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Effects of Transglutaminase Catalysis on the Functional and Immunoglobulin Binding Properties of Peanut Flour Dispersions Containing Casein

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The functionality of light roasted peanut flour (PF) dispersions containing supplemental casein (CN) was altered after polymerization with microbial transglutaminase (TGase). The formation of high molecular weight covalent cross-links was observed with likely development of PF-PF, PF-CN, and CN-CN polymers based on Western blotting patterns visualized using antiserum directed against Ara h 1, Ara h 2, Ara h 3, or casein. The gelling temperature of TGase-treated PF dispersions containing 2.5% CN was significantly raised compared to the nontreated PF-CN control solutions. Furthermore, the gel strength and water holding capacity of cross-linked PF-CN test samples containing 5% CN was increased, while the yield stress and apparent viscosity were lowered compared to control dispersions. The immunological staining patterns were also changed where, in some cases, IgE binding to TGase-treated PF-CN fractions appeared less reactive compared to equivalent polymeric PF dispersions lacking supplemental CN and non-cross-linked PF-CN samples. Perhaps, covalent modification masked IgE peanut protein binding epitopes, at least to some degree, on an individual patient basis. Casein proved to be an effective cosubstrate with PF for creating Tgase modified PF-CN dispersions for use as a novel high protein food ingredient.

KEYWORDS: Peanut proteins; peanut flour; transglutaminase; casein; rheology; antibody reactivity; IgE binding

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. Commercial flours typically contain 40−50% protein, 10−30% fat, and 20−30% carbohydrate/fiber (*1*), compared to roasted peanuts comprised of 25−30% protein, 50% fat, and 20% carbohydrate/fiber (*2*). Currently, peanut flour is used in manufacturing protein bars, confections, cakes, cookies, crackers, and other baked goods.

Oftentimes, the rheological characteristics of a particular ingredient are important for predicting the performance traits in a given food product. Thus, apparent viscosity measurements and dynamic oscillatory analyses, for example, can serve as a means to evaluate operational parameters, such as flow behavior and gelation. Most foods are neither ideal solids nor liquids but are instead considered "viscoelastic", meaning that they exhibit both viscous (fluid-like) and elastic (solid-like) properties (*3*). Such attributes can be quantitatively evaluated based on instrumental determinations of the storage modulus (G'), representing the energy stored or the elastic response, and the loss modulus (G''), defined as the energy lost and equated with a viscous response (*4*).

Previously, Davis and others (*5*) described some of the rheological characteristics associated with light and dark roasted peanut flour dispersions containing 12 and 28% fat at pH 8.0. These results established that low fat flours were more viscous than high fat flours, at equal solids concentrations, under large-strain deformation experimental conditions. Furthermore, upon heating, gelation was observed when the PF concentration was adjusted to 20% (w/w total solids), as determined by small strain oscillatory analyses under the conditions specified.

Enzyme modification plays an important role in altering the functionality of numerous food products and microbial transglutaminase (TGase) has been previously identified by several independent investigators to catalyze protein cross-linking reactions using soy, whey, and casein substrates (*6*−*8*). The mechanism of this reaction involves covalent bond formation,

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achieved through acyl-transfer reactions between glutaminyl residues (donor) and primary amines (acceptor; (*9*)). Most recently, Clare and others (*10*) observed the polymerization of PF dispersions with TGase as qualitatively determined by SDS-PAGE and quantitatively measured using *ortho*-phthaldialdehyde (OPA) assays. Furthermore, it was noted that the apparent viscosity of PF dispersions was diminished after treatment with the enzyme compared to nontreated dispersions. Presently, the enzyme is utilized in the food industry to modify various functional attributes including texture, water holding capacity, gelation temperature, and viscosity (*11*).

TGase polymerization of PF dispersions containing amidated pectin (AP) was recently studied as well (*12*); however, OPA assay data established that ~40% protein coupling was achieved in PF dispersions lacking supplemental pectin compared to ~20% for those containing the carbohydrate under equivalent experimental conditions. The apparent viscosity was increased in all test samples containing AP, and small-strain data revealed higher gelation temperatures in TGase-treated PF-AP dispersions (~78 °C) compared to noncross-linked dispersions (~68 °C).

Herein, PF dispersions were supplemented with milk casein prior to treatment with the enzyme. Casein (CN) is principally composed of four major subcomponents, including alpha (s_1), alpha (s_2), β-casein, and κ-casein, accounting for ~80% of the total protein in bovine milk (*13*). Casein fractions are also used in a variety of foods to impart structure, texture, and nutritive value. Furthermore, Schorsch et al. (*8*) and Lee et al. (*14*) noted that TGase-treated CN dispersions formed even stronger gels after enzyme catalysis. Hence, a major objective for these experiments was focused on identifying the functional changes that occurred in a mixed PF-CN protein system after TGase modification in which PF-CN hybrids, PF-PF, and CN-CN polymers were likely to be formed.

Peanuts are considered one of the major allergenic foods and, so far, nine separate proteins have been identified with respect to promoting immune responses (*15*). Ara h 1, Ara h 2, and Ara h 3 are considered to be the major allergens because they are recognized by serum IgE in over 50% of peanut allergic individuals (*16*−*18*). In previous work, Maleki and others (*19, 20*) established that the IgE binding properties of peanuts were enhanced during the roasting process and that formation of oligomers occurred through covalent linkage via the Maillard reaction. Therefore, while the addition of casein to test PF dispersions might improve rheological and functional attributes, it was also important to evaluate any potential changes that may occur with regard to IgE binding versus Ara h 1, Ara h 2, and Ara h 3 after PF-CN polymerization reactions were accomplished.

Hence, the overall aim for this study was focused on evaluating the rheological characteristics of aqueous, heated, TGase-treated PF dispersions containing supplemental CN, and to establish whether or not the creation of covalent cross-links between PF and CN fractions might significantly impact the overall immunoglobulin binding activity with respect to peanut protein epitopes.

MATERIALS AND METHODS

Materials. Light roast, 12% fat PF was generously provided by the Golden Peanut Company (Alpharetta, GA), while casein (CN; 82.4% protein) 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 reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

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 resuspended in deionized water, the pH was initially adjusted to 8.0 with 2 N NaOH, stirred for 1 h, and then readjusted to pH 8.0.

