JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Reactions between β-Lactoglobulin and Genipin: Kinetics and Characterization of the Products

Phoebe X. Qi,*[,†] Alberto Nuñez,[‡] and Edward D. Wickham[†]

[†]Dairy and Functional Foods Research Unit, and [‡]Office of Center Director, Eastern Regional Research Center (ERRC), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, United States

Supporting Information

ABSTRACT: In this paper, we present the first detailed study of the reaction kinetics and the characterization of the products from the endothermic reactions between β-lactoglobulin and genipin. The effects of the concentration, temperature, and pH were investigated. In the temperature range studied, the reaction was approximately a pseudo-first-order with respect to genipin and 0.22-order and −0.24-order with respect to β-lactoglobulin for pH 6.75 and 10.5 with corresponding activation energy (E_a) estimated to be 66.2 ± 3.8 and 9.40 ± 0.36 kJ/mol, respectively. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis studies, validated by matrix-assisted laser desorption ionization−time of flight mass spectrometry, showed the presence of oligomeric, i.e., di-, tri-, quadri-, and pentameric, forms of cross-linked β-lactoglobulin by genipin at neutral but not alkaline pH; however, an extensive cross-linked network was not observed, consistent with the atomic force microscopy images. It was demonstrated that the reaction temperature and the concentration of genipin but not that of β-lactoglobulin positively affected the extent of the cross-linking reactions.

KEYWORDS: *β-Lactoglobulin, genipin, cross-linking reaction, kinetics, MALDI−TOF mass spectrometry, AFM*

INTRODUCTION

Genipin (GP, Figure 1) can be easily obtained from its parent compound geniposide, which is extracted from the fruit of

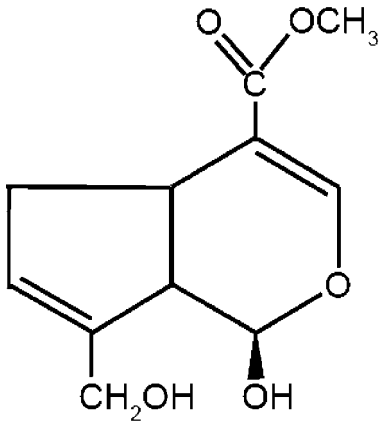

Figure 1. GP molecule ($C_{11}H_{14}O_5$, molecular weight of 226.2 g/mol). International Union of Pure and Applied Chemistry (IUPAC) name: methyl (1*R*,2*R*,6*S*)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]-nona-4,8-diene-5-carboxylate.

Gardenia jasminoides Ellis,[1,2] a traditional Chinese herbal medicine. In addition to its known medicinal and therapeutic properties, such as anti-inflammatory[3] and anti-angiogenesis,[4] GP has been identified[2,5,6] as an effective naturally occurring cross-linking agent and is capable of reacting with amino acids or proteins containing residues with primary amine groups, such as lysine, hydroxylysine, or arginine.[7] Both *in vitro* and *in vivo* studies have shown[8−11] that GP is 10 000 times less cytotoxic than the commonly used glutaraldehyde when used as a cross-linking reagent for tissue fixation, drug carrying, and nerve regeneration.

Despite its numerous beneficial and advantageous properties, GP as a cross-linking reagent has only been limited to uses with gelatin and chitosan for the development of hydrogels, tissue scaffolds, and certain biodegradable materials.[12−17] More importantly, the details involved in the cross-linking reaction mechanism between GP and the primary amine-containing biomolecules remain poorly understood,[6,18−20] although the complexity has been speculated.[6,21] To the best of our knowledge, there has been thus far no report on the reaction kinetics between protein and GP and the in-depth characterization of the reacted products is absent.

The most abundant protein in the whey of bovine milk, β-lactoglobulin (β-LG) represents about ∼12% of total milk protein and nearly 50% of total whey protein.[22,23] This small protein (ca. 18.3 kDa) consists of 162 amino acid residues, including 15 lysine and 3 arginine residues, whose side chains may be susceptible to reacting with GP depending upon the pH conditions. Native β-LG contains five cysteine residues, four of which are engaged in intrachain disulfide bridges. The free sulfhydryl group (Cys121) is thought to be completely buried under the C-terminal α-helix,[24] whose reactivity is pH-dependent and may be oxidized to form intermolecular disulfide bonds or participate in a thiol disulfide interchange

Received: October 6, 2011
Revised: March 22, 2012
Accepted: April 1, 2012
Published: April 1, 2012

dx.doi.org/10.1021/jf300311k | J. Agric. Food Chem. 2012, 60, 4327−4335

reaction when the structure is perturbed, such as when heated.[25,26]

Although its biological function remains rather undefined, β-LG has been used as a paradigm in extensive research effort[27−34] on protein folding. It has been established[30,32,35−37] that, at room temperature and under physiological conditions (neutral pH and concentration > 50 μM), β-LG exists predominantly as a stable noncovalently associated homodimer but displays complex association equilibria at pH above 6.5, shifting between monomer, dimer, tetramer, octamer, and monomer as a function of the concentration, ionic strength, and pH. It dissociates into a monomer at acidic pH (<3), retaining its native structure when heated to 80 °C.[38] At neutral pH (6.0−7.5), the dissociation and conformational changes of β-LG display increased concentration dependence as a function of the temperature[37,39,40] and become somewhat irreversible because of the involvement of the disulfide exchange reactions between the free thiol group and one of the disulfide bonds, leading to the formation of soluble aggregates at above 65 °C.[25] Although the folding behavior of β-LG at above pH 9 is less well-studied,[30,33,41] it is suggested that β-LG undergoes irreversible base-induced denaturation, with global disruption of secondary and tertiary structures even at 20 °C. In general, when proteins are subjected to chemical reactions, their previously buried side-chain groups sustain extensive solvent exposure, leading to increased reactivity and hydrophobicity.[41]

