JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Modification of the Rheological Properties of Whey Protein Isolate through the Use of an Immobilized Microbial Transglutaminase

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A process was developed in which calcium-independent, microbial transglutaminase (mTgase) was immobilized to controlled-pore glass. Avidin was adsorbed to glass beads that had been derivatized and biotinylated. The enzyme was biotinylated and adsorbed to the avidin affinity matrix. Solutions of 8% whey protein isolate (WPI) were then incubated with the mTgase beads, resulting in limited cross-linking of whey proteins. As incubation time increased, intrinsic viscosity increased, gelation temperature decreased, and stronger, more brittle gels were formed upon heating. This process allowed for recycling of the enzyme, eliminated the requirement for a downstream inactivation step, and permitted control over the extent of cross-linking. The functional properties of several batches of WPI were modified using $<$10 mg of the same enzyme, illustrating the capacity of immobilized enzymes to be used more frequently in applications of this nature.

KEYWORDS: Immobilization; transglutaminase; viscosity; whey protein isolate; gelation; cross-linking

INTRODUCTION

The desire of the food industry to convert waste products into value-added, high-priced 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*−*10*). Treatments can enhance the ability of the proteins to form gels or give them better water-holding or foaming properties (*11*, *12*).

Transglutaminase is a transferase that forms isopeptide bonds between lysyl and glutaminyl residues. The mammalian tissue transglutaminase has been extensively characterized (*13*−*16*) but is limited in its usefulness due to its high cost of extraction and calcium dependency. Microbially produced transglutaminase (mTgase) is distinct from its mammalian counterpart in that it is smaller, more stable, and calcium-independent (*17*). Therefore, its use in food systems has become of great interest. mTgase has been used to modify a number of proteins for improvement of functionality (*18*, *19*). In this paper, we report the development of a method to modify the viscous and elastic properties of whey protein isolate (WPI) using an immobilized form of the enzyme. The method allowed for easy separation of catalyst and substrate and eliminated any requirements for a downstream inactivation step.

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MATERIALS AND METHODS

Materials. Controlled-pore glass was purchased from CPG Inc. (Lincoln Park, NJ). Celite was purchased from Manville Inc. (Lompac, CA). WPI was obtained from Davisco (Minneapolis, MN). Immunopure avidin, biotinylation reagents, and the BCA protein assay reagents were purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. *Matrix Preparation.* Five milliliters of controlled-pore glass beads (200 nm mean pore diameter) were cleaned with nitric acid, aminopropylated with 10% 3-aminopropyltriethoxysilane, pH 4.0, and biotinylated with 0.5 mg/mL NHS-LC-biotin reagent. Biotin concentration was measured by displacement of HABA from an avidin−HABA complex by biotin as described by Swaisgood et al. (*20*). Avidin was adsorbed to the biotinylated matrix by overnight recirculation of 1 mg/mL avidin in 50 mM phosphate, pH 6.0, containing 0.9% NaCl.

Biotin-Binding Sites. Determination of biotin binding was performed by incubating 100 μL of beads with 500 μL of 0.5 mM biotin in 50 mM sodium phosphate, pH 6.0, containing 0.9% NaCl, for 5 min. Twenty-five microliters of the supernatant was added to 1.025 mL of an HABA−avidin complex formed by combining 1 mL of 0.5 mg/mL avidin in 50 mM phosphate buffer, pH 6.0, and 0.9% NaCl with 0.025 mL of 10 mM HABA in 10 mM NaOH. The presence of biotin displaces HABA, which exhibits an absorption at 500 nm when complexed with avidin. Therefore, biotin can be measured by the decrease in absorbance at 500 nm (*21*).

Biotinylation of mTgase. NHS-LC biotin (2 mg/mL) was prepared in 50 mM bicarbonate, pH 6.0. Enzyme solution was dialyzed against

10.1021/jf0117154 CCC: $22.00 © 2002 American Chemical Society
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the same buffer prior to biotinylation. A 10 mg/mL sample of transglutaminase was mixed with 2 mg/mL of NHS-LC biotin in 50 mM bicarbonate, pH 8.5, and allowed to react overnight. Excess reagent was removed by dialysis against 50 mM phosphate, pH 6.0. Biotinylated enzyme was recirculated over the prepared beads for 12 h. The beads were washed with 1 M urea and 50 mM phoshate, pH 6.0.