TGase Activity. TGase was supplied as a dried powder mixed with maltodextrin, rehydrated in 1.0 M Tris-acetate buffer, pH 6.4, and enzymatic activity assayed according to the method of Folk and Chung (*9*) with minor modification by Truong and co-workers (*7*). The stock used for these experiments was identical to the one previously characterized by Wilcox and Swaisgood (*21*) in which the purity was shown to be >90% according to electrophoretic data. To verify enzyme activity, *N*-carbobenzoxy (CBZ)-glutaminyl-glycine and hydroxylamine were tested, initially, at 37 °C, in which reagent concentrations were prepared 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 reaction conditions, one unit of enzyme activity is defined as an absorbance change of 0.29/min at 525 nm, pH 6.0, forming 1 μmol of hydroxamate/min.

Rheological Analyses. PF (20% w/w) dispersions, (±)CN (1.0, 2.5, or 5.0%, w/w), and (±)TGase were preincubated for 6 h at 37 °C with shaking, prior to immediately performing rheological measurements. Microbial transglutaminase, added at a ratio of ~5 units/g of dried peanut flour, was slowly mixed with the protein substrate in a rotating incubator at 37 °C. A stress-controlled rheometer (StressTech, Rheologica Instruments AB, Lund, Sweden) equipped with a CC25 serrated cylinder was utilized to characterize PF-based dispersions under both small and large-strain deformation conditions. All test solutions were added to the cup in sufficient volume to cover the bob, and then a thin layer of mineral oil was overlaid to limit vapor loss. After a 60 s equilibrium time at 40 °C, dispersions were presheared at 50 s^{-1} for 30 s to ensure complete mixing and establish a similar shear history for all test samples. A stress setting of 1.5 Pa was used during small strain deformation, a parameter identified to be within the linear viscoelastic region (LVER), based on stress sweeps of these samples performed at 10 Hz (40 °C).

The dispersions were also 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 samples were held at 90 °C. Large strain deformation, or the apparent viscosity, was measured during heating and cooling of the samples from 40° - 90 °C at a rate of 1 °C/min using a rotational shear rate of 50 s^{-1}. Moreover, during large strain analyses, rotational shear rates, ranging from 0.1−100 s^{-1}, were applied to each experimental sample. All rheological analyses were conducted in duplicate.

SDS-PAGE Protocols. Initially, test samples were assayed for total protein content using the bicinchoninic acid method (BCA assay kit, Pierce Inc., Rockford, IL) 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), 0.9 M 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 protein bands were visualized using a colloidal Coomassie Blue staining reagent (Invitrogen Corp., Carlsbad, CA).

Water Holding Capacity. The water holding capacity (WHC) of non-enzyme-treated PF (20% solids, w/w) and PF dispersions containing 5% CN (w/w) was compared to equivalent TGase-modified PF and PF + 5% CN mixtures according to the centrifugation method of Fleming and co-workers (*22*) with minor modification by Resch and others (*23*). All experimental samples were incubated for 6 and 15 h at 37 °C and centrifuged at 635*g* for 15 min. Supernatants were carefully removed and the water held per gram of solid was determined by calculated weight differences. Test dispersions were analyzed in triplicate.

SDS-PAGE for Western Blots. The SDS-PAGE methods for the Western blotting experiments were slightly different than previously described. All test samples were microcentrifuged for 5 min and separated into the supernatant and pellet fractions. Each portion was then mixed with 3x sample loading buffer (Invitrogen Corp.), according to the manufacturer's instructions, incubated for 10 min at 65 °C, and subjected to electrophoresis using a 4−20% Novex Tris-Glycine HCl

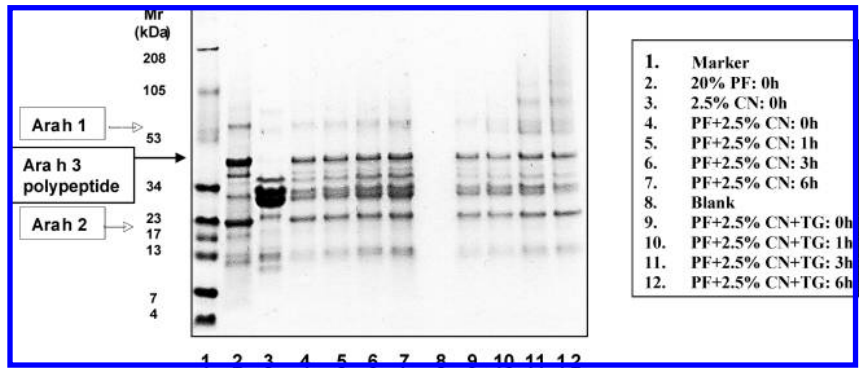

Figure 1. SDS-PAGE protein banding pattern of 20% PF (w/w) dispersions containing 2.5% CN (w/w), plus and minus TGase treatment at 37 °C, pH 8.0. Approximately 30 μg of protein were loaded per lane.

precast gel (Invitrogen Corp.). Individual proteins were separated according to size and stained with Gel-Code Blue (Pierce) for 1 h, destained according to the manufacturer's instructions, and photographed.

Antibodies Used in Western Blotting Experiments. Anti-Ara h 1, Ara h 2, and Ara h 3 chicken IgY antibodies were custom manufactured by Sigma Immunosys (The Woodlands, TX). Sigma Inc. provided the yolks from an immunized animal, which was subjected to acetone precipitation and drying. A total of 1 μg of solid acetone precipitated material was then dissolved in 1 mL of phosphate buffered saline (PBS) and used at stated dilutions.

For most blotting experiments, IgE fractions were pooled using serum collected from 15 peanut allergic individuals, who were affiliated with the University of Arkansas for Medical Sciences (Little Rock, AR), and approved in accordance with the rules and regulation of the review board for this institution. A radioallergosorbent test (RAST), which quantitatively detects the amount of IgE in a patient's blood that specifically reacts with a suspected or known allergen (peanut protein in this case), was not performed on all subjects; however, of those examined, the levels measured >15 IU/mL. All patients used in this study were previously identified as having peanut allergies.

