Properties of biopolymers produced by transglutaminase treatment of whey protein isolate and gelatin

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ABSTRACT

Byproduct utilization is an important consideration in the development of sustainable processes. Whey protein isolate (WPI), a byproduct of the cheese industry, and gelatin, a byproduct of the leather industry, were reacted individually and in blends with microbial transglutaminase (mTGase) at pH 7.5 and 45 °C. When a WPI (10% w/w) solution was treated with mTGase (10 U/g) under reducing conditions, the viscosity increased four-fold and the storage modulus (G′) from 0 to 300 Pa over 20 h. Similar treatment of dilute gelatin solutions (0.5–3%) had little effect. Addition of gelatin to 10% WPI caused a synergistic increase in both viscosity and G′, with the formation of gels at concentrations greater than 1.5% added gelatin. These results suggest that new biopolymers, with improved functionality, could be developed by mTGase treatment of protein blends containing small amounts of gelatin with the less expensive whey protein.

1. Introduction

Whey and gelatin are both proteinaceous agricultural byproducts. Whey is an abundant, inexpensive and readily available byproduct of the cheese industry. The United States is the world’s largest whey exporter; latest available figures from the US Dairy Export Council (USDEC, 2006) show an increase in exportation by 104% between 2001 and 2006. Despite the exports, a considerable amount of whey is wasted through disposal, and would be available for potential new uses. These uses could be for food or non-food products.

Animal hides, skins and bones, major byproducts of the meat industry, are rich sources of collagen, the structural protein of connective tissue. Gelatin, produced by partial hydrolysis of collagen, is a polydisperse mixture of varying sized collagen fragments. Gelatins are classified by type, edibility, and gel strength. Type A gelatin is obtained by acid treatment usually of pigskins, while type B is obtained by alkaline treatment of cattle hides or bones (Rose, 1992). An early step in leather manufacturing is the liming of the hide to assist in hair removal and opening up of the collagen fiber bundle for access by tanning chemicals. The hide may then be split; the upper layer would be tanned to make leather, and the lower layer would provide a rich source for the extraction of type B gelatin. Gelatin extracted from limed hides is edible, and is used in sausage casings, as an ingredient in a variety of food products, as well as in pharmaceuticals. Inedible gelatin may be surplus lower grade material from production of edible gelatins or may be from an inedible source such as leather waste (Taylor et al., 1994). The highest value market for inedible gelatin traditionally was the photographic industry, where the demand for gelatin has declined markedly in recent years. The Bloom value, a measure of gel strength of a 6.67% (w/w) gelatin, is often used to predict the behavior of a gelatin in a particular application. Gelatin suppliers typically give a nominal Bloom value for the gelatins they market, in this case 75 g. High Bloom (> 225 g) gelatins are taken from the highest molecular weight fraction of crude gelatin and are relatively uniform. High and low molecular weight gelatin fractions are blended to produce low Bloom (<125 g) gelatins, and variation between lots may be expected.

Transglutaminase (TGase, EC 2.3.2.13) is a calcium-dependent enzyme that catalyzes an acyl transfer reaction between the γ-carboxyamide of a protein or peptide bound glutamine and a primary amine (Folk and Chung, 1973). An ε(γ-glutamyl)lysine bond is formed when the primary amine is the ε-amino group of a lysine residue. In 1989, Ando et al. used Streptoverticillium mobaraense to produce a calcium-independent form of the enzyme, namely microbial transglutaminase, mTGase.
A variety of individual food proteins, including gelatin, casein and whey proteins have been polymerized by the formation of mtGase-mediated intermolecular crosslinks (Sakamoto et al., 1994; Rodríguez-Nogales, 2006). Biopolymers formed by the enzymatic crosslinking of dissimilar proteins have the potential for generating novel products (Motoki and Nio, 1983; Yildirim and Hettiarachchy, 1997; Oh et al., 2004). The most abundant proteins in whey, \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin, are globular and contain two and four disulfide bonds, respectively (Farrell et al., 2004). The reduction of disulfide bonds in globular proteins, prior to mtGase treatment, has been shown (Færgemand et al., 1997) to increase access to primary amino groups. Dithiothreitol (DTT) is generally the reductant of choice for cleaving disulfide bonds, given that it is needed in much lower concentration than other reductants to complete the reaction (Grazú et al., 2003).