Determination of Free NH_2. The relative concentration of free amino groups was used to demonstrate cross-linking. A modified OPA procedure was used, according to the method of Church et al. (*22*). Estimation of moles of NH_2 per mole of protein was based on an estimated average molecular weight of 17000 Da for whey protein.

Covalent Attachment on Controlled Pore Glass or Celite. mTgase was also attached covalently to CPG and Celite. Aminopropylated CPG or Celite was treated with 1% triethylamine in acetone, containing 10% succinic anhydride. The beads were frequently degassed to ensure that all of the pore surfaces were exposed. The beads were activated by placing them in a solution of freshly prepared 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (EDC) in 0.2 M sodium phosphate, pH 4.75; the mixture was stirred with nitrogen gas for 30 min at room temperature. The beads were rapidly washed with 400 mL of ice-cold 47 mM sodium phosphate, pH 7.0, followed by recirculation of mTgase (6 mg/mL in phosphate buffer) overnight. The beads were washed with 2 M urea in phosphate buffer and stored in phosphate buffer, pH 7.0.

Transglutaminase Activity. Activity measurements for mTgase were performed at 37 °C using CBZ-glutaminyl-glycine and hydroxylamine as substrates. A change in absorbance of 0.29 at 525 nm was defined as a unit of activity, corresponding to the formation of 1 mmol of hydroxamate/min at pH 6.0 (*23*). The pH optimum was established by measuring the activity as stated, adjusting for a pH range between 3 and 9.

Protein Concentration. Immobilized protein concentration was determined using either a BCA assay (Pierce) with BSA as the standard or the spectrophotometric OPA assay described by Janolino and Swaisgood (*24*).

Electrophoresis. SDS-PAGE was performed using a Novex Xcell II Mini-Cell system. NuPAGE Bis-Tris gels with a 4−12% gradient were utilized and run in MES buffer. Staining and destaining of gels were performed using standard protocols.

Cross-Linking of WPI. WPI was solubilized to a final protein concentration of 8% in water containing 10 mM DTT and 5 mM $CaCl_2$ or 50 mM sodium sulfite and 5 mM $CaCl_2$, pH 6.85. Batches of 1 L each were held at 40 °C while being continuously stirred by a suspended stirring apparatus. One milliliter of active beads was added to the batch, after which 100-mL samples were withdrawn after 30, 60, 90, 270, and 1200 min for analysis.

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 of the sample. Using the Huggins equation, which states $\eta_{sp}/c = [\eta] + k[\eta]^2 c$, 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 (*25*).

Turbidity. Turbidity at 600 nm was determined for samples (2 mg/mL) in triplicate.

Viscosity under Shear. 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, and measurements were taken in triplicate at shear rates of 25, 50, and 100 s^{-1}. Shear rate ramps (0−100 s^{-1}, 1 s^{-1}/s) were performed at 25 °C on the Stress Tech to generate stress versus shear rate curves. The differences that were observed in the stress values between the up and down shear rate ramps demonstrated hysteresis. The area between the curves was calculated using a compensating polar planimeter. Flow behavior was calculated using the power law-based model

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

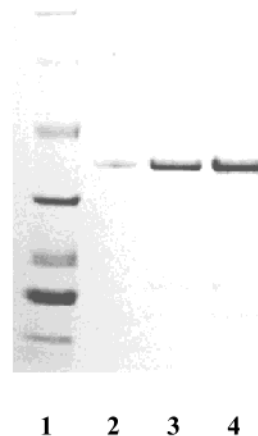

Figure 1. mTgase purity (SDS-PAGE of commercially available microbial tranglutaminase): lane 1, molecular weight markers (from bottom: 7, 11, 20, 32, 54, 98, and 150 kDa); lanes 2–4, increasing concentrations of mTgase.

where σ = shear stress, η = apparent viscosity, γ = shear rate, n = the flow behavior index, and k = the consistency coefficient.

Small-Strain Rheology. Gel point temperatures were determined using a temperature ramp protocol on a Reologica Stress Tech rheometer with a bob and cup attachment. The temperature range was 25−85 °C (held at 85 °C for 980 s), with a constant oscillation of 0.1 Hz and a stress of 0.25 Pa. The temperature at which G' and G'' crossed was considered to be the gel point for reference. Frequency sweeps were performed in the linear range from 0.01 to 20 Hz at 25 °C, and phase angles were compared to investigate the mechanical spectra.