Modification of β-LG by an enzyme, such as transglutaminase, has resulted in improved functional properties of dairy-based ingredients, including solubility, heat stability, foaming capability, gelation, emulsion formation and stabilization, and thermorheological properties, all of great importance to the food industry.[42−45] Other means of food-grade modification of β-LG, in addition to the manipulation of disulfide bonds and the naturally occurring Maillard reactions,[46] have largely gone undocumented.

In this work, we attempt to investigate and characterize the cross-linking reaction kinetics between β-LG and GP to determine the optimal reaction conditions for producing novel multimeric protein-based biocompatible products for potential food and biomedical applications, as well as to gain insight into the mechanism of the complex endothermic process.

MATERIALS AND METHODS

Materials. All chemicals, including NaCl, KCl, and buffer reagents, used in these studies, unless otherwise noted, were of analytical grade or "ACS certified" from Sigma-Aldrich (St. Louis, MO).

GP (Figure 1) was purchased from Challenge Bioproducts Co., Ltd. (Yun-Lin Hsien, Taiwan, Republic of China) with purity at least 98% by the high-performance liquid chromatography (HPLC) method (http://www.genipin.org) and used without further purification.

Bovine β-LG was a generous gift from Dr. Harold M. Farrell of this laboratory. It was purified via 3× crystallization and found to contain 95% β-LG (total), 83% A and 12% B genetic variants, as determined by a reversed-phase HPLC unit (Varian ProStar 230, Pola Alto, CA) equipped with a Varian ProStar 325 ultraviolet−visible (UV−vis) detector and a C-4 column (Vydac 214TP54, 4.6 × 250 mm, The Separations Group, Hesperia, CA), using the same separation protocol as published previously.[47]

Reactions between β-LG and GP. Reactions between β-LG and GP were investigated at pH 6.75 and 10.5 using buffer systems consisting of 25 mM piperazine-1,4-bis(2-ethanesulfonic acid) dipotassium salt (PIPES) and 8 mM NaCl (I = 50 mM) and 22 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) and 25 mM KCl (I = 50 mM), respectively. The stock solution of β-LG was typically at ∼20 mg/mL (∼1.1 mM), and the exact concentration was determined by measuring the absorption at 280 nm using ε = 0.96 L g^{-1} cm^{-1} [35] and molecular weight = 18.3 kDa. A stock GP solution was made by stirring in Milli-Q deionized water at 10 mg/mL (∼44 mM) at room temperature (20 °C) for a few hours before each use, and a clear solution was obtained.

Four reaction mixtures with varying concentrations of β-LG and GP (mM/mM), 0.025:0.5, 0.025:0.1, 0.5:0.1, and 0.5:0.01, equivalent to molar ratios of 1:20, 1:4, 5:1, and 50:1, respectively, were used to conduct the reaction kinetics studies in each buffer solution (described above) at temperatures of 50, 60, 70, and 80 °C. The reaction was monitored by UV−vis spectroscopy (UV−vis Bio-100 spectrometer, Varian, Palo Alto, CA) scanned in the range of 800−450 nm every 1−10 min at pH 6.75, and a fixed wavelength (nm) was set to record the absorbance at 595 nm every 5−10 s at 80 °C and pH 10.5 when the reaction was too fast for a full range scan. The maximum absorbance (A_{max}) of the reacted product(s) shifted from ∼580 to ∼590 nm and to ∼600 nm with an increasing protein concentration, temperature, and pH.

All reaction kinetics experiments at each condition of varying reactant concentrations, temperatures, and pH were performed in triplicate, and the average was used in the final analysis.

Reaction Kinetics and Activation Energy (E_a) Calculations. For a generic cross-linking reaction consisting of two reactants at a given temperature (and pH), the following reaction scheme can be established assuming that the reverse reaction is negligible, i.e., an irreversible process:

$$\text{reactant 1} + \text{reactant 2} \xrightarrow{k} \text{products} \quad (1)$$

On the basis of the differential rate law,[48] the cross-linking reaction rate (r) between β-LG and GP can be expressed as

$$r = \frac{\mathrm{d}[\text{products}]}{\mathrm{d}t} = -\frac{\mathrm{d}[\text{GP}]}{\mathrm{d}t} = -\frac{\mathrm{d}[\beta\text{-LG}]}{\mathrm{d}t} = k[\beta\text{-LG}]^a[\text{GP}]^b \quad (2)$$

where k represents the reaction rate constant and is a function of the reaction temperature. The variables, a and b, denote the order of the reaction with respect to the concentration of β-LG and that of GP, respectively, and the sum of a and b yields the overall reaction order.

