undesirable affects in food products and decreased solubility.

Aboumahmoud and Savello (1990) found that TGase, with the inclusion of dithiothreitol (DTT), crosslinked whey and formed polymers. In this event, DTT acted as a denaturant, reducing disulfide bonds in whey proteins, a process that promotes unfolding and promotes interaction of TGase with the protein substrate.

Truong and co-workers (2004) investigated the use of TGase in crosslinking with whey proteins to form protein-protein hybrids. The results of this study demonstrated that with increased enzyme to substrate ratio (soluble TGase at 10-25 units/g WPI proteins), some functional properties decreased such as gel strength, whereas gel point temperature

increased. This was in contradiction to previous work that reported an increase in gel strength and decrease in gel point temperature using TGase treated whey protein isolate (Wilcox and Swaisgood, 2002; Wilcox et al., 2002).

Ikura and co-workers (1980) found that casein is a good substrate for TGase. Traore and Meunier (1991) demonstrated that the different casein proteins (α-, β-, and κ-) behaved differently when crosslinked with TGase due to enzyme specificity and glutamyl and lysine residue participation. β-casein and κ-casein were better substrates than α-casein.

Schorsch and co-workers (2000^{A}) also investigated casein dispersions (5% w/w) crosslinked with TGase and their results indicated that gelation occurs at 50°C at pH < 5.4. This was important as the addition of TGase allowed the gel to form much more quickly than a pure acid gel with the times being 20 hr in the absence of TGase and 6-7 hr with TGase. A low pH was required to minimize the electrostatic repulsion which causes an inhibitory effect on gelation. Additionally, Schorsch and co-workers (2000^{B}) demonstrated that TGase treated casein micelles form covalent crosslinked gels which are generally stronger and form more rapidly than gels formed by renneting and/or acidification. Furthermore, the gels were not temperature (heating) dependent and did not show signs of exudation of liquid after lengthy storage times.

Liu and Damodaran (1999) used SDS-PAGE to demonstrate the effect of polymerization when TGase was used to crosslink β-casein. Native β-casein migrated to a

relative molecular weight of 31 kDa. The samples that were crosslinked with TGase formed new protein bands that had molecular weight of approximately 72, 110, and 150 kDa. The resulting new protein bands appeared to be dimers, trimers, and tetramers. The dimer band intensity increased with time up to 40 minutes then the intensity decreased until vanishing after 3 hr. The trimer protein band emerged at 20 minutes, remained briefly visible until 90 minutes, and then vanished. The tetramer band appeared briefly between the time intervals of 40 minutes and 90 minutes. High molecular weight bands that could not migrate through the gel were evident at time intervals of 3, 5, and 24 hours of polymerization. This confirmed that TGase had crosslinked the β-casein and that β-casein is a good substrate for TGase.

TGase has been used to form heterologous conjugate proteins (defined as using proteins as a substrate for TGase to crosslink with other proteins). Yildirim and Hettiarachchy (1997) crosslinked the 11S globulin of soy with whey proteins and demonstrated that the addition of TGase with whey and soy resulted in the formation of both heterologous and homologous biopolymers. Furthermore, crosslinked conjugates exhibited improved heat stability suggesting that biopolymers will stay in solution at higher temperatures as a result of heat treatment, a process that generally leads to a loss of solubility.

Yildirim and Hettiarachchy (1998) found that whey and soybean 11S globulin proteins crosslinked with TGase formed films that were resistant to solubilization in aqueous buffers at different pH values. Presumably, heterologous conjugates have

increased tensile strength compared to homologous protein-protein conjugates. Such a finding would likely benefit the food industry with respect to protein conjugate formation, by improving the quality of some food products.

Ramirez-Suarez and Xiong (2003) studied the crosslinking of TGase with myofibrillar and soy protein combinations. The outcome of their investigation demonstrated that when soy and myofibrillar proteins were polymerized with TGase (4.5% total protein, heat to 80ºC/4hr, 0.5% TGase), a rigid mixed protein gel was produced at decreased myofibrillar concentrations. Interestingly, this conjugate mixture increased denaturation temperatures and also elevated elasticity of the conjugate protein gels. This finding was significant since restructured low-salt muscle foods may need less total myofibrillar protein in order to form a gel.

IMPETUS FOR FURTHER STUDY

We were unable to find publication materials that describe polymerization of peanut proteins with TGase. Generally, most of the TGase research has utilized whey, soy, casein, and myofibril proteins as substrates. Moreover, the potential formation of protein conjugates with peanut proteins using TGase has not been examined and provides opportunity to the food industry. Continued investigation suggests the potential application of TGase treated peanut protein dispersions and peanut protein conjugates for use in peanut-base food products, including protein bars, shakes, ground beef patties, hot dogs, deli meats, analogs for the meat industry, and value added baked goods.

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Chapter 2

Some Rheological Properties of Aqueous Peanut Flour Dispersions

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Abstract

The rheological behaviors of aqueous peanut flour dispersions were characterized across a range of conditions, including controlled heating and cooling rates under both large and small-strain deformations. Fat content of the dry flours influenced rheological changes, as dispersions of higher fat flours were less viscous than lower fat flours on an equal weight basis. A roast effect was also apparent, especially for dispersions of the higher fat flours in which light roast flours were more viscous than dark roast flours. Dispersions of toasted soy flour were more viscous at lower temperatures (< 75°C), but at higher temperatures, low fat peanut flour dispersions were more viscous than soy. Centrifugal separation revealed that the insoluble material of all dispersions primarily contributed to the observed rheological responses. SDS-PAGE was used to characterize the soluble and insoluble proteins making up the various dispersions and these results are discussed in terms of observed rheological phenomena.

Keywords: Peanut, Peanut Flour, Peanut Protein, Rheology, SDS-PAGE, Toasted Soy Flour, Soy Flour, Flour, Flour Rheology

Introduction

Peanut flour is a dry powder prepared after partial extraction of peanut oil from roasted peanut seed. The typical composition of peanut flour is approximately 40-50% protein, 10-30% fat and 20-30% carbohydrate/fiber (Prinyawiwatkul *et al.* 1993; Suknark *et al.* 1997), compared to mature peanut seed, which are approximately 25-30% protein, 50% fat and 20% carbohydrate/fiber (Basha *et al.* 1976; Hoffpauir 1953; Jonnala *et al.* 2005). Current or potential products that may contain peanut flours include protein bars, confections, cakes, cookies, crackers, and other baked goods, many of which are heat processed. Thermal processing significantly influences food texture, which is one of the principal quality factors of foods, along with flavor, appearance, and nutrition (Bourne 2002). The rheological profile of a food largely influences the human perception of texture (Pollen *et al.* 2004). Therefore, rheological characterization of foods and their individual ingredients can greatly aid food scientists in optimizing product texture. Fundamental rheological measurements also provide information needed to solve various process engineering equations (Steffe 1996). However, there is a limited amount of published information pertaining to the heat induced rheological changes associated with peanut flours and/or isolated peanut proteins.

The primary protein fraction of peanut seed, arachin, forms heat reversible gels at pH values < 3.8 and concentrations above approximately 7.5% w/v (Kumar *et al.* 1980). That is, the protein solutions gel upon heating and subsequent cooling, and the gels will subsequently dissipate upon reheating. An empirical system originally developed to characterize gelatin gels, which are similarly thermolabile, was used to characterize these

arachin gels (Kumar *et al.* 1980), but no fundamental rheological data was presented. Substitution of peanut flour at levels ≥ 25% within whey protein concentrate (WPC) solutions formulated at 10% protein, significantly reduced the gel forming capacity of the whey protein (Schmidt *et al.* 1978). Empirical methods were utilized to qualitatively and quantitatively characterize the gelling properties of these WPC/peanut flour blends, but no fundamental rheological measurements were presented.

Soybean is a legume like peanut and the two share many similarities, including homologous proteins that are present in similar amounts (Casey 1999; Prakash and Rao 1986). There is a far greater abundance of published data regarding the rheological/gelation behaviors of soybean flour and/or soybean protein dispersions, with a few examples given here (Bhattacharya and Jena 2007; Puppo and Anon 1998; Renkema *et al.* 2000; van Vliet *et al.* 2002). These data have undoubtedly facilitated the utilization of soybean flour/protein in various formulated foods and a similar approach for collecting data on peanut flours is expected to improve the marketability of these ingredients.

The objective of the current work was to fundamentally characterize several rheological properties of aqueous dispersions prepared from commercially available peanut flours. Specific tests included small-strain dynamic oscillatory measurements to observe structural transitions during defined heating conditions while minimally disrupting the dispersions. Large strain deformations, i.e. constant rotational shear measurements, were also conducted to gauge the flow behaviors of the dispersions in the absence of heat, as

well as structural transitions occurring in the dispersions in the presence of a shear field. Variables of interest included 1) the total solids concentration (w/w), 2) fat content and 3) the degree of roast for the various flours. Rheological data for the peanut flours were also compared to the rheological behavior of toasted soy flour at equivalent solids concentration. Chemical analyses of these flours, including total Nitrogen, lipid content, soluble protein and protein electrophoresis, were included to aid rheological data interpretation.

Materials

Peanut flours, obtained from Golden Peanut Company (Alpharetta, GA, USA), were classified by the manufacturer according to 1) roast level, either light or dark and 2) fat content, either 12% or 28% of the dry weight. The following abbreviations are used to designate these flours: Light Roast-12% Fat = LR12, Dark Roast-12% Fat = DR12, Light Roast-28% Fat = LR28 and Dark Roast-28% = Fat DR28. Toasted Soy Flour was obtained from Archer Daniels Midland Company (Decatur, IL, USA) and is abbreviated TSF. Table 1 summarizes chemical analyses of the various flours. Virginia peanuts (2002 US Harvest) were blanched at ~90°C for 45 minutes (to remove testae) and served as electrophoresis standards. All other chemicals were reagent grade and purchased from Sigma Chemical Company.

Methods

Hydration of Flours. Flours were dispersed in deionized water under vigorous stirring using a magnetic stir plate. Previous work established that the solubility of peanut protein (as determined by total nitrogen solubility) in peanut flour dispersions was

greatest at pH levels above 7.0 (McWatters and Holmes 1979). Accordingly, dispersions were adjusted to pH 8.00 for this work, as increased protein solubility is often associated with improved ingredient functionality (Kinsella 1976; McWatters and Holmes 1979).

Within either the 12 or 28% fat peanut flours, initial pH values were lower for DR dispersions compared to LR dispersions (Table 2). Sugars and amino acids participate extensively in Maillard browning reactions during peanut roasting (Mason *et al.* 1969; Newell *et al.* 1967), and acidic compounds are generated during these reactions (Cabodevila *et al.* 1994; Rich and Foegeding 2000), likely explaining the lower initial pH of the DR dispersions. Dispersion pH was quantitatively adjusted to 8.00 with 2 N NaOH after an initial stirring of 10-12 min. The pH of these dispersions was not stable after adjustment to 8.00, as seen by measurements after 1 hr (Table 2). A possible explanation for this downward pH drift is that the acidic molecules produced during the Maillard reaction slowly liberate H^+ ions as the flours are exposed to water. Readjustment to pH 8.00 1 hr after the initial adjustment resulted in a more stable pH, as seen by pH measurements at 2 hr (Table 2). This 1 hr, two-step adjustment to pH 8.00 was used for all preparations. The pH of 20% TSF dispersions was also unstable (Table 2), but less so than any of the peanut flour dispersions.

Rheological Analyses. Two fundamentally different types of rheological analyses were utilized to characterize the flour dispersions, those involving either small-strain or large-strain deformations. Small-strain testing minimally modifies the material of interest, whereas large strain analyses disrupt the experimental sample (Daubert and Foegeding

1998). Therefore, small strain testing best relates to rheological changes a material experiences in the absence of a shear field, for example, the heating of an unstirred vat of liquid. Large strain measurements best relate to rheological changes that materials experience in the presence of a shear field, for example the heating of a fluid during pipe flow.

A stress-controlled rheometer (StressTech, Rheologica Instruments AB, Lund, Sweden) equipped with a CC25 serrated cylinder geometry (cup and bob) was utilized to characterize flour dispersions under both small and large-strain deformations. Dispersions were prepared daily, and continuously stirred until rheological testing. Dispersions were added to the cup, such that they just covered the bob, and a layer of mineral oil was then added over the dispersion to minimize vapor loss. After a 5 min equilibrium time at 40°C, samples were pre-sheared at 50 1/s for 30 s to ensure thorough mixing and similar shear histories for all tests. The stress used during small strain testing was 1.5 Pa, a value identified to be within the linear viscoelastic region of stress response for all dispersions based on stress sweeps at 10 Hz in the unheated samples. Dispersions were heated and cooled between 40 and 90°C at a rate of 1°C/min during constant oscillatory testing at a frequency of 0.1 Hz. Frequency sweeps from 0.1 to 10 Hz were also applied to samples held at 90°C. During large strain testing, rotational shear rates from 0.1 to 100 1/s were applied to dispersions. A rotational shear rate of 50 1/s was selected for large-strain heating measurements, as this frequency is within the relevant range for many food processes, including extruding, mixing and pipe flow (Daubert and

Foegeding 1998). All rheological measurements were replicated a minimum of three times.

Chemical Analyses. Total nitrogen content of the dry powders was determined using a PE 2400 Series II CHN Elemental Analyzer (Perkin-Elmer; Wellesley, MA, USA). Fat and moisture content of the dry powders was measured using a Maran pulsed proton NMR (Resonance Instruments, Witney, Oxfordshire, UK) by the Field Induction Decay-Spin Echo procedure (Rubel 1994). After dispersion preparation, centrifugation at 2600g for 30 min was used to separate soluble (supernatants) and insoluble fractions of 20% w/w preparations (stirred $\geq$ 2 hr). Soluble protein content of the supernatants was determined with a BCA Protein Assay Kit (Pierce; Rockford, IL, USA) and total soluble nitrogen was measured by a Shimadzu (Columbia, MD, USA) Total Organic Carbon analyzer equipped with Nitrogen analysis capacity. All chemical analyses were replicated a minimum of three times.

The XCell Sure Lock^{TM} Mini Cell system (Invitrogen, Carlsbad, CA, USA) was used for SDS PAGE analyses according to the manufacturer's recommendations. Gels were NuPage® (Invitrogen) 4-12% Bis-Tris polyacrylamide and the running buffer was a solution 2-(N-morpholino)ethanesulfonic acid- sodium dodecylsulfate (MES-SDS) prepared according to the manufacturer's recommendations. Samples were solubilized in a lithium dodecylsulfate buffer with added reducing agent according to the manufacturer's recommendations. Defatted (hexane extraction) peanut meal prepared from blanched (non-roasted) Virginia style peanuts served as a reference. This defatted

meal, and the insoluble fractions of the dispersions, was suspended directly in the electrophoresis sample buffer. Total protein content in the electrophoresis sample buffer was normalized (10 □g per lane) after analysis with the EZQ® protein quantification kit (Molecular Probes-Invitrogen). The molecular weight standards were the SeeBlue® Plus2 Pre-Stained Standards (Invitrogen). Proteins were visualized by staining with a Colloidal Blue Staining Kit (Invitrogen).

Results and Discussion

Peanut flours were readily dispersed in water but did not form true solutions, as separation was visually apparent 30 min after 20% w/w preparations had been transferred to 100 ml graduated cylinders (inner diameter 28 mm). The first indication of separation was a thin layer of emulsified oil (< 1% volume) forming at the top of each dispersion. By 1 hr, this layer was ~1.5 and 1.0% of the total volume for the 28 and 12% fat flours respectively. After 24 hr, a semitransparent aqueous layer with a fatty layer on top (~1% volume for all) was visible above each preparation, and was ~7, 8, 11 and 13% of the total volume for LR12, DR12, LR28 and DR28 dispersions respectively. This data suggested that separation was most rapid for preparations of the higher fat flours and/or darker roasts. From these initial observations and protein solubility data discussed subsequently, it was concluded that peanut flour preparations were best described as semi-stable dispersions of insoluble material mixed within an aqueous protein solution, but are simply referred to as "dispersions" throughout this paper. In contrast, 20% w/w TSF dispersions were more stable to separation, as by 24 hr the unstirred samples still appeared homogenous.

Small strain rheological responses of 20% w/w flour dispersions were characterized from 40 to 90°C while heating at 1°C/min (Figure 1). The point at which the storage modulus (G′) crosses over the loss modulus (G″) was used as an empirical measure of the gel point, and represents the temperature at which the material transitions from a liquid to a more solid-like material (Almdal *et al.* 1993; Truong *et al.* 2004). Comparison of panel A and B reveal the gel point for 12% fat peanut flours occurred at lower temperatures (~65-72°C) as compared to the 28% fat peanut flours (> 80°C). This is at least partially attributable to the lower protein content (total and soluble) of the 28% fat flours (Table 1), as the protein fraction of the flours is expected to be a major contributor to the gel network (Kinsella 1976; Martinez 1979), and gelation rates typically increase with higher protein concentrations in model systems (Clark and Ross-Murphy 1985). With regard to the fat contents of the flours, the addition of emulsified oil (protein covered) to model protein gels can increase the gelation rate and increase the final gel moduli, if the proteins adsorbed on the fat globules interact with the proteins that makeup the gel network (Kim *et al.* 2001; Vanvliet 1988). However, dispersions prepared from the higher fat flours did not gel more rapidly (Figure 1), suggesting that the lipid in these flours is not behaving similarly to the emulsified oil of the previously described model systems. Lipid association with protein can delay the onset of heat-induced molecular changes (Cheftel and Cuq 1985), which are a typical prerequisite in heat-induced gel formation of proteins. Furthermore, if the fat interacts hydrophobically with the proteins participating in the gel network, this would lessen the likelihood of protein-protein interactions, ultimately delaying the onset of gelation. Lipid extraction in wheat dough systems has also been

shown to increase G′ during small strain testing, with a potential explanation being fat extraction increases friction between starch granules (Georgopoulos *et al.* 2006).

LR dispersions had slightly lower gel points than DR dispersions when compared across equivalent fat levels (Figure 1A or 1B). Work with model systems in which the Maillard reaction occurs *during* gelation indicated that this reaction promoted gelation as it results in the formation of more covalent cross links (Rich and Foegeding 2000). However, in the current dispersions, the proteins had already participated in this reaction, i.e. during roasting, which seems to limit their gel forming capacity within the newly forming network. Arachin has been shown to aggregate during oil roasting (Basha and Young 1985), and these aggregates, if present, may be less efficiently incorporated into a newly forming gel network due to their larger size, i.e. smaller diffusion coefficients, possibly explaining the reduced gelation rate of DR dispersions.

In contrast to the peanut flours, 20% TSF was notably more viscous at lower temperatures (< 75°C), as seen by higher magnitudes of G′ and G″ across this temperature regime (Figure 1). Furthermore, at temperatures < 75°C, G′ and G″ were roughly equivalent for TSF dispersions, indicating the overall structure was less liquid-like than all 20% peanut flour dispersions. At temperatures > ~75°C, G′ increased over G″ for TSF, indicative of a more solid-like response. The lower fat content of TSF likely contributed to the increased viscous nature of these dispersions.

Dynamic oscillatory frequency sweeps were used to further characterize small strain rheological behavior of dispersions held at 90°C (Figures 2A, 2B and 2C). G′ was greater than G″ by roughly an order of magnitude across the frequency range, and the two responses were essentially frequency independent, indicating an established gel network(Almdal *et al.* 1993) for the 12% peanut flours (Figure 2A) and the 20% TSF dispersion (Figure 2C). The higher moduli of the 12% fat peanut flours indicated these gels were more firm than the TSF dispersions. At higher frequencies, G′ and G″ became frequency dependent for the 28% fat flours, indicative of incomplete gel formation (Figure 2B). Figures 2D-2F show the same dispersions as they are cooled from 90 to 40°C at 1°C/min during dynamic oscillatory testing (same as Figure 1, except cooling). The 12% fat peanut flour dispersions formed gels (Figure 2D) as did the LR28 dispersion (Figure 2E) and TSF dispersion (Figure 2F). In contrast, the DR28 dispersion did not gel upon cooling, as indicated by the similar magnitudes of G′ and G″ (Figure 2E). Contributing factors for this observation include the lower protein content, higher fat content, and more extensive roast of the DR28 flour when compared to other samples.

To better understand the effect of peanut flour concentration on heat-induced, small-strain rheological changes, 10 to 25% w/w dispersions were analyzed while heating from 40 to 90°C at 1°C/min (Figure 3). The beginning of gel formation was observed at concentrations as low as 15% w/w for the 12% fat flours, yet a minimum of 20% solids was needed for the higher fat flours. This data confirmed earlier findings that the higher protein content and lower fat levels of the 12% fat flours promoted a more efficient gel formation. At 25% solids, gels formed rapidly for all flours, with formation occurring at

lower temperatures for the low fat flours. Therefore, if enough total solids are present, gelation will occur for all peanut flours under the tested conditions.