Large-Strain Rheology. Gels were formed by holding the protein solutions in tightly covered glass containers 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 strain were calculated according to the method of Daubert et al. (*26*).

RESULTS AND DISCUSSION

The purity of commercially available mTgase was determined to assess whether additional steps were required prior to biotinylation and immobilization. Commercially available mTgase was found to be >90% pure and suitable for immobilization purposes (**Figure 1**). Upon biotinylation and immobilization, mTgase retained most of its activity and was stable during several months of storage at 4 °C.

The immobilized enzyme showed a bimodal activity profile with optima at pH 7 and 8.5, which was significantly more basic than that observed for the soluble enzyme (*17*) but similar to the profile observed by Huang et al. (*15*) using immobilized guinea pig liver transglutaminase. The activity profile was advantageous because the WPI substrate had a pH of 6.85 upon solubilization.

Covalent attachment of the enzyme on CPG and Celite was performed to compare activities with those of the bioselectively adsorbed enzyme. Expense is often a limiting factor in the use of immobilized enzymes. Therefore, direct covalent attachment has certain advantages over adsorption using avidin and biotin, namely, expense of reagents (*20*). Historically, however, covalent attachment has resulted in a dramatic loss of enzyme activity and prevented easy regeneration of the matrix. In addition, because the process is not selective, a relatively pure enzyme solution must be used to obtain high specific activities. The mTgase enzyme appeared to be a good candidate for

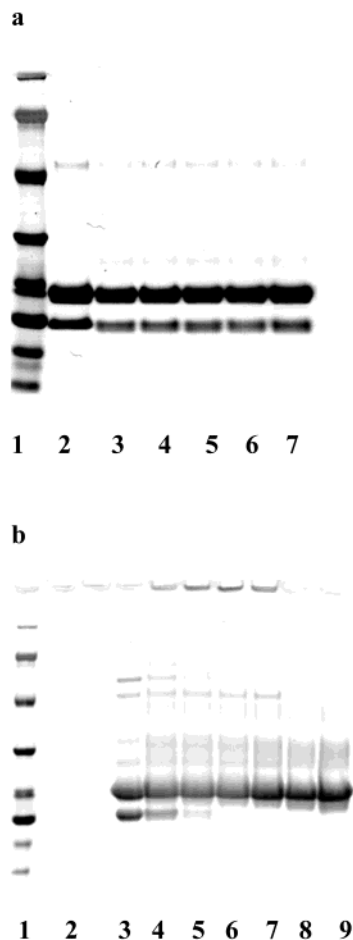

Figure 2. 1% WPI treated in the absence (a) and presence (b) of reducing agent: lane 1, molecular weight markers (from top: 150, 98, 52, 35, 20, 11, and 7 kDa); lane 2a, native WPI; lane 3b, native WPI; lanes 3–7a, treatment times of 30, 60, 90, 270, and 1200 min; lanes 4–9b, treatment times of 30, 60, 90, 270, 1200, and 1440 min.

covalent attachment because it was a fairly pure, commercially available enzyme with a relatively low molecular weight (increased stability). Specific activity of the enzyme on CPG was comparable to that immobilized by bioselective adsorption (data not shown). Slightly lower activities for covalently bound enzyme may be due to increased steric hindrance. The total activity was much higher, a result of higher loading on the beads. Celite as a support gave exceptionally poor results. Future work may include stability characterization and possible applications for the covalently bound mTgase. However, this paper focuses on the application of bioselectively adsorbed enzyme to impart the desired effects.

The WPI solutions were incubated with the active beads in a constant-temperature vessel with a suspended stirring apparatus. This process simulated a stirred-tank reactor and was preferred over recirculation because of fouling concerns and scale-up feasibility. After each treatment, the beads were simply allowed to settle. The substrate was then decanted, and the beads were washed with water and/or 1 M urea.