The casein antibody fraction was prepared in a rabbit and received as a gift from Dr. Samuel Godefroy and Michael Abbott (Health Canada, Food Allergens Program, Food Research Division).

Horseradish peroxidase (HRP)-conjugated (1) goat antichicken IgY, (2) goat antirabbit IgG, and (3) goat antihuman IgE were purchased from Sigma Chemical Co. (St. Louis, MO). The ECL Western Substrate kit was obtained from Amersham Bioscience Corp. (Piscataway, NJ).

Western Blotting Protocols. After electrophoresis, the protein constituents were transferred to a PVDF membrane for Western blotting analyses. The membrane was blocked for 1 h using 5% Blotto [5% dry milk dissolved in phosphate buffered saline (PBS) containing 0.05% TWEEN (PBST)] prior to incubation with the primary antiserum, either chicken IgY (Ara h 1, Ara h 2, Ara h 3), rabbit IgG (casein), or human IgE (peanut protein). A 2% bovine serum albumin (BSA) blocking reagent was prepared with PBST to screen for casein antigen.

After blocking, the primary antibodies, chicken (IgY) anti-Ara h 1, Ara h 2, and Ara h 3, were diluted 1:5000 in 5% blotto, added to the membrane, and incubated for 1 h. For IgE analysis, the membranes were blocked in 2% blotto for 15 min and incubated overnight with a 1:10 dilution (PBST) of patient sera, collected from peanut allergic individuals. The casein antibody probe was diluted 1:10,000 in 2% BSA prepared with PBST.

After incubation with the identified primary antibody, the membranes were washed three times with PBST and further incubated for 30 min with a secondary HRP labeled antibody directed against (1) chicken IgY (1:100,000 in 2% blotto), (2) human IgE (1:10,000 in 2% blotto), or (3) rabbit IgG (1:100,000 in 2% BSA, PBST).

Afterward, the membrane was washed three times with PBST and two times with PBS, followed by a final incubation with ECL-Plus Western substrate (Amersham Bioscience Corp., Piscataway, NJ). The signal was visualized using a CCD camera system (Fuji Photo Film Co., Ltd., Duluth, GA).

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

RESULTS AND DISCUSSION

In this work, PF dispersions were initially covalently linked in the presence of casein fractions (2.5%, w/w) after enzyme catalysis for 1, 3, and 6 h at 37 °C, likely forming hybrid (PF-CN) protein polymers, together with homologous PF-PF and CN-CN cross-links (**Figure 1**, lanes 10−12). SDS-PAGE banding profiles revealed (*i*) the formation of a new midsized protein band, which migrated just below that of Ara h 1, ~64 kDa (*24*), after a 3 h incubation period, (*ii*) the disappearance of other minor peanut protein bands together with casein components, and (*iii*) increased smearing of the protein staining dye toward the top of the gel, indicating polymer formation (**Figure 1**, lane 12 vs lane 9). This particular pattern differed from the configuration previously seen by Clare et al. (*10*) in which Tgase-treated PF fractions, lacking casein, exhibited a broad size range without forming distinct higher molecular weight conjugates. Furthermore, Ara h 2, ~20 kDa (*25*), and a polypeptide of Ara h 3 (45 kDa; (*26*)), did not appear to be cross-linked to the same extent as Ara h 1, a finding reported in prior work (*10, 12*).

The impact of TGase catalysis on functional parameters, such as gelation was then examined. Gelation temperatures may be empirically defined as the point at which G' values, reflecting the storage modulus, cross G'' readings, or loss modulus, based on a log plot of [G', G''] versus temperature (*3*). In this study, small strain rheological data established that gelation occurred at a higher temperature for enzyme-treated PF dispersions containing 2.5% CN (~82 °C; **Figure 2B**), indicative of increased heat stability, compared to the control sample which gelled at ~73 °C (**Figure 2A**). Such changes were not so pronounced in TGase modified PF fractions prepared with 1% supplemental CN compared to equivalent nonenzyme-treated samples. Moreover, it appeared that the gel strength (G') was slightly lowered in PF-CN (2.5%) fractions after enzyme catalysis, compared to PF-CN (2.5%) per se, such that the apparent structural modifications impeded adequate unfolding which may, in turn, limit formation of a gelling network (**Figure 2B** vs **Figure 2A**).

In prior studies, where whey protein isolates and β-lactoglobulin were polymerized with TGase, the authors proposed that extensive cross-linking increased gelling temperatures and limited the exposure of hydrophobic portions within the gel matrix during heat denaturation (*7, 27, 28*). Similarly, we hypothesized that similar effectors would play a significant role in defining the formation of this protein network as well.

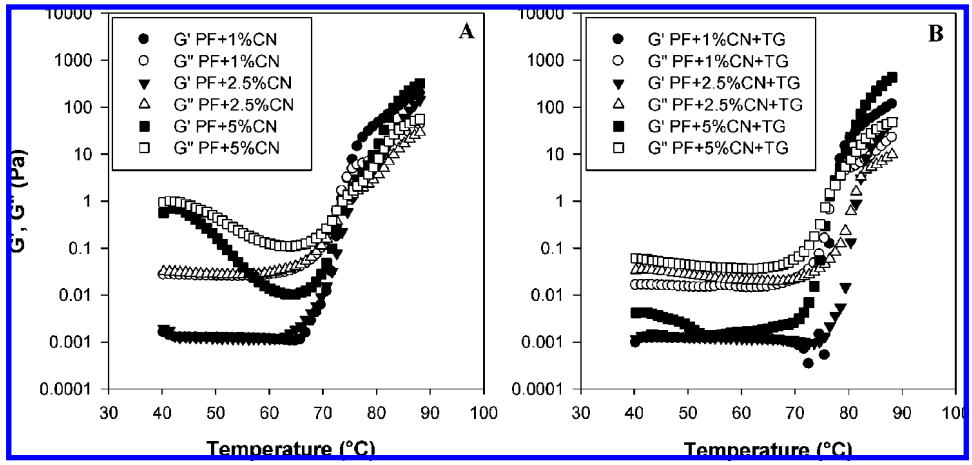

Figure 2. Small-strain, constant oscillatory testing of **(A)** PF + 1, 2.5, or 5% CN; **(B)** PF + 1, 2.5, or 5% CN + TGase. All PF dispersions were prepared at 20% total solids (w/w) with indicated casein levels, pH 8.0, and incubated at 37 °C for 6 h (±)TGase prior to measurement. Stress setting = 1.5 Pa, frequency setting = 0.1 Hz, heating ramps (1 °C/min).

