JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 3723−3730 **3723**

Immobilization and Utilization of the Recombinant Fusion Proteins Trypsin−Streptavidin and Streptavidin−Transglutaminase for Modification of Whey Protein Isolate Functionality

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A method was developed for the production of a hydrolyzed/polymerized whey protein derivative with altered solution and gelation properties using a combination of recombinant DNA and immobilized enzyme technologies. The recombinant fusion proteins trypsin−streptavidin (TrypSA) and streptavidin−transglutaminase (cSAcTG) were produced in *Escherichia coli*, extracted, and then immobilized by selective adsorption on biotinylated controlled-pore glass. Recirculation through a TrypSA reactor induced limited proteolysis of whey proteins. Hydrolysates were then recirculated through a cSAcTG reactor for incremental periods of time to arrive at increasing degrees of polymerization. The polymers were subsequently analyzed for viscosity/flow behavior, gelation properties, and fracture properties using shear rate ramps/intrinsic viscosity, small-strain oscillatory rheology, and vane viscometry, respectively. By combining limited proteolysis with controlled cross-linking, it was possible to create derivatives of whey proteins with enhanced functional properties. Increases in the degree of whey protein modification were correlated with greater apparent viscosity and intrinsic viscosity, lowered gel point temperatures, and stronger, more brittle gels. This method allowed for recycling of the enzyme, eliminated the requirement for a downstream inactivation step, and permitted control over the extent of modification. Utilization of a similar process may allow for the production of designer proteins engineered with specific functionalities.

KEYWORDS: Immobilization; trypsin; transglutaminase; whey protein; functionality

INTRODUCTION

The desire of the food industry to convert waste products into value-added commodities has inspired a growing interest in the development of processes for the enhancement of whey protein functionality (*1*, *2*). Whey has most commonly been used as a protein supplement, thickening agent, emulsifying agent, gelling agent, foam stabilizer, and filler/water binder. The modification of whey proteins to improve their functional properties in specific food systems has become a focus of current research. Whey proteins have been both chemically and physically treated by a number of methods that include acidification, heating, enzymatic cleavage, and cross-linking, all in the presence or absence of different salts (*3*−*12*). Treatments can enhance the ability of the proteins to form gels or give them better water-holding or foaming properties (*13*, *14*).

Transglutaminase is a transferase that forms isopeptide bonds between lysyl and glutaminyl residues. The mammalian tissue transglutaminase has been extensively characterized (*15*−*24*). Trypsin is a proteinase specific for the hydrolysis of peptide bonds that follow the amino acids lysine and arginine and has also been well-characterized (*25*−*29*).

The avidin−biotin interaction is one of the strongest noncovalent associations known, having a K_d of $\sim 10^{-15}$ M (*30*). This interaction is stable to changes in pH and ionic strength of fluid streams (*21*, *31*). By using streptavidin as the affinity domain in a fusion protein, it is possible to bioselectively adsorb the enzyme of interest to the biotinylated matrix (*32*−*38*). A similar approach has been used by Le et al. (*39*), in which a cellulose-binding domain was fused with streptavidin to immobilize biotinylated alkaline phosphatase.

Our laboratory has developed a system by which enzymes can be simultaneously purified and immobilized, decreasing the cost of purification and eliminating the requirement for a downstream enzyme inactivation treatment (*33*, *36*, *38*, *40*). In addition, the extent of the reactions can be controlled, and the enzyme can be reused when properly stored. Using affinity adsorption, the support can be easily regenerated, further

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10.1021/jf011603c CCC: $22.00
Published on Web 05/21/2002

lowering the costs associated with the process (*34*). In this paper, we report the development of a method to modify the viscous and elastic properties of WPI using immobilized forms of recombinant trypsin and transglutaminase. These two enzymes were chosen for the fusion constructs on the basis of their potential for use in food systems.

MATERIALS AND METHODS

Materials. Controlled-pore glass (CPG) was obtained from CPG Inc. Whey protein isolate (WPI) was purchased from Davisco International (Minneapolis, MN). Immunopure avidin, biotinylation reagents, and BCA protein assay reagents were purchased from Pierce Chemical Co. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. *Derivatization of the Matrix.* After firing in a muffle furnace (600 °C) overnight and cleaning with concentrated nitric acid for 2 h at 95 °C, CPG beads (CPG, Inc.) were derivatized by addition to a 10-fold excess of 10% 3-(aminopropyl)triethoxysilane, pH 4.0. The beads were held at 70 °C for 3 h, after which time they were dried at 110 °C overnight.