Cross-linking was first attempted with 1% WPI in the absence of reducing agent (DTT) to determine the ability of the enzyme to act on proteins in their native state. As **Figure 2a** shows, no observable cross-linking occurred under nonreducing conditions. However, inclusion of 10 mM DTT allowed significant cross-linking, believed to be a result of partial unfolding of the substrate proteins and increased enzyme access to NH_2 groups

Table 1. OPA Measurement of Available NH_2 After Treatment with Immobilized MTgase

destabilizing agent	treatment time (min)	$[NH_2]$ (μM)	mol of NH_2/mol of protein[a]
DTT	0	68.4	11.6
	30	59.7	10.1
	90	58.8	10.0
	270	57.9	9.8
	1200	52.9	9.0
sulfite	0	69.6	11.8
	30	58.9	10.0
	90	58.6	10.0
	270	57.8	9.8
	1200	N/A[b]	N/A[b]

[a] Based on an average molecular weight of 17000 Da. In all cases, the standard error was <0.05. [b] N/A represents that a cold-set gel had formed and no measurement could be taken.

(**Figure 2b**) (*27*). 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). OPA determination of free NH_2 (**Table 1**) groups verified that as incubation time increased, available NH_2 groups decreased, a sign that cross-linking had occurred. Sodium bisulfite is food-grade and had been shown to destabilize protein structures in a similar manner (*28*, *29*). Therefore, WPI solutions were prepared with 100 mM bisulfite to determine if cross-linking would occur. On the basis of the decrease in free amino groups (**Table 1**), it was apparent that bisulfite had an impact on protein stability similar to that of DTT. Further studies could be performed to assess concentration effects and the use of other salts to increase enzyme accessibility.

The stability of mTgase immobilized on glass was apparent in the absence of concentrated protein substrates. The enzyme 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 8% WPI consistently lost 50% of its activity, even after washing with appropriate regeneration agents. This phenomenon was not observed when 1% WPI solutions were treated in earlier work (data not shown). It is believed that the high protein concentration resulted in clogging of the pores and/or the 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.

Intrinsic viscosity (η) and shear viscosity (Pa s) were used to determine flow behavior of the cross-linked WPI prior to heating and gel formation. Intrinsic viscosity is a measure of the molecular properties of each biopolymer in solution. It is related to the hydrodynamic volumes of the biopolymers in solution and is affected by the molecular weight and shape of the molecules. 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 or less spherical biopolymers were being formed. The viscosities at shear rates of 25, 50, and 100 s^{-1} demonstrated the shear thinning (**Figure 3**) 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 caused an increase in the area of hysteresis, calculated from stress versus shear rate curves. This was due to the alignment of protein molecules with the shear field and exposure of their

Table 2. Solution Characteristics of 8% WPI Modified Using Immobilized mTgase[a]

treatment	intrinsic viscosity[b] (mL/g)	viscosity (Pa s) at various shear rates (1/s)			hysteresis[c] (mm^2)	turbidity[d] (OD_{600})
		25	50	100		
untreated	17.2 ± 0.3	0.047 ± 0.000	0.033 ± 0.000	0.023 ± 0.000	3.5 ± 0.2	0.17 ± 0.00
30 min, mTgase	22.9 ± 0.7	0.078 ± 0.001	0.052 ± 0.000	0.035 ± 0.000	6.5 ± 0.8	0.21 ± 0.01
60 min, mTgase	27.2 ± 0.2	0.183 ± 0.003	0.111 ± 0.001	0.065 ± 0.000	20.1 ± 2.7	0.28 ± 0.00
90 min, mTgase	28.3 ± 0.3	0.228 ± 0.004	0.141 ± 0.002	0.082 ± 0.000	25.7 ± 3.4	0.36 ± 0.01
270 min, mTgase	31.9 ± 0.4	0.344 ± 0.003	0.201 ± 0.001	0.111 ± 0.001	40.1 ± 4.7	0.44 ± 0.02

[a] All treatments were carried out in the presence of DTT. Standard error is given with each entry. [b] Based on extrapolation of η_{sp}/c vs c from the Huggins equation. [c] Area between up and down stress vs shear rate curves, measured using a Compensating Polar Planimeter (Keuffel and Essex Co.) One rev = 10 in^2. [d] Measured at 0.2% protein.

Table 3. Flow Behavior of 8% WPI Treated with Immobilized mTgase

treatment time (min)	consistency coefficient, *k*[a]	flow behavior index, *n*[a]
0	0.26 ± 0.03	0.47 ± 0.02
30	0.54 ± 0.02	0.41 ± 0.01
60	1.81 ± 0.05	0.28 ± 0.00
90	2.13 ± 0.07	0.30 ± 0.01
270	3.64 ± 0.07	0.26 ± 0.00

[a] Mean ± standard error.