Continuous rotation shear rate ramps were used to characterize the large strain response of dispersions at 40°C (Figure 4). Application of the Herschel-Bulkley model:

$$\sigma = K(\dot{\gamma})^n + \sigma_0$$

to the data in Figure 4, where σ and $\dot{\gamma}$ are the shear stress and shear rate respectively, allows one to solve for n, K and σ_0, which are the flow behavior index, consistency index and yield stress respectively (Steffe 1996). These parameters are summarized in Table 3. All values of n were < 1, meaning all dispersions were shear thinning, with lower values of this parameter indicative of a more extensive change in apparent viscosity with shear rate. K values reflect the apparent viscosity of the materials across the entire shear rate range, accordingly the value for TSF was greatest, followed by values for the 12% fat peanut flour dispersions and then 28% fat peanut flour dispersions. Yield stress was also greatest for the TSF dispersion, followed by values for the 12% fat peanut flour dispersions and then 28% fat peanut flour dispersions. Yield stress is the finite stress required to achieve flow (Steffe 1996) and below this stress, particles within a dispersion will resist settling. Yield stress data supports settling observations previously mentioned, specifically higher dispersion yield stress is associated with increased stability to settling.

Samples were heated from 40 to 90°C at 1°C/min, held for 30 min at 90°C, and cooled from 90 to 40°C at 1°C/min during continuous rotation (50 1/s) to understand heating effects while in the presence of a shear field (Figure 5). Apparent viscosity increased

during heating, holding and cooling for all dispersions, meaning heat induced interactions occurred while dispersions were being sheared at 50 1/s. Comparison of the 12% peanut fat flours (Figure 5A) and the 28% fat peanut flours (Figure 5B) revealed higher apparent viscosities for the lower fat flours, in agreement with small-strain rheological data. Within the 12% fat flours, apparent viscosity was initially greater for the light roast upon heating; however, upon holding and cooling, the DR flour dispersions were more viscous. A similar phenomenon was observed in the small-strain data (Figure 2), as DR12 consistently formed gels that were more firm after the initial heating ramp as compared to LR12 dispersions. Apparent viscosity of the 20% TSF flours also increased with heating, holding and cooling (Figure 5C), with final viscosity ranges lower than 12% fat peanut flours.

Dispersions were centrifugally separated (see Methods) and the protein and total nitrogen contents of the supernatants were determined (Table 1). Previous work established that the solubility of protein extracted from peanut seed was reduced by more than 50% when seed were dry roasted (Neucere *et al.* 1969) (Aminigo and Ogundipe 2003). Maillard browning reactions involve the formation of covalently bound protein-sugar or amino acid-sugar adjuncts (Basha and Young 1985; Maleki *et al.* 2000; Rodriguez *et al.* 1989), and these adjuncts may be less soluble than the reactants from which they are derived (Armstrong *et al.* 1994). Therefore, the reduced protein solubility of DR12 compared to LR12 is likely a function of the more extensive roast, despite the higher initial nitrogen content of DR12 flour (Table 1). However, for the 28% fat flours, DR protein solubility was greater, which may simply be a function of its higher initial nitrogen content (Table

1). Soluble protein concentration in TSF supernatants was approximately twice that of the highest peanut flour (Table 1). The much lower fat level of the dry TSF powder may explain the increased protein solubility of this material, as fat can hydrophobically interact with protein, making it less soluble (Cheftel and Cuq 1985). Furthermore, since the specific heating conditions utilized during the industrial production of these flours are unknown, it may be the TSF received a less extensive heat process.

SDS-PAGE was applied to better understand characteristics of the proteins present in the soluble (Figure 6A) and insoluble fractions (Figure 6B) of these flour dispersions. Comparison of the peanut flours with the protein extracted from the blanched, non-roasted peanut (VA-02), showed that conarachin, also known as Ara h1 by the International Union of Immunological Societies nomenclature, which is present at approximately 64 kDa (Shokraii *et al.* 1985; Wichers *et al.* 2004) , was essentially absent in the peanut flours (soluble or insoluble fraction) but was present in VA-02. The heavy band(s) between 38 and 49 kDa present in VA-02 represent fractions of the acidic subunit of Ara h3 (Piersma *et al.* 2005), and the presence of this band(s) was noted in LR12 for both the soluble and insoluble fractions, but the band was present in much lower quantities for the other peanut flours (Figure 6).

Maillard reaction products, i.e. protein-sugar derivatives are expected to be present in the peanut flours due to the roasting of these ingredients. Generally speaking, smearing (poorly resolved protein) in the gels was more prevalent for the peanut flours as compared to either the raw peanut protein extract or the TSF protein. Smearing is likely

due to the presence of protein-sugar derivatives, which will likely have a range of molecular weights and hence smear. Smearing was greatest in the lower MW region for the soluble material (Figure 6A) as opposed to the insoluble proteins (Figure 6B) where smearing was more prevalent in the lower MW region. For the resolvable proteins, similar banding patterns were generally observed for the soluble and insoluble protein fractions for both the peanut and soy flours (Figure 6). Comparison of the banding patterns for the protein extracted from the TSF dispersion (both soluble and insoluble fractions) revealed distinct banding patterns as compared to the peanut protein.

The soluble and insoluble fractions of 12% fat peanut flour dispersions were rheologically characterized to assess contributions of the individual fractions. It was hypothesized that the soluble fractions should be a primary contributor to rheological measurements, since the soluble components should contribute to 3-D networks more effectively as these materials are in solution. Supernatants (soluble fractions) were collected and weighed to the nearest 0.1 g. Equivalent amounts of deionized water were then returned to the collected pellets, followed by thorough mixing to prepare new dispersions. Figure 7 summarizes small strain data for the parent and reconstituted dispersions. Soluble fractions displayed no detectable rheological changes upon heating (data not shown). In contrast, the reconstituted dispersions displayed rheological behaviors essentially equivalent to those of the parent dispersions, indicating that the insoluble fractions primarily contribute to rheological changes. This data suggested that for the original dispersions, the initial stirring suspends insoluble aggregates within the aqueous protein medium. The heating step then induces molecular changes, such as

protein denaturation, which promotes new interactions among aggregates and with soluble material, until a network is formed that resists settling and separation. We hypothesize that protein, carbohydrate and fat all makeup this molecular mesh, with earlier data suggesting fat weakens the overall 3-D structure, as compared to protein or carbohydrate. The larger size of the protein-sugar Maillard products present in the insoluble fraction (Figure 6B) should also be more effective at contributing to gel formation as compared to the lower MW protein-sugar adjuncts found in the soluble fraction (Figure 6A). Frequency sweeps and cooling data confirmed equivalent gel formation for the parent and reconstituted LR12 and DR12 dispersions (data not shown).

Further complicating the gelation picture is the highly organized subcellular microstructures of peanut seed and other oilseeds, which include protein bodies and lipid bodies among others (Young *et al.* 2004). These microstructures have been shown to survive past the defatting stage of flour processing in oilseed other than peanuts (Wolf and Baker 1975). These microstructures, if present, would impact gelation properties and future work will be designed to investigate this possibility. Gelation properties of these flours are also expected to be a function of the various complex carbohydrates and fibers present in the flours, with examples being hemicellulose and pectin (Martinez 1979). The total carbohydrate fraction of the 12% fat flours is typically 30% dry weight, as opposed to 20% dry weight for the 28% fat flours (as reported by the manufacturer). Therefore, assuming the complex carbohydrate and fiber content is also higher in the 12% fat flours, the presence of these polymeric molecules may also contribute to the better gel forming properties of the low fat peanut flours.

Conclusion

This paper provides some fundamental rheological data pertaining to aqueous peanut flour dispersions, both in the presence and absence of heating. Higher levels of fat in the dry powders inhibited the capacity of the dispersions to form gels. Flours which had been roasted were more viscous or formed gels more slowly than flours which had been roasted less extensively, although roast effects were less pronounced for 12% fat peanut flours as compared to 28% fat peanut flours. Settling rate observations for these dispersions were explained by comparison with rheological data; specifically, an increase in yield stress was associated with decreased settling. Centrifugation to remove insoluble particles from the flours revealed that the insoluble material most extensively contributed to the rheological behavior of the dispersions, with minor, if any, contributions from the soluble fractions. SDS-PAGE data found electrophoretic smearing to be more evident for protein solubilized from peanut flour dispersions, indicating the presence of Maillard reaction adjuncts. Electrophoresis of the soluble fractions showed higher contents of low MW smears, whereas electrophoresis of the insoluble material, which primarily contributed to gel formation in these flours, found a higher concentration of high MW smears. This data as a whole suggests the potential to collect the soluble fraction of such dispersions and use these solutions for appropriate applications, i.e. beverage systems requiring soluble protein. The insoluble material could then be utilized as a thickening agent in applications requiring an increase in viscosity.

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Disclaimer

The use of trade names in this publication does not imply endorsement by the United States Department of Agriculture- Agricultural Research Service.

TABLE 1.

CHEMICAL ANALYSES OF PEANUT AND SOY FLOURS

	dry flours		*supernatants*	
Ingredient	**% Protein[1]**	**% Fat[2]**	**Total N (mg/ml)[3]**	**Protein –BCA (mg/ml)[4]**
LR12	50.7 +/- 0.2	12.3 +/- 0.1	2.4 +/- 0.1	20.9 +/- 2.1
DR12	55.2 +/- 0.1	11.6 +/- 0.1	1.4 +/- 0.1	13.0 +/- 1.4
LR28	46.3 +/- 0.2	25.4 +/- 0.1	1.0 +/- 0.1	9.7 +/- 1.5
DR28	49.2 +/- 0.2	24.2 +/- 0.3	1.3 +/- 0.3	13.8 +/- 1.7
TSF	53.5 +/- 0.2	2.40 +/- 0.1	4.6 +/- 0.3	38.6 +/- 0.7

Values are means +/- the standard deviation of at least 3 replications 1. % protein of flours as determined by combustion (N X 6.25). 2. % fat of flours as determined by NMR. 3. Total nitrogen in supernatants of 20% w/w dispersions as determined by micro-Kheldal. 4. Protein in supernatants of 20% w/w dispersions as determined by BCA Assay. See methods for separation conditions. LR12, DR12, LR28, DR28 and TSF are light roast-12% fat peanut flour, dark roast-12% fat peanut flour, light roast-28% fat peanut flour, dark roast-28% fat peanut flour and toasted soy flour respectively.

TABLE 2.

pH VALUES FOR 200 g OF 20% w/w DISPERSIONS
OF PEANUT AND SOY FLOURS

Ingredient	*Initial pH*	*2 N NaOH (mL)*[1]	*pH - 1 hr*	*2 N NaOH (mL)*[2]	*pH – 2 hr*
LR12	6.31 +/- 0.02	2.60 +/- 0.10	7.74 +/- 0.04	0.10 +/- 0.05	7.89 +/- 0.02
DR12	6.16 +/- 0.03	3.60 +/- 0.05	7.76 +/- 0.03	0.25 +/- 0.05	7.89 +/- 0.04
LR28	6.20 +/- 0.03	2.70 +/- 0.10	7.76 +/- 0.05	0.15 +/- 0.05	7.90 +/- 0.03
DR28	6.09 +/- 0.02	3.60 +/- 0.10	7.74 +/- 0.04	0.25 +/- 0.05	7.85 +/- 0.03
TSF	6.54 +/- 0.03	3.00 +/- 0.10	7.74 +/- 0.04	0.20 +/- 0.05	7.95 +/- 0.03

Values are means +/- the standard deviation of 3 replications. 1. The amount of 2 N NaOH necessary to adjust the pH of the dispersions to 8.00 +/- 0.03 after the initial 10-12 min stirring period. 2. The amount of 2 N NaOH necessary to readjust the pH of the dispersions to 8.00 +/- 0.03 after stirring for 1 hr after the initial pH adjustment. LR12, DR12, LR28, DR28 and TSF are light roast-12% fat peanut flour, dark roast-12% fat peanut flour, light roast-28% fat peanut flour, dark roast-28% fat peanut flour and toasted soy flour respectively.

TABLE 3.

HERSCHEL-BULKLEY PARAMETERS FOR 20% DISPERSIONS OF PEANUT AND SOY FLOURS.

Dispersion	***K*** ***(Pa·s^n)***	***n***	***σ_0*** ***(Pa)***
LR12	0.10 +/- .02	0.79 +/- .03	0.37 +/- .03
DR12	0.06 +/- .01	0.90 +/- .09	0.33 +/- .11
LR28	0.04 +/- .01	0.78 +/- .06	0.13 +/- .02
DR28	0.04 +/- .01	0.85 +/- .03	0.13 +/- .01
TSF	0.49 +/- .04	0.66 +/- .02	2.29 +/- .28

Values are means +/- the standard deviation of at least 3 replications. K, n and σ_0 are the flow behavior index, consistency index and yield stress respectively. LR12, DR12, LR28, DR28 and TSF are light roast-12% fat peanut flour, dark roast-12% fat peanut flour, light roast-28% fat peanut flour, dark roast-28% fat peanut flour and toasted soy flour respectively.

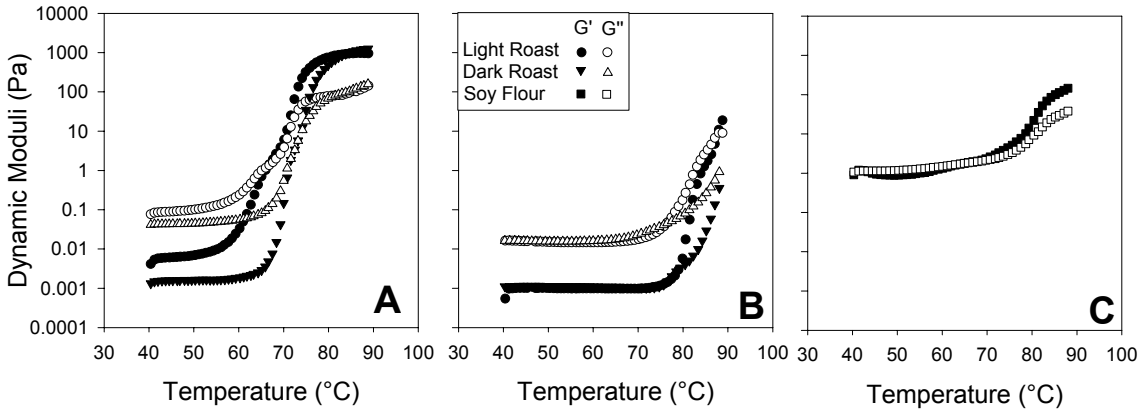


Figure 1. Small-strain, constant oscillatory testing; stress = 1.5 Pa, Frequency = 0.1 Hz. G' and G" are the storage modulus and loss modulus respectively. Heating ramp (1°C/min) of commercially available flour dispersions. Dispersions were 20% w/w total solids and adjusted to pH 8.00. A = 12% fat peanut flour, B = 28% fat peanut flour, C = toasted soy flour. Symbols appear on the graph.

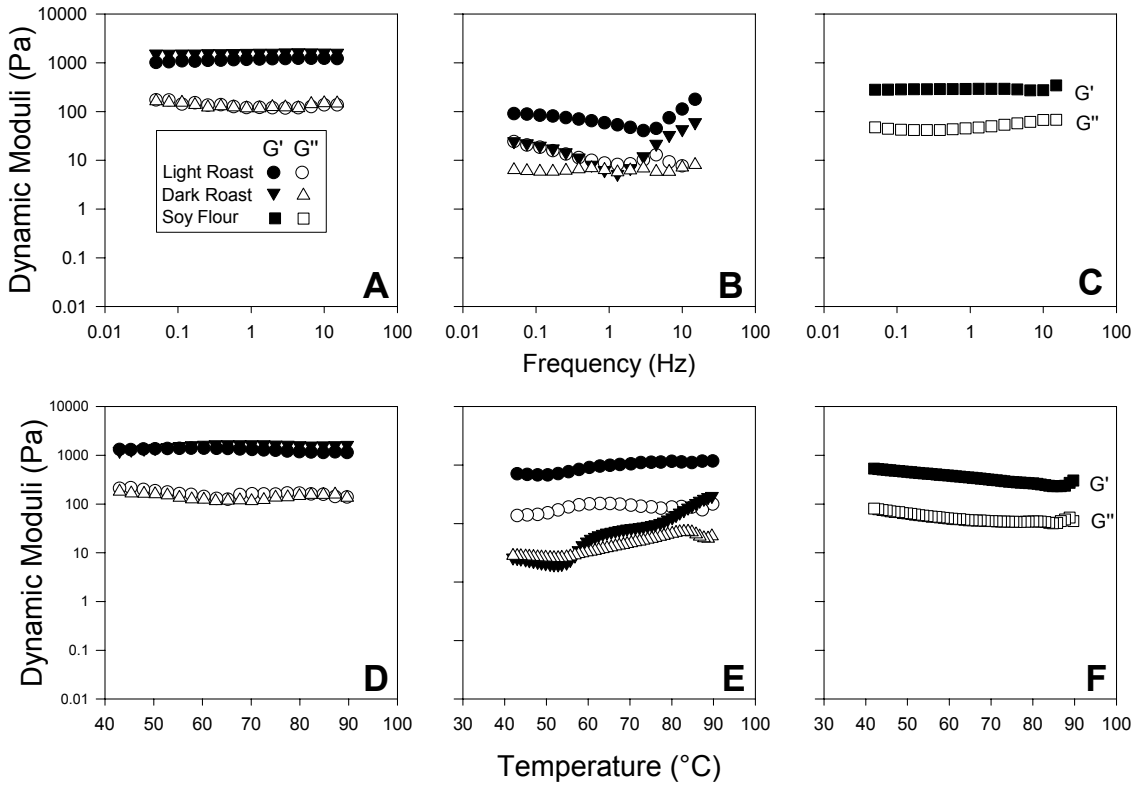


Figure 2. Small-strain, constant oscillatory testing of commercially available flour dispersions; stress = 1.5 Pa. G' and G" are the storage modulus and loss modulus respectively. Dispersions were 20% w/w total solids and adjusted to pH 8.00. A-C are frequency sweeps at a constant temperature of 90°C. D-F are cooling ramps (1°C/min) at a constant frequency of 0.1 Hz. A and D are 12% fat peanut flours, B and E are 28% fat peanut flours. C and F are toasted soy flour. Symbols appear on the graph.

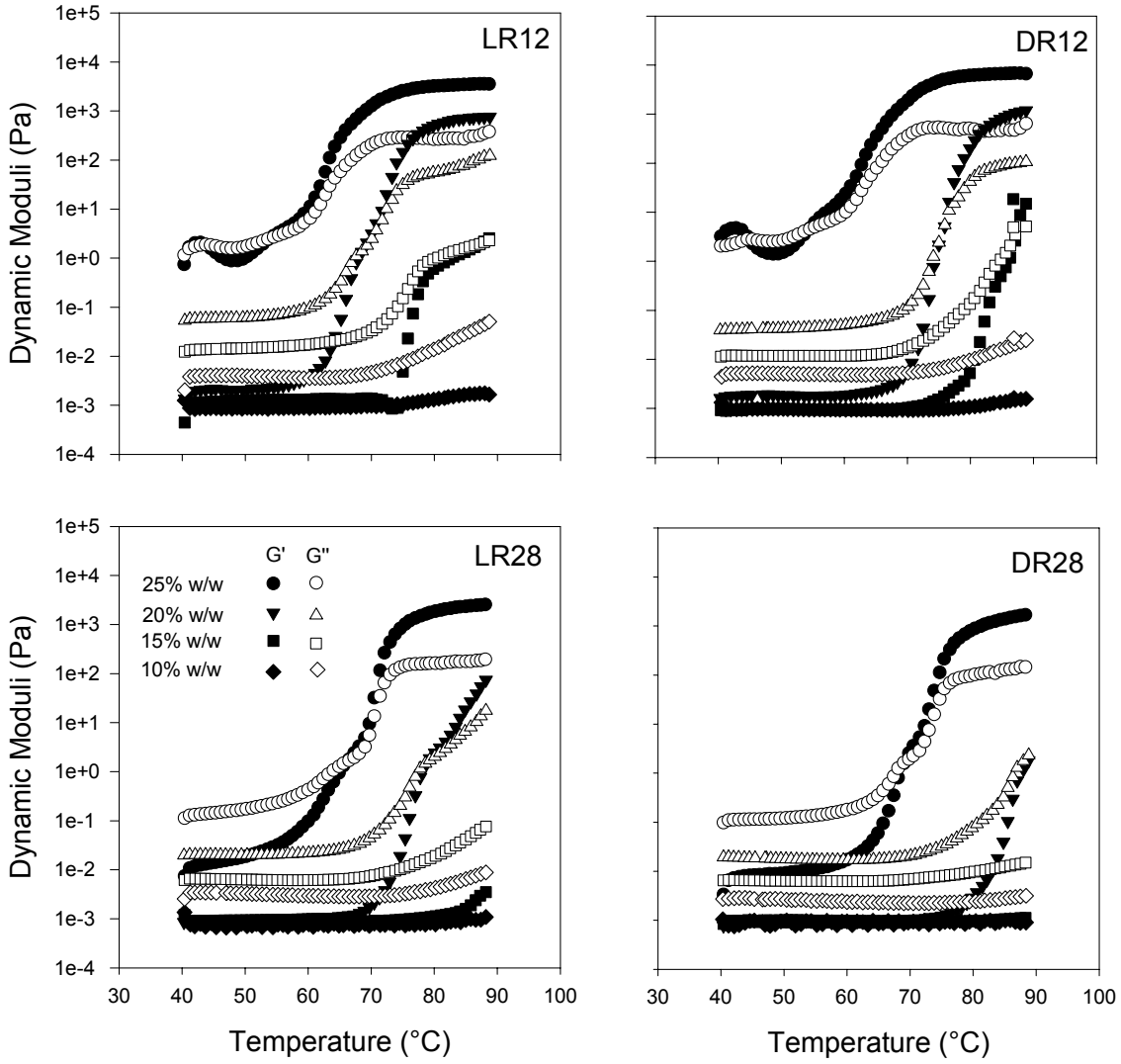


Figure 3. Small-strain, constant oscillatory testing of commercially available peanut flours as a function of dispersion concentration; stress = 1.5 Pa, Frequency = 0.1 Hz. Heating ramp (1°C/min) of various dispersions, all adjusted to pH 8.00. LR12, DR12, LR28 and DR28 are light roast-12% fat peanut flour, dark roast-12% fat peanut flour, light roast-28% fat peanut flour and dark roast-28% fat peanut flour. Symbols appear on the graph.