Biotinylation of the Matrix. The derivatized beads were added to a 10-fold excess of precooled biotinylation reagent composed of 0.5 mg/mL sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC biotin) in 50 mM bicarbonate, pH 8.5. The reagent was recirculated over the beads overnight (12 h) and washed extensively with 50 mM phosphate buffer, pH 6.0. The beads were stored in the wash buffer containing 0.02% sodium azide.

Biotin Concentration. The biotin concentration was checked to ensure that adequate sites were present. A 25-μL aliquot of 4-hydroxyazobenzene-2′-carboxylic acid (HABA) was mixed with 1.0 mL of avidin solution in a 1-mL cuvette. The absorbance was measured at 500 nm. Fifty microliters of biotinylated beads was added to the solution and mixed. Five minutes was allowed for the beads to settle before the reading was taken at 500 nm. The difference between the first and second readings is a result of the displacement of HABA from the avidin by the immobilized biotin. The immobilized biotin concentration is given by (*41*)

$$\mu\text{mol of biotin/mL of beads} = [((A_1 - A_2) \times 1.025)/34] \times \frac{1}{0.05 \text{ mL of beads assayed}}$$

where 34 is the millimolar absorptivity of the avidin−HABA complex.

Cloning and Construction of Plasmids (Trypsin−Streptavidin). Full-length streptavidin cDNA was isolated from the plasmid pStp4 described by Walsh and Swaisgood (*33*). The gene for rat anionic trypsin was isolated from a plasmid kindly donated by Dr. Craik at the University of California, San Francisco (*25*). A detailed description of the fusion construct design has been published (*40*).

Core Streptavidin−Core Transglutaminase. The streptavidin gene was originally excised from the plasmid pStp4 (*33*). The region containing amino acid residues 14−136 was incorporated into the pET26b vector (Novagen). Such "core" streptavidin retains its high-affinity biotin-binding domain (*42*). The human tissue transglutaminase gene was a gift from Dr. Davies at the University of Texas (*23*). The gene, excised from the plasmid provided, was ligated to the 3′-end of streptavidin in the pET26b vector. The resulting plasmid was used to transform *Escherichia coli* BL21 cells. For the core transglutaminase construct, the upstream, noncoding sequence was removed to yield the mature protein (*43*). This construct is also currently being expressed in the BL21−pLysS system.

Trypsin−Streptavidin Expression. A detailed description of the expression and extraction protocols has been published (*40*).

Core Streptavidin−Core Transglutaminase Expression. Freshly streaked LB-agar plates containing kanamycin (60 μg/mL) and chloramphenicol (35 μg/mL) were incubated at 37 °C overnight. Four liters of autoclaved LB-medium containing the appropriate antibiotics was prepared for growing cultures. Each of four flasks containing 1 L of medium was inoculated with a colony from the plates. The cultures were grown at 30 °C with constant shaking for ~8 h or until the OD_{660} reached 0.4, at which time IPTG was added to give a final concentration of 1 mM. Induction proceeded for 2 h. The cells were pelleted by centrifugation at 4 °C and 7000 rpm using a Sorvall centrifuge. The supernatant was discarded, and the cells were resuspended in 100 mL of 50 mM Tris, pH 8.0, containing 1 mM dithiothreitol (DTT) and 0.5 mg of lysozyme/100 mL of cell culture.

The suspension was incubated at room temperature for 10 min and placed on ice for 30 min prior to sonication for 10 s. The sample was again chilled on ice for 10 min followed by sonication for 10 s. Centrifugation at 18000 rpm for 2 h at 4 °C pelleted insoluble material. The soluble proteins were removed and dialyzed against the sonication buffer excluding lysozyme. The remaining insoluble protein was partially solubilized with 2 M GdnHCl overnight. The resultant suspension was centrifuged at 15000 rpm for 30 min at 4 °C. The supernatant was dialyzed against the 1000 volumes of buffer and stored at 4 °C until needed.

Immobilization of pTrypSA Fusion Protein on Biotinylaminopropyl CPG Beads. The 2 and 4 M GdnHCl-solubilized fractions containing most, if not all, of the trypsin activity were recirculated through the biotinylated beads overnight (*40*). The beads were washed and equilibrated with Tris buffer, pH 3.5, until needed.

Purification of cSAcTG Fusion Protein Using GTP-Agarose. Dialyzed protein fractions from the sonication and GdnHCl extractions were pooled and applied to a column containing 5 mL of cross-linked GTP-agarose (*43*) that had been equilibrated with binding buffer (50 mM Tris, pH 8.0, containing 1 mM DTT, and 0.1% P-9-L). The matrix was washed with 6 column volumes of binding buffer, followed by elution with 100 mM $CaCl_2$ in the same buffer. Fractions (5 mL) were collected during application and elution for activity and protein assays.