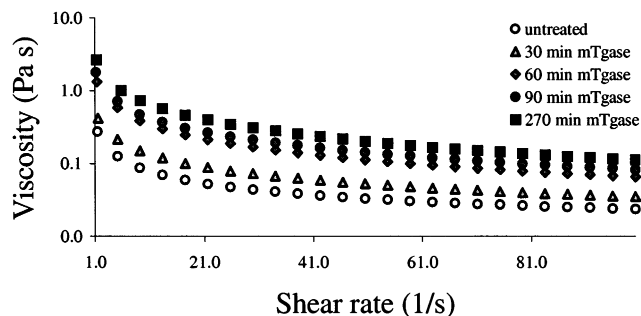

Figure 3. Effect of shear rate on apparent viscosities of mTgase-treated WPI. Each point represents the mean of three replicates.

lowest hydrodynamic radius. Also shown in **Table 2** is the trend toward increased turbidity as treatment time was increased. Light scattering due to increased aggregate size upon cross-linking is the proposed cause.

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 increased cross-linking was 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 as in Munro et al. (*30*). For comparative purposes, the gel point temperature was recorded as the intersection of G' and G'' (**Table 4**). Although not the true definition, this point represented quite accurately the transition point from what was perceived to be a liquid to a more solidlike material. Once again a trend was observed that was directly related to treatment time. Gel point temperatures were lower for samples that had more extensive cross-linking. This observation is not surprising because less heat would be required to form a network that was already partially formed enzymatically than would be necessary to form a network from "scratch". A representative profile of a temperature ramp for bisulfite-containing WPI demonstrates the behavior of the material as it is heated to 85 °C (**Figure 4**).

Table 4. Gelation Temperatures of 8% WPI Treated with Immobilized mTgase

destabilizing agent	treatment time (min)	gelation temp[a] (°C)
DTT	0	no gel
	30	85.00 ± 0.00
	90	82.67 ± 0.07
	270	81.83 ± 0.03
	1200	68.73 ± 0.03
sulfite	0	85.00 ± 0.00
	30	73.33 ± 0.07
	90	72.77 ± 0.19
	270	71.37 ± 0.03
	1200	N/A[b]

[a] Identified by the convergence of G' and G''. [b] Gel was formed prior to heating.

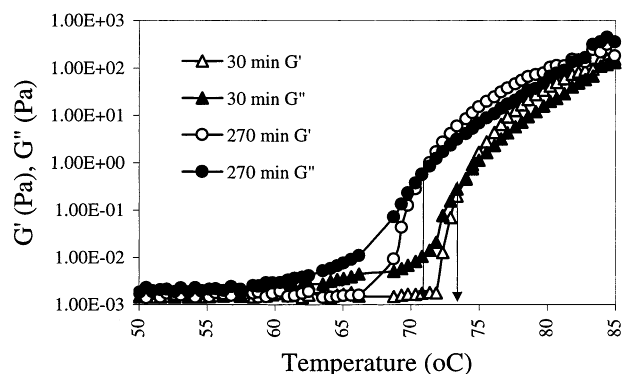

Figure 4. Representative temperature ramps on 8% WPI treated with immobilized mTgase in the presence of 50 mM sulfite. Arrows approximate the temperature at which G' and G'' intersect.

Frequency sweeps on the cross-linked, heat-gelled WPI demonstrate the differences in phase angle (solid character) among samples (**Figure 5**). In the range of 5−20 Hz, the phase angles were basically frequency-independent. Phase angle, which is directly related to the energy lost over the energy stored, decreased as cross-linking treatment time increased, indicating that more energy was being stored by the material. In other words, the treatments gave rise to gels with more solidlike characteristics. Additional analysis supported this finding. Fracture properties of the gelled samples were obtained by recording time and torque at fracture using a vane technique. Samples that had been enzymatically cross-linked 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