The initial reaction rate method was used in this work to determine the initial reaction orders for the cross-linking of β-LG by GP. Dark-blue-colored pigments were generated from the reactions with an absorption peak centered in the region of 580−600 nm (dependent upon the concentration of β-LG, temperature, and pH). The reaction kinetics was followed by monitoring changes in this absorption as a function of time, in minutes or seconds, depending upon the conditions as discussed below. To determine the initial reaction rate (r) based on eq 2, the concentration of either the remaining GP or that of the pigments formed (at time t) is needed. In the absence of the extinction coefficients for both GP and the blue pigments in the literature, the UV−vis absorption spectra of GP at pH 6.75 and 10.5 (at a carefully prepared concentration of 0.025 mg/mL) (see the Supporting Information) were used to calculate the extinction coefficients, $\varepsilon_{240\ nm}$ = 7620 mol^{-1} L cm^{-1} at pH 6.75 and $\varepsilon_{268\ nm}$ = 6240 mol^{-1} L cm^{-1} at pH 10.5.

Using the results by Park et al.,[49] where they observed the formation of an isosbestic point in the reactions between methylamine and GP at pH 7.0, we estimated the concentration of the remaining GP in each reaction mixture (at time t) using the observed A_{max} [of the product(s)] and the calculated $\varepsilon_{240\ nm}$ (for pH 6.75), and $\varepsilon_{268\ nm}$ (for pH 10.5) as discussed above, assuming that the contribution of β-LG (at these two wavelengths) remained unchanged. The initial reaction rate (r) was thus obtained using the linear portion of the kinetics plot, starting at time t = 0. The reaction order with respect to β-LG and GP, a and b, respectively (in eq 2), and the initial reaction rate constant (k) were solved using these experimentally estimated reaction rates when the concentrations of the reactants were varied.

The Arrhenius equation (eq 3) generally correlates the dependency of the reaction rate constant, k, on the reaction temperature, T.

$$k = Ae^{-E_a/RT} \quad (3)$$

In this work, the reaction rate constant, k, was also found to be pH-dependent, as discussed below. The activation energy (E_a) for the reactions between β-LG and GP can be obtained by a different form as the following, i.e., as the slope of plot ln k versus $1/T$:

$$\frac{d \ln k}{d(1/T)} = \frac{E_a}{R} \quad (4)$$

Extent of the Reactions between β-LG and GP. The extent of the reactions for each condition, i.e., concentrations of β-LG and GP, temperature, and pH, was determined by the Sigma Aldrich product N 7285 ninhydrin reagent solution (2%).[50] All reacted mixtures were prepared at the concentrations of β-LG and GP, as described above, and allowed to react at temperatures of 50, 60, 70, and 80 °C for up to 20 h at pH 6.75 and 10.5, respectively. The mixture was diluted by 10 times (1:10) prior to being heated with the ninhydrin solution at 100 °C for 10 min. The unreacted β-LG (~0.35 mg/mL) dissolved in the corresponding buffer system was used as the reference to obtain the percentage of decrease in the available primary amine. A stock solution of leucine at a concentration of 1.0 mM in 0.05 M glacial acetic acid was used to prepare the standard curve according to the reagent instructions, and the absorbance was recorded at 570 nm (UV−vis Bio-100, Varian, Palo Alto, CA).

Differential Scanning Calorimetry (DSC). DSC was used to evaluate the increase in thermal stability of reacted β-LG with GP. A multi-cell scanning calorimeter (model CSC-4100, Calorimetry Sciences Corp., Lindon, UT) was equipped with four demountable 1 mL Hasteloy ampules. One reference along with three samples can be studied under identical conditions. The reacted β-LG with GP at pH 6.75 and 10.5, respectively, was prepared as described above using concentrations at ~20 mg/mL (~1.1 mM) for β-LG and 5.0 mg/mL (22 mM) for GP and allowed to react at 70 °C for up to ~20 h. The unreacted β-LG solution at 20 mg/mL in both buffer systems was also measured as a comparison. A total of 1 mL of each solution was placed in preweighed DSC pans, which were hermetically sealed and weighed accurately. The samples were scanned from 30 to 150 °C at a programmed heating rate of 1 °C/min. For each run, a sample pan containing the buffer used for dissolving or reacting the protein was used as a reference.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of reacted and unreacted β-LG was carried out on a Phast-System (Pharmacia, Piscataway, NJ) with a PhastGel homogenous 20% acrylamide (Amershan Biosciences AB, Uppsala, Sweden). The cross-linked β-LG with GP was prepared as described above, by allowing 3.5 mg/mL β-LG (~0.2 mM) and 0.9 mg/mL GP (~4.0 mM) to react at pH 6.75 and 10.5, respectively, at 70 °C overnight. A dilution of 1:2 was made to yield a protein concentration of 1.8 mg/mL (appropriate for SDS gel analysis) in a solvent system containing 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 2.5% SDS, and 0.01% bromophenol blue dye at pH 8.0. Samples in buffer with or without 50 mM dithiothreitol (DTT), providing reducing and nonreducing conditions respectively, were boiled for 5 min. The unreacted β-LG was dissolved at ~1.8 mg/mL in the two buffer solutions used in this work and also heated at 70 °C overnight. Gels were stained with Coomassie Brilliant Blue R250 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), followed by destaining in a solution containing 30% methanol and 10% acetic acid until a desired color density level. A low-molecular-weight marker kit (14.4−97 kDa) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was used as a standard.