Immobilization of cSAcTG Fusion Protein on Biotinylamidopropyl CPG Beads. GdnHCl-solubilized fractions that tested positive for transglutaminase by Western analysis were pooled and recirculated overnight (12 h) through an affinity column containing 7 mL of biotinylated CPG beads that had been equilibrated with 2 M GdnHCl in 50 mM Tris, pH 8.0. The column was washed extensively with the buffer, followed by a wash with 1 M GdnHCl in 50 mM Tris, pH 8.0, containing 10 mM $CaCl_2$ and then with 50 mM Tris, pH 8.0, containing 10 mM $CaCl_2$ and 1 mM DTT.

Transglutaminase Activity. Transglutaminase activity measurements were based on the methods described by Folk and Chung (*15*). The reaction buffer contained the following reagents: 0.1−0.2 mL of 50 mM Tris, pH 8.0, 0.150 mL of H_2O, 75 μL of 0.2 M CBZ glutaminyl-glycine, 25 μL of 2.0 M hydroxylamine, 25 μL of 100 mM $CaCl_2$, 25 μL of DTT, and 0.01−0.1 mL of sample or beads to give a final volume of 0.5 mL. The mixture was incubated at 37 °C for 1−6 h, and 0.5 mL of an equal mixture of 5% $FeCl_3$, 2.5 M HCl, and 15% TCA was added to each tube. A_{525} absorbances of samples were measured and compared to those of the negative and positive controls. Negative controls used buffer in place of sample, whereas positive controls used various quantities of microbial or guinea pig liver transglutaminase.

Trypsin Activity. Trypsin activity was determined at pH 8.1 using 0.01 M *p*-toluenesulfonyl-L-arginine methyl ester (TAME) as the substrate as described in the Worthington manual (*44*).

Protein Assay. The bicinchoninic acid protein assay was used with bovine serum albumin as the protein standard (Pierce Chemical Co.) for determination of soluble protein. The OPA method developed in our laboratory was utilized for determination of protein bound to CPG beads (*45*). In addition, the OPA method according to Church et al. (*46*) was used to determine available amino groups in solution.

SDS-PAGE. All electrophoreses were performed using the Novex NuPAGE Bis-Tris gel system and standard SDS-PAGE protocols utilizing 4−12% gradient gels.

Western Analysis. Antibodies to both transglutaminase and streptavidin were used to differentiate target proteins in the SDS-PAGE gels. Standard protocols were followed according to the method of Maniatis et al. (*47*).

Whey Protein Treatment. WPI was BiPRO obtained from Davisco International and determined to consist of 93.2% protein. The protein, mineral, and moisture contents of the powder were taken into consideration when 10% protein solutions were prepared in distilled, deionized H_2O containing 10 mM DTT and 5 mM $CaCl_2$, pH 6.8. The

solutions were allowed to equilibrate overnight prior to the addition of the salt and reducing agent and prior to any analysis.

Limited Proteolysis. A 1-L sample of 10% WPI solution was introduced into a large glass beaker containing 1 mL of TrypSA−CPG beads. The WPI was then stirred with a suspended stirring apparatus in a constant-temperature water bath at 40 °C for 1 h. The treatments were in batch format. Between treatments, the matrix was washed with 10 bed volumes of equilibration buffer (10 mM DTT, 5 mM $CaCl_2$, pH 6.8).

Limited Cross-Linking. The TrypSA-hydrolyzed WPI solution was introduced into a large beaker containing 1 mL of cSAcTG−CPG beads equilibrated with H_2O containing 10 mM DTT and 5 mM $CaCl_2$, pH 6.8. The WPI was then stirred in a constant-temperature water bath at 40 °C for preset times of 1, 2, 4, and 8 h. The treatments were in batch format with 100-mL aliquots removed at the appropriate times. Between each treatment, the matrix was washed with 10 bed volumes of equilibration buffer.

Intrinsic Viscosity. Intrinsic viscosities were determined using a Cannon−Fenske capillary viscometer immersed in water maintained at 25 °C. Protein concentrations of 5, 10, and 15 mg/mL were prepared and analyzed in triplicate for each treatment. Specific viscosity (η_{sp}) was calculated from

$$\eta_{sp} = (t - t_0)/t_0$$

where t_0 = the efflux time of water and t = the efflux time of the sample. Using the Huggins equation that states $\eta_{sp}/c = [\eta] + k[\eta]^2c$, where c is defined as the concentration of protein (g/mL), intrinsic viscosity $[\eta]$ was determined by extrapolation of the plot of η_{sp}/c versus c (*48*). Turbidity at 600 nm was determined for samples (2 mg/mL) in triplicate.