In this study, biopolymers produced by treating gelatin or whey protein isolate (WPI) and blends of WPI and gelatin with mtGase under reducing or nonreducing conditions are characterized. In blends, WPI, the less expensive of the protein sources, is the primary component and the focus is on the effect of including small amounts of gelatin with the WPI in these biopolymers.

2. Experimental

2.1. Materials

mtGase, Activa TC-TI (Ajinomoto USA Inc., Paramus, NJ) with an active range of pH 4.0–9.0 at 0–70 °C was used without further purification. The mtGase activity, in the presence of the maltodextrin, carrier, under the assay conditions of Folk and Chung (1985) was approximately 10 U/g. Type B gelatin, alkaline extracted from bovine skin, was obtained from Sigma (St. Louis, MO), and characterized in this laboratory as 115 g Bloom. WPI, Alacen 895, containing 93.2% protein (manufacturer’s data), was generously supplied by NZMP (formerly New Zealand Milk Products; Lemoine, PA). DTT was obtained from Calbiochem (San Diego, CA). All other chemicals were reagent grade and used as received.

2.2. Sample preparation

Solutions of gelatin or WPI ranging from 1% to 10% (w/w) were prepared by suspending protein powder in the required weight of deionized water. WPI–gelatin solutions (10% WPI with 0.5–3% gelatin) were prepared by suspending the required amounts of WPI and gelatin powders in deionized water and stirring for about 30 min to ensure a uniform suspension. To test the effect of a reducing environment, a 10% DTT (w/v) solution was prepared and the volume necessary to give a concentration of 10 mg DTT per g protein was included in the preparation of selected samples. Samples were allowed to swell at room temperature for 4 h, and then adjusted to pH 7.5 with 1 N NaOH or 1 N HCl. Samples were then heated at 38 °C for 1 h, cooled to room temperature and stored overnight at 4 °C.

Typical conditions for mtGase-mediated crosslinking of gelatin are pH 6.5 and 50 °C for 4 h (Taylor et al., 2001). For whey proteins, the optimum conditions are pH 7.5 and 40 °C for 8 h (Truong et al., 2004). To provide a basis for comparison, all reactions in this study were performed at pH 7.5 and 45 °C for 5 h, considering that WPI was the major protein component in the mixtures.

For crosslinking experiments, protein samples were prepared in 10 ml less than the required volume. The appropriate concentration of mtGase was then prepared in 10 ml of water and this solution added with stirring to gelatin, WPI, or WPI–gelatin solutions to give the desired final protein concentration. Samples were readjusted to pH 7.5 and incubated for 5 h at 45 °C in a shaker bath under mild agitation. Reaction products were then heated at 90 °C for 10 min to deactivate the mtGase. Control samples, without mtGase, were subjected to the same thermal treatment.

Gelatin and WPI solutions (1–10%) were treated with mtGase at 10 U/g of protein. The effect of enzyme concentration (0–10 U/g of protein) was also investigated for gelatin and WPI solutions at 10%. Finally, the effect of gelatin (0.5–3%) was tested by adding gelatin to a 10% WPI sample containing mtGase at either 0 or 2 U/g of total protein.