Mass Spectrometry Analysis. The reacted products between β-LG and GP were analyzed using a 4700 matrix-assisted laser desorption ionization−time-of-flight (MALDI−TOF) mass spectrometer instrument (Applied Biosystems, Framingham, MA). The unreacted β-LG (A) was also analyzed as a reference. Spectra were obtained by acquiring 10 000 spectra in the positive linear mode. Conversion of the time-of-flight to mass (kDa) for the average mass, $[M + H]^+$, was based on the calibration of the instrument with a protein standard calibration kit (Applied Biosystems, Framingham, MA).

An aliquot of 100 μL of reacted mixture, obtained by allowing 3.5 mg/mL β-LG (~0.2 mM) and 0.9 mg/mL GP (~4.0 mM) to react at 70 °C overnight, at pH 6.75 and 10.5, respectively, was precipitated by adding 3 μL of 10% formic acid solution. After centrifugation, the supernatant was removed and the pellet was resuspended in 100 μL of 0.1% trifluoroacetic acid (TFA) and then separated by centrifugation. The process was repeated 5 times, and the final cleaned and desalted solid was redissolved in 100 μL of acetonitrile/water solvent (50:50, and 0.1% TFA). A total of 2 μL of resulting solution was then mixed with 10 μL of matrix solution containing α-cyano-4-hydroxycinamic acid (5.0 mg/mL), also in acetonitrile/water solvent (50:50, and 0.1% TFA), and spotted on a MALDI plate for mass spectrometry analysis.

Atomic Force Microscopy (AFM). β-LG (A) was dissolved at 3.5 mg/mL directly into the PIPES buffer at pH 6.75 (I = 50 mM) and the CAPS buffer at pH 10.5 (I = 50 mM) used in this study. An aliquot of 3.0 μL of 1:20 diluted β-LG sample was applied to a freshly cleaved muscovite mica (Pelco International, Redding, CA) substrate.

The reacted β-LG with GP at pH 6.75 and 10.5 was prepared as described above using concentrations at 3.5 mg/mL for β-LG (~0.2 mM) and 4.0 mM for GP and incubated at 60 °C for up to ~20 h. The reacted mixture was diluted at 1:20 and deposited onto the highly oriented pyrolytic graphite (HOPG), ZYA grade, a highly nonpolar substrate (Structure Probe, Inc., West Chester, PA). Because of the highly hydrophobic nature of the reacted products between β-LG and GP, the HOPG substrate rather than the regular mica was used for imaging the reacted mixtures. Both the mica surface and the HOPG surface were rinsed with Millipore-filtered water (100 mL) to remove loosely bound protein and air-dried. This procedure was repeated 4 times. The sample was then imaged immediately using a Nanoscope IIIa controller (Veeco Metrology, Inc., Santa Barbara, CA) with a multimode scanning probe microscope equipped with an E-scanner. All measurements were carried out in the tapping mode under ambient conditions using single-beam silicon cantilever probes. A nominal tip radius of correction was 1−5 nm.

RESULTS AND DISCUSSION

Analysis of Initial Reaction Kinetics between β-LG and GP. In the kinetics studies, changes in the absorbance in the visible region, indicative of the formation of the blue-colored pigments, were monitored and the maximum (A_{max}) was plotted as a function of time, shown in the Supporting Information. For reasons of clarity, only reactions occurring at 0.025 mM β-LG and 0.5 mM GP at the temperatures and pH conditions studied in this work were displayed. The initial reaction rate (r) with varying concentrations of the reactants, temperatures, and pH, were estimated as described in the Materials and Methods, and the results were summarized in the Supporting Information.

Clearly, the initial reaction rate (r) is directly proportional to the concentration of GP for each given β-LG concentration in all reactions, as demonstrated in the Supporting Information. When the GP concentration was kept constant, it (r) exhibited a slight increase as a function of the β-LG concentration only at pH 6.75 and an inverse relationship was observed at pH 10.5. Using the initial reaction rate along with the concentrations of each reactant, the reaction order with respect to the concentration of β-LG and GP, a and b (eq 2), was calculated and averaged over all temperatures used in this work to be 0.22 ± 0.02 and 1.01 ± 0.02 at pH 6.75 and −0.24 ± 0.01 and 1.01 ± 0.02 at pH 10.5, respectively. These unusual reaction orders clearly reflected the complex and multistep nature[51] of the reactions between β-LG and GP. As discussed in the Introduction, the intricate pH- and temperature-dependent conformational changes in β-LG are likely the main cause for

the multifaceted reaction kinetics. Moreover, recent reports[52,53] suggested that that GP undergoes severe degradation and self-polymerization prior to participation in cross-linking reactions at basic pH and elevated temperature, which would undoubtedly further complicate the reaction mechanism as well.

The initial reaction rate constant for each reaction, k, was obtained using the initial concentrations of both reactants, β-LG and GP, along with the estimated apparent reaction orders, $a \approx 0.22$ and $b \approx 1$ (pH 6.75) and $a \approx -0.24$ and $b \approx 1$ (pH 10.5), and the results were also given in the Supporting Information. Clearly, k was dependent upon not only the temperature but more so the pH, which also intensified at elevated temperatures. At 50 °C, the lowest reaction temperature used in this work, k assumed a value of ~1.0, regardless pH. As the reaction temperature was increased to 80 °C, k increased by more than 8-fold at pH 6.75 compared to a mere ~1.5-fold at pH 10.5.