Shear Rate Ramps. Viscosities (Pa s) of treated samples were determined at various shear rates using a bob and cup attachment on a Reologica Stress Tech rheometer at 25 °C. Measurements were taken in triplicate at shear rates of 25, 50, and 100 s^{-1}. The flow behavior was calculated using the power law-based model

$$\sigma = k\gamma^n \quad \text{or} \quad \eta = k\gamma^{n-1}$$

where σ = shear stress, η = apparent viscosity, γ = shear rate, n = the flow behavior index, and k = the consistency coefficient. Shear rate ramps were performed on the StressTech to generate stress versus shear rate curves. Curves were used to determine the area of hysteresis. The area was calculated using a compensating polar planimeter.

Small Strain. The StressTech rheometer utilizing the bob and cup attachment was used for oscillatory analysis measurements. Approximately 12 mL of native or treated WPI solution was loaded into the cup. Temperature ramps, frequency sweeps, and stress sweeps were carried out under the following conditions.

(a) Temperature Ramps. Temperature was increased at 0.5 °C/min from 25 to 85 °C while a constant frequency of oscillation of 0.1 Hz was maintained. The shear stress was also kept constant at 1.0 Pa. G' (storage modulus), G'' (loss modulus), G^*, V^* (apparent viscosity), and Δ (phase degree) were monitored to note changes in gelation behavior and gel characteristics.

(b) Frequency Sweeps. Frequency sweeps were performed in the linear viscoelastic range. In other words, the gel acted independently of frequency. During frequency sweeps, the stress was held constant while the frequency was increased from 0.01 to 20 Hz. The same parameters were monitored as before during the temperature ramps.

(c) Vane Analysis. Gels were formed by holding the protein solutions at 85 °C for 1 h in a forced-air oven. Yield stress analysis was performed on formed gels using a vane attachment on the Haake VT 550 viscometer operating at 0.5 rpm and 25 °C. The vane dimensions were 10 mm high and 5 mm in diameter. Time and peak torque at fracture were recorded in triplicate. Stress and vane rotation (strain) at fracture were calculated according to the method of Daubert et al. (*49*).

RESULTS AND DISCUSSION

The pTrypSA fusion protein was located in cell-free extracts and in the GdnHCl-solubilized fraction as identified by both trypsin activity and binding of anti-streptavidin antibodies (*40*). The crude protein solutions were passed through biotinylated CPG beads, resulting in selective adsorption of TrypSA yielding a biocatalyst with an activity of 0.516 unit/mL of beads, or ~1.5 units/g of beads.

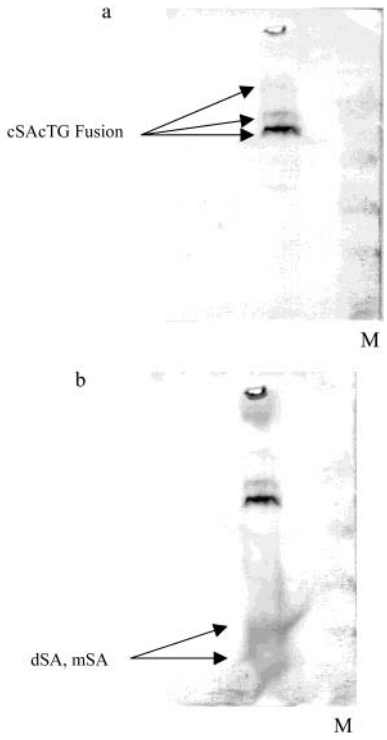

Figure 1. Electrophoresis of 2 M GdnHCl-solubilized cell extracts: (a) Western blot using antibodies to transglutaminase (Attention is directed to positive bands in the center region of the gel. For perspective, the prominent positive band falls between the 54 and 98 kDa marker proteins.); (b) Western blot using both antibodies to transglutaminase and streptavidin (Attention is directed to the positive bands located at approximately 11 and 21 kDa, believed to be monomer and dimer streptavidin, respectively). M represents molecular mass markers from bottom (11, 20, 32, 54, and 98 kDa).