2.3. Analyses

The gelatin used for this study was characterized by the standard Bloom test (AOAC Method 948.21, 1990). The strength of a gel formed in a standard 59 mm Bloom jar from a 6.67% (w/w) solution of gelatin at pH 6.5 was measured on a TA.XT2 Texture Analyzer (Stable Micro Systems, Surrey, UK). Gel strengths of experimental samples were determined by the modified small sample Bloom test (Wainewright, 1977). Samples were transferred to 39 mm weighing bottles, to a height of 40 mm, cooled to room temperature and then chilled for 17 h at 10 °C in a constant temperature bath. Each sample was then placed under a 0.5 in. diameter analytical probe, which then was driven into the sample to a depth of 4 mm at 1 mm/s. The measured force, using the correction factor (1.389) previously determined for small samples (Taylor et al., 1994), was expressed in grams. After the determination of gel strength, samples were melted at 60 °C for viscosity measurements.

Viscosity was determined using a Model LV 2000 Rotary Viscometer (Cannon, State College, PA) equipped with a low Centipoise Adapter and a jacketed sample chamber connected to a refrigerated bath circulator, Model RTE-8 (Neslab, Portsmouth, NH). An 18 ml aliquot of each sample was added to the sample chamber and equilibrated for at least 10 min. Viscosity was measured at a spindle speed of 60 rpm, corresponding to a shear rate of 73.42 s⁻¹. After the viscometer had been stabilized for one minute, readings were taken at 60 °C for gelatin as recommended by Rose (1992) and at 25 °C for WPI and blended biopolymer solutions.

Dynamic oscillatory rheometric measurements were carried out in a controlled stress AR-2000 Rheometer (TA Instruments, New Castle, DE) at room temperature (21 ± 2 °C) after enzyme deactivation and equilibration of samples for 1 h. The sample was placed between parallel 25 mm diameter plates and the gap between them was set to 2.5 mm. Excess sample was trimmed off and a thin layer of mineral oil applied to the exposed free edges of the sample to prevent moisture loss. Time sweep measurements were used to study the evolution of the storage modulus \( G’ \) as a function of time. A strain sweep test was performed at a constant frequency of 0.1 Hz to find an oscillation stress value that was within the linear viscoelastic range. This value, 0.5 Pa, was then used to perform time sweep measurements at a constant 0.1 Hz frequency. Storage modulus \( G’ \) was recorded and analyzed over 20 h, at a rate of two points per hour.

Inter-protein crosslinking was evaluated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS–PAGE) (Laemmli, 1970) using precast gradient gels (4–15%). Gels were calibrated using the broad range (BRM) SDS calibration standard (Bio-Rad, Hercules, CA) that contains a mixture of nine proteins ranging in size from 6.5 to 200 kDa. Samples (approximately 0.5 mg) of lyophilized protein dissolved in sample buffer (10 mM Tris–HCl at pH 8.0 containing 1 mM EDTA, 25 mg/ml SDS, 50 μl/ml β-mercaptoethanol and 0.1 μl/ml bromphenol blue) were heated at 40 °C for 4 h. Separation was achieved using a Phast System (Pharmacia Biotech Inc., Piscataway, NJ). Gels were stained with Coomassie Blue.
2.4. Statistical modeling

Because % plots of the data points always showed curvature, second order models in %gelatin or %WPI were generated by regression analysis of viscosity and gel strength data for products from the treatment of combinations of gelatin, WPI, and blends with mTGase and DTT. These models are of the form: Response = a + b*Gelatin + c*Gelatin^2, where a, b, and c are regression coefficients. Analysis of covariance (ancova) was performed on the models to determine whether the coefficients were significantly different between treatments. Comparisons between treatments of the slopes and second order coefficients were performed using orthogonal contrasts (Littell et al., 2002). All tests of significance were performed at the P < 0.05 level.