When ln k was plotted versus $1/T$ (Figure 2), the average was fitted (at each pH) with a linear relationship, as indicated

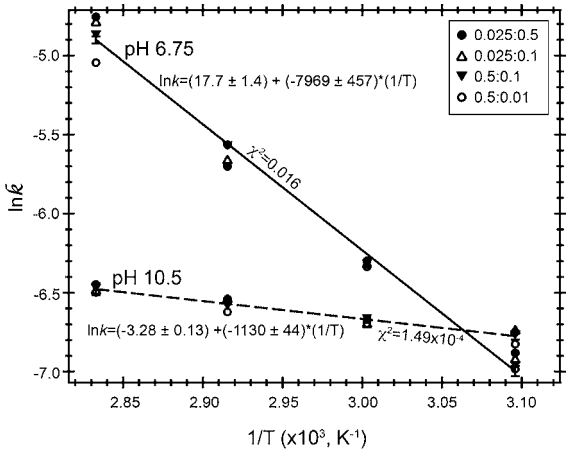

Figure 2. ln k versus $1/T$ plot for the cross-linking reactions for four concentrations of [β-LG]/[GP] (mM/mM) as indicated in numbers at the two pH values. The average ln k values were fitted with a linear relationship (solid and dashed lines) as shown on each line. The standard deviation (versus the average) was also represented as ± error bars.

on the graph. The slope of the line allowed for the apparent activation energy (E_a) of the reaction to be calculated according to eq 4, i.e., 66.2 ± 3.8 kJ/mol for pH 6.75, and 9.40 ± 0.36 kJ/mol for pH 10.5, suggesting a greater energy barrier for the reaction to occur at pH 6.75 than at pH 10.5. As mentioned above, the more open and extended conformation of monomeric β-LG at alkaline pH favored a higher starting energy level along the reaction coordinate, resulting in lowered E_a.

In addition to the apparent effects of reaction conditions (concentration, temperature, and pH) on the initial reaction rate (r), the initial reaction rate constant (k), the reaction order with respect to the concentration of protein (a), and the activation energy (E_a) for the reactions between β-LG and GP, the dark-blue-colored pigments, reacted products, exhibited changing absorption behavior as the reaction conditions were varied as well. A gradual shift toward a shorter wavelength accompanied by a broadened peak width was observed as the reactions proceeded at lower β-LG concentrations, reaction temperatures, and pH 6.75, as shown in the Supporting Information. In contrast, this spectral change disappeared when the concentration of β-LG (relative to GP) and temperature were both elevated, as was the case for all of the reactions that occurred at pH 10.5. This reaction-condition-dependent behavior in the absorption of the reacted products provided further evidence for the complexity of the reaction kinetics, as well as the heterogeneity of the products. Although the kinetics of a chemical reaction, in principle, bears little impact on the products formed,[51] the reactions between β-LG and GP are anything but "conventional", as demonstrated in this work.

Extent of the Reactions between β-LG and GP. The ninhydrin assay method was used to determine the extent of the reactions between β-LG and GP, indicative of the completeness after an extended reaction time (>20 h), using the unreacted β-LG as the reference. The effects of the concentration, temperature, and pH were studied, and the results are shown in Figure 3. All reactions remained

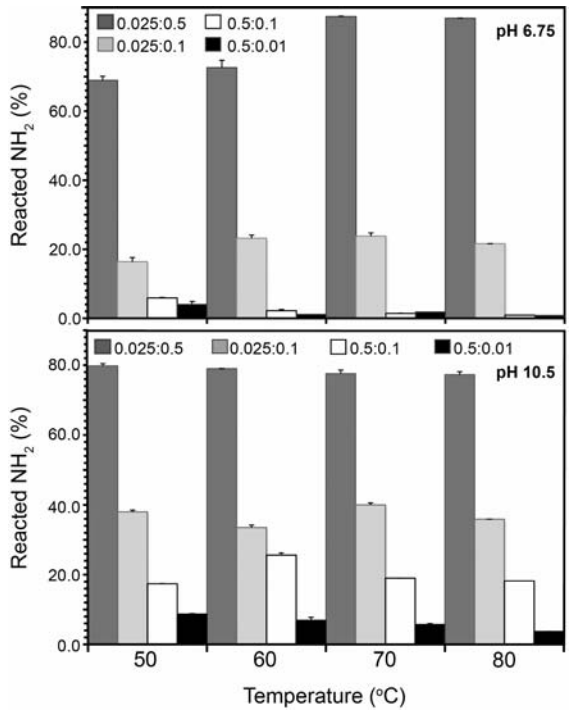

Figure 3. Effects of the concentration, temperature, and pH on the extent of cross-linking between β-LG and GP by the ninhydrin assay method. Reactant concentrations, [β-LG]/[GP] (mM/mM), were as indicated at pH 6.75 and 10.5, and temperatures were represented as the x axis. All data were collected in triplicate with the average and standard deviations plotted.

incomplete, <100%, even at elevated concentrations of GP, temperatures, and pH, which would argue against the irreversibility of the reactions between β-LG and GP based on Helfferich's treatment[51] on complex multistep chain reactions; i.e., the assumed reaction scheme in eq 1 may need to be revised to include a slight reverse reaction. Furthermore, even though β-LG (A) contains 15 lysine and 3 arginine residues, whose $-NH_2$ group on the side chains may be available for reactions with GP, 3 of the lysine residues (~16% total $-NH_2$) are known to be inaccessible for modification,[54] which would also account for, in part, the observed incompleteness of the reactions.