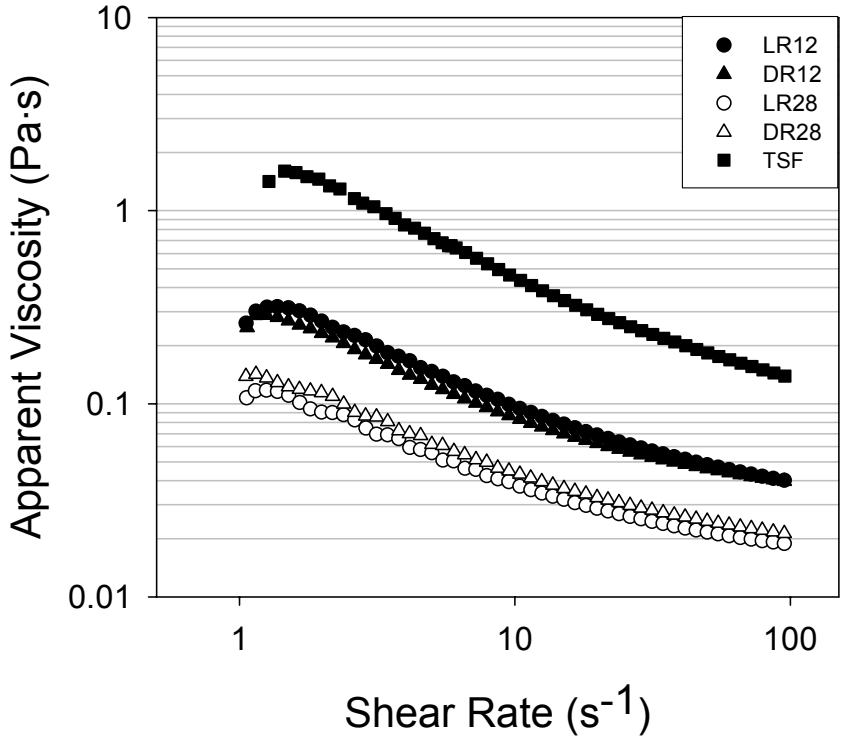


Figure 4. Rotational shear rate ramps at 40°C for 20% w/w flour dispersions adjusted to pH 8.00. Shear rate was increased as the test proceeded. LR12, DR12, LR28, DR28 and TSF are light roast-12% fat peanut flour, dark roast-12% fat peanut flour, light roast-28% fat peanut flour, dark roast-28% fat peanut flour and toasted soy flour respectively.

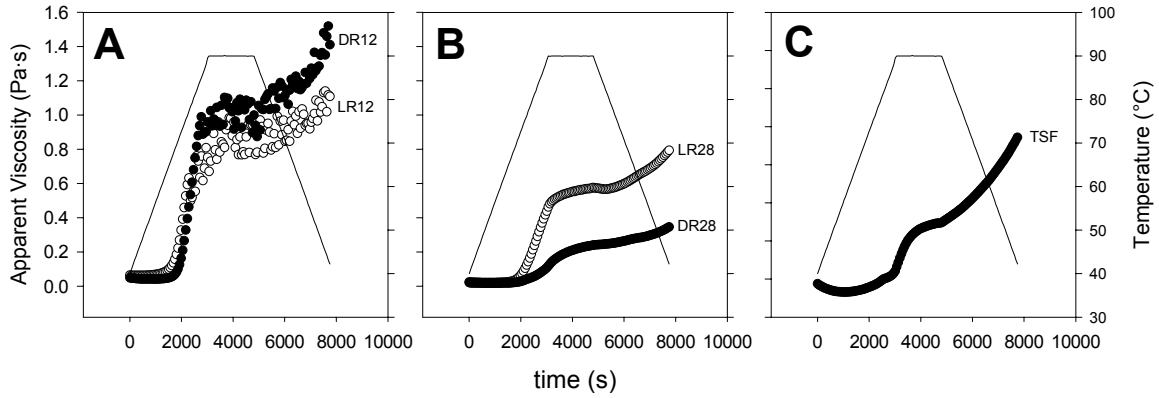


Figure 5. Rotational viscosity testing of 12% fat peanut flour (A), 28% fat peanut flour (B), and toasted soy flour (C). Shear rate was 50 1/s. Dispersions were 20% w/w total solids and adjusted to pH 8.00. Heating and cooling rates were 1°C/min, samples were held at 90°C for 30 minutes. Solid lines represent the temperature traces. LR12, DR12, LR28, DR28 and TSF are light roast-12% fat peanut flour, dark roast-12% fat peanut flour, light roast-28% fat peanut flour, dark roast-28% fat peanut flour and toasted soy flour respectively.

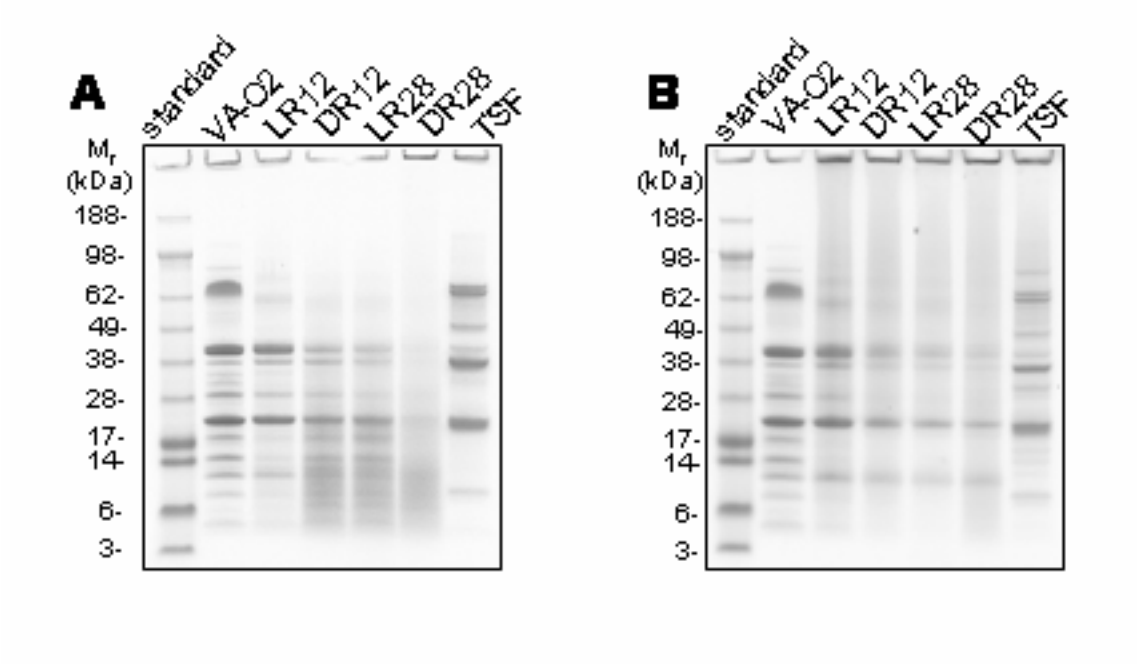


Figure 6. SDS PAGE of soluble (A) and insoluble (B) fractions of peanut and soy flour dispersions. Standard is the SeeBlue® Plus2 Pre-Stained Standard. M_r is the relative molecular weight. VA-02 is defatted, blanched Virginia style peanuts. LR12, DR12, LR28, DR28 and TSF are light roast-12% fat peanut flour, dark roast-12% fat peanut flour, light roast-28% fat peanut flour, dark roast-28% fat peanut flour and toasted soy flour respectively. Approximately 10 μg of protein were loaded per lane. See methods for details.

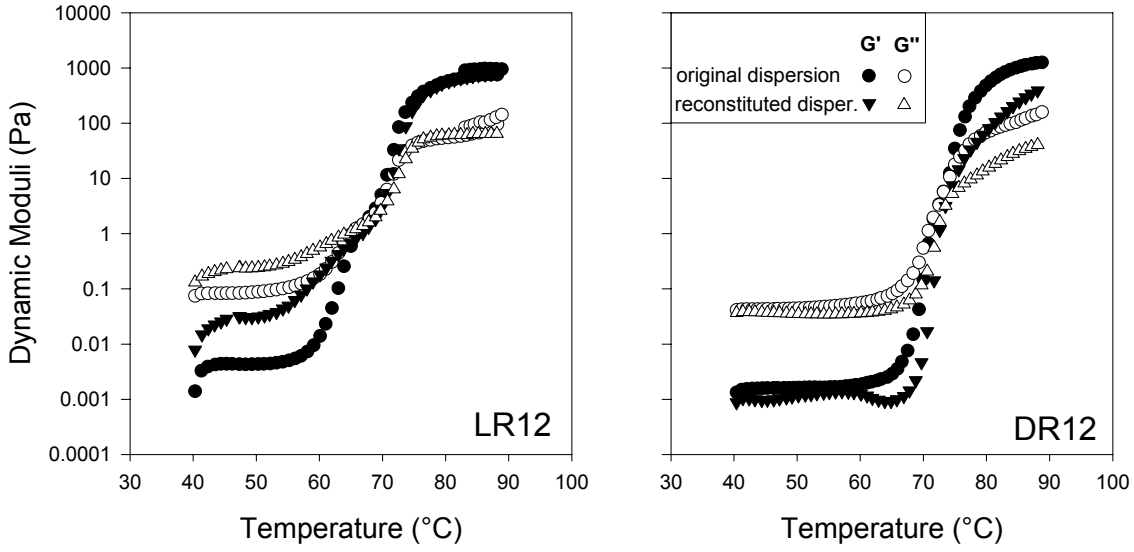


Figure 7. Small-strain, constant oscillatory testing; stress = 1.5 Pa, Frequency = 0.1 Hz. Heating ramp (1°C/min) of original and reconstituted dispersions of 12% fat peanut flours (see text for details). LR12 and DR12 are light roast-12% fat peanut flour and dark roast 12% fat peanut flour respectively.

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Chapter 3

Transglutaminase Polymerization of Peanut Proteins

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Transglutaminase Polymerization of Peanut Proteins

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Transglutaminase promotes protein cross-linking reactions through an acyl transferase mechanism involving protein-bound glutaminyl residues and primary amines including the ϵ-amino group of lysine residues in soy, myosin, gluten, oat globulin, casein, and whey. Herein, we present a first report of exogenous transglutaminase catalysis of several peanut protein fractions, including purified Ara h 1. In most cases, SDS-PAGE banding patterns revealed the formation of high molecular weight polymers while catalysis of Ara h 1 resulted in distinct dimer formation. Cross-linking effects were accomplished in the presence and absence of the reducing reagent, dithiothreitol. Ortho-phthaldialdehyde assays, used to quantify the degree of polymerization, indicated ~21% and ~30% coupling over a similar time interval, using either cold hexane extracted peanut protein fractions or lightly roasted flour dispersions, respectively. Rheological measurements established that transglutaminase-modified peanut extracts exhibited lowered viscosity readings compared to nontreated dispersions. Peanut protein polymers and glycoprotein conjugates, created by covalent linkage between protein substrates and monosaccharide amino sugars, exhibited similar IgE binding activity, compared to control solutions. These results suggested that potential allergic responses were not enhanced after enzymatic modification. Ultimately, these approaches may provide novel peanut-based food ingredients with unique functional characteristics for expanded applications within the world marketplace.

KEYWORDS: Transglutaminase; peanuts (*Arachis hypogaea Linn*); protein functionality; food allergies

INTRODUCTION

The production of peanuts on a worldwide scale is estimated to be approximately 20.4 million metric tons. In the United States alone, 1.8−2.0 million metric tons are currently utilized for food applications (*1*). Presently, this commodity is added to numerous edible foods including peanut-buttered snacks, ice cream, candies, beverages, and confections (*2*). However, on a global basis, peanuts are primarily grown to extract oil for cooking purposes, and during this process, residual protein-rich "pucks" are generated that may be used to manufacture partially defatted peanut meals for nutritive usages. Oftentimes, these materials can be prepared for human consumption in the form of commercial flours, peanut protein concentrates and isolates, flakes, or grits if suitable handling and food-processing conditions are maintained (*3*).

Peanut-based food ingredients can be modified using both chemical means and physical manipulations including acidification, heating, proteolysis, and enzymatic cross-linking, all in the presence and absence of different salts. Previous work had already demonstrated that transglutaminase [protein-glutamine: amine γ-glutamyl-transferase, EC 2.3.2.13] promoted cross-linking reactions of soy, myosin, gluten, oat globulin, casein, and whey proteins (*4*−*8*); therefore, to test the potential reactivity of the enzyme with peanut protein substrates, several experimental samples were prepared under a variety of conditions. These fractions included raw peanuts, cold and hot hexane extracted fractions, preheated protein solutions (60 °C), dried peanut flours, and purified Ara h 1. To our knowledge, this report represents a first documentation of peanut protein catalysis by an exogenous transglutaminase.

In this work, we have characterized protein−protein interactions that occurred on the basis of the nature of initial starting materials. In addition, experiments were designed to measure the effects of polymerization on peanut protein solubility, to illustrate the SDS-PAGE protein-banding patterns for all conjugates, and to quantify the degree of cross-linking. Rheological changes were assessed upon monitoring the changes in apparent viscosity after treatment of peanut protein dispersions with the enzyme. IgE binding parameters, a measure of immunogenic activity, were calculated for polymeric protein and glycoprotein conjugates, generated by covalent linkages between peanut protein substrates with monosaccharide amino sugars.

Allergenicity remains a major hurdle with respect to ensuring the safe ingestion of peanut-based food products for some consumers. With these studies, we have demonstrated that transglutaminase modification of dried flour materials did not lower IgE binding to peanut protein epitopes; however, it may be possible to create alternative enzyme-modified protein

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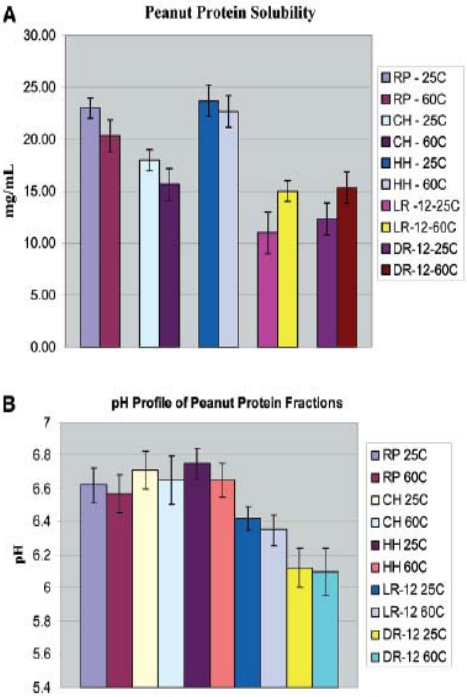


Figure 1. (A) Solubility study. Raw (RP), cold (CH) and hot (HH) hexane extracted peanut protein fractions, and two peanut flours, light roast, 12% fat (LR-12), and dark roast, 12% fat (DR-12), were prepared at 10% solids (w/v) in deionized water at 25 °C and 60 °C as described in Materials and Methods. (B) pH study. The pH of the solution was measured directly.

fractions that elicit diminished immunogenic responses using different starting materials or variant experimental reaction conditions. Since we established that transglutaminase reacted with peanut proteins under a wide range of solution parameters, ongoing research will be directed toward optimization of enzyme-treated protein fractions, exhibiting superior performance traits, for utilization as a new food ingredient.

With the relative abundance of underutilized peanut protein resources, there is a critical need for the development of innovative strategies that promote new usages within the food industry. Ultimately, we anticipate that these approaches will afford increased utility for peanut proteins by expanding its functional effectiveness within a given food product as previously demonstrated in other protein-based nutritional systems (*3*, *9*−*12*).

MATERIALS AND METHODS

Materials. *Peanut Protein Resources.* Raw peanuts (both runner and Virginia types) were obtained for these studies. Light and dark roasted, partially defatted (12% fat content), peanut flours were provided as a gift from Golden Peanut Company (Alpharetta, GA). Amidated pectin, GENU Type LM-20AS, was contributed by CP Kelco (Wilmington, DE). *N*-Carbobenzoxy (CBZ)-glutaminyl-glycine, catalog #6154, was obtained from Sigma-Aldrich (St. Louis, MO) as were all other reagent-grade chemicals.

Transglutaminase. Purified microbial transglutaminase was donated by Ajinomoto Food Ingredients LLC (Japan) and was stored at −20 °C prior to use. The enzyme was supplied as a solid powder, mixed with maltodextrin. The specific activity was measured as 800 units per gram/powder using *N*-carbobenzoxy (CBZ)-glutaminyl-glycine and hydroxylamine upon assay according to the method of Folk and Chung

Table 1. Summary of Peanut Protein Fractions Polymerized by Transglutaminase

peanut protein dispersions[a]	heat treatment	reducing solution conditions, 10 mM DTT	pH
I. raw peanuts: Runner and Virginia lines	25 °C 60 °C	(+) DTT	6.62
II. cold hexane extracted: Runner and Virginia, ~4% fat	25 °C 60 °C	(±) DTT	6.71
III. hot hexane extracted: Runner and Virginia, ~4% fat	25 °C 60 °C	(±) DTT	6.74
IV. peanut flours:			
light roast, 12% fat	25 °C 60 °C	(±) DTT	6.41 and 8.0
dark roast, 12% fat	25 °C 60 °C	(±) DTT	6.12 and 8.0
V. purified Ara h 1	25 °C	(±) DTT	7.0

[a] Peanut protein dispersions (10% solids, w/v) were prepared at 25 °C and 60 °C as detailed in Materials and Methods. Most samples were supplemented with 10 mM dithiothreitol (+). Reaction assay mixtures lacking 10 mM DTT are denoted by a minus sign (−). Ara h 1 was purified as described (*16*).

(*4*) with slight modification by Truong et al. (*13*). More specifically, the final reagent concentrations in the assay mixture were as follows: 100 mM hydroxylamine, 15 mM CBZ-glutaminyl-glycine, 5 mM calcium chloride, 10 mM dithiothreitol in 200 mM Tris acetate buffer, pH 6.0. Under these experimental conditions, one unit of enzyme activity was defined as an absorbance change of 0.29/min at 525 nm at pH 6.0, forming 1 μmol of hydroxamate/min, at 37 °C. The purity was >90% according to electrophoretic data (*14*).

Methods. *Hydration of Peanut Flour.* Peanut flour, containing 12% fat content, was dispersed in deionized water to either 10% or 20% (w/v). In some cases, the pH was adjusted to 8.0 with 2 N NaOH and stirred continuously for 2 h, at which time the pH was readjusted to 8.0 if required. All peanut protein suspensions were then stored overnight at 4 °C before analysis.

Cold Hexane Extraction (Defatting Method). Runner and Virginia type peanuts were ground using a standard homogenizer (Braun, Boston, MA), were placed into a large centrifuge tube at one-third filled capacity, and hexane (Fisher Scientific) was added to the top of the tube. The contents were then shaken for 30 min and centrifuged at 2600*g* for 15 min to separate soluble fractions from insoluble particulate matter. This process was repeated three times (3×). The defatted peanut meal was then air-dried under a chemical fume hood for 16 h (*15*).

Hot Hexane Extraction (Defatting Method). Runner and Virginia type peanuts were ground using a standard homogenizer (Braun, Boston, MA), and the homogenate was placed into a cellulose thimble, 25 mm × 80 mm (Whatman, Maidstone, England), filled to approximately three-fourths capacity (45.0 g). Soxhlet hexane extraction of lipid materials was then conducted at ~50 °C for 6 h. Peanut meals, with reduced fat content, were air-dried under a chemical fume hood for 16 h (*15*).