3. Results and discussion

3.1. Gel strength

The effects on gel strength of increasing concentrations of gelatin (1–10%) in the presence or absence of mTGase and DTT were investigated. The second order model showed that gel strengths of all samples significantly increased (P < 0.05) with increasing gelatin concentration and were greatest at each concentration for samples containing gelatin alone (Fig. 1a). Because type I collagen, the primary source of gelatin, has no disulfide bonds, the addition of DTT to gelatin was not expected to have a noticeable effect on gel strength and at concentrations below 6% gelatin, the addition of DTT did not impact the gel strength. At higher concentrations of gelatin (6–10%), however, gel strengths were significantly (P < 0.05) reduced by up to 15%. One possible explanation is the presence of type III collagen, which is generally co-located with type I collagen in connective tissue (van der Rest et al., 1990). The individual chains of type III collagen are disulfide-linked (Boudko and Engel, 2004), and separation of these chains may have interfered with the partial renaturation that normally accompanies gelation. Inclusion of mTGase in dilute (1–5%) gelatin solutions, either with or without DTT, resulted in gel strengths significantly (P < 0.05) lower than for gelatin alone (Fig. 1a). The formation of intramolecular crosslinks, which would inhibit the attainment of collagen-like structure, is favored over intermolecular ones in dilute solutions (Sakamoto et al., 1994). At higher (6–10%) gelatin concentrations where mTGase-mediated intermolecular crosslinking is more favorable, the resulting gel strengths were about 90% of those for gelatin alone.

When the effect of mTGase (1–10 U/g gelatin) was determined, no trend could be observed in the gel strength of a 10% gelatin (data not shown). The range of values was between 188 and 225 g, with an average gel strength of 207 ± 12 g, significantly (P < 0.05) lower than the 254 ± 1 g determined for 10% gelatin without enzyme. In the presence of the reducing agent, DTT, the activity of mTGase was enhanced, as reported by Kolodziejska et al. (2006), and gel strengths (263 ± 14 g) were comparable to those for gelatin alone.

The formation of mTGase-mediated WPI gels has been previously reported at WPI concentrations greater than 10% (Faergeemand et al., 1997). Under the conditions of these experiments, no gel formation was observed for WPI alone at concentrations up to 10%, with or without mTGase, under reducing or nonreducing conditions. Gels did form when small amounts of gelatin (0.5–3%) were included in 10% WPI solutions (Fig. 1b). Gelatin alone at these concentrations forms only very weak gels, and so long as the reaction conditions were not reducing, the gel strength was not significantly altered whether the gelatin was alone or in a 10% WPI solution, with or without mTGase at 2 U/g WPI-gelatin.

Under reducing conditions, a dramatic increase in gel strength was seen for mTGase-treated WPI–gelatin blends, the significance of which was confirmed by the second order statistical model (P < 0.05). Because reducing conditions and gelatin were essential for enhancing the gel strength, it is likely that crosslinking was between whey proteins and gelatin chains. The reduction of disulfide bonds in the globular whey proteins by the action of DTT would expose reactive groups to the action of the mTGase. In the absence of DTT, the WPI proteins were not sufficiently unfolded to serve as effective substrates for mTGase.

3.2. Viscosity

The viscosities at 60 °C, of gelatin solutions (1–10%), with and without DTT and mTGase, were determined (Fig. 2a). Neither DTT nor mTGase alone had a significant effect on gelatin viscosity at this temperature. Similar to the case for gel strength, the effect of mTGase under reducing conditions was not significant at gelatin
concentrations in the 1–6% range. Above 7% gelatin, the effect was a significantly dramatic increase \((P < 0.001)\) in viscosity to values greater than 1800 mPa s, beyond the limit of the viscometer. These results are consistent with the formation of intramolecular cross-links at low gelatin concentrations and both intra- and intermolecular crosslinks at higher concentrations (Clark and Courts, 1977; Yi et al., 2006).

The viscosities at 25 °C of solutions of WPI (1–10%) with and without DTT and mTGase were determined (Fig. 2b). At WPI concentrations less than 7%, neither DTT nor mTGase, alone or together, had any significant effect on the viscosity of the solution. Between 7% and 10% WPI, mTGase alone did not affect the viscosity (data not shown), and by inference had little ability to crosslink the proteins. In this concentration range, the viscosity was decreased slightly when disulfide bonds were reduced and the protein conformation was less well defined. A highly significant effect on the viscosity \((P < 0.001)\) was observed as a result of the treatment that combined the action of DTT and mTGase on a 10% WPI solution; a four-fold increase in viscosity was attained.