As seen in Figure 3, the most important factor affecting the extent of the reactions between β-LG and GP was the concentrations of the reactants. The highest level of completion was less than 90% and was achieved by the reactions occurring at the highest GP concentration (0.5 mM) and relatively low level of β-LG (0.025 mM), pH 6.75, and elevated temperatures (70 and 80 °C). The same reaction mixture at pH 10.5, on the other hand, resulted in only 80% reduction of $-NH_2$. When the concentration of β-LG was kept constant (0.025 mM), a moderate decrease in the concentration of GP, from 0.5 to 0.1 mM, apparently resulted in a sharp reduction in the completeness of the reaction at all temperatures. Contrarily, increasing the concentration of β-LG imparted an adverse effect, more so under neutral than alkaline conditions.

It should be pointed out that, at low temperatures, 50 and 60 °C, and neutral pH, it is possible that the reactions were still ongoing, even after 20 h, leading to the lowered level of reaction extent (Figure 3A). Overall, the temperature is a less significant factor affecting the extent of the reactions between β-LG and GP compared to the concentration and pH.

Characterization of the Reaction Products between β-LG and GP. From the kinetics analysis discussed above, we chose to investigate and characterize, in detail, the reacted products from the reactions when β-LG was limiting and GP was in excess, i.e., the molar ratio of β-LG/GP = 1:20. The reaction temperature was selected to be 70 °C to balance a practical reaction time frame and an acceptable level of completion (Figure 3). DSC, SDS–PAGE, MALDI–TOF mass spectrometry, and AFM were used to study to the effect of pH on the reacted products to gain insight on the complex reaction mechanism between β-LG and GP.

Table 1 showed the DSC results of reacted β-LG with GP at pH 6.75 and 10.5 compared to the unreacted β-LG at the corresponding pH conditions. The transition temperature (T_m) of β-LG at pH 6.75 (PIPES buffer, I = 50 mM) was determined to be ~76 °C, which is in close agreement with the value published previously for β-LG (A) in the phosphate buffer system.[55] For the reacted mixture, a well-defined T_m transition was not possible to obtain, suggesting the heterogeneous nature of the cross-linked products. At alkaline pH (10.5), on the other hand, a distinct transition point (T_m) could not be determined for the unreacted β-LG, because β-LG exists predominately in the denatured monomeric state.[30] The T_m was measured to be ~128 °C for the reacted β-LG and GP mixture, supporting the notion that the reaction products possess greatly increased thermal stability than the native form of β-LG (at pH 6.75).

Table 1. DSC Results for β-LG Solution Reacted with GP at 60 °C Overnight Compared to Unreacted β-LG

pH	β-LG (mM)	GP (mM)	transition T_m (°C)	ΔH (kcal/mol)	ΔS (kcal/mol)
6.75	1.1	22			
	1.1		75.9	16.4	0.047
10.5	1.1	22	127.7	44.1	0.11
	1.1				

At neutral pH and below 80 °C, β-LG exists predominately as a dimer, nearly ~100%,[40] even at a relatively low ionic strength (I = 50 mM) and low concentration, such as that (≤0.2 mM) used in this work. When heated at above 60 °C, β-LG undergoes disulfide exchange reactions between the free thiol group (Cys121) and one of the disulfide bonds, leading to the formation of large soluble aggregates, as shown in lane 1 (Figure 4). In addition, the dimers formed through intermolecular S–S bonds of the free thiol group, were also evident. These aggregates and dimers were nearly completely converted into monomers by DTT reduction (lane 4). For the reacted β-LG and GP mixture, at least four distinct bands with estimated molecular weights of ~36, ~54, 73, and 92 kDa were visible in lanes 2 and 5 (Figure 4). These bands are attributed to the di-, tri-, quadri-, and pentameric forms of β-LG, evidently caused by the cross-linking reactions with GP. Larger molecular-weight species do not appear to be present in either lane 2 or 5, demonstrating that the growth of the polymeric chains is limited. Further work is needed to gain a comprehensive understanding of the molecular mechanism involved in these cross-linking reactions.

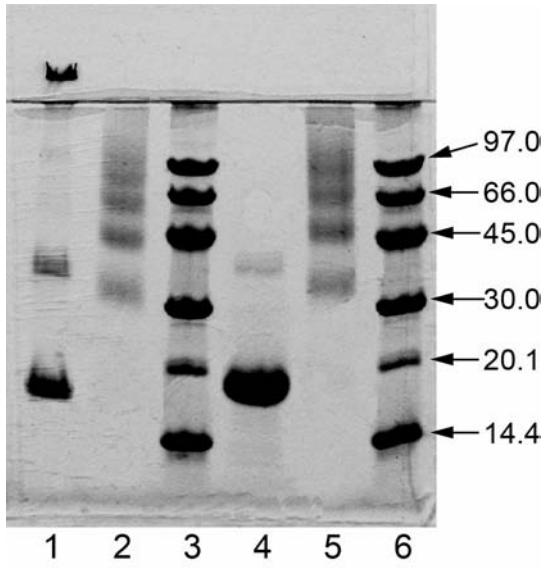

Figure 4. SDS–PAGE patterns of β-LG at a concentration of 1.8 mg/mL and pH 6.75. Samples were loaded as the following designated lanes: 1, unreacted and heated at 70 °C in PIPES buffer overnight; 2, reacted with GP at 70 °C overnight; 3 and 6, low-molecular-weight markers (kDa); 4, same as lane 1 followed by reduction with DTT; and 5, same as lane 2 followed by reduction with DTT.