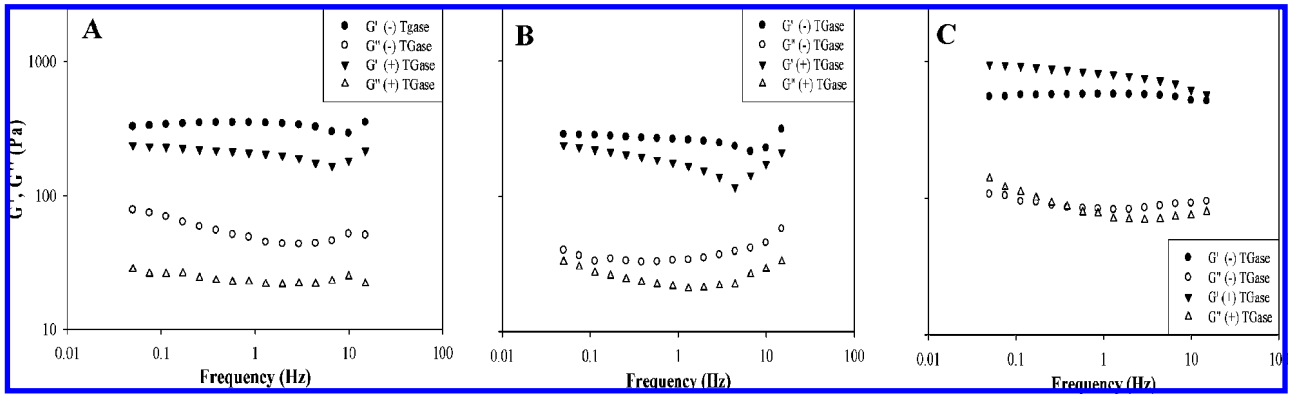

Figure 3. Small-strain, oscillatory testing of **(A)** PF-CN (1%, w/w, ± TGase), **(B)** PF-CN (2.5%, w/w, ±TGase), and **(C)** PF + CN (5%, w/w, ±TGase). All PF dispersions were prepared as 20% total solids with indicated levels of casein, pH 8.0, and preincubated at 37 °C for 6 h prior to measurement. The data in panels **A**−**C** represent frequency sweeps taken at a constant temperature of 90 °C. Stress setting = 1.5 Pa.

Small-strain deformation experiments established that the elastic component (G') of control PF dispersions containing 5% CN was initially the same as the viscous module (G'') at 40 °C; however, with continued heating, the G' value decreased dramatically with increasing temperature until ~73 °C, at which point gelation occurred (**Figure 2A**). This was not the case for the equivalent enzyme-treated dispersion in which $G'' > G'$ at the start of the measurement (40 °C), signifying that preprocessing with TGase (6 h, 37 °C) produced a more fluid sample. However, with additional heating, G' values crossed those of G'' at ~77 °C, and by the end of the experiment, the gel strength of PF-CN (5%) dispersions was actually higher than PF fractions containing either 1 or 2.5% CN. This finding may reflect the formation of increased amounts of PF-CN and/or CN-CN conjugates, thus promoting development of a more favorable gel template. Again, Schorsch et al. (*8*) and Lee et al. (*14*) previously noted that TGase catalysis of CN dispersions produced stronger gels after enzyme processing.

To further elucidate the small strain characteristics of PF dispersions containing CN, (±)TGase treatment, dynamic oscillatory sweeps were conducted at 90 °C at a constant stress setting of 1.5 Pa (**Figure 3**). As before, these measurements were made after a 6 h preincubation step at 37 °C to mimic the experimental conditions previously employed. The G' values of control and TGase modified PF dispersions containing 1% CN were essentially equivalent to those containing 2.5% CN (**Figure 3A**,**B**). Moreover, the gel strength was higher in the nonenzyme-treated samples. In contrast, the G' values of PF-CN samples containing 5% CN were elevated compared to those dispersions containing less CN, and a stronger gelling network was formed after enzyme modification (**Figure 3C**).

These results were in contrast to trends previously observed in PF dispersions prepared with amidated pectin (AP), (±)TGase catalysis, under similar testing conditions; however, in the present case, PF-PF, hybrid PF-CN, and CN-CN polymers were likely formed compared to the generation of homogeneous PF-PF cross-links in the earlier experiments (*12*). For the most part, these measurements appeared to be independent of frequency, suggesting the establishment of a relatively stable network (*3*), although at higher frequencies, there were minor changes in the data curves (**Figure 3**).

Upon cooling, there was a significant increase in the G' values of enzyme polymerized PF-CN samples containing 5% CN compared to PF-CN (5%) control solutions and TGase-treated PF dispersions prepared with lower amounts of CN (1% or 2.5%; **Figure 4**). Presumably, the degree of cross-linking between PF and CN and/or CN-CN fractions was augmented in the presence of higher concentrations of the CN substrate. Similar observations were reported by Zhu and co-workers (*11*), who noted an increase in gel strength after enzyme processing of various single component casein fractions. Treatment of PF, alone, with TGase lowered G' values (**Figure 4B**) compared to the control dispersions (**Figure 4A**), while the gel strength of