The cSAcTG fusion protein was expressed at modest levels. The only viable method to identify cSAcTG after extraction was GdnHCl solubilization followed by Western analysis (**Figure 1**). The method did not allow for accurate quantitation and did not account for unrecovered, insoluble protein. Therefore, estimates of fusion protein expression had to be made on the basis of the amount of protein immobilized to the affinity matrix after extraction. In that case, ~0.98 mg of protein/mL of beads was recovered. Considering that 4 L of cells was grown, 7 mL of beads was used, and recovery was optimistically ~50%, we concluded that ~3.5 mg of fusion protein was expressed per liter of cell culture. An argument could be made for higher expression levels, considering that the recovery may have been much lower than 50%. However, because streptavidin was observed upon desorption with 6 M GdnHCl and Western analysis (**Figure 2**), it is possible that the immobilized protein concentration included a fair amount of free streptavidin in place of fusion protein. The overall conclusion was that we had milligrams per liter levels of expression. There was no activity detected in any of the soluble cell extracts. In addition, attempts to purify the fusion using GTP-agarose were unsuccessful. Upon immobilization using a biotinylated matrix, the cSAcTG fusion protein exhibited activity. There was an average of 0.011 unit/mL of beads and 0.012 unit/mg of protein. Activity was

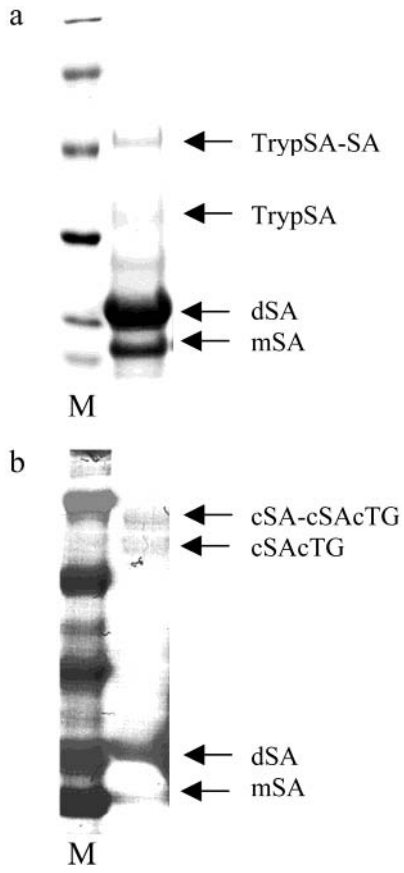

Figure 2. SDS-PAGE of fusion proteins desorbed with 6 M GdnHCl: (a) fusion protein that had been immobilized to CPG, demonstrating the presence of TrypSA of correct size and also excess streptavidin; (b) cSAcTG fusions that had been immobilized, excess streptavidin is also present. M represents molecular mass markers from bottom (11, 20, 32, 54, 98, and 150 kDa).

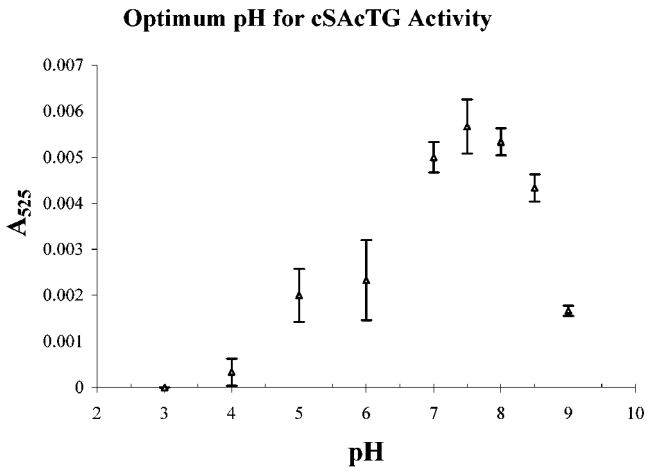

Figure 3. Activity profile for immobilized cSAcTG over the pH range of 3–9. Points plotted are the average of three replicates, demonstrating a relatively broad optimum between 7 and 8.5. Error bars represent the standard error.

Table 1. OPA Measurement of Available NH_2 after Treatment with Immobilized Fusion Enzymes

treatment	$[NH_2]$ (μM)	mol of NH_2/mol of protein[a,b]
none	74.0	12.6
1 h, TrypSA	74.2	12.6
1 h, cSAcTG	51.0	8.7
2 h, cSAcTG	49.1	8.3
4 h, cSAcTG	47.3	8.0

[a] Based on an average molecular mass of 17000 Da. [b] In all cases, the standard error was <0.05.

calculated from the millimoles of hydroxamate formed per minute. The activity profile over a wide pH range is shown in **Figure 3**. The profile demonstrates that the immobilized cSAcTG had optimal activity around pH 8.0. However, relatively high activity was also observed at pH 7.0, in the range of the present study. These values are consistent with those reported previously for soluble mammalian transglutaminase (*23*).