To provide a visual understanding of the reacted products between β-LG and GP, AFM studies were conducted to obtain the topographical images in comparison to the unreacted protein, as shown in the Supporting Information. The immediate observation was that the reaction mixture (of all conditions) was so hydrophobic that it failed to bind to the commonly used mica surface (normally hydrophilic), and the HOPG substrate had to be used for the AFM experiments. This implied a reverse in the hydrophilic property of the native protein solution and could bear implications for potential use in biomaterials. The AFM images of the unreacted native β-LG (see the Supporting Information) closely resembled those published previously,[40] where its self-association caused the individually well-defined spheres to pack more closely and densely. In contrast, the reacted products (see the Supporting Information) were far less densely populated and showed a range of particles of various shapes with diverse and limited sizes, representing the oligomeric and heterogeneous nature. The AFM images are consistent with the results of SDS–

PAGE, in which no extensive cross-linked polymeric network was observed (see the Supporting Information).

Figure 5 shows the SDS−PAGE profiles of β-LG at pH 10.5 and 70 °C. β-LG assumes an extended and denatured

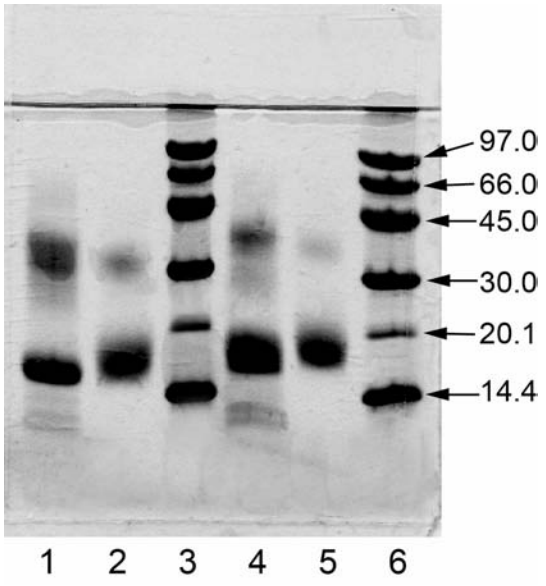

Figure 5. SDS−PAGE patterns of β-LG at a concentration of 1.8 mg/mL and pH 10.5. Samples were loaded as the following designated lanes: 1, unreacted and heated at 70 °C in CAPS buffer overnight; 2, reacted with GP at 70 °C overnight; 3 and 6, low-molecular-weight markers (kDa); 4, same as lane 1 followed by reduction with DTT; 5, same as lane 2 followed by reduction with DTT.

conformation, with the majority of its residues exposed to the solvent at this condition, resulting in its failure to undergo similar disulfide exchange reactions occurring at pH 6.75, thus the large soluble aggregates in lane 1 of Figure 4 were not observed in lane 1 of Figure 5. The dimers formed through intermolecular S−S bonds between the free thiol groups (Cys121) were still detectable (lane 1 of Figure 5) and were significantly reduced but not eliminated by the addition of DTT (lane 4). The reacted mixtures, lanes 2 and 5, yielded somewhat surprising results, in which no cross-linking reactions seemingly occurred. However, a closer examination of the electrophoretic patterns revealed that the band located in the proximity of monomeric β-LG represents a slightly larger species with a molecular weight of 20.6 kDa, as determined by mass spectrometry (see the discussion below). When the patterns of unreacted β-LG and reacted mixture, i.e., lane 1 versus lane 4 and lane 2 versus lane 5, were compared, it was apparent that the intermolecular S−S bond formation was significantly inhibited by the reactions with GP.

These results clearly contradicted the findings at pH 6.75 and led to the assumption that other reactions and processes preceded and dominated the cross-linking of β-LG by GP at pH 10.5. In light of the recent reports[52,53] on the sustained reactivity of GP, despite degradation and self-polymerization, particularly at high pH and temperature, we speculate that the reaction between β-LG and GP likely produced a simple addition of monomeric β-LG onto polymerized GP, which would increase the molecular weight of the products (from β-LG alone) but not significantly. Quantitative analysis by MALDI−TOF mass spectrometry, discussed below, provided further insight on the details of the reactions between β-LG and GP.

The AFM studies of the reacted products obtained at pH 10.5 (see the Supporting Information) appeared to be less conclusive with seemingly more ordered, more tightly bound, and linearly stranded species (see the Supporting Information) that look similar to the smaller set of spherical particles seen in the Supporting Information, presumably the dissociated β-LG monomers. We postulate that these beaded strands are the self-polymerized GP with the addition of a simple monomeric β-LG. The following results from mass spectrometry will provide independent and quantitative validation of this assumption.