Viscosities measured at 25 °C for blends of gelatin (0.5–3%) with 10% WPI were always higher than those for gelatin alone (Fig. 2c). When mTGase was included in blends of 10% WPI with gelatin, the increase in observed viscosity was not significant. When both DTT and mTGase were included in the blend, their effect on viscosity became more significant \((P < 0.001)\). In fact, viscosities for blends of 10% WPI with more than 1.5% gelatin could not be measured because the solution formed a gel that did not melt below 60 °C.

### 3.3. Rheological properties

The storage modulus \((G')\) was determined for 10% WPI, blends of 10% WPI with 3% gelatin, and samples treated with mTGase under reducing or nonreducing conditions (Fig. 3). A 10% WPI solution, which remained liquid at 20 °C, exhibited a constant value less than 1 for \(G'\). In contrast, for a 10% WPI solution treated with mTGase under reducing conditions, \(G'\) remained nearly constant and low for the first 10 h and then increased to 300 Pa between 10 and 20 h, suggesting the formation of a stable and permanent gel structure (Comfort and Howell, 2002). Because \(G'\) can be related to the amount of crosslinking, the similar and flat response of the WPI sample in a reducing environment and the WPI sample with mTGase under nonreducing conditions suggest that no significant crosslinking occurred under these conditions. WPI–gelatin blends under reducing conditions without mTGase or with mTGase in a nonreducing environment showed a small increase in \(G'\) between 10 and 20 h, possibly due to noncovalent interactions between gelatin and whey protein molecules. Chen and Dickinson (1999) suggested that electrostatic interactions, hydrogen bonding or hydrophobic interactions that have a physical nature and are temperature-dependent might contribute to the appearance of weak gel-like behavior. A similar study of 3% gelatin samples with or without mTGase (2 U/g gelatin) produced a constant \(G'\) value near zero (data not shown). Blends of 10% WPI with 3% gelatin after

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**Fig. 2.** Viscosities of solutions of (a) gelatin, (b) WPI and (c) WPI–gelatin blends prepared as described for Fig. 1. Viscosities were measured at 60 °C for gelatin solutions and at 25 °C for WPI or WPI–gelatin blends. Abbreviations are as reported for Fig. 1.

**Fig. 3.** Time sweep analysis of 10% (w/w) WPI and WPI–gelatin blends. Blends contain 10% (w/w) WPI and 3% (w/w) gelatin, mTGase was 10 U/g protein for WPI and 2 U/g protein for WPI-gelatin blends, DTT was 10 mg/g protein. Abbreviations are as designated for Fig. 1.
incubation with mTGase (2 U/g protein) under reducing conditions showed an exponential increase of $G'$ from approximately 70 Pa to approximately 5000 Pa within the first 10 h. This remarkable gain suggests the existence of an initial gelled network that became more reticulated and stable with time (Eissa et al., 2004). Similar enhancements in storage modulus were reported for blends of gelatin with small amounts of chitosan, a larger polymer, and mTGase (Chen et al., 2003).