Purification of Ara h 1. Five grams of raw, defatted, peanut material was added to ~40 mL of 0.1 M phosphate buffer, pH 8.0, and the samples were incubated at 37 °C/3.5 h while shaking. Subsequently, Ara h 1 was purified according to the method of Basha and Cherry (*16*).

Solubility and pH Studies. Raw peanuts, cold and hot hexane extracted, and two peanut flours [light roast, 12% fat (LR-12), and dark roast, 12% fat (DR-12)] were dissolved in deionized water at 10% solids (w/v) at 25 °C and 60 °C. Heated samples were maintained at 60 °C for ~3 h and were held overnight at 4 °C prior to use. Afterward, all samples were centrifuged at 11950*g* for 30 min at 4 °C, and the protein concentration was measured according to bicinchoninic acid (BCA) methodologies developed by Pierce Inc. (Rockford, IL). The pH of all test solutions was measured directly using an Orion pH meter, model 405 (Fisher Scientific). Solubility and pH measurements were made in triplicte.

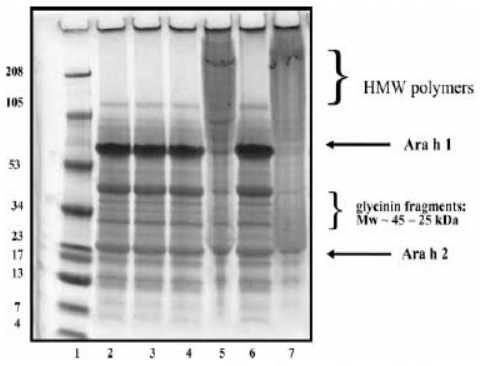


Figure 2. SDS-PAGE banding pattern of transglutaminase-treated cold hexane extracted peanut protein (CH–PP) fractions. CH–PP samples were incubated with transglutaminase (TG) at 37 °C for the times indicated. Lane 1: Marker (kDa); lane 2: CH–PP/0 h; lane 3: CH–PP + TG/0 h; lane 4: CH–PP/1 h; lane 5: CH–PP + TG/1 h; lane 6: CH–PP/7 h; lane 7: CH–PP + TG/7 h. HMW: high molecular weight.

Ortho-Phthaldialdehyde (OPA) Analysis. Experimental samples were appropriately diluted with deionized water and microcentrifuged for 5 min. Twenty microliters of the resultant supernatant was then added to 2.0 mL of the OPA reagent, and the sample was incubated for 2 min at 25 °C. Then the absorbance was read at A_{340} nm using a Gilford 2600 spectrophotometer. All final readings fell within the range of [0.1−1.0]. This procedure was adapted from the method of Church et al. (*17*). Test samples were analyzed in triplicate.

Enzymatic Polymerization of Peanut Protein Substrates. Peanut protein dispersions were typically prepared at 10% or 20% solids content (w/v), pH 8.0, as previously described. Microbial transglutaminase, added at a ratio of ~5 units/gram of dried peanut flour, was slowly mixed with the protein substrate in a rotating incubator at 37 °C. Aliquots were removed at timed intervals for SDS-PAGE and OPA analyses. In most cases, soluble supernatants, removed after centrifugation at 11950*g*, were tested. Also, BCA methods were used to measure residual protein content in transglutaminase-treated samples maintained at 37 °C for 24 h. Control solutions, devoid of enzyme, were evaluated as well.

SDS-PAGE Electrophoresis. All samples were initially assayed for protein content (BCA method) to ensure equivalent loading amounts into each lane. The fractions were appropriately diluted and mixed (1:1, v/v) with sample buffer [8% SDS, 0.9 M Tris buffer containing 5.0% β-mercaptoethanol (InVitrogen Inc., Carlsbad, CA)]. Each sample was then heated at 100 °C for 10 min, a process which also stops the enzymatic reaction, prior to loading onto 10−20% Tris-Tricine gradient polyacrylamide gels (InVitrogen, Inc). After electrophoresis, the samples were stained directly for visualization of proteins using a colloidal Coomassie Blue staining reagent (InVitrogen, Inc.).

Apparent Viscosity. A 20% (w/v) dispersion of LR-12 was prepared in deionized water at pH 8.0. Dispersions containing microbial transglutaminase, and nontreated control samples, were then incubated at 37 °C with agitation for 6 h prior to rheological analysis using a stress-controlled rheometer (StressTech, Reologica Instruments AB, Lund, Sweden). The apparent viscosity was monitored as the test fraction was heated from 40° to 80 °C at 1 °C/min at a constant shear rate of 50 1/s.

ELISA Methodology. LR-12 dried flours were prepared at 20% solids content (w/v) in deionized water, the pH was adjusted to 8.0, and the sample was centrifuged at 11950*g* for 30 min prior to testing. Supernatant fractions included (1) transglutaminase-treated LR-12 peanut protein samples, (2) glycoprotein conjugates formed by enzymatic catalysis between 20% LR-12 dispersions, pH 8, mixed with either 5% glucosamine or galactosamine (w/v) at 37 °C for 24 h, and (3) 20% LR-12 suspensions containing 1%-amidated pectin (w/v) that were heated for 3 h at 60 °C.

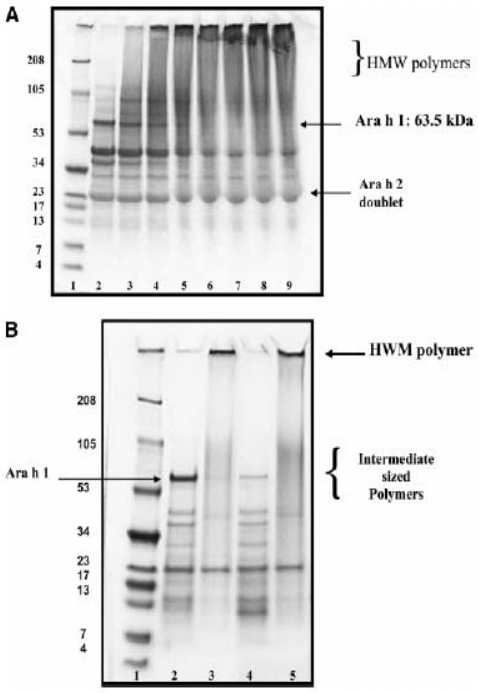


Figure 3. (**A**) SDS-PAGE banding pattern of transglutaminase-treated LR-12 peanut flour dispersions. LR-12 dispersions, prepared in deionized water containing 10 mM dithiothreitol, pH 8.0, were incubated with transglutaminase at 37 °C for the times indicated. Lane 1: Marker (kDa); lane 2: 0 h; lane 3: 0.5 h; lane 4: 1 h; lane 5: 2 h; lane 6: 4 h; lane 7: 6 h; lane 8: 9 h; lane 9: 24 h. (**B**) Effect of preheating on LR-12 peanut protein polymer formation. LR-12 flour dispersions were prepared in deionized water, pH 8.0, at 25 °C and 60 °C (maintained for 3 h) as described in Materials and Methods. Test fractions were treated with transglutaminase (TG) for 3 h at 37 °C. Lane 1: Marker (kDa); lane 2: LR-12, 25 °C; lane 3: LR-12, 25 °C (+) TG; lane 4: LR-12, 60 °C; lane 5: LR-12, 60 °C (+) TG.

Table 2. Degree of Protein Polymerization

peanut protein sample[a]	time point	absorbance 340 nm	standard deviation
CH 60 °C	0 h	0.443	±0.05
(+) TG	0.5 h	0.398	±0.02
(+) TG	1 h	0.374	±0.01
(+) TG	3 h	0.35	±0.01
(+) TG	6 h	0.343	±0.02
LR-12, 25 °C	0 h	0.84	±0.04
(+) TG	2 h	0.68	±0.05
(+) TG	4 h	0.58	±0.03

[a] Peanut protein dispersions, CH–PP (10% solids, w/v), and LR-12 (20% solids, w/v), pH 8.0, were prepared at 60 °C and 25 °C as detailed in Materials and Methods. Soluble supernatant fractions were used directly for enzymatic cross-linking at 37 °C. LR-12 dispersions contained 10 mM dithiothreitol. The degree of polymerization was determined by OPA analysis.

All test samples were diluted to an equivalent protein concentration on the basis of BCA protein data. Glucosamine/galactosamine glycoprotein conjugates were adjusted to comparable values on the basis of an A_{280} nm reading of ~0.02 since reducing sugar components interfered with BCA assay protocols. ELISA methodologies were then performed according to the instructions provided in the Veratox Peanut Allergen

Table 3. Loss of Soluble Protein After Treatment of LR-12 and DR-12 with Transglutaminase

peanut protein sample[a]	time point	protein mg/mL	standard deviation
LR-12, 37 °C	0 h	11.97	1.41
(+) TG	24 h	8.63	1.13
DR-12, 37 °C	0 h	11.46	1.40
(+) TG	24 h	5.83	0.85

[a] Peanut protein dispersions, LR-12 (10% solids, w/v) and DR-12 (10% solids, w/v), pH 8.0, were prepared at 25 °C. After treatment of each solution with transglutaminase for 24 h at 37 °C, soluble supernatant fractions were measured for residual protein concentrations using BCA methods (Materials and Methods).

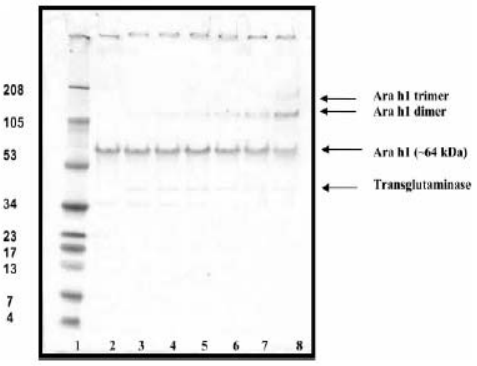


Figure 4. SDS-PAGE banding pattern of transglutaminase-treated Ara h 1. Ara h1 was incubated with transglutaminase (TG), and cross-linking reactions were performed at 37 °C in the presence of 10 mM dithiothreitol for the times indicated. Lane 1: Marker (kDa); lane 2: 0 h; lane 3: 0.5 h; lane 4: 1 h; lane 5: 2 h; lane 6: 3 h; lane 7: 5 h; lane 8: 20 h.

test kit, specifically designed for quantitative analysis of IgE binding activity (Neogen Corporation, Lansing, MI).

RESULTS AND DISCUSSION

Characterization of Peanut Protein Substrate Fractions. To address the potential reactivity of transglutaminase polymerization of various peanut protein fractions, we first assessed general solubility parameters in an aqueous medium using raw peanuts, cold and hot hexane extracted samples, and two peanut flour dispersions prepared at either 25 °C or 60 °C. We hypothesized that heated solutions may slightly denature the protein substrate, providing increased reactive sites for enzyme catalysis. Also, under these experimental conditions, additional glycoconjugates might have formed as a result of Maillard reactivity given that native carbohydrate was present in some of these test samples.

As shown in **Figure 1A**, raw and hexane-extracted peanut preparations contained approximately 1.5−2.0 times more soluble protein than the flours at equivalent total solids content (10%, w/v). Furthermore, native pH values varied slightly, especially between differentially roasted flour samples where longer heating treatments, used in preparing dark roasted flours, caused increased acidity (**Figure 1B**).

Enzymatic Catalysis of Minimally Processed Peanut Protein Substrates. Initially, only soluble peanut protein substrates were tested for transglutaminase catalysis under a variety of experimental conditions (**Table 1**). In fact, polymerization was observed in raw peanut fractions, cold and hot hexane extracted protein solutions, and peanut flour suspensions

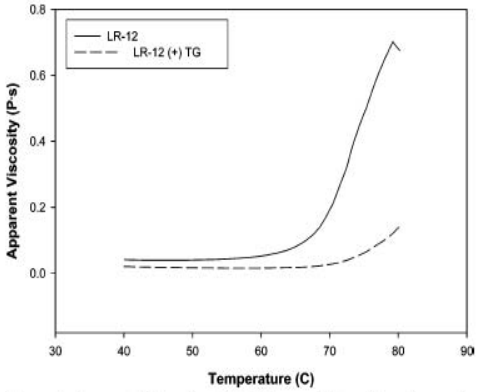


Figure 5. Apparent viscosity of LR-12 peanut flour dispersions after polymerization with transglutaminase. LR-12 protein dispersions, pH 8.0, were incubated with transglutaminase at 37 °C with agitation for 6 h prior to rheological analysis.

prepared over a pH range of 6−8. Furthermore, catalysis was accomplished in the presence and absence of dithiothreitol (DTT), a reducing denaturant. In one example, cold hexane extracted fractions, preheated at 60 °C for 3 h, were extensively cross-linked after a 60-min incubation period even in the absence of DTT (**Figure 2**). Concomitantly, high molecular weight polymers were observed at the top of the gel, near the interface.

Transglutaminase Reactivity with Roasted Peanut Flours. After determining that minimally processed peanut protein samples, such as raw peanuts and cold/hot hexane extracted materials were polymerized by the enzyme, solution parameters were further refined to study the effects of enzymatic catalysis on peanut flours, a readily available commercial source of protein starting material. Peanut flour extracts, per se, were devoid of endogenous transglutaminase activity using CBZ-glutaminyl-glycine and hydroxylamine substrates under the reaction conditions described. In first experiments, dithiothreitol was added to the lightly roasted flour dispersions, an effect previously reported to increase transglutaminase activity by maintaining the redox status of the cysteine residue residing at the active site (*14*).

Under these solution conditions, SDS-PAGE results of soluble LR-12 flour dispersions revealed considerable formation of high molecular weight polymers, demonstrated by amplified smearing at the top of the gel (**Figure 3A**). Furthermore, these reaction end products ranged in their size distribution in contrast to major reaction end products generated by transglutaminase catalysis of dark roasted dispersions (DR-12), containing dithiothreitol, that almost exclusively produced highly cross-linked protein polymers at the gel interface (data not shown). This latter result might be attributed to larger-sized protein substrates already present in DR-12 flour materials that were generated under higher thermal processing parameters (e.g., increased temperatures, longer heating times) as a result of the Maillard reaction.

As evidenced in **Figure 3B**, electrophoresis data illustrated that heating alone diminished the amount of Ara h 1 that was visible in the peanut flour test sample prepared at 60 °C (lane 4) versus the dispersion dissolved at room temperature, 25 °C (lane 2). Furthermore, in the absence of dithiothreitol, the rate of the transglutaminase-catalyzed reaction was slower, resulting in the formation and visualization of intermediately sized polymers using substrates that were thermally denatured (60 °C, 3 h) prior to exposure to the enzyme (lane 5). This

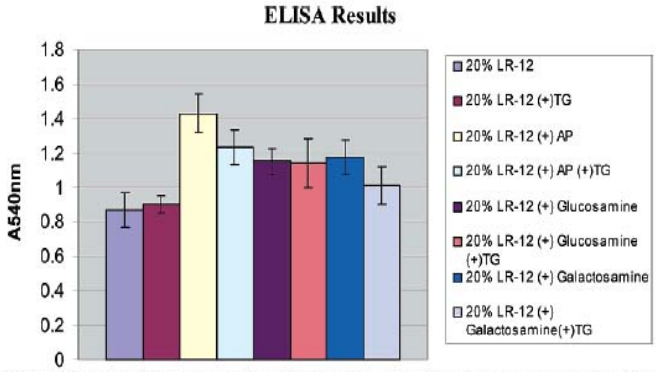


Figure 6. IgE binding activity of transglutaminase-treated peanut protein fractions. Transglutaminase treated peanut protein test suspensions included (1) LR-12, (2) glycoprotein conjugates, and (3) LR-12 suspensions containing 1% amidated pectin (w/v). All protein samples were diluted to an equivalent protein concentration. ELISA protocols were performed according to the instructions provided in the Veratox Peanut Allergen test kit.

phenomenon was also noted in enzyme-modified fractions prepared at 25 °C (lane 3), although to a lesser degree. These findings supported the observations of other investigators who recently reported that transglutaminase reactivity was enhanced upon utilization of preheated protein substrates (*18*). Prolonged incubation of the enzyme with all flour dispersions (e.g., 24 h at 37 °C) resulted in significant precipitation, a finding which suggested decreased solubility of extensively cross-linked protein materials. This hypothesis was confirmed by quantitative protein analyses of supernatant fractions obtained after removal of an insoluble pellet (**Table 3**). Taken together, these data demonstrated that the specific form of the polymeric end product differed considerably on the basis of the variability between preprocessed protein-starting materials.

Interestingly, a couple of these banding areas were not extensively cross-linked under these reaction conditions. For example, the subunits ranging from ~28 to 45 kDa, likely representing glycinin fragments (*19*), were not polymerized, at least to the same degree, as Ara h 1 at equivalent time intervals (**Figure 3A**). Furthermore, Ara h 2, another major peanut allergen, reportedly migrates as a doublet (*20*) [22 and 20 kDa] and appeared to remain essentially intact even after a 2−3 h treatment with the enzyme (**Figure 3A**). Since both of these peanut protein fractions contain glutaminyl and lysyl residues, as discerned from a search of the protein sequence data base provided by the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/), it may be that they are folded in such a way so as to prevent their ready accessibility to the enzymatic catalytic site. Alternatively, enzyme/substrate binding affinities or concentration effects may limit polymerization under these reaction conditions. In contrast, the disappearance of Ara h 1 subunits in these fractions was essentially complete after a 4-h incubation period (**Figure 3A**).

Transglutaminase Polymerization of Ara h 1. In earlier work, Ara h 1, a major protein found in peanut extracts, was reported to be among the most reactive antigens (*21*), and the results of these investigations demonstrated that transglutaminase catalysis of Ara h 1 resulted in distinct dimer formation (**Figure 4**). Concurrently, the density of the Ara h 1 subunit, itself, was diminished. Notably, there was only slight evidence of trimer formation (192 000 Da), while tetramer assembly (256 000 Da) and so forth was not observed during this time frame. Perhaps, in this case, the accessibility of polymeric substrates, such as Ara h 1 dimers, to the enzymatic catalytic site was more limited.

OPA Assay Results. The degree of covalent cross-linking was quantitatively analyzed using OPA assay methods, a test that measures the disappearance of free amino groups. As summarized in **Table 2**, a decrease of ~21% was noted in the enzyme-treated cold hexane extracted peanut protein suspension (10% solids, w/v) that was preheated to 60 °C for 3 h prior to exposure to the enzyme. Similarly, a LR-12 dispersion (20% solids, w/v), pH 8.0, was more highly cross-linked, ~30%, during a similar incubation period. In this latter instance, however, the experimental sample contained supplemental dithiothreitol, known to improve the catalytic parameters of transglutaminase (*14*).

Viscosity Measurements of Transglutaminase-Modified Peanut Proteins. Since transglutaminase is currently used in numerous functional food applications (*6*−*8*, *10*−*12*), experiments were designed to investigate the potential effect of enzymatic cross-linking reactions on the apparent viscosity of peanut protein solutions. Typically, the flow characteristics of a particular protein are influenced by the molecular shape, charge, size, and water-holding capacity; therefore, experimental parameters such as pH, temperature, ionic strength, concentration, and preprocessing treatments, including enzyme modification, can affect the "thickness" of any given solution (*22*).

LR-12 dispersions, pH 8.0, were incubated with transglutaminase for approximately 6 h at 37 °C, and the results indicated that the apparent viscosity of enzyme-treated samples was lower than that of the controls over a broad temperature range (~60−80 °C). For example, at ~74 °C, the magnitude of this difference was ~5 fold. Also, a significant increase in the flow characteristics of the untreated sample was observed at ~67 °C as compared to a less dramatic rise at ~72 °C, for enzyme-modified LR-12 dispersions, suggesting delayed network formation (**Figure 5**). Taken together, these data may reflect relevant differences in diffusion parameters between high molecular weight protein polymers as compared to non-cross-linked proteins that exhibit a wider range in their size distribution. Recently, Eissa and Khan reported that transglutaminase-treated whey protein isolate (WPI) dispersions also exhibited a lowered viscosity that was on the order of several magnitudes (*23*). Furthermore, they proposed that enzymatic cross-linking minimized hydrophobic interactions, causing formation of compact, structured, protein polymers as a result of inter- and intramolecular bonding. Alternatively, these rheological findings may simply reflect a lowered "soluble" protein content since long term incubation of these samples with transglutaminase eventually caused significant precipitation of the treated material. Typically, viscosity readings are correlated with the total amount of protein in any given solution.

Allergenicity. Peanut proteins remain one of the most important food allergens, and the heat-stable glycoproteins, Ara h 1 (63.5 kDa) and Ara h 2 (20, 22 kDa), are considered to be the major immunogenic epitopes (*21*, *24*, *25*). Potentially, cross-linking reactions may alter in vivo allergic responses, as noted in a previous study. Chung et al. examined the effects of peroxidase treatment on IgE binding to peanut protein epitopes and reported that immunogenic responses of roasted fractions, especially Ara h 1 and Ara h 2, were diminished upon treatment with this enzyme (*26*).