WPI treatments to obtain significant cross-linking were performed in the presence of a reducing agent (DTT) that is believed to cause partial unfolding of the substrate proteins and increase enzyme access to glutaminyl and NH_2 groups (*50*). Previous work in our laboratory (paper submitted) showed the requirement of DTT for cross-linking of WPI by an immobilized form of microbial transglutaminase (mTgase). DTT had the added benefit of maximizing mTgase activity by maintaining the active site sulfhydryl in the reduced state. As a result, overall reaction rates increased by 10−25% (data not shown). The effect of DTT on trypsin activity was determined to be minimal, most likely because of the internal location of disulfides and low concentration of reducing agent used. Calcium levels were chosen such that enzyme activity was maximized but the salt-induced effects on the WPI network were minimized.

Good stability of the immobilized fusion enzymes was observed in the absence of concentrated protein substrates. The enzymes lost no activity over several months of storage. However, the activity decreased significantly after treatment of concentrated solutions of WPI. One milliliter of active beads used to treat 1 L of 10% WPI consistently lost between 50 and 75% of its activity, even after washing with appropriate regeneration agents. This phenomenon was not observed when 1% WPI solutions were treated in earlier work. It is believed that the high protein concentration resulted in clogging of the pores and/or formation of a monolayer of protein on the bead surface, limiting diffusion into the pores. This caused the irreversible inactivation of a significant percentage of the enzyme.

Proteolysis by the TrypSA fusion protein was not apparent using electrophoresis or OPA determination of free NH_2 groups (**Table 1**), due to the low percentage of hydrolysis that occurred (<1%). However, significant changes in the solution characteristics were apparent after 1 h. These changes were not observed when WPI solutions were incubated in the presence of beads void of enzyme, demonstrating that hydrolysis was indeed affecting the observed changes. The significant increase in viscosity and aggregate size that were observed after such a minimal degree of hydrolysis deserves some discussion. Previous work has demonstrated that much higher levels of hydrolysis were required to observe such effects. However, no one has duplicated our conditions. For example, Huang et al. (*28*, *51*) obtained hydrolysis levels that resulted in a decrease of >30% of the intact protein, but treated only 1% protein solutions. Chen et al. (*52*) used trypsin to hydrolyze 35% of the native β-lactoglobulin in a 7% solution. Other research has shown that limited hydrolysis with trypsin actually decreased WPI gelation and increased solubility, but not in the presence of DTT (*53*−*55*). It is our belief that the DTT had a multiplicative effect on network formation and aggregation. In the presence of DTT, the whey proteins partially unfolded due to reduction of disulfide bonds. In addition, the unfolding increased protease access to

Table 2. Solution Characteristics of 10% WPI Modified Using Immobilized Fusion Enzymes

treatment	intrinsic viscosity[b] (mL/g)	viscosity (Pa s) at various shear rates[a] (1/s) 25	50	100	hysteresis[c] (mm^2)	turbidity[d] (OD_{600})
none	0.6 ± 0.1	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.0 ± 0.0	0.22 ± 0.01
TrypSA (1 h)	22.1 ± 1.1	0.130 ± 0.007	0.080 ± 0.004	0.050 ± 0.002	5.7 ± 1.2	0.40 ± 0.00
cSAcTG (1 h)	36.5 ± 0.2	0.251 ± 0.005	0.149 ± 0.001	0.086 ± 0.000	22.9 ± 3.9	0.43 ± 0.02
cSAcTG (2 h)	50.7 ± 0.9	0.515 ± 0.011	0.313 ± 0.004	0.175 ± 0.001	38.6 ± 2.1	0.48 ± 0.02
cSAcTG (4 h)	81.1 ± 1.4	0.575 ± 0.010	0.333 ± 0.003	0.179 ± 0.001	45.2 ± 3.5	0.55 ± 0.01

[a] ± Standard error. [b] Based on extrapolation of η_{sp}/c vs c from the Huggins equation. [c] Area between up an down stress vs shear rate curves, measured using a planimeter. [d] Measured at 0.2% protein.