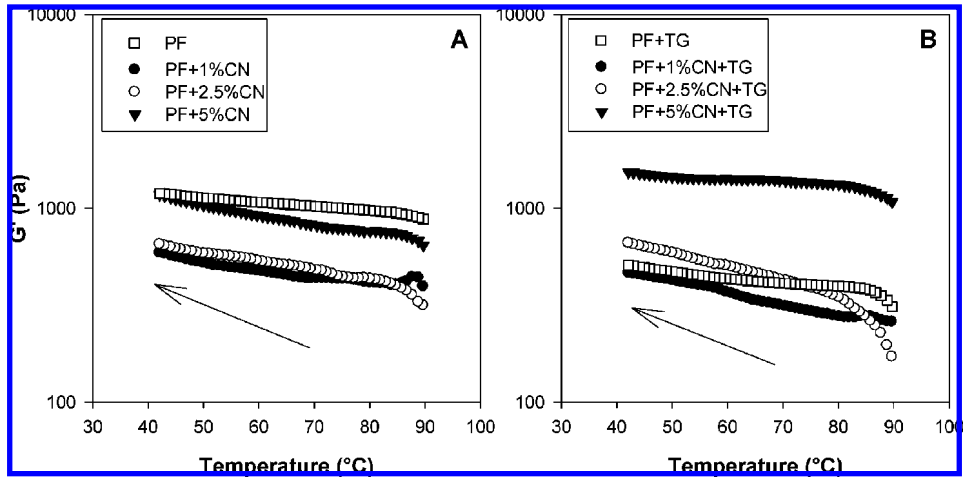

Figure 4. Small-strain, oscillatory testing of **(A)** PF and PF-CN (1, 2.5, or 5% CN, w/w); **(B)** PF and PF-CN (1, 2.5, and 5% CN, w/w) + TGase. All PF dispersions were prepared at 20% total solids (w/w), with indicated CN levels, pH 8.0, and preincubated at 37 °C for 6 h prior to measurement. Arrows indicate cooling data. Stress setting = 1.5 Pa, frequency setting = 0.1 Hz. *G′* data were collected upon cooling from 90−40 °C at a rate of 1 °C/min.

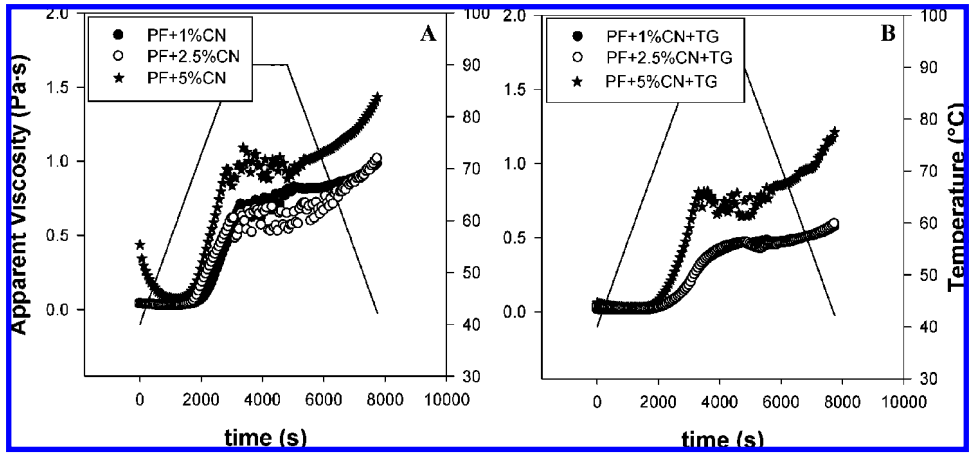

Figure 5. Apparent viscosity testing of: **(A)** PF-CN (1, 2.5, or 5% CN, w/w) and **(B)** PF-CN (1, 2.5, or 5% CN, w/w) + TGase. All PF dispersions were prepared as 20% total solids (w/w) with indicated casein levels, pH 8.0, and preincubated at 37 °C for 6 h prior to measurement. Heating and cooling rates were achieved at 1 °C/min, and the dispersions held at 90 °C for 30 min. The shear rate was set at 50 s^{-1}.

the PF-CN (1 and 2.5%) samples remained essentially the same with or without enzyme processing.

Perhaps this increase in *G′* may be attributed to a more open structure of casein micelles, resulting in easier access to reactive glutamine and lysine residues (*29*). Conversely, decreased *G′* values might be ascribed to the compact structure of peanut proteins per se (*30*), with limited entry at the TGase catalytic site, thereby restricting polymerization. Hence, the degree of PF-PF cross-linkages versus hybrid protein (PF-CN) and CN-CN polymer formation should have a significant impact on the final gelling characteristics as a result of the actual content of resultant end products.

Large strain deformation experiments, performed at a continuous shear of 50 s^{-1}, were used to characterize the apparent viscosity of this mixed protein system during heating, holding, and cooling cycles. Test dispersions were heated from 40 to 90 °C, held at 90 °C for 30 min, then cooled from 90 to 40 °C at a rate of 1 °C/min. After heating, TGase-treated PF-CN dispersions containing either 1, 2.5, or 5% CN were less viscous than untreated samples, especially during the later cooling stages (**Figure 5B**). A similar trend was noted in previous studies using either light roast PF-12% fat or PF-AP dispersions after enzyme modification (*10, 12*).

Table 1. Flow Properties of Peanut Flour, Peanut Flour-Casein, and Transglutaminase-Treated Peanut Flour and Peanut Flour-Casein Dispersions

test sample	K (Pa·s^n)	n	σ_0 (Pa)
PF[a]	0.11 ± 0.02	0.76 ± 0.03	0.34 ± 0.03
PF + 1% CN	0.07 ± 0.01	0.84 ± 0.02	0.13 ± 0.02
PF + 2.5% CN	0.07 ± 0.01	0.84 ± 0.02	0.13 ± 0.02
PF + 5% CN	1.21 ± 0.02	0.63 ± 0.01	1.90 ± 0.03
PF + TGase	0.05 ± 0.01	0.84 ± 0.02	0.14 ± 0.02
PF + 1% CN + TGase	0.02 ± 0.01	0.89 ± 0.02	0.05 ± 0.02
PF + 2.5% CN + TGase	0.05 ± 0.01	0.90 ± 0.03	0.17 ± 0.03
PF + 5% CN + TGase	0.09 ± 0.02	0.87 ± 0.03	0.27 ± 0.04

[a] Peanut flour (PF) dispersions were prepared at 20% solids (w/v) as detailed in the Materials and Methods. Herschel−Bulkley parameters were determined for PF, PF containing added casein (CN) at 1, 2.5, and 5% levels (w/w), and equivalent transglutaminase (TGase)-treated PF and PF-CN samples, pH 8.0. All test fractions were preincubated for 6 h at 37 °C prior to measurement. Listed values represent averages of duplicate sample readings.