Herein, protein polymerization was accomplished using microbial transglutaminase, and the antigenic characteristics of several conjugates were evaluated. Test samples included (1) lightly roasted protein polymers created through inter- and intramolecular bond formation, (2) transglutaminase-generated protein−carbohydrate complexes, formed as a result of covalent linkages between peanut proteins with either glucosamine or galactosamine, and (3) peanut flour dispersions, mixed with amidated pectin, under Maillard reaction conditions (60 °C, 3 h, pH 8.0).

ELISA test results indicated that IgE binding was higher, to varying degrees, in most experimental fractions supplemented with carbohydrate, as compared to the control, lightly roasted flour dispersions (**Figure 6**). Furthermore, IgE readings were essentially equivalent in transglutaminase cross-linked protein samples as compared to nontreated fractions. Peanut flour dispersions, prepared with amidated pectin, exhibited the highest IgE response of all. Glycoproteins, generated by conjugation of peanut proteins with either glucosamine or galactosamine, exhibited elevated IgE binding capacities compared to peanut flour dispersions per se, although this data was not significantly different from control samples containing equivalent amounts of the monosaccharide amino sugar.

In other systems, however, transglutaminase modification was shown to have a diminished effect on immunogenic responses. Previously, Watanabe et al. concluded that treatment of either soft or hard wheat flour with the enzyme decreased allergenicity using both salt-soluble and salt-insoluble fractions, an observation attributed to a masking of IgE antibody binding sites as a result of cross-linking (*27*). Also, Babiker and co-workers examined the effect of enzymatic processing on soy protein immunogenic responses and observed that acid-precipitated soy proteins were less immunogenic after polymerization with transglutaminase than control samples (*28*, *29*); however, both were considerably less effective at lowering IgE responses than soy−galactomannan conjugates prepared via the Maillard reaction.

To our knowledge, this study provides the first report of peanut protein catalysis by an exogenous transglutaminase. Moreover, enzymatic cross-linking of these substrates was achieved in the presence and absence of DTT although the rate of the reaction varied. Since initial rheological data suggested that enzymatic modification impacted functional characteristics, such as the final viscosity of a given solution, future studies will be directed toward developing peanut protein-based food ingredients that deliver superior performance characteristics.

Allergenicity remains one of the key limitations with regard to the utilization of peanut materials in the food industry, and these results established that transglutaminase catalysis of peanut flour protein solutions did not diminish IgE binding responses. It remains to be seen, however, whether or not these approaches might be used to decrease/eliminate potential allergic responses by creating alternative peanut protein derivatives. In this event, the expanded use of peanut protein resources in the world marketplace can be safely achieved.

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Chapter 4

The effect of transglutaminase crosslinking on the rheological characteristics of peanut flour dispersions

The content of this paper has been submitted for publication:

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ABSTRACT

Peanut flour (PF) is a high protein ingredient prepared after partial extraction of oil from roasted peanut seed. Microbial transglutaminase (TGase) catalyzes protein crosslinking via acyl-transfer reactions, resulting in modification of functional properties such as viscosity, gelation, solubility, and water holding capacity. These experiments were conducted with either TGase treated or untreated 20% w/w PF dispersions containing 0.5% or 1.0% w/w amidated pectin (AP). Dispersions were heated from 40 to 90ºC at 1°C/min and subsequently cooled to 40ºC at a rate of 1ºC/min while continuously monitoring rheological changes under both small and large strain deformation conditions. Gelation occurred at higher temperatures (~78°C) using PF dispersions treated with TGase compared to dispersions devoid of enzyme (~68°C). The addition of 0.5% AP to PF dispersions minimized shifts in gel point temperature and increased the apparent viscosity of all samples, especially those treated with TGase. High molecular weight polymers were formed in TGase treated PF dispersions both in the presence and absence of AP; however, polymer formation was more rapid in PF dispersions without AP. Ortho-phthaldialdehyde assays, used to quantitatively determine the degree of polymerization, indicated ~40% protein coupling in PF dispersions treated with TGase compared to ~20% in those containing both AP and TGase. Collectively, these data suggest potential applications of TGase treated PF dispersions, both in the presence and absence of AP, for use in peanut-base food products, including protein bars, shakes, and value added baked goods.

Keywords: Peanut Proteins, Peanut Flour, Transglutaminase, Amidated Pectin, and Rheology.

INTRODUCTION

Peanuts (*Arachis hypogaea L.*) are a staple food commodity around the world. In the last few decades, peanut flour has increasingly been used as a food ingredient. Peanut flour is a dry powder manufactured after the partial extraction of peanut oil from roasted peanut seed. Peanut flour is typically composed of approximately 40-50% protein, 10-30% fat, and 20-30% carbohydrate/fiber (Suknark and others 1997) compared to roasted peanuts which are approximately 25-30% protein, 50% fat, and 20% carbohydrate/fiber (Basha and others 1976; Hoffpauir 1953).

Heat induced rheological modifications of protein based formulations are important to the texture and stability of numerous food products, and previous research established that solutions of peanut protein isolate will form gels under the appropriate conditions. Arachin, the primary protein fraction of peanut seed forms a heat-reversible gel at pH <3.8 and at concentrations above 7.5% w/v (Kumar and others 1980). Kella and Poola (1985) reported that arachin (15% w/v) forms a gel when heated at 90°C for 15 minutes and then cooled to 5°C for 24 hr.

Recently, rheological analyses were conducted for a range of aqueous dispersions prepared from commercially available peanut flours (Davis and others 2007). Flours varied according to the degree of roast, either light or dark, and by the residual fat content present in the flours, either 12% or 28%. Small and large strain rheological measurements were used to characterize aqueous flour dispersions at pH 8.0. Low fat flours were more viscous than high fat flours on an equal weight basis. Gelation was also

observed in these dispersions as determined by small strain oscillatory analyses while heating at 1°C/min. The 28% fat and/or dark roast flours formed gels less effectively than 12% fat and/or light roast flours, with residual fat content most affecting gelation properties. The optimum PF gelation concentration was determined to be 20% w/w total solids under the specified conditions.

Clare and others (2007) recently reported that light roast peanut flour-12% fat (PF) dispersions were polymerized by TGase as determined by qualitative (SDS-PAGE) and quantitative (OPA assays) analyses. Furthermore, these studies confirmed that PF dispersions did not contain endogenous TGase activity. Microbial transglutaminase (TGase) catalyzes protein crosslinking through acyl-transfer reactions between glutaminyl residues (donor) and primary amines (acceptor) (Folk and Chung 1985).

TGase is used in the food industry to impart important functional characteristics such as particle size, stability, texture, formation of heat and water resistant films, water holding capacity, gelation, viscosity, and solubility to finished products (Truong and others 2004; Zhu and others 1995). Currently, TGase is used to increase dough elasticity in baked goods. It is also used to improve the elasticity and texture of meat proteins, fish paste texture, and the texture of wheat proteins (Zhu and others 1995). Common substrates for TGase include whey, casein, and soy protein (Tang and others 2006; Truong and others 2004; Schorsch and others 2000).

The addition of polysaccharides to manufactured food products containing high protein content affects the functionality of the ingredient (Barrera and others 2002). Polysaccharides reportedly reduce *in vivo* and *in vitro* protein digestibility by various proteases and numerous mechanisms have been put forth to explain this reduction in protease activity, including direct protease-carbohydrate or carbohydrate-substrate interactions which could inhibit protein-protease interactions (Mouecoucou and others 2004). Viscosity increases resulting from carbohydrate addition are also hypothesized to reduce protease activity due to diffusion considerations (Larsen and others 1994).

Amidated pectin, which is a type of low methoxy pectin, is used in the food industry to form gels, increase viscosity, and perform as a stabilizer (Barrera and others 2002). Previous data suggested that low methoxy pectin formed ionic conjugates with amino acids and/or peptides derived from peanut proteins (Mouecoucou and others 2004). In this event, protein-polysaccharide interactions result from electrostatic attractions between the negatively charged polysaccharides and positively charged areas of the protein (Xia and Dubin 1994; Imeson and others 1977).

Therefore, the objective of this research was to evaluate the effect of exogenous TGase on the rheological characteristics of peanut flour dispersions prepared with and without amidated pectin.

MATERIALS AND METHODS

Materials. Light roast peanut flour-12% fat (PF) was provided by Golden Peanut Company (Alpharetta, Ga). Amidated pectin (AP), GENU® Type LM-20AS, was contributed by CP Kelco (Wilmington, DE). Purified microbial transglutaminase was donated by Ajinomoto Food Ingredients LLC (Japan) and stored at -20°C prior to use. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were reagent grade.

Methods. *Hydration of peanut flours and amidated pectin.* PF (20% w/w total solids) was mixed in deionized water to form a dispersion. The pH of the PF dispersion was adjusted initially to 8.0 with 2N NaOH then stirred for 1 hr and readjusted to pH 8.0. AP was added to a final concentration of 0.5 and 1.0% w/w before pH adjustment.

TGase Activity. TGase was supplied as a powder mixed with maltodextrin. TGase was solubilized in 1.0 M Tris-acetate buffer, pH 6.4. Soluble activity of TGase was assayed according to the method of Folk and Chung (1985) with minor modification by Truong and others (2004). The purity of TGase was > 90% according to electrophoretic data presented by Wilcox and Swaisgood (2002). N-carbobenzoxy (CBZ)-glutaminyl-glycine and hydroxylamine were used to measure enzymatic activity at 37ºC. Reagent concentrations in this assay mixture included: 100mM hydroxylamine, 15 mM CBZ-glutaminyl-glycine, 5 mM calcium chloride, 10 mM dithiothreitol in 200 mM Tris acetate buffer, pH 6.0. Under the above conditions, one unit of enzyme activity was defined as an

absorbance change of 0.29/min at 525 nm at pH 6.0, forming 1 μmol of hydroxamate/min and specific activity of TGase was determined to be 600 units per gram of powder.

Rheological Analysis. PF dispersions +/- AP (0.5% w/w for rheological testing) or +/- TGase were incubated at 37°C while shaking. Control dispersions (no TGase) were also incubated at 37°C for equivalent times. Rheological measurements were begun immediately after TGase incubation. Using a stress-controlled rheometer (StressTech, Rheologica Instruments AB, Lund, Sweden) equipped with a CC25 serrated cylinder, apparent viscosities of peanut flour dispersions were determined while heating the samples from 40°C to approximately 90ºC at a rate of 1ºC/min with a rotational shear rate of 50 1/s. Small strain, constant oscillatory testing was performed at a stress of 1.5 Pa and a frequency of 0.1 Hz during heating of the samples as described. The stress, 1.5 Pa, was identified to be within the linear viscoelastic region (LVER) based on stress sweeps at 10 Hz for samples at 40°C, meaning this stress was within the LVER for all other tests. Rheological tests were conducted in duplicate.

Solubility Measurements. PF (20% solids w/w) and AP (1.0 % w/w) were mixed in deionized water and incubated at 60°C for 3 hr, then stored overnight at 4°C. Dispersions were then re-heated to 37°C, and in identified samples TGase was added. All samples were centrifuged at 11,950 x g for 30 min at 4°C prior to measuring the protein concentrations according to bicinchoninic acid (BCA) methodologies developed by Pierce Inc. (Rockford, IL). All samples were measured in duplicate.

Enzymatic Polymerization of Peanut Flour Dispersions. TGase was added to PF dispersions (20% w/w, pH 8.0) at a ratio of ~5 units/g PF while AP was added at 1.0%. The dispersions were slowly mixed in an incubator at 37°C and the soluble supernatants were removed after centrifugation at 13,600 x g and evaluated for: *(i)* the degree of cross-linking by means of OPA measurements, and (*ii*) protein banding patterns after SDS-PAGE electrophoresis. Aliquots were removed at timed intervals for electrophoresis and OPA analyses. Control dispersions, without TGase and AP, were evaluated as well.

O-Phthaldialdehye Analysis (OPA). All samples were diluted with deionized water and microcentrifuged at 13,600 x g for 5 minutes. Twenty microliters of the resultant supernatants were collected, added to 2.0 ml of the OPA reagent, and incubated for 2 min at 25°C. Measurements were then taken at A_{340} nm using a Gilford 2600 spectrophotometer and all readings fell within an absorbance range from 0.1 – 1.0 as described by Church and others (1983). Samples were analyzed in triplicate.

SDS-PAGE. PF supernatants were assayed for protein content to ensure that equivalent amounts were loaded into each gel lane. Samples were mixed with 8% sodium dodecyl sulfate (SDS), 0.9M Tris sample buffer containing 5.0% β-mercaptoethanol and heated at 100°C for 10 min prior to loading onto 10-20% Tris-Tricine gradient polyacrylamide gels. After electrophoresis was completed, the samples were stained for visualization of protein content using a colloidal Coomassie Blue staining reagent (Invitrogen Inc., Carlsbad, CA).

Water Holding Capacity. The water holding capacity of PF dispersions, with and without TGase and/or AP (0.5% w/w), were determined according to the centrifugation method of Fleming and others (1974) with minor modification by Resch and others (2005). Control and enzyme-treated dispersions were incubated for 6 hr and 24 hr at 37°C, then centrifuged at 635 x g/15 min. Supernatants were carefully removed and the water held per gram of solid was determined by calculated weight differences. Test samples were analyzed in triplicate.

Statistical Analysis. Statistical analyses of water holding capacity were analyzed by analysis of variance using SAS statistical software (version 8.0; SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

In prior studies, we observed that protein solubility of PF dispersions generally increased when dispersions were heated to 60°C for ~3 hr (Clare and others 2007). Notable precipitation was observed in dispersions treated with TGase for 24 hr, suggesting decreased solubility of extensively crosslinked proteinaneous materials. The addition of 1% AP decreased precipitation as seen by the amount of soluble protein remaining after centrifugation (Figure 1) compared to TGase treated samples in which AP was not added. Increased solubility could result from the increased viscosity of the continuous phase and/or electrostatic interactions between the PF protein and the soluble carbohydrate. Moreover, the degree of catalysis may be lower in the PF-AP dispersion due to altered viscosity.

SDS-PAGE data confirmed the formation of high molecular weight (MW) polymers using PF dispersions that were treated with TGase for 3, 6, and 24 hr at 37°C (Figure 2A). Crosslinking was indicated by increased smearing, the disappearance of Ara h 1, ~ 64 kDa (Wichers and others 2004), the disappearance of other minor bands, and the accumulation of high MW protein polymers unable to enter the gel (Figure 2A, lanes 8-10). This pattern was not observed in control, non-treated, PF dispersions (lanes 2-5, Figure 2A). TGase activity was also observed in the presence of AP (1%) (Figure 2B); however, the rate of polymerization was reduced. Smearing as a result of TGase crosslinking was less prevalent in PF + AP dispersions after a 6 hr treatment (Figure 1B, lanes 7-9 compared to lanes 2-4); however, smearing at the gel interface was more prevalent after 24 hr revealing substantial amount of high MW polymers (Figure 2B-lane10). Also, Ara h 1 was not visible after an extended treatment with TGase (Figure 2B-lane 10). Of particular interest was the observation that Ara h 2, ~20 kDa (Koppelman and others 2001) did not appear to be crosslinked under these experimental conditions.

Quantitative measurements were accomplished using OPA assays, a testing method based on the loss of free amino groups. These results revealed approximately 40% crosslinking in TGase treated PF dispersions that lacked AP (1%) compared to 20% for those containing the carbohydrate after a 6 hr incubation period at 37°C (Figure 3).

This slower rate of TGase crosslinking in PF containing AP may be attributed to electrostatic interactions between PF protein constituents and AP, causing limited

accessibility of the non-covalently linked [PF protein-AP] conjugate to the catalytic site on the enzyme. Protein-polysaccharide complexes can form as a result of electrostatic interactions and/or hydrogen bonding between the negatively charged carboxylic groups present in the polysaccharide and positive amino acid residues, such as lysine and arginine. Hence, such associations could diminish the rate of crosslinking; however, earlier work indicated that pectin addition enhanced TGase crosslinking of soy proteins (Mariniello and others 2003). In this latter case, electrostatic interactions between added pectin with the soy protein were hypothesized to promote a preferred conformation of the soy protein substrate, resulting in increased TGase polymerization. The differing TGase response between these two studies could be attributed to the different type of pectins that were used. In the current work, AP was added to PF dispersions while Apple Pectin USP was utilized in the work of Mariniello and others (2003). Furthermore, different protein substrates, soy vs. peanut, were reactants.

Initially, we tested PF containing 1% w/w AP dispersions for general solubility studies; however, we discovered that these dispersions were too viscous for rheological measurements. Therefore, we used 0.5% w/w AP for these types of experiments. Small strain rheological data was obtained by continuously monitoring viscoelastic changes in these dispersions under controlled heating conditions. Previously, gelation was observed for equivalent PF dispersions under similar testing conditions, and the gel point being empirically defined as the point at which the storage modulus (G') crosses over the loss modulus (G") (Davis and others 2007). Gelation occurred at higher temperatures (~78°C; Figure 4B) using TGase treated dispersions compared to the controls (~68°C; Figure 4A).

Similar data trends were previously noted by Truong and others (2004), who studied the gelation characteristics of WPI solutions. These findings might be explained by the extensive crosslinking of the resultant end products such that adequate unfolding and network development of the PF matrix was compromised. Accordingly, previous work with both WPI and β-lactoglobulin, the primary protein in whey, confirmed that extensive crosslinking via TGase limited exposure of hydrophobic portions within the whey proteins during heat denaturation (Eissa and Khan 2006; Eissa and others 2006). Ultimately, this process resulted in decreased gelation properties with WPI and β-lactoglobulin due to limited hydrophobic interactions within the gel network. We hypothesize that a similar phenomenon seems to be occurring within these PF gels (Figure 4).

Dynamic oscillatory frequency sweeps at 90°C were used to further characterize the small strain rheological characteristics of PF +/- TGase (Figures 4C & 4D). G' was greater than G" by roughly one order of magnitude across the frequency range tested in untreated PF dispersions (Figure 4C). These responses were essentially frequency independent, indicative of an established gel network (Steffe 1996). G' and G" were greater in control PF dispersions compared to the enzyme treated dispersions across this frequency range, indicative of a more rigid structure. However, PF dispersions treated with TGase displayed some frequency dependence at higher frequencies, also indicative of a weaker structure (Figure 4D).

Small strain rheological data was collected over the heating range 40°-90°C for PF dispersions +/- AP +/- TGase as summarized in Figure 5. The addition of AP (Figure 5B) to the PF dispersion (Figure 5A) resulted in an increase in viscoelasticity that was most notable at temperatures < ~60°C. These data were in agreement with previous researchers who reported that pectin, protein, and cellulose interactions affect rheological properties and that the addition of soluble, polymeric molecules, such as pectin, increased solution viscosity (Hoskins and others 1996; Brown and Stein 1977). A more gradual transition from a viscous fluid to primarily elastic gel was also observed in the PF + AP dispersion (Figure 5B) compared to the PF dispersion, alone (Figure 5A). As discussed previously, TGase addition to the PF dispersions shifted the gel point to higher temperatures (Figures 5A and 5C). However, a similar shift in gel point was observed in PF + AP dispersions upon TGase addition (Figures 5B and 5D), although this shift was less obvious (Figures 5B and 5D) compared to enzyme treated dispersions lacking AP (Figures 5A and 5C). This shift in gelation temperature may be explained by the slower rate of polymerization that occurred with AP.

Under large strain deformations (continuous shear at 50 s^{-1}), PF + AP dispersions were more viscous than PF dispersions, especially under low heating (<70°C) conditions (Figure 6). Samples treated with TGase for 6 hr prior to rheological analysis were less viscous, both in the presence and absence of AP (Figure 6B). This data is in agreement with recently published research (Clare and others 2007) and small strain oscillatory analyses (Figures 4-5). At higher temperatures (~77-90°C), the apparent viscosity of dispersions without TGase was not stable at a shear rate of 50 sec^{-1}. We hypothesize this

instability might be due to the break-down and re-formation of the protein network in the presence of the shear field (Figure 6). The decreased rate of TGase crosslinking observed in PF + AP dispersions (Figure 1B) as compared to PF dispersions (Figure 1A) may also be attributed to the increased viscosity of the PF-AP dispersion, as increased viscosity slows the diffusibility of both the substrates and enzyme (Larsen and others 1994).

The water holding capacity (WHC) of PF dispersions +/- AP +/- TGase (24 hr incubation) is summarized in Table 1. In other studies, a positive correlation was seen between the WHC of proteins and increased viscosity (Ragab and others 2004; Khalid and others 2003; Idris and others 2003). Accordingly, the increased viscosity that was observed in PF dispersions containing AP, both in the presence and absence of TGase, (Figure 5 and 6), correlates with an increased WHC of PF dispersions containing AP (Table 1). Previous researchers have also noted that the addition of TGase has been shown to improve the WHC in a variety of prepared foods (Kuraishi and others 2001; Zhu and others 1995). Likewise, the addition of TGase to these PF dispersions, both in the presence and absence of AP, significantly ($p<0.001$) increased WHC (Table 1); however, no significant differences were noted among PF +/- AP dispersions after incubation for 6 hr with TGase (Data not shown). Therefore, these approaches may be used in future applications to manipulate the WHC in peanut-based food ingredients.