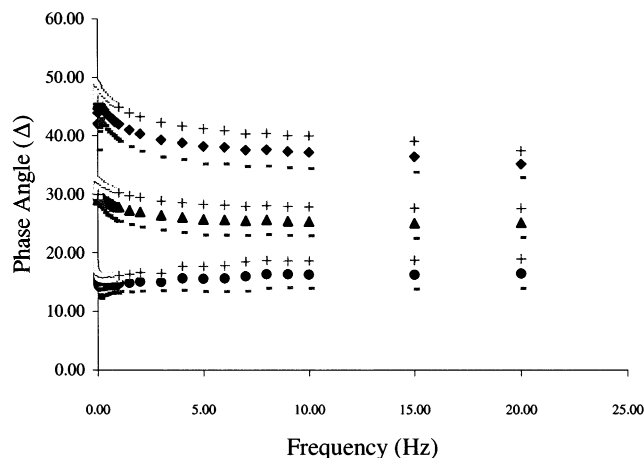

Figure 5. Frequency sweep analysis of WPI that had been treated for various durations with immobilized mTgase in the presence of DTT. 8% WPI in the presence of 10 mM DTT was treated for 30 (◆), 270 (▲), or 1200 (●) min with 1 mL of mTgase immobilized on CPG beads. The frequency sweep was performed after ramping to 85 °C and cooling to 25 °C. The (+) and (–) symbols represent the 95% confidence interval calculated from the standard deviation of three replicates.

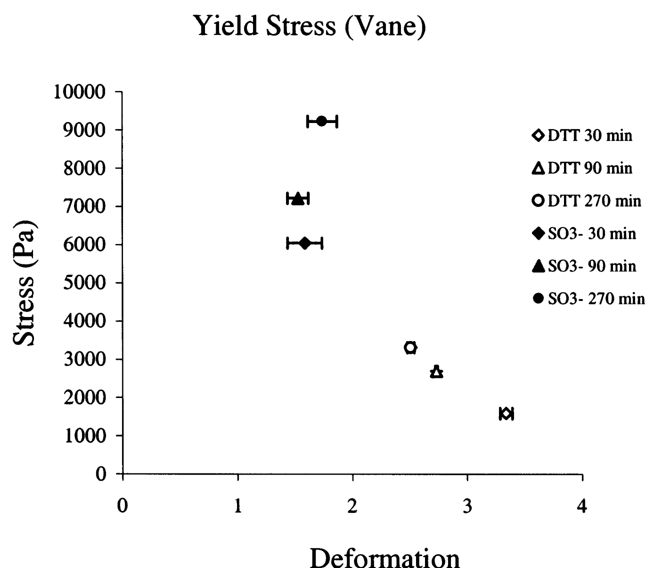

Figure 6. Vane fracture analysis of mTgase-treated 8% WPI gels containing DTT or sulfite.

brittle gels. It should be noted that greater cross-linking resulted in particulate gels, whereas fine-stranded gels were formed by native WPI. When compared to DTT, addition of bisulfite led to stronger, more brittle gels. This was attributed to the increased salt concentration and the formation of particulate gels even in the absence of cross-linking.

CONCLUSIONS

The avidin−biotin interaction is one of the strongest non-covalent associations known, having a K_d of $\sim 10^{-15}$ M. This interaction is stable to changes in pH and ionic strength of fluid streams (*15*). By covalently attaching the robust biotin molecule to porous glass and subsequently adsorbing avidin, it was possible to bioselectively adsorb the biotinylated enzyme of interest. This allowed for stable immobilization while permitting easy regeneration of the matrix by desorption with 6 M guanidinium chloride and/or readsorption of avidin and the biotinylated enzyme. The biotin−avidin complex also acted as a spacer between the matrix and the enzyme, decreasing steric effects.

Because most improvements are seen with only a limited degree of modification, the control afforded by immobilized enzymes makes them highly desirable. Immobilized enzymes offer several advantages over free enzymes when used for treatment of process streams or in a batch format. The requirement for a downstream enzyme inactivation treatment is eliminated, the extent of the reactions can be precisely controlled, and the enzyme may be reused indefinitely when properly stored (*31*, *32*). These characteristics help in lowering the costs associated with the process while minimizing the amount of undesirable product degradation (e.g., thermal inactivation) that could cause loss of functionality, off-flavors, and odors.

Future work will include solving the enzyme inactivation problem associated with treating high concentrations of protein.

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.

ACKNOWLEDGMENT

We thank Dr. Chris Daubert and Dr. Allen Foegeding for suggestions in the preparation of this paper. Also, 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 28, 2001. Revised manuscript received June 6, 2002. Accepted June 19, 2002. Paper FSR 02-22 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.

JF0117154