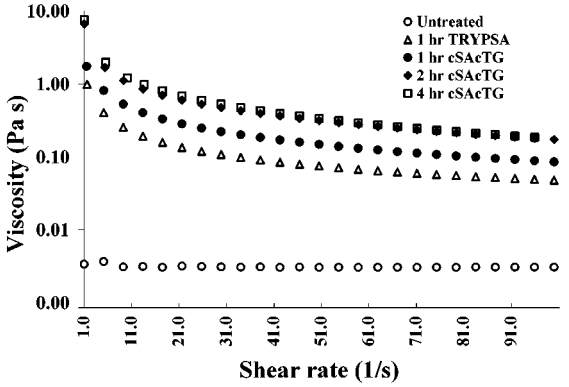

Figure 4. Shear rate effects on apparent viscosity for native and fusion enzyme-treated WPI solutions.

internal cleavage sites. A limited amount of hydrolysis therefore produced domains that quickly associated at exposed hydrophobic regions. The presence of calcium enhanced the aggregation by ion bridging and electrostatic shielding as discussed by Ju and Kilara (*8*). It should be noted once again that whey proteins showed no change when incubated with nonactive beads under these conditions.

Cross-linking by cSAcTG was apparent using the OPA analysis, which verified that as incubation time increased, available NH_2 groups decreased (**Table 1**). Intrinsic viscosity (η) and viscosity under shear (Pa s) were used to determine the flow behavior of the proteolyzed and cross-linked WPI prior to heating and gel formation. Intrinsic viscosity is a measure of the molecular size, or hydrodynamic volume, and shape of each biopolymer in solution. In general, the larger and/or less spherical a biopolymer becomes, the greater its intrinsic viscosity. In this study, an increase in intrinsic viscosity for solutions that had been treated for longer times (**Table 2**) indicated that on average, larger, less spherical biopolymers were being formed. The viscosities at shear rates of 25, 50, and 100 s^{-1} were determined on the StressTech to demonstrate the shear-thinning (**Figure 4**) and hysteresis behavior of treated WPI. As seen in **Table 2**, higher shear rates led to decreased apparent viscosity, whereas increased treatment times resulted in higher observed viscosities. In addition, longer treatment times brought about an increase in the area of hysteresis, calculated from stress versus shear rate curves. The increase indicated that a more extensive network was being formed over time, which was disrupted when exposed to shearing conditions. It is believed that the increased viscosity of hydrolysates can be attributed to protease-induced aggregation of proteins due to exposure of otherwise buried hydrophobic regions. Also shown in **Table 2** is the trend toward increased turbidity as treatment time was increased, which was caused by increased light scattering due to increased aggregate size after treatment.

Table 3. Flow Behavior of 10% WPI Treated with Immobilized Fusion Enzymes

treatment	consistency coefficient, k[a]	flow behavior index, n[a]
none	0.01 ± 0.00	0.89 ± 0.05
1 h, TrypSA	1.51 ± 0.14	0.28 ± 0.01
1 h, cSAcTG	2.77 ± 0.23	0.26 ± 0.02
2 h, cSAcTG	6.25 ± 0.30	0.23 ± 0.01
4 h, cSAcTG	6.29 ± 0.03	0.24 ± 0.01

[a] Mean ± standard error.

Table 4. Gelation Temperatures of 10% WPI Treated with Immobilized Fusion Enzymes

treatment	gelation temp[a] (°C)
none	70.33 ± 0.03
1 h, TrypSA	66.67 ± 0.03
1 h, cSAcTG[b]	66.33 ± 0.03
2 h, cSAcTG	66.17 ± 0.03
4 h, cSAcTG	65.93 ± 0.03
8 h, cSAcTG	N/A[c]

[a] ± standard error. [b] All cSAcTG treatments had first been treated with TrypSA for 1 h. [c] Gel formation occurred prior to heating.

The flow behavior index (n) and consistency coefficient (k) were also obtained from the stress versus shear rate curves. It should be noted that when $n = 1$, solutions are considered to be Newtonian. As n approaches 0, the solution becomes less and less Newtonian. The consistency coefficient is directly correlated with viscosity. In **Table 3**, it is apparent that increases in the degree of WPI modification, whether achieved by proteolysis or cross-linking, were correlated with a steady progression toward non-Newtonian properties and increased consistency coefficient (viscosity).

Small-strain oscillatory rheology in the form of temperature ramps with a bob and cup attachment was performed on modified WPI to avoid disruption of networks formed upon heating (*56*). Gel point temperatures were recorded from the intersection of G' and G'' (**Table 4**). Once again, a trend was observed that was directly related to treatment time. Gel point temperatures were lower for samples that had been hydrolyzed and cross-linked. A representative graph demonstrating the modified proteins' response to temperature is depicted in **Figure 5**. This observation was expected because less heat would be required to form a network that was already partially formed due to aggregation and/or limited cross-linking.