CONCLUSION

Enzymatic polymerization of PF dispersions was achieved in the presence and absence of AP, however, polymer formation was more rapid in PF dispersions without

AP. In these experiments, the rheological data suggested that enzymatic modification altered functional characteristics such as apparent viscosity, gelation, and WHC of TGase treated PF dispersions with and without AP.

These data suggest potential applications of polymerized PF dispersions in peanut-based food products, including protein bars, shakes, and value added baked goods.

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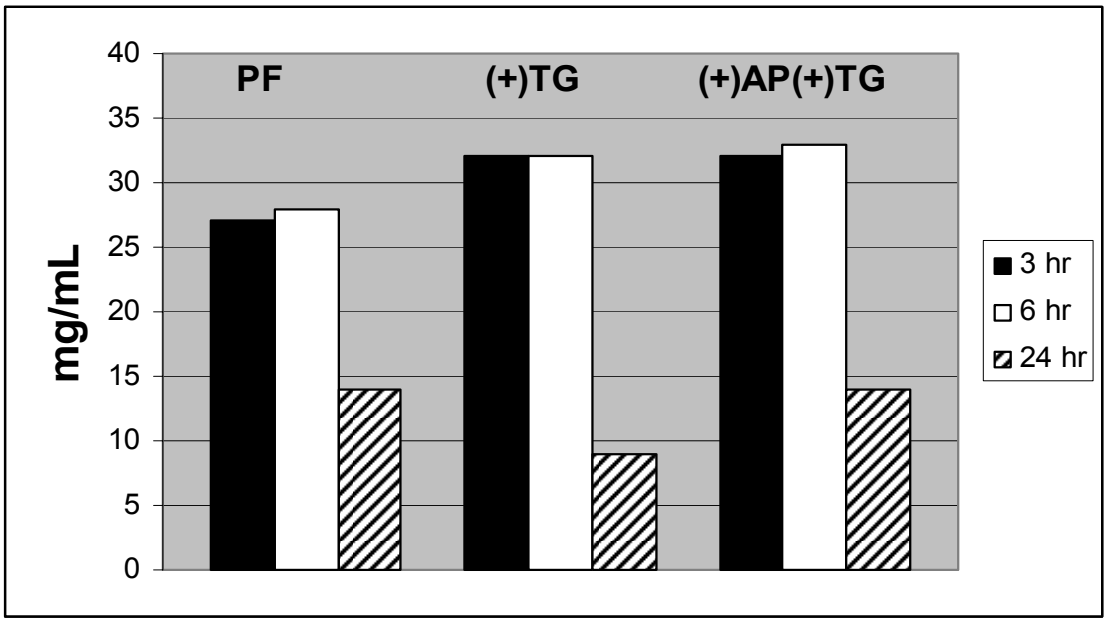
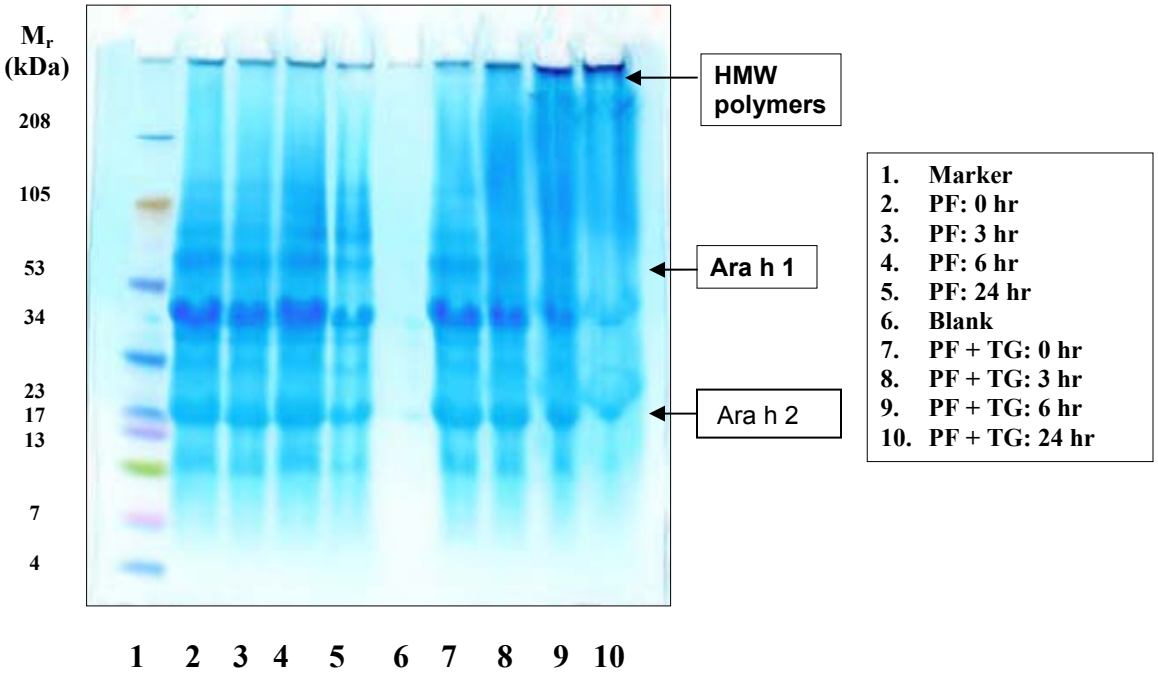


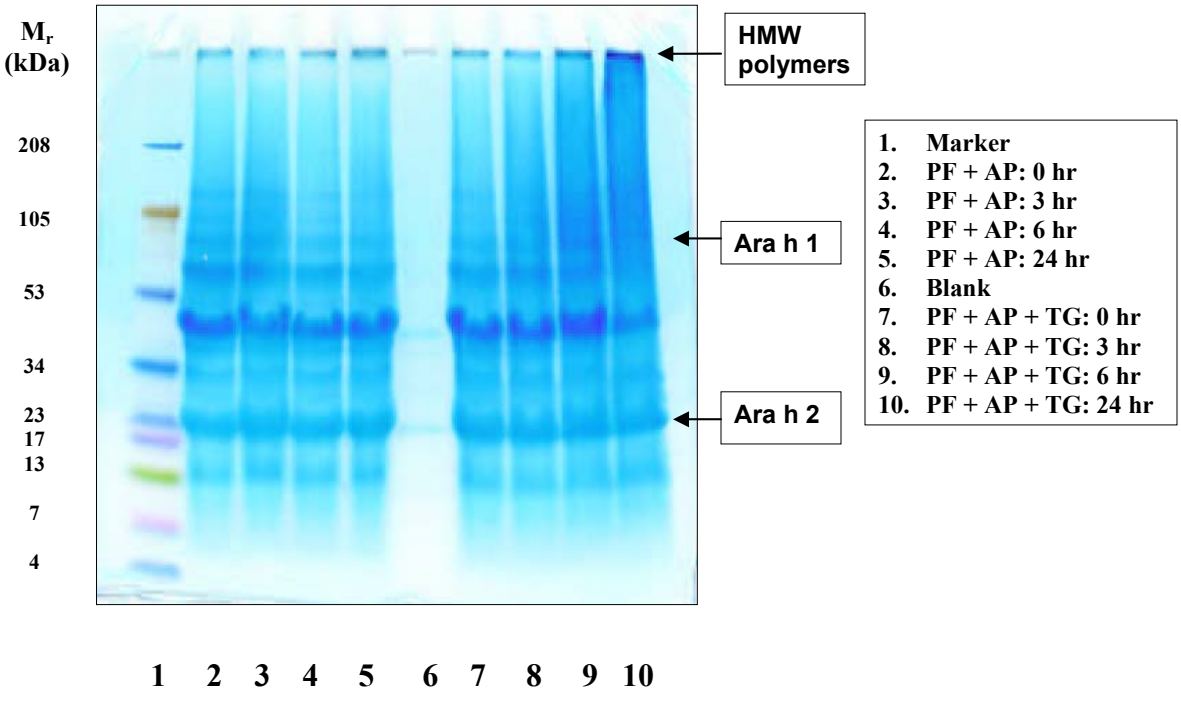
Figure 1. Solubility of 20% w/w PF dispersions +/- 1.0% w/w AP, prepared at 60°C/3 hr and stored overnight at 4°C. Dispersions were re-incubated at 37°C and in specified samples treated with TGase, pH 8.0. Values are averages done in duplicate. Bovine serum albumin was used as the standard.

Figure 2. TGase Polymerization of 20% w/w PF (A) and 20% w/w PF + 1.0% w/w AP (B), pH 8.0. Approximately 30 μg of protein were loaded per lane.

A


Mr (kDa)
208
105
53
34
23
17
13
7
4
HMW polymers
Ara h 1
Ara h 2
1. Marker
2. PF: 0 hr
3. PF: 3 hr
4. PF: 6 hr
5. PF: 24 hr
6. Blank
7. PF + TG: 0 hr
8. PF + TG: 3 hr
9. PF + TG: 6 hr
10. PF + TG: 24 hr
1 2 3 4 5 6 7 8 9 10

B


Mr (kDa)
208
105
53
34
23
17
13
7
4
HMW polymers
Ara h 1
Ara h 2
1. Marker
2. PF + AP: 0 hr
3. PF + AP: 3 hr
4. PF + AP: 6 hr
5. PF + AP: 24 hr
6. Blank
7. PF + AP + TG: 0 hr
8. PF + AP + TG: 3 hr
9. PF + AP + TG: 6 hr
10. PF + AP + TG: 24 hr
1 2 3 4 5 6 7 8 9 10

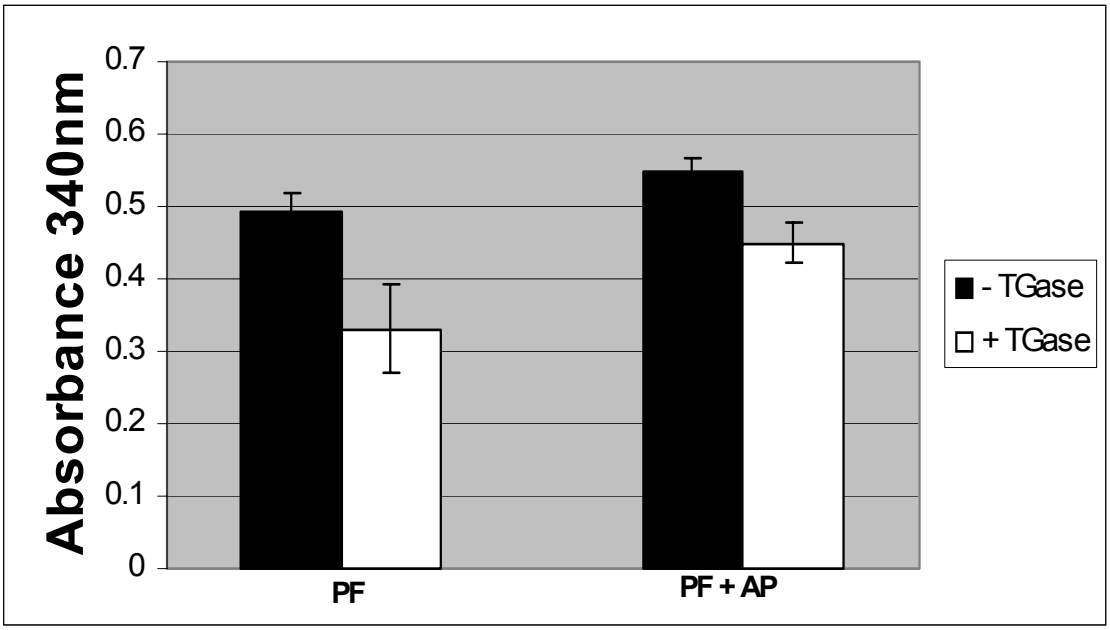
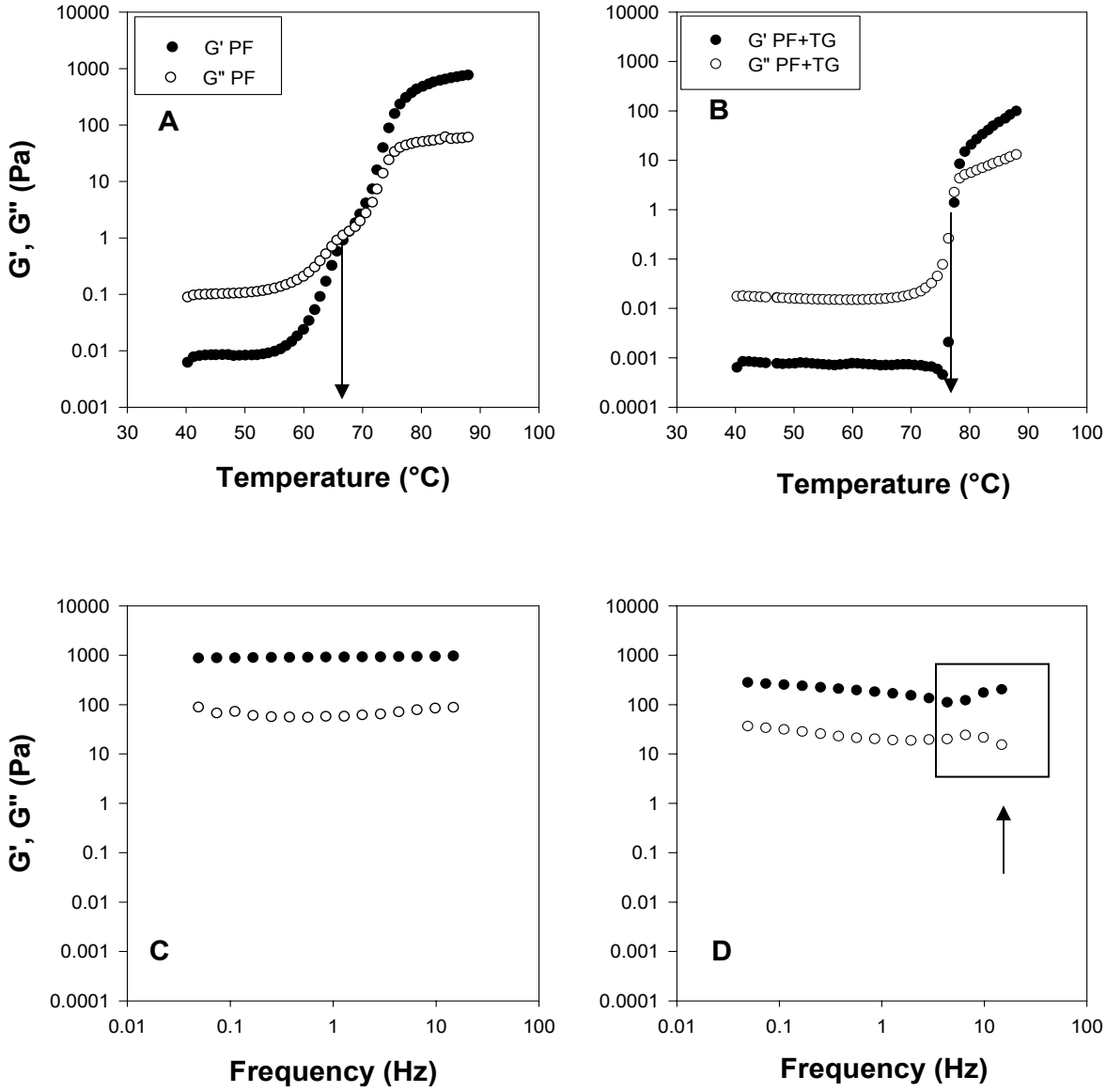


Figure 3. OPA assay for 20 % w/w PF dispersions +/- 1.0% w/w AP +/- TGase and adjusted to pH 8.0 after reaction at 37°C for 6 hr.

Figure 4. Small-strain, oscillatory testing of PF + TGase dispersions; stress = 1.5 Pa. PF dispersions were prepared as 20% total solids w/w and adjusted to pH 8.0. A & B represent heating ramps (1°C/min) at a constant frequency of 0.1 Hz after a 7hr pre-incubation with TGase at 37°C. C & D depict frequency sweeps at a constant temperature of 90°C after a similar incubation time. PF dispersions were prepared as 20% total solids w/w +/- 0.5% w/w AP and adjusted to pH 8.0. Arrows were added to aid in data interpretation. Arrows were added to aid data interpretation.


G' PF
G" PF
A
G', G" (Pa)
Temperature (°C)
G' PF+TG
G" PF+TG
B
Temperature (°C)
C
G', G" (Pa)
Frequency (Hz)
D
Frequency (Hz)

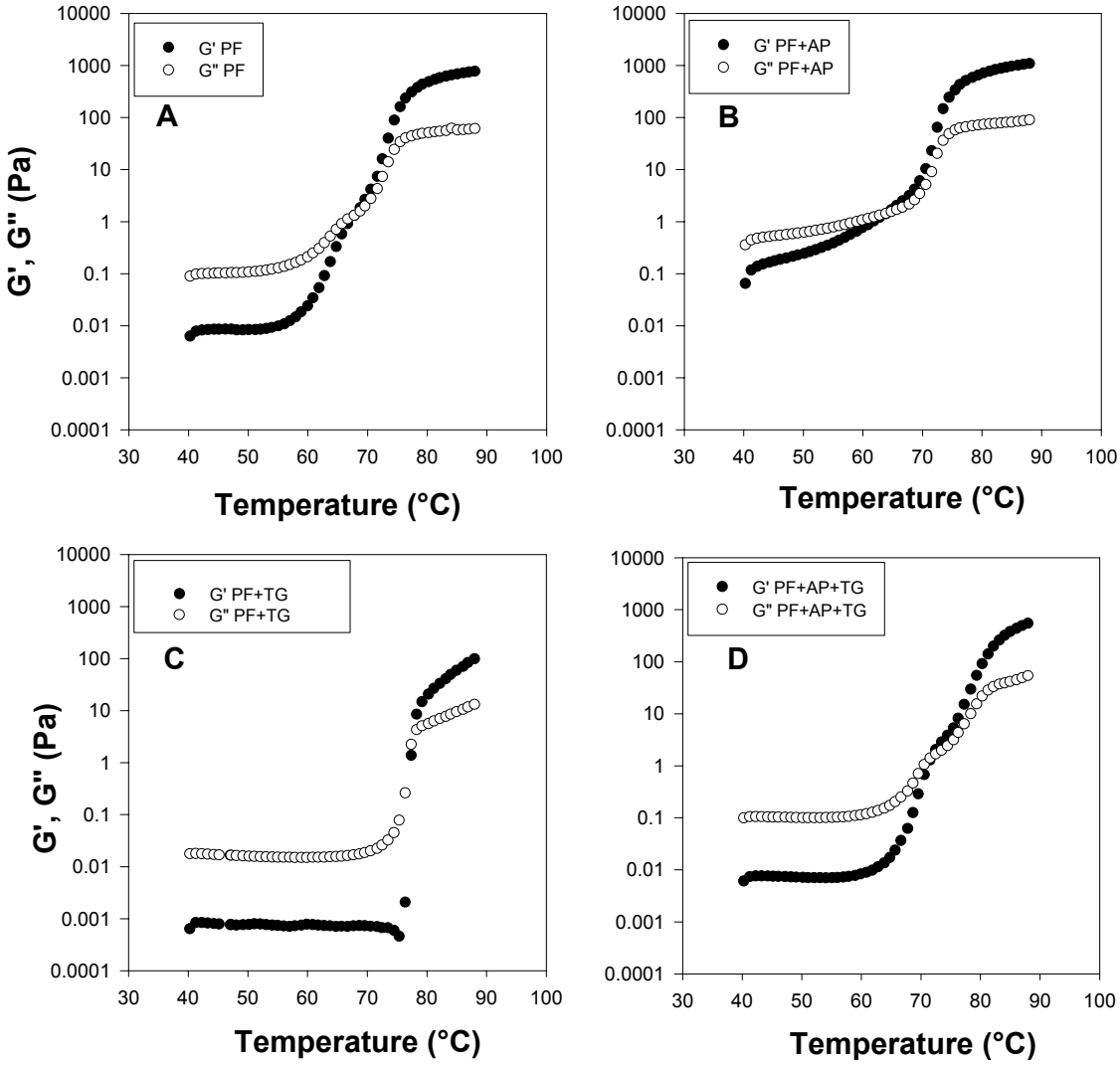


Figure 5. Small-strain, constant oscillatory testing of PF (A), PF + AP (B), PF + TGase (C), and PF + AP + TGase (D); stress = 1.5 Pa, Frequency = 0.1 Hz, heating ramps (1°C/min). Rheological testing began immediately after 7 hr incubation with TGase. PF dispersions were prepared as 20% total solids w/w +/- 0.5% w/w AP and adjusted to pH 8.0.

Figure 6. Rotational viscosity testing of PF, PF + TG, PF + AP, PF + AP + TG. The shear rate was 50 1/s. PF dispersions were prepared as 20% total solids w/w +/- 0.5% w/w AP and adjusted to pH 8.0. The heating rate was 1°C/min. A = dispersions at 0 hr time. B = dispersions after a 6 hr pre-incubation with TGase.