At higher temperatures (>60 °C), data scattering was apparent over the temperature range of 60−75 °C, in both control and enzyme polymerized PF samples containing 5% CN (**Figure 5A**), which may be attributed to the breakdown and reformation

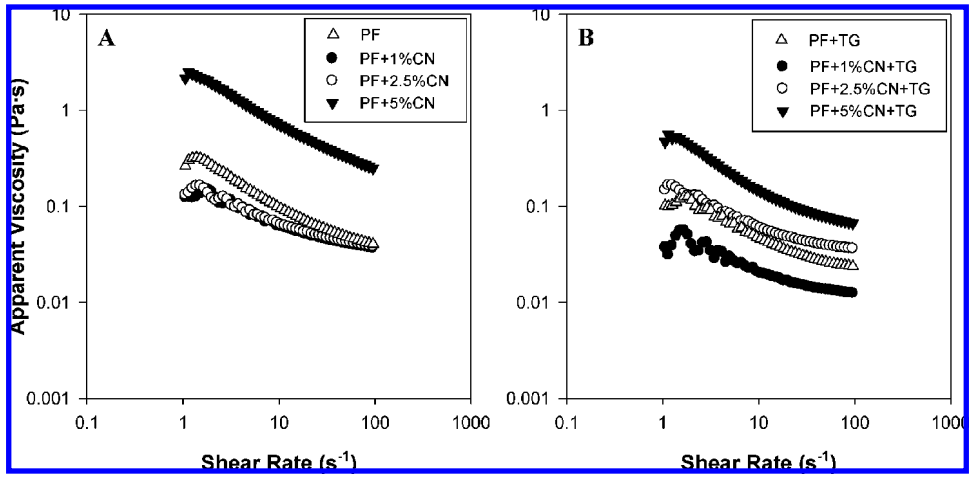

Figure 6. Rotational shear rate ramps at 40 °C for **(A)** PF, PF-CN (1, 2.5, or 5% CN, w/w); **(B)** PF, PF-CN (1, 2.5, or 5% CN, w/w) + TGase. All PF dispersions were prepared as 20% total solids (w/w) with indicated casein levels, pH 8.0, and preincubated at 37 °C for 6 h prior to measurement.

Table 2. Water Holding Capacity of Peanut Flour, Peanut Flour-5% Casein, and Transglutaminase-Treated Peanut Flour and Peanut Flour-5% Casein Dispersions

peanut flour sample	water holding capacity[b] (g water held/g solid)
PF[a]	5.64±0.10[a]
PF + TGase	6.05±0.05[b]
PF + 5% CN	7.05±0.12[c]
PF + 5% CN + TGase	7.19±0.05[d]

[a] Peanut flour (PF) dispersions were prepared at 20% solids (w/v) as detailed in the Materials and Methods. The water holding capacity was determined for control PF and transglutaminase (TGase)-treated PF and PF dispersions containing 5% supplemental casein (w/w). All samples were incubated for 15 h at 37 °C, pH 8.0. [b] Mean ± standard deviation. Means in a column followed by a different letter are significantly different ($p < 0.05$). All measurements were made in triplicate.

of the protein network in the presence of the shear field (*12*). The apparent viscosity of PF dispersions containing 5% CN was higher; overall, a finding impacted by the higher total protein content. Because TGase is active over a wide temperature range (*31*), a continued covalent coupling of the protein substrates likely occurred during the initial heating cycle from 40−70 °C. However, at 90 °C, enzyme inactivation would prevent further catalysis such that the data collected during the cooling phase reflected only the overall flow behavior of the final end products.

In prior studies, Davis and co-workers (*5*) established that various peanut flours, light roast (12 and 28% fat) and dark roast (12 and 28% fat), exhibited a pseudoplastic or shear thinning behavior based on the Herschel−Bulkley equation

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

in which σ and γ represent the shear stress and shear rate, respectively, while σ_o denotes yield stress. This mathematical relationship defines the general characteristics exhibited by many fluids, over a continuous rotational shear rate ramp (*3*). Thus, the consistency coefficient (K value) and flow behavior indexes (n value) may be calculated as the y-intercept and slope, respectively, obtained from a logarithmic plot of shear stress versus shear rate (*32*).

In this work, the K and n readings were determined for PF and PF + CN dispersions under large-strain deformation conditions at 37 °C (**Table 1**). With one exception, that is, PF-5% CN samples, the n values were <1 and, thus, considered pseudoplastic (*3*). The apparent viscosity of such solutions decreases with increasing shear rate, exhibiting shear thinning flow behavior. Furthermore, K values represent the apparent viscosity measured across a defined shear range, and the highest readings were observed in PF fractions containing 5% CN (**Table 1**). Notably, these numbers decreased dramatically from 1.21 to 0.09 Pa·s^n after enzyme catalysis, reflecting increased fluidity. Similar trends were observed in TGase-treated, versus nontreated PF and PF-CN (1%) samples; whereas, essentially no differences were detected after enzyme modification of PF-CN (2.5%) samples.

Yield stress is a term generally used to describe the finite force needed to achieve flow (*3*), and in these experiments, the control PF-CN (5%) sample exhibited the highest σ_o value, which was significantly lowered after enzyme processing for 6 h at 37 °C (**Table 1**). Again, the yield stress decreased following TGase catalysis in most test fractions, with the exception of PF dispersions containing 2.5% CN. As stated previously, the actual protein composition of the starting material may impact the final ratio of product formed, that is, nonpolymerized versus polymerized PF-PF, PF-CN, and CN-CN cross-links as generated under the given experimental conditions.

Previously, a positive correlation was made between the viscosity of various protein solutions with the water holding capacity (WHC; *12, 33*−*35*). In this work, the apparent viscosity was highest in PF dispersions containing 5% CN, (±)enzyme modification (**Figure 6**); although, the values for the cross-linked dispersions were lower at a given shear rate across the range examined. Accordingly, the water holding capacity was elevated in PF-CN (5%) fractions, (±)enzyme catalysis, for 15 h at 37 °C compared to either PF or TGase-treated PF dispersions, lacking supplemental CN (**Table 2**). No differences were seen among PF-CN (5%) samples (±)TGase treatment compared to PF dispersions (±)TGase after a more limited 6 h incubation period at these temperatures (data not shown).