3.4. SDS–PAGE

Gelatin, WPI, and blended biopolymers were analyzed by SDS–PAGE. Ten percent gelatin (Fig. 4, lane 2) reflects a polydisperse mixture of different sized collagen fragments with some aggregated material in the stacking gel at the top, and faint bands visible through the general smear in the separating gel. Although more distinct bands can be seen in SDS–PAGE patterns for gelatins isolated from a specific source (Taylor et al., 1994) or high Bloom (250 g) commercial gelatins (Tosh et al., 2003), the pattern seen here is typical of low Bloom (115 g) commercial gelatins (Taylor et al., 2004), which are sold on the basis of gel strength, and are blends of gelatins from different preparations. The pattern for 10% gelatin treated with mTGase under reducing conditions (Fig. 4, lane 3) shows only a faint smear, representing small collagen fragments that most likely lack the lysine or glutamine sidechains necessary for mTGase-mediated crosslinking. Although samples prepared for lanes 2 and 3 were of the same weight, the faintness of the pattern in lane 3 and the lack of material in the stacking gel and the upper range of the separating gel suggests the formation of aggregates so large they could not penetrate the stacking gel (Taylor et al., 2004). A similar observation (Sharma et al., 2002) was interpreted to suggest that the protein had become so highly polymerized that it could not penetrate the stacking gel; they also demonstrated that although the highly polymerized products did not appear on the gels, their presence could be confirmed by chromatography. The SDS–PAGE patterns for 10% WPI alone (Fig. 4, lane 4) or after incubation with mTGase 5 U/g under nonreducing conditions (Fig. 4, lane 5) show the major whey proteins, $\beta$-lactoglobulin (MW approximately 18 kDa) and $\alpha$-lactalbumin (MW approximately 14 kDa) as well as faint bands for bovine serum albumin (MW approximately 66 kDa) and lactoferrin (MW approximately 86 kDa). Incubation of 10% WPI with mTGase 5 U/g under reducing conditions (Fig. 4, lane 6) resulted in bands, above both the separating gel and the stacking gel, showing formation of high molecular weight polymers in addition to the typical WPI bands. An earlier study (Færgemand et al., 1997) produced similar results.

The addition of 1.5% gelatin to 10% WPI had little effect on the gel pattern (Fig. 4, lane 7). In an earlier study, when small amounts of WPI were added to 10% gelatin samples (Taylor et al., 2006), the whey component was clearly visible in the gel patterns, and its participation in the formation of crosslinked biopolymers could easily be monitored. Here, the lack of defined molecular weight bands in patterns for gelatin and the low concentration of gelatin in the blends contribute to its invisibility. Incubation of the WPI–gelatin blend with mTGase under nonreducing conditions resulted in the appearance of a high molecular weight band, possibly representing crosslinked gelatin fragments, but had little effect on the WPI pattern (Fig. 4, lane 8). The gel pattern (Fig. 4, lane 9) for a similar sample incubated under reducing conditions shows a decrease in intensity of the WPI bands, an increase in high molecular weight aggregates and a decrease in total protein. The pattern is consistent with the formation of WPI–gelatin biopolymers in addition to aggregates of the individual biopolymers. Although it is clear from the gel strengths and rheological data that the inclusion of 1.5% gelatin in 10% WPI induces gel formation and enhances physical properties, the biopolymers formed range in size from those formed by WPI when treated with mTGase under reducing conditions to the much larger aggregates that do not penetrate the gel.

4. Conclusions

Both gelatin (ICIS, 2006) and whey (NASS, 2006) are relatively low value byproducts of the American food industry. While the actual prices vary over time the ratio is relatively constant at approximately 5:1 (gelatin:whey). The addition of minor amounts of relatively low quality gelatin to whey protein improves the strength and stability of gels formed by the action of mTGase in a reducing environment. As a byproduct of the meat industry, and a breakdown product of collagen, gelatin comes in a range of qualities. The higher quality gelatins find a variety of uses in food, pharmaceutical and industrial products and are heavily traded. Whey is a lower value byproduct that can be recovered from the waste stream of the cheese industry. The use of mTGase to catalyze the formation of crosslinks in and between protein molecules is well established, as is the efficacy of its usefulness with either gelatin or WPI. When a small amount of gelatin was added to WPI, before mTGase treatment under reducing conditions, a dramatic rise in viscosity, higher gel strengths, and the appearance of high molecular weight bands due to inter-protein crosslinking in SDS–PAGE gel patterns than for either gelatin or WPI treated separately were observed. These results suggest that the reducing environment partially unfolds the whey proteins, increasing access to glutamine and lysine side chains, and that the gelatin chains crosslink the whey proteins to form a network. The improvement in physical properties over either protein component, given the same treatment, suggests the possibility of greater utilization and new products from these byproducts.

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References