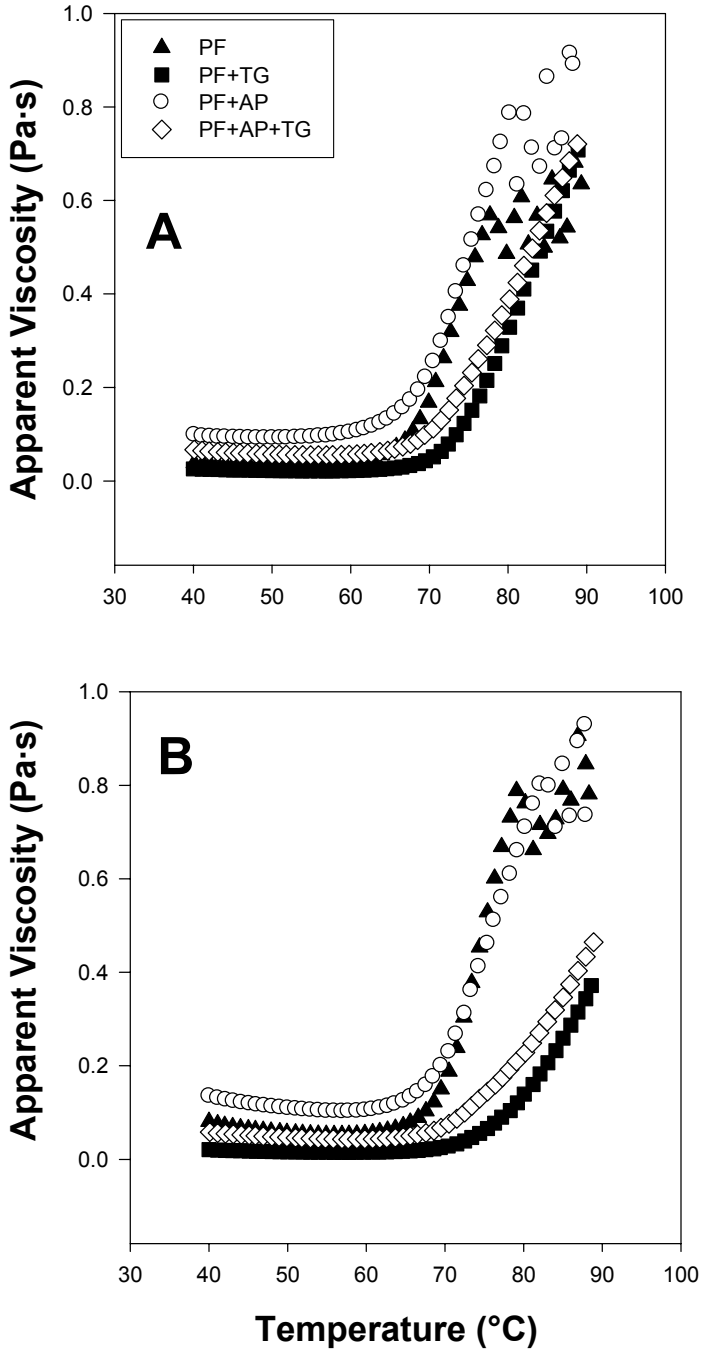

PF
PF+TG
PF+AP
PF+AP+TG
A
B
Apparent Viscosity (Pa·s)
Temperature (°C)
1.0
0.8
0.6
0.4
0.2
0.0
30
40
50
60
70
80
90
100

Table 1.

Water holding capacity of PF +/- TGase +/- AP dispersions after incubation for 24 hr at 37°C. PF dispersions were prepared as 20% w/w +/- 0.5% w/w AP and adjusted to pH 8.0.

Peanut Flour Sample	**Water holding capacity[1] (g water held/g solid)**
PF	5.64±0.10[a]
PF + TGase	6.05±0.05[b]
PF + AP	7.04±0.05[c]
PF + AP + TGase	7.60±0.03[d]

[1] Mean ± standard deviation. Means in a column followed by a different letter are significantly different ($p < 0.001$).

Chapter 5

The effects of transglutaminase crosslinking on the functional properties of peanut flour dispersions containing casein

ABSTRACT

Light roast-12% fat peanut flour (PF) is a high protein food ingredient. Recently, we observed that microbial transglutaminase (TGase) crosslinked peanut proteins and changed the functional characteristics of the final product. Polymer formation was observed in TGase treated PF-casein (CN) dispersions and occurred more rapidly with increasing concentrations of CN. Upon heating, the gelation temperature of all TGase-treated PF-CN dispersions increased. Moreover, the viscosity and yield stress of TGase treated PF-CN dispersions were less than in non-TGase treated controls. TGase treated PF + 5% CN dispersions increased in water holding capacity after 24 hr incubation at 37°C. Casein proved to be an effective co-substrate with PF, and enzymatic processing changed several rheological characteristics including gelation temperature, viscosity, yield stress, and WHC. These data suggest the potential use of PF-CN and PF-PF polymers in high protein peanut-based food products.

Keywords: Peanut Proteins, Peanut Flour, Transglutaminase, Casein, and Rheology.

INTRODUCTION

Light roast-12% fat peanut flour (PF) is a high protein, dry powder, food ingredient manufactured after the partial extraction of peanut oil from roasted peanuts. Commercially available peanut flour typically contains 40-50% protein, 10-30% fat, and 20-30% carbohydrate/fiber (Suknark et al., 1997) compared to roasted peanuts which contain 25-30% protein, 50% fat, and 20% carbohydrate/fiber (Basha et al., 1976). Currently, peanut flour is used as an ingredient in protein bars, confections, cakes, cookies, crackers, and other baked goods.

Rheological characteristics of food and/or food ingredients are important in the manufacture of numerous products. Dynamic oscillatory testing is one of the methods used to study viscosity and gelation properties. When a force is applied to an elastic solid the deformation is proportional to the applied force when performed within the viscoelastic region, where the observed rheological responses are independent of stress and strain. Moreover, the application of force to a liquid result in flowing of the material and the flow is proportional to the force applied. Most foods do not fall within the ideal solid or liquid categories but instead are viscoelastic, meaning that they have both viscous (fluid) and elastic (solid) properties (Steffe 1996). Quantification measurements obtained by rheological analyses are the storage modulus (G') which represents the energy stored or the elastic response, and the loss modulus (G") which is the energy lost and equals the viscous response (Daubert and Foegeding, 1998).

Davis and others (2007) analyzed the rheological characteristics of light and dark roast and 12 and 28% fat peanut flours at pH 8.0. On an equal weight basis under large-strain deformation the low fat flours were more viscous than high fat flours. Upon heating the dispersions at 1°C/min, gelation was observed as determined by small strain oscillatory analyses. Gelation was observed at an optimum PF concentration of 20% w/w total solids under the specified conditions.

Previous studies demonstrated that microbial transglutaminase (TGase) catalyzes protein crosslinking through acyl-transfer reactions between glutaminyl residues (donor) and primary amines (acceptor) of casein, soy, and whey (Tang et al., 2006; Truong et al., 2004; Schorsch et al., 2000; Folk and Chung 1985). Currently, the food industry uses TGase to modify functional characteristics such as texture, water holding capacity, gelation temperature, and viscosity (Zhu et al., 1995). We recently reported polymerization of PF by TGase as determined by SDS-PAGE and Ortho-phthaldialdehyde (OPA) analyses (Clare et al., 2007). Moreover, we noted that PF dispersions treated with TGase were less viscous than non-treated dispersions under large-strain deformation.

Further, we observed the polymerization of PF dispersions containing amidated pectin (AP) treated with TGase (Gharst et al., submitted). In this work, high molecular weight polymers were formed as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). OPA assays indicated ~40% protein coupling in PF dispersions treated with TGase compared to ~20% in those with both AP and TGase.

Small-strain analyses indicated that gelation in PF dispersions with TGase was higher (~78°C) compared to dispersions without the enzyme. With large-strain deformation, the addition of AP increased apparent viscosity of all samples tested.

Caseins (CN), composed principally of four major fractions: alpha(s1) and alpha(s2)-caseins, β-casein, and κ-casein, account for ~80% of the total protein in bovine milk. They are used in manufactured foods to impart structure, texture, and nutritive value (Smithers et al., 1991). Research conducted using casein treated with TGase demonstrated the formation of gels (Schorsch et al., 2000). In another related study, frequency and temperature sweeps were used to characterize sodium caseinate treated with TGase. Frequency sweep (0.01 to 10 Hz) results indicated minimal frequency dependence as well as stronger gel formation due to covalent bonding. Temperature sweeps using a constant frequency (1 Hz) and strain (0.1%) during a heating ramp, 25° - 90°C at 6.5°C/min, revealed that G' and G" were nearly constant indicating that heat did not alter the covalent bonds; however, under similar conditions, a purely physical gel, normally collapses because covalent bonding does not occur (Lee et al., 2006).

Based upon findings in our previous research, the objective of this study was to investigate the rheological characteristics of TGase treated peanut flour dispersions with added CN.

MATERIALS AND METHODS

Materials. Light roast peanut flour-12% fat was provided by the Golden Peanut Company (Alpharetta, GA). CN was contributed by Fonterra (Lemoyne, PA). Purified microbial TGase was donated by Ajinomoto Food Ingredients LLC (Japan) and stored at -20°C prior to use. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were reagent grade.

Methods. *Hydration of peanut flours and casein.* PF (20% w/w total solids) and CN (final concentrations of 1.0%, 2.5%, and 5.0% w/w total solids) were mixed in deionized water to form a dispersion. The pH of the PF dispersion was adjusted initially to 8.0 with 2N NaOH then stirred for 1 hr and readjusted to pH 8.0.

TGase Activity. TGase was supplied as a powder mixed with maltodextrin. TGase was solubilized in 1.0 M Tris-acetate buffer, pH 6.4. Soluble activity of TGase was assayed according to the method of Folk and Chung (1985) with minor modification by Truong and co-workers (2004). The purity of TGase was > 90% according to electrophoretic data presented by Wilcox and Swaisgood (2002). N-carbobenzoxy (CBZ)-glutaminyl-glycine and hydroxylamine were used to measure enzymatic activity at 37ºC. Reagent concentrations in this assay mixture included: 100mM hydroxylamine, 15 mM CBZ-glutaminyl-glycine, 5 mM calcium chloride, 10 mM dithiothreitol in 200 mM Tris acetate buffer, pH 6.0. Under the above conditions, one unit of enzyme activity was defined as an absorbance change of 0.29/min at 525 nm at pH 6.0, forming 1 μmol of hydroxamate/min and specific activity of TGase was determined to be 600 units per gram of powder.

Rheological Analyses. PF (20% w/w) dispersions + CN (1.0%, 2.5%, and 5.0% w/w) and +/- TGase were incubated for 6 hr at 37°C while shaking. Rheological measurements were performed immediately after incubation with TGase. A stress-controlled rheometer (StressTech, Rheologica Instruments AB, Lund, Sweden) equipped with a CC25 serrated cylinder was utilized to characterize PF dispersions under both small and large-strain deformations. All dispersions were added to the cup in sufficient volume to cover the bob, and then a thin layer of mineral oil was added to limit vapor loss. After a 60 s equilibrium time at 40°C, dispersions were pre-sheared at 50 s^{-1} for 30 s to ensure complete mixing and similar shear histories for all experiments. A stress of 1.5 Pa was used during small strain deformation. This parameter was identified to be within the linear viscoelastic region (LVER) based on stress sweeps at 10 Hz for samples at 40°C, meaning this stress was within the LVER for all test conditions. Dispersions were heated and cooled from 40° - 90°C at a rate of 1°C/min during constant oscillatory testing at a frequency of 0.1 Hz. Frequency sweeps were performed from 0.1 - 10 Hz while the samples were held at 90°C. Large strain deformation of dispersions was determined while heating and cooling the samples from 40° - 90ºC at a rate of 1ºC/min with a rotational shear rate of 50 s^{-1}. Moreover, during large strain testing, rotational shear rates from 0.1 - 100 s^{-1} were applied to dispersions. Rheological tests were conducted in duplicate.

SDS-PAGE. Initially, all samples were assayed for total protein content using the bicinchoninic acid (BCA) method to ensure that equivalent amounts were loaded into

each gel lane. Samples were properly diluted and mixed (1:1 v/v) with 8% sodium dodecyl sulfate (SDS), 0.9M Tris sample buffer containing 5.0% β-mercaptoethanol and heated at 100°C for 10 min prior to loading onto 10-20% Tris-Tricine gradient polyacrylamide gels. After electrophoresis was completed, the samples were stained for visualization of protein content using a colloidal Coomassie Blue staining reagent (Invitrogen Inc., Carlsbad, CA).

Water Holding Capacity. The water holding capacity (WHC) of TGase-treated and non-treated PF + CN dispersions was determined according to the centrifugation method of Fleming and co-workers (1974) with minor modification by Resch and others (2005). Control and enzyme-treated dispersions were incubated for 6 hr and 24 hr at 37°C, then centrifuged at 635 x g/15 min. Supernatants were carefully removed and the water held per gram of solid was determined by calculated weight differences. Test samples were analyzed in triplicate.

ELISA Methodology. PF dispersions were prepared at 20% w/w total solids content in 50 mM phosphate buffer, the pH adjusted to 8.0, then dispersions were stored overnight at 4°C. CN was added to specified samples at a final concentration of 2.5% w/w. All samples were diluted (1:20) and then microcentrifuged at 13,600 *x g* for 5 minutes prior to BCA assay. In order to ensure equivalent protein concentrations, the TGase and control samples were diluted (1:80) before ELISA testing. ELISA methodologies were conducted according to the instructions provided in the Veratox Peanut Allergen test kit,

which is specifically designed for quantitative analysis of IgG binding activity (Neogen Corporation, Lansing, Michigan).

Statistical Analysis. Water holding capacity data were analysed by analysis of variance using SAS statistical software (version 8.0; SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Recent studies established the polymerization of peanut protein with TGase (Clare et al., 2007). In an extension of that observation, the data presented, herein, indicates the polymerization of PF dispersions containing 2.5% w/w CN after treatment with TGase for 1, 3, and 6 hr at 37°C (Figure 1, lanes 10-12). SDS-PAGE banding profiles indicate crosslinking based on the formation of new protein bands, the disappearance of Ara h 1, ~ 64 kDa (Wichers et al., 2004), the disappearance of other minor bands, and increased smearing (Figure 1, lanes 9-12 compared to lanes 4-7). This pattern was more prevalent after extended treatment of PF + CN dispersions with the enzyme (Figure 1, lanes 11-12 compared to lanes 9-10). Further, we observed that Ara h 2, ~20 kDa (Koppelman et al., 2001), did not appear to be crosslinked under these experimental conditions, a finding which supported observations made in earlier work (Clare et al., 2007 and Gharst et al., submitted). Clare and co-workers (2007) reported that Ara h 2 contained both glutaminyl and lysyl residues, which potentially serve as reactive amino acid residues that participate in crosslinking reactions; however, the protein could be folded in such a way that accessibility to the catalytic site is limited, thus minimize crosslinking. Interestingly, CN bands, alone (Figure 1, lane 3), were not prominently displayed in PF + 2.5% CN

dispersions (Figure 1, lanes 4-7). This phenomenon may be due to CN not being fully solubilized because of the lack of tertiary structure and resulting exposure of hydrophobic residues which impede reactions that solubilize CN in the aqueous system used (Lee et al., 2006).

Gelation temperature may be empirically defined as the point where the storage modulus (G') crosses over the loss modulus (G") (Steffe 1996). Small strain rheological measurements were performed while continuously observing viscoelastic changes. Gelation occurred at higher temperatures for all TGase treated samples. PF dispersions containing 2.5% CN and treated with TGase formed a gel at the highest temperature (~82°C; Figure 2B) compared to control non-enzyme treated dispersions (~72°C; Figure 2A). Previous work using whey protein isolate and β-lactoglobulin established that extensive crosslinking by TGase increased gelation temperatures and limited exposure of hydrophobic portions within the gel matrix during heat denaturation (Eissa and Khan 2006; Eissa et al., 2006; Truong et al., 2004). Similarly, we hypothesize that these gelation temperature data may be due to limited hydrophobic interactions and extensive crosslinking of the potential polymeric endproducts: PF-PF, PF-CN, and CN-CN, thus preventing adequate unfolding and gel network development. Small-strain deformation initially indicated an elastic behavior in PF containing 5% CN dispersions, where G' was essentially the same as G". Upon increased heat, shear thinning occurs until ~73°C where the dispersions began to form a gel (Figure 2A). Similar increased viscosity measurements, under small-strain deformations, were observed in PF dispersions containing AP (Gharst et al., submitted).

In order to further evaluate small strain characteristics of PF containing CN +/- TGase, dynamic oscillatory sweeps were conducted at 90°C using a constant stress of 1.5 Pa (Figure 3). Under these conditions, G' was greater than G" and increased with higher protein concentrations using PF dispersions containing 1% and 2.5% CN across the frequency range tested (Figures 3A & 3B). Moreover, in PF dispersions with 1% and 2.5% CN, G' and G" were greater compared to enzyme treated samples, suggesting a firmer network in the control dispersions (Figure 3A & 3B). However, G' was slightly greater in TGase treated PF dispersions containing 5% CN compared to G' in the untreated sample (Figure 3C) and in contrast to the results obtained with PF + AP +/- TGase dispersions under similar testing conditions (Gharst et al., submitted). Collectively, these rheological parameters appeared to be frequency independent which suggested establishment of a gel network (Steffe 1996). Although at higher frequencies, some frequency dependence was displayed in both treated and untreated PF dispersions containing CN thus indicating a slightly weaker structure (Figure 3).

The effects of TGase upon the gel strength of food proteins have been previously documented. Zhu and co-workers (1995) reported TGase crosslinking of milk proteins, such as casein and whey, with resulting modified functional properties including increased gel strength. On the other hand, we observed an increase in the G' values of all the PF + CN + TGase samples (Figure 4B) compared to the non-enzymatically treated samples (Figure 4A) upon cooling of the dispersions. However, PF + TGase dispersions had lower G' values (Figure 4B) compared to PF dispersions, alone (Figure 4A). The

increase in G' readings may be attributed to the more open structure of the casein micelle, resulting to easier access to glutamine and lysine residues (Bonisch et al., 2004), while the decrease in G' values may be due to the compact structure of peanut proteins that do not allow easy access to TGase catalytic sites (Sen et al., 2002) thus limiting crosslinking by the enzyme. For these studies, we propose that similar changes in G' values may occur within the structure of PF and PF + CN gels (Figure 4).

Large strain deformation conditions (continuous shear at 50 s^{-1}), involving heat, hold, and cooling cycles, were used to determine viscosity of PF + CN dispersions. Dispersions were heated from 40° - 90°C, held at 90°C for 30 minutes, then cooled from 90° - 40°C using a rate of 1°C/min. Upon heating to 90°C, PF + CN dispersions incubated with TGase were less viscous than untreated samples (Figure 5B). We observed similar data trends using light roast-12% fat peanut flour treated with TGase and PF containing AP treated with TGase (Clare et al., 2007; Gharst et al., submitted). Moreover, we observed, at higher temperatures (>60°C), the instability of non-enzyme treated dispersions at a shear rate of 50 s^{-1} (Figure 5A) which may be attributed to the break-down and re-formation of the protein network in the presence of the shear field (Gharst et al., submitted). Interestingly was the observation that PF + 5% CN + TGase was more viscous than the other enzyme treated dispersions and displayed some instability at this particular shear rate (50 s^{-1}). Presumably, the increased apparent viscosity may be attributed to the increase in total protein and the instability of the dispersion explained by the break-down and re-formation of the protein network in the presence of the shear field the slow (Gharst et al., submitted).

In prior studies, Davis and co-workers (2007) established that various peanut flours: light roast (12% and 28% fat) and dark roast (12% and 28% fat), had a pseudoplastic or shear thinning behavior based on the Herschel-Bulkley model.

$$\sigma = \sigma_o + K\gamma^n$$

This equation defines the general relationships for many fluids, and continuous rotational shear rate ramps (Steffe 1996). We designed additional experiments using PF and PF + CN dispersions under large-strain deformation conditions at 40°C where σ and γ are the shear stress and shear rate. With this linear relationship, we were able to solve for n (flow behavior index), K (consistency coefficient), and σ_o (yield stress) (Steffe 1996) (Table 1). Values of n were all <1, consequently all dispersions were defined as pseudoplastic. Lower values of n indicated a change in apparent viscosity with shear rate. Values of K represent the apparent viscosity across the shear rate range tested in which PF + 5% CN was observed to be the greatest (Figure 6). The addition of TGase to PF + 5% CN resulted in a decrease in K values as compared to PF + 5% CN (0.09-1.21 $Pa \cdot s^n$). Moreover, TGase treated PF and PF + 2.5% CN dispersions had the same K values and essentially the same apparent viscosities across the shear rate range performed. Yield stress measurements, parameters that generally describes the finite stress needed to achieve flow (Steffe 1996) confirmed that after 6 hr incubation, PF + 5% CN had the highest σ_o value and that TGase treated samples were generally lower (Figures 6A & 6B).