Most likely, at least some of these rheological characteristics can be attributed to the interplay between the soluble and insoluble portions of these dispersions. With extended enzymatic processing, the formation of covalently linked protein polymers generally caused increased precipitation, or insolubility, of the end products. In earlier work, Davis et al. (*5*) proposed that the soluble portion of heated PF samples played a relatively minor role with respect to altered functionality. Additionally, other researchers have discovered that the insoluble fraction of alternative protein solutions, such as soy, ultimately affected the viscosity and water-imbibing capacities of the system (*36*).

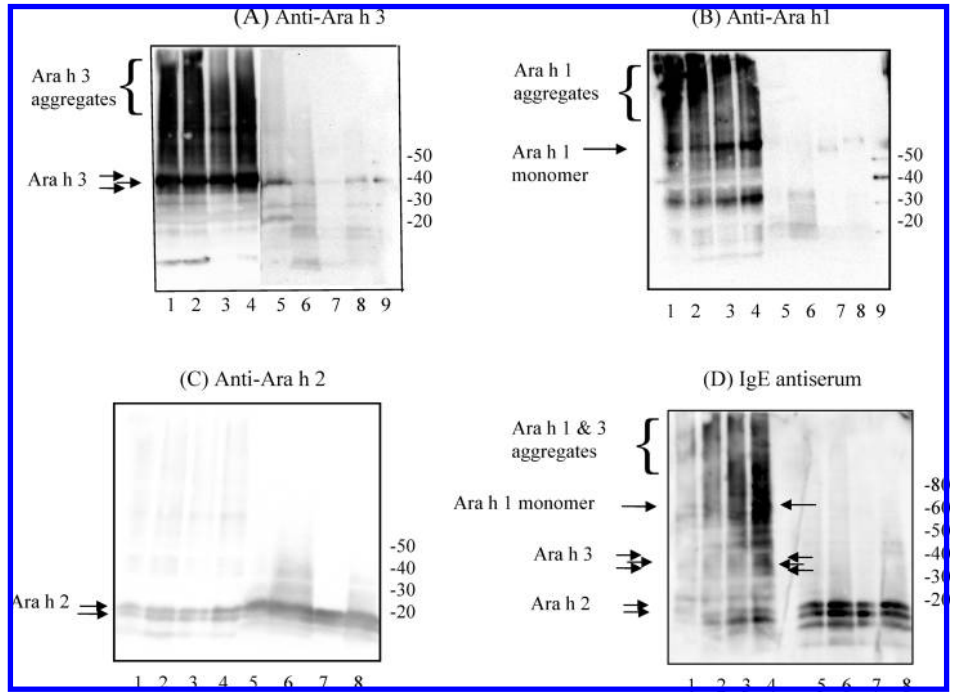

Figure 7. IgE binding to test peanut flour (PF) fractions, (±) 2.5% casein (CN), and (±)TGase treatment for 15 h at 37 °C. Lane 1: PF + CN + TGase pellet; lane 2: PF + TGase pellet; lane 3: PF + CN pellet; lane 4: PF pellet; lane 5: PF + CN + TGase supernatant; lane 6: PF + TGase supernatant; lane 7: PF +CN supernatant; lane 8: PF supernatant; lane 9: marker. Ara h 1 and Ara h 3 are identified by arrows on the left margin and right side of lane 4 in panel **D** to distinguish their presence in all pellet fractions. The IgE antiserum was collected and pooled from 15 patients.

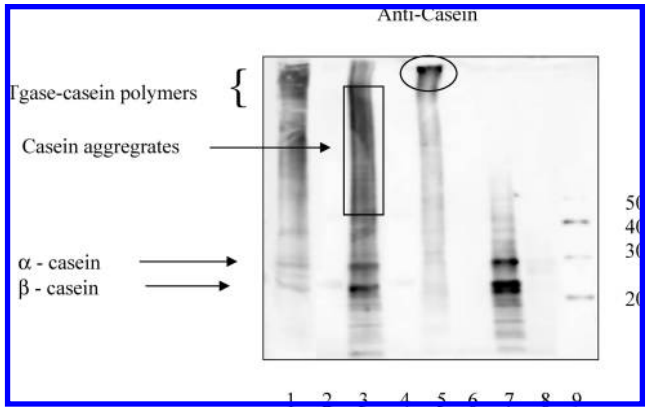

Figure 8. Casein antibody binding to test peanut flour (PF) fractions, (±)2.5% casein (CN), and (±)TGase treatment for 15 h at 37 °C. Lane 1: PF + CN + TGase pellet; lane 2: PF + TGase pellet; lane 3: PF + CN pellet; lane 4: PF pellet; lane 5: PF + CN + TGase supernatant; lane 6: PF + TGase supernatant; lane 7: PF + CN supernatant; lane 8: PF supernatant; lane 9: marker.

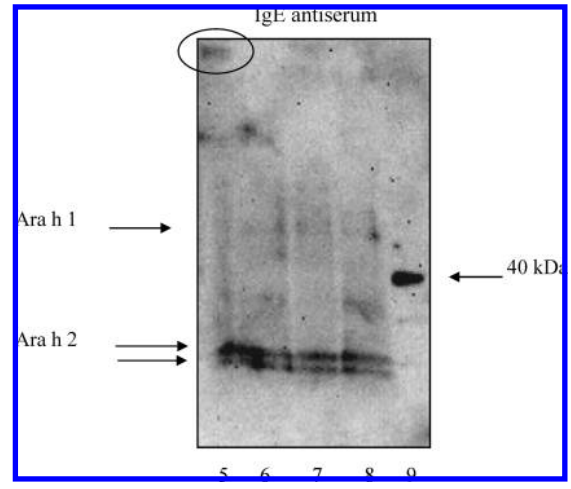

Figure 9. IgE binding to soluble test peanut flour (PF) fractions, (±)2.5% casein (CN), and (±)TGase treatment for 15 h at 37 °C using antiserum collected from a single patient. Lane 5: PF + CN + TGase supernatant; lane 6: PF + TGase supernatant; lane 7: PF +CN supernatant; lane 8: PF supernatant; lane 9: marker. The IgE antiserum was collected from a single patient.