Fracture properties of the gelled samples were obtained by recording time and torque at fracture using a vane technique (*49*). Samples that had been hydrolyzed were much more brittle than the native protein gels. The enzymatically cross-linked gels

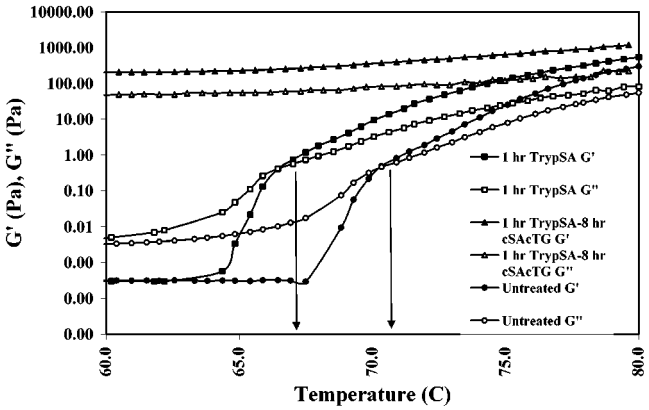

Figure 5. Small-strain temperature ramps: gelation characteristics of WPI treated with the recombinant fusion enzymes TrypSA and cSAcTG.

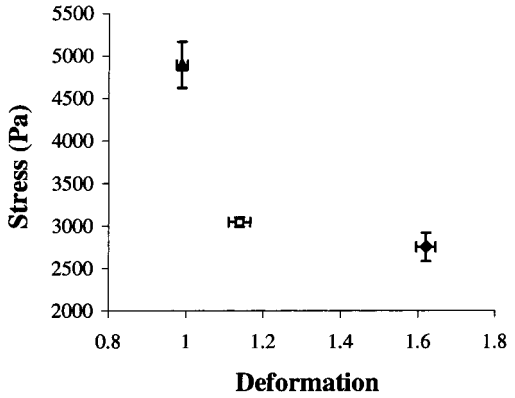

Figure 6. Vane fracture analysis of 10% WPI gels that had been treated with the immobilized fusion enzymes TrypSA and cSAcTG: (◆) 10% protein untreated; (□) 10% protein treated for 1 h with TrypSA; (▲) 10% protein treated for 1 h with TrypSA followed by 1 h with cSAcTG. Values were derived from measuring time and torque at the point of fracture. Error bars were calculated from the standard error of three replicates.

had higher torque (stress) values than the untreated WPI. Also, the time of fracture (strain) decreased as cross-linking increased (**Figure 6**). In general, more cross-linking resulted in stronger, more brittle gels. It should be noted that greater modifications resulted in more opaque, particulate gels, whereas fine-stranded gels were formed by native WPI.

The focus of this study was to examine the possibility of designing protein functionality by limited cross-linking with immobilized transglutaminase, which required breaking the disulfide bonds of the whey proteins for maximal activity. This was achieved by reduction with DTT; however, this can be accomplished by various means. For example, in a companion study (unpublished results), we have obtained similar results by cleavage of the disulfides by treatment of WPI with sodium bisulfite prior to cross-linking with immobilized microbial transglutaminase.

CONCLUSIONS

Limited enzymatic treatment (proteolysis or cross-linking) of whey proteins has been shown, under the appropriate conditions, to enhance existing functional properties. A decrease in the gel point temperature, stronger or more elastic gels, and changes in the infrastructure of the gel network may be viewed as enhancements depending on the application. Using this immobilized enzyme system eliminated the need for a heat inactivation step, avoiding any detrimental effects such as loss of desired functionality and production of off-flavors and odors. Also, because significant changes were seen with only a limited degree of modification, the control afforded by immobilized enzymes makes them highly desirable in such applications. Future work must include manipulation of substrate concentrations/conditions to avoid potential problems associated with scaling-up of the process. In addition, utilization of enzymes from extremophiles should be considered, as they offer greater stability under food-processing conditions.

ABBREVIATIONS USED

NHS-LC biotin, succinimidyl 6-(biotinamido)hexanoate; HABA, 2-(hydroxyazobenzene-4′-carboxylic acid); OPA, *o*-phthaldialdehyde; DTT, dithiothreitol; MES, 2-(*N*-morpholino)-ethanesulfonic acid; CBZ, carbobenzyloxy; IPTG, isopropyl β-D-thiogalactopyranoside; WPI, whey protein isolate.

ACKNOWLEDGMENT

We thank Dr. Chris Daubert and Dr. Allen Foegeding for their suggestions in the preparation of this paper. We are grateful to the Food Rheology Laboratory at NCSU, especially Heather Hudson, for assistance through the course of this work.

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Received for review December 5, 2001. Revised manuscript received April 17, 2002. Accepted April 17, 2002. Paper FSR 01 of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of products named or criticism of similar ones not mentioned.

JF011603C