A positive interrelation between WHC of proteins and increased viscosity has been reported (Idris et al., 2003; Khalid et al., 2003; Ragab et al., 2004; Gharst et al.,

submitted). The water holding capacity (WHC) of PF + CN dispersions +/- TGase (24 hr incubation) is summarized in Table 2. We observed that increased viscosity of PF + 5% CN dispersions after 6 hr incubation at 37°C (Figures 5 & 6) were correlated with an increased WHC of PF + 5% CN dispersions after 24 hr incubation at 37°C (Table 2). Similarly, we observed that the WHC was increased significantly ($p<0.05$) in PF + 5% CN + TGase dispersions after 24 hr incubation at 37°C (Table 2); although, no differences were noted among PF + CN dispersions after incubation for 6 hr with TGase (Data not shown). These data are in agreement with similar findings in which case test dispersions were prepared with AP +/- TGase (Gharst et al., submitted).

In order to evaluate changes that may occur in soluble peanut protein antigenicity studies, we examined the IgG binding properties of these test fractions using the Veratox Peanut Allergen test kit. ELISA data indicated a 25% reduction in IgG binding in TGase treated peanut protein samples containing CN compared to the non-enzyme treated fractions (Figure 7). However, we observed a slight increase in IgG binding using PF + TGase fractions compared to non-enzyme treated PF dispersions (Figure 7). Other researchers have reported that peroxidase treated peanut protein fractions lower IgE binding responses to Ara h 1 and Ara h 2 epitopes (Chung et al., 2004) and that TGase modification of salt-soluble and salt-insoluble fractions using soft or hard wheat flour systems also lowered IgE responses as a result of crosslinking (Watanabe et al., 1994).

Casein proved to be an effective co-substrate with PF, and under these experimental conditions, enzymatic processing changed the rheological characteristics

such that gelation temperature, viscosity, yield stress, and WHC were altered. Furthermore, antigenicity was somewhat diminished in PF dispersions containing CN after incubation with TGase compared to non-treated samples. Taken together, these data suggest the potential use of PF-CN polymers for use in high protein peanut-based food products, such as protein bars and shakes.

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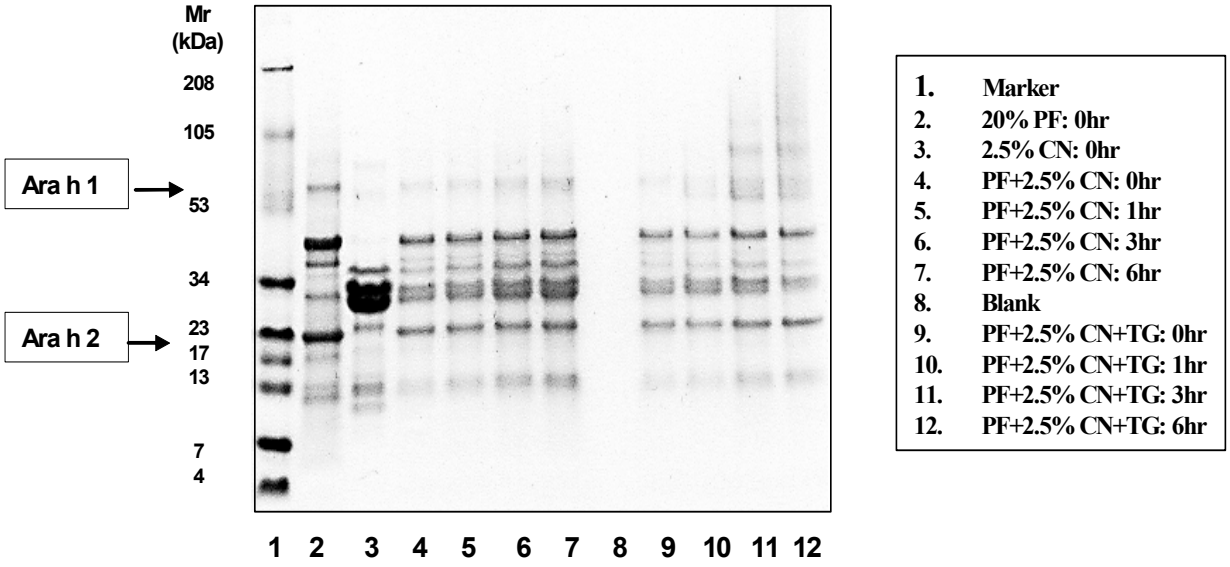


Figure 1. TGase Polymerization of 20% w/w PF + 2.5% w/w CN +/- TGase, pH 8.0. Approximately 30 µg of protein were loaded per lane.

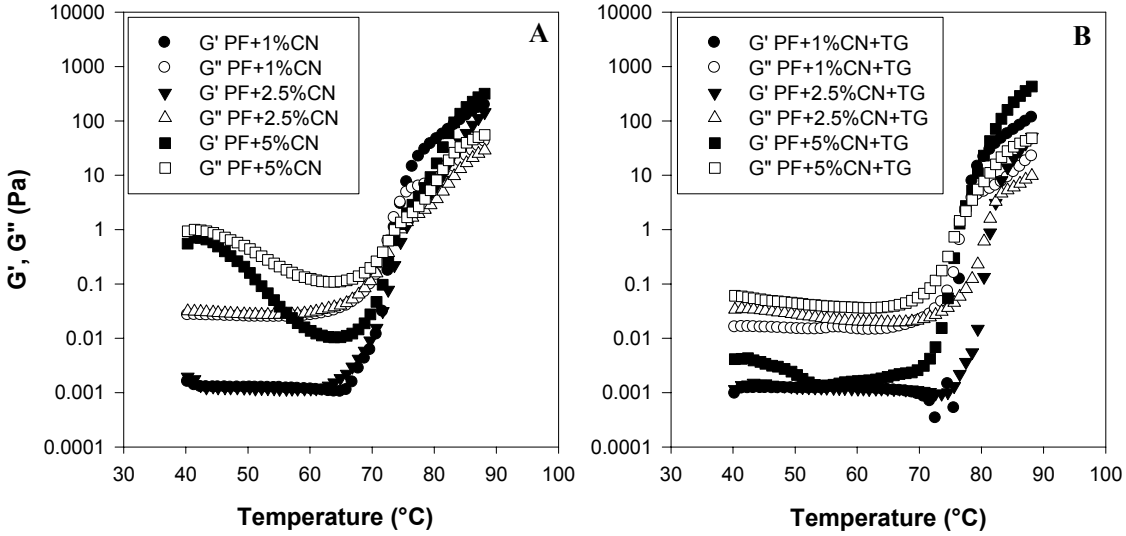


Figure 2. Small-strain, constant oscillatory testing of: A = PF + 1%, 2.5%, and 5% CN; B = PF + 1%, 2.5%, and 5% CN + TGase; stress = 1.5 Pa, Frequency = 0.1 Hz, heating ramps (1°C/min). Rheological testing began immediately after 6 hr incubation with TGase. PF dispersions were prepared as 20% w/w total solids + 1%, 2.5%, and 5% w/w CN and adjusted to pH 8.0.

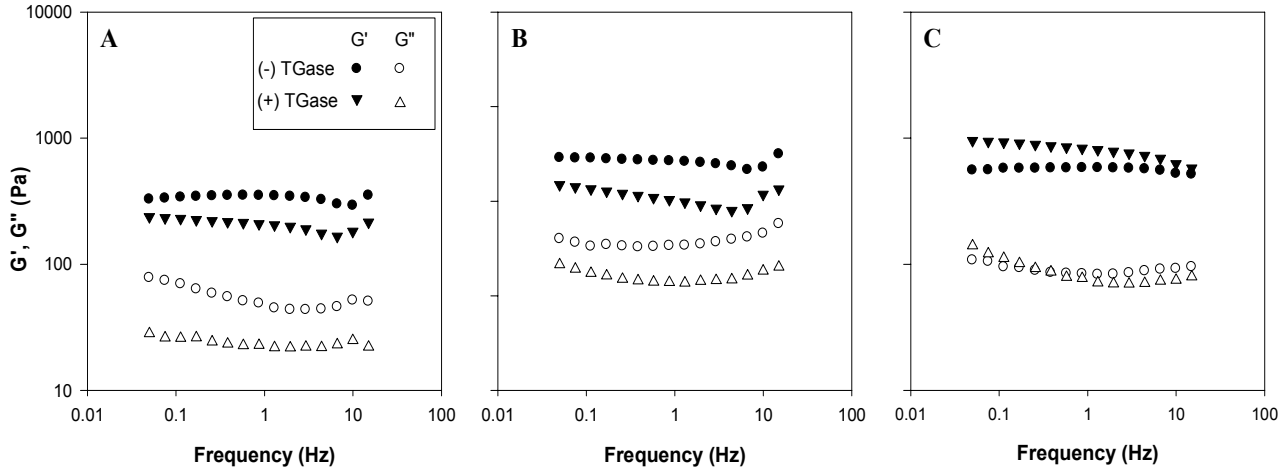


Figure 3. Small-strain, oscillatory testing of: A = PF + 1% CN +/- TGase, B = PF + 2.5% +/- TGase, C = PF + 5% CN +/- TGase; stress = 1.5 Pa. PF dispersions were prepared as 20% total solids w/w and 1%, 2.5%, and 5% w/w CN and adjusted to pH 8.0. A - C indicates frequency sweeps at a constant temperature of 90°C after a 6 hr pre-incubation time.

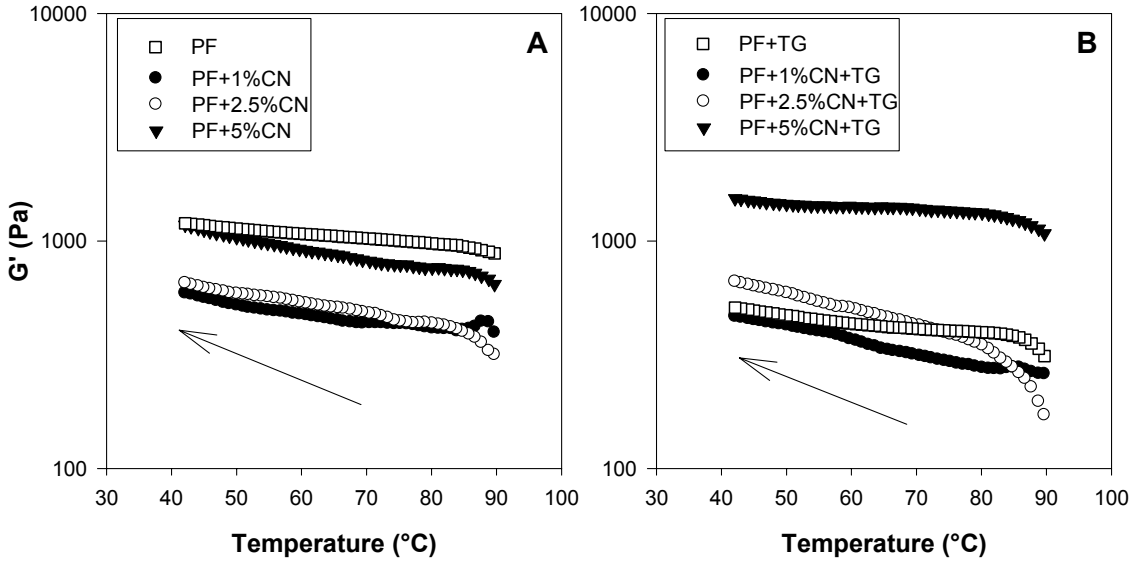


Figure 4. Small-strain, oscillatory testing of: A = PF, PF + 1%, 2.5%, and 5% CN; B = PF, PF + 1%, 2.5%, and 5% CN + TGase; stress = 1.5 Pa. PF dispersions were prepared as 20% w/w total solids + 1%, 2.5%, and 5% w/w CN and adjusted to pH 8.0. G' data was collected upon cooling from 90° - 40°C at1°C/min. A & B = dispersions after a 6 hr pre-incubation time. Arrows were added to aid data interpretation.

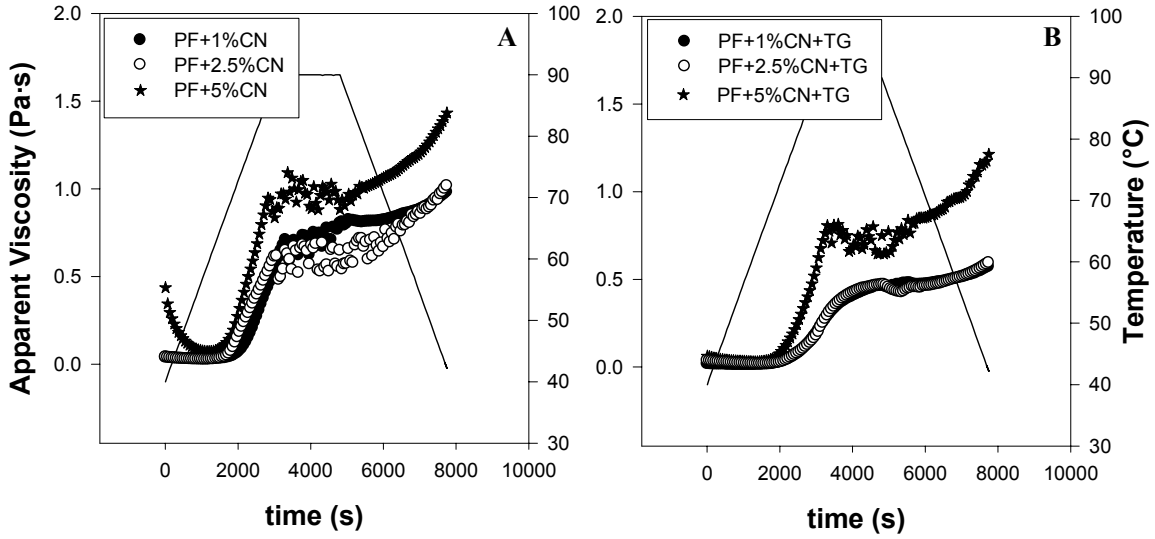


Figure 5. Rotational viscosity testing of: A = PF + 1%, 2.5%, and 5% CN; B = PF + 1%, 2.5%, and 5% CN + TGase. The shear rate was 50 1/s. PF dispersions were prepared as 20% w/w total solids + 1%, 2.5%, and 5% w/w CN and adjusted to pH 8.0. Heating and cooling rates were 1°C/min, samples were held at 90°C for 30 minutes. A & B = dispersions after a 6 hr pre-incubation time.

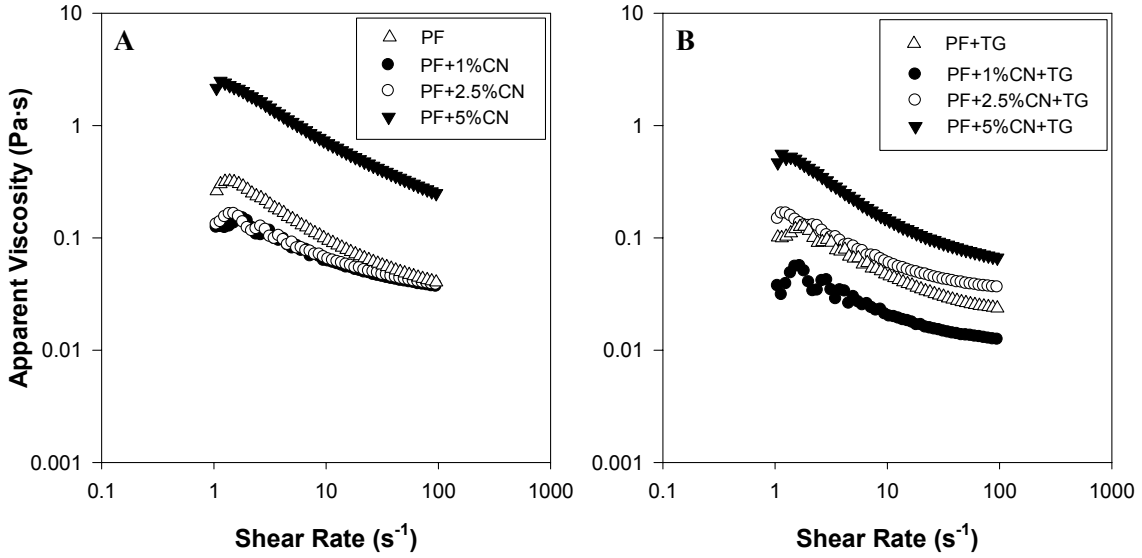


Figure 6. Rotational shear rate ramps at 40°C for: A = PF, PF + 1%, 2.5%, and 5% CN; B = PF, PF + 1%, 2.5%, and 5% CN + TGase. PF dispersions were prepared as 20% w/w total solids + 1%, 2.5%, and 5% w/w CN and adjusted to pH 8.0. Shear rate was increased as the test proceeded. A & B = dispersions after a 6 hr pre-incubation time.

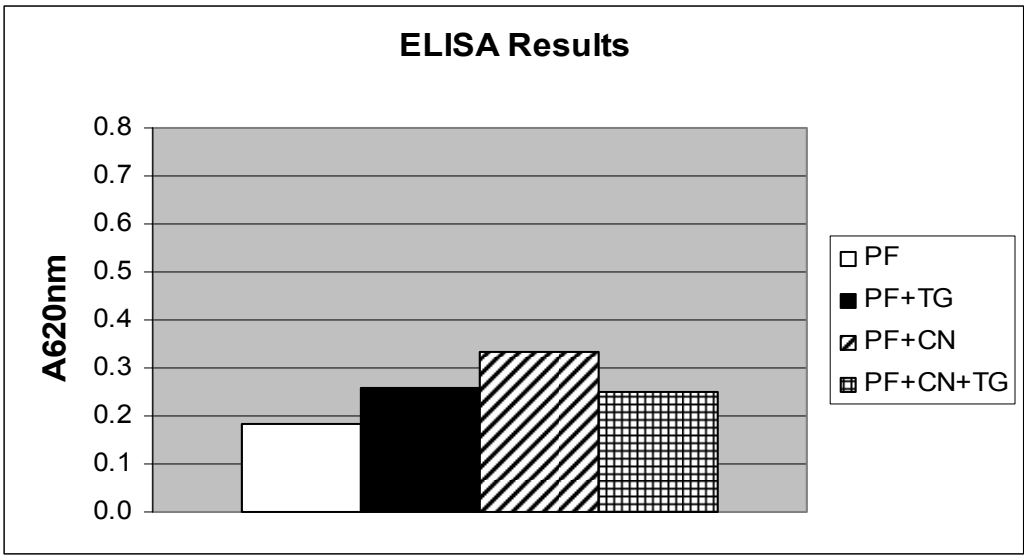


Figure 7. IgG binding activity of TGase treated peanut protein fractions. TGase treated peanut protein test dispersions after incubation for 24 hr at 37°C included: (i) 20% w/w PF, (ii) 20% w/w PF + TGase, (iii) 20% w/w PF + 2.5 % w/w CN, and (iv) 20% w/w PF + 2/5% w/w CN + TGase. All protein samples were diluted to an equivalent peanut protein concentration. ELISA protocols were performed according to the instructions provided in the Veratox Peanut Allergen test kit.

Table 1.

Herschel-Bulkley Parameters for PF, PF + CN, PF + TGase, and PF + CN + TGase after incubation for 6 hr at 37°C. Dispersions were prepared as 20% w/w total solids + 1%, 2.5%, and 5% w/w CN and adjusted to pH 8.0. Values are averages done in duplicate.

Dispersion	**K** **($Pa \cdot s^n$)**	**n**	**σ_0** **(Pa)**
PF 6 hr	0.11 +/- .02	0.76 +/- .03	0.34 +/- .03
PF + 1% CN 6hr	0.07 +/- .01	0.84 +/- .02	0.13 +/- .02
PF + 2.5% CN 6hr	0.07 +/- .01	0.84 +/- .02	0.13 +/- .02
PF + 5% CN 6hr	1.21 +/- .02	0.63 +/- .01	1.90 +/- .03
PF + TG 6 hr	0.05 +/- .01	0.84 +/- .02	0.14 +/- .02
PF + 1% CN + TG 6hr	0.02 +/- .01	0.89 +/- .02	0.05 +/- .02
PF + 2.5% CN + TG 6hr	0.05 +/- .01	0.90 +/- .03	0.17 +/- .03
PF + 5% CN + TG 6hr	0.09 +/- .02	0.87 +/- .03	0.27 +/- .04

Table 2.

Water holding capacity of PF, PF + TGase, and PF + 5% CN +/- TGase dispersions after incubation for 24 hr at 37°C. PF dispersions were prepared as 20% w/w + 1%, 2.5%, and 5% w/w CN and adjusted to pH 8.0.

Peanut Flour Sample	**Water holding capacity[1] (g water held/g solid)**
PF	5.64±0.10a
PF + TGase	6.05±0.05b
PF + 5% CN	7.05±0.12c
PF + 5% CN + TGase	7.19±0.05d

[1] Mean ± standard deviation. Means in a column followed by a different letter are significantly different ($p < 0.05$).