Thus, the final operative parameters will be influenced, at least to some degree, by the extent of polymerization and ensuing precipitation reactions that occur.

To determine whether or not oligomer formation, and the antibody binding activity versus Ara h 1, Ara h 2, and Ara h 3 were significantly different between the various PF and PF-CN, (±)TGase, treatment regimens, Western blotting analyses were performed. In addition, human sera, collected from 15 peanut allergic individuals, were combined and used to evaluate IgE binding levels with respect to PF fractions, (±)2.5% CN and (±)TGase modification (**Figure 7**), while casein antibodies were employed to detect the presence of milk antigen (**Figure 8**). The supernatant, obtained after microcentrifugation, represents the extractable or soluble protein content while pelleted materials denote insoluble constituents.

Soluble monomeric Ara h 3 was detected in all samples, to varying degrees (**Figure 7A**), although most of the oligomer/polymer staining was observed in the insoluble portion of the sample. The manufacturing process used to formulate the peanut flour, itself, caused some degree of Ara h 3 oligomerization as seen in PF pellets containing supplemental CN and control PF fractions (**Figure 7A**, lanes 3, 4). However, the formation of even higher molecular weight cross-links was seen in TGase modified PF fractions lacking CN (**Figure 7A**, lane 2), and to a much lesser extent, a slightly positive "fuzzy" band was noted at the gel interface in PF-CN pellets collected after enzyme catalysis (**Figure 7A**, lane 1).

Analogous trends were noted upon western analysis of PF-based pellets using the Ara h 1 antibody probe, where extensive TGase catalysis of Ara h 1 occurred, particularly in the presence of CN (**Figure 7B**, lane 1). In this case, the broad smearing patterns reflected the formation of a wide range of large-sized, insoluble cross-links, followed to a lesser degree by PF + TGase samples < PF + CN ≤ PF, itself (**Figure 7B**, lanes 1−4). With respect to anti-Ara h 1 binding in the soluble supernatants, residual monomer was faintly seen only in the control PF supernatant (**Figure 7B**, lane 8), while a breakdown fragment of Ara h 1 was visualized in the PF sample containing CN (**Figure 7B**, lane 7).

Ara h 2, is a smaller protein and more soluble than either Ara h 1 or Ara h 3. Again, the level of Ara h 2 in all samples was mostly unaffected by the various treatment regimens, suggesting that Ara h 2 was not polymerized by the enzyme, at least to the same extent, as either Ara h 1 or Ara h 3 (**Figure 7C**). Similar conclusions were previously reached by Clare et al., (*10*) based on results obtained with SDS-PAGE techniques; however, it should be noted that these antibody probes were even more sensitive in detecting the potential formation of TGase-generated Ara h 2 conjugates.

Human IgE binding patterns also verified protein aggregate formation during roasting, independent of TGase catalysis, based on the Western blotting results obtained with insoluble PF fractions (±)2.5% CN (**Figure 7D**, lanes 3,4). Again, Ara h 2 was essentially the only major peanut antigen detected in each of the soluble supernatants, with essentially no evidence of cross-linking. However, IgE reactivity with insoluble PF protein constituents, (±)2.5% CN and (±)TGase treatment, revealed a difference with respect to the intensity of the staining pattern, as reflected by the overall color development from light (weakest) to dark (strongest). Based on the immuno-binding properties of a pooled IgE serum, the order of reactivity was established as TGase-PF-CN < TGase-PF < PF-CN < PF (**Figure 7D**, lanes 1−4).

Western blotting experiments were also performed using IgE antiserum collected from a single patient, where the insoluble portion of PF exhibited the most positive staining pattern with relatively minor differences noted between nontreated and TGase-treated PF samples (±)2.5% CN (data not shown). Hence, the overall IgE binding activity with respect to these types of peanut protein dispersions may vary from person to person. Previously, Chung and others reported that peroxidase-treated peanut protein fractions promoted cross-linking via oxidation reactions and lowered IgE binding responses to Ara h 1 and Ara h 2 epitopes (*37*), while Watanabe and others observed that TGase modification of salt-soluble and salt-insoluble fractions, prepared from soft or hard wheat flour systems, decreased IgE reactivity as well (*38*). Taken together, these results suggested that at least in some individuals, there may be a decrease in the IgE binding properties with respect to TGase-treated PF-CN protein fractions compared to noncoupled PF-CN mixtures.

Casein antibodies were also utilized to probe for the presence of milk antigen, and it was observed that the pellets collected from the nonenzyme-treated PF-CN (2.5%) dispersions evidenced formation of insoluble CN aggregates over a broad size range (**Figure 8**, lane 3). However, the largest polymeric group appeared only after enzyme processing (**Figure 8**, lane 1). Furthermore, Ara h 1 was prominently stained in this region at the gel interface (**Figure 7B**, lane 1), suggesting the likely formation of Ara h 1-CN hybrid linkages. Of interest was the appearance of a high molecular weight, CN positive band, seen in the supernatant portion of the TGase-treated PF-CN (2.5%) sample (**Figure 8**, lane 5), which also reacted with IgE serum collected from a second individual patient (**Figure 9**, lane 5). This particular staining pattern was not visualized upon screening with the pooled IgE sera. Again, IgE reactivity is unique and highly specific on an individual patient basis.

Overall, casein proved to be an effective cosubstrate with PF in that enzymatic processing of PF-CN dispersions altered important rheological characteristics, including gelation temperatures, viscosity, yield stress, and WHC. Moreover, upon extended treatment with the enzyme, extensive polymerization occurred, which resulted in the formation of large molecular weight aggregates, altering the ratio of soluble versus insoluble protein amounts. Ultimately, such changes will have a direct impact on the functional properties of the protein system. Also, at least in some individuals, the IgE binding activity of TGase-treated PF-CN mixtures was lowered, compared to appropriate control dispersions, which might be useful in creating novel, peanut protein-based foods with reduced IgE responses associated with allergic reactions. Thus, the utilization of enzyme modified PF-CN fractions in manufacturing high protein food products, such nutrition bars and liquid shakes, affords a feasible option for future development.

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