The reaction products between GP and β-LG were examined in detail using MALDI−TOF mass spectrometry, as shown in Figure 6. At pH 6.75, an ion peak at 20.9 kDa was detected, and

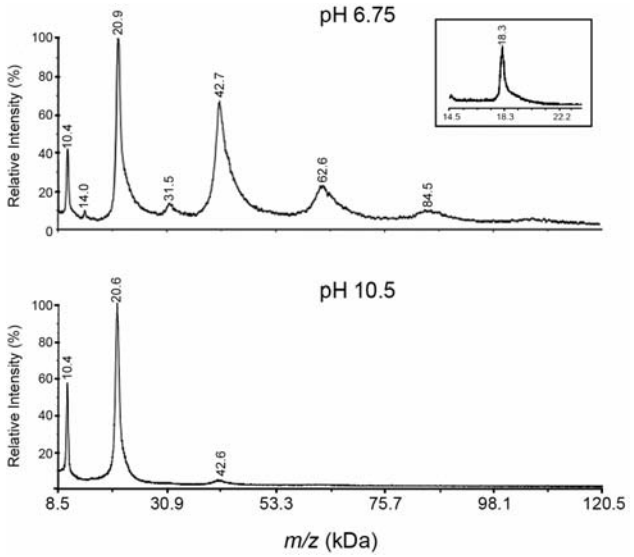

Figure 6. MALDI−TOF mass spectra of reacted products of β-LG and GP at pH 6.75 and 10.5. Reaction conditions were as described in the Materials and Mathods. The MALDI−TOF mass spectrum of unreacted β-LG was shown as an inset for referencing.

its double-charged ion, $[M + 2H]^{2+}$, appeared at 10.4 kDa. As discussed above, β-LG exists nearly completely as a dimer at neutral pH, which was evidenced by the high intensity of the ion peak at 42.7 kDa. This dimeric species should generate a double-charged ion, at 20.9 kDa, and a triple-charged ion as well, at ∼14.0 kDa in Figure 6. The presence of tri- and quadrimeric cross-linked β-LG by GP is positively established by the ion peaks at 62.6 kDa (with its double-charged ion at 31.5 kDa) and 84.5 kDa, respectively. The double-charged ion of the latter should be located precisely at 42.7 kDa. In addition, the spectrum also shows a broadened peak at ∼100 kDa (not labeled), indicating the formation of a pentameric cross-linked product. The results in Figure 6 are in close agreement with the observation made by SDS−PAGE, as in Figure 4, which also showed that the dimeric form of β-LG was the main product. Moreover, MALDI−TOF mass spectrometry proved to be a far more sensitive detection technique and provided more quantitative analysis than SDS−PAGE.

The spectrum of the reacted products at pH 10.5 (lower panel in Figure 6) showed the formation of a low-concentration product with an ion peak at 42.6 kDa and a base ion peak at 20.6 kDa, much the same as pH 6.75. Because both single- and

multiple-charged ions, likely the double-charged species $[M + 2H]^{2+}$, can be generated in proteins by the MALDI process, the observed 42.6 kDa ion is considered to be a close approximation to the $[M + 2H]^{2+}$ ion at 20.6 kDa. Although the ion at 10.3 kDa can easily be accounted for as a double-charged species at 20.6 kDa, it is inconsistent with the triple-charged form at 42.6 kDa. This led us to conclude that the ion peak at 20.6 kDa may only correspond to a single-charged species, consistent with the observations in Figure 5.

The MALDI−TOF analysis of unreacted β-LG showed a single-charged ion at 18.3 kDa (inset of Figure 6), comparable to the molecular weight reported for β-LG (A) in the literature.[22] This provided a strong basis to attribute the ion at 20.6 kDa (pH 10.5, Figure 6) to the products lacking extensive cross-linking from the reactions between β-LG and GP. It should be noted that the instrumental error was well within a single GP molecule (226 Da). The mass difference between the monomeric β-LG (18.3 kDa) and the observed value of 20.6 kDa is precisely an equivalent of 10× GP molecules. Similarly, the species at 42.6 kDa (with a lesser amount) may be due to a product involving a dimeric β-LG with about 26 GP molecules added covalently.

In summary, the results presented in this work showed highly complex and multistep reaction kinetics for the reactions between β-LG and GP, likely caused by the temperature- and pH-dependent conformational transitions occurring in β-LG, complicated by the intricate degradation and self-polymerization behavior of GP (at alkaline pH). The reaction order with respect to the concentration of β-LG was found to be pH-dependent, 0.22 for pH 6.75 and −0.24 for pH 10.5, while the reaction order with respect to the concentration of GP remains a pseudo-first-order, independent of pH. The activation energy (E_a) was derived to be 66.2 ± 3.8 and 9.40 ± 0.36 kJ/mol for pH 6.75 and 10.5, respectively.

The products formed from the reactions, characterized by SDS−PAGE, mass spectrometry, and AFM methods, showed the presence of oligomeric, i.e., di-, tri-, quadri-, and pentameric, forms of cross-linked β-LG by GP only at neutral pH. The absence of a definitive transition point (T_m) in the DSC studies, likely resulting from the heterogeneous distribution of the products, evidently corroborated this finding. At alkaline pH, the degradation and self-association of GP appear to precede and dominate all other reactions involving the monomeric and extended form of β-LG. Despite such a "by-process", GP remains reactive with β-LG and the products displayed increased thermostability (relative to the native protein), $\Delta T_m > 50$ °C, as demonstrated by DSC.

Taken together, the following reaction schemes were proposed on the basis of eq 1:

At neutral pH

$$n\beta\text{-LG} + m\text{GP} \rightleftharpoons \{\beta\text{-LG}\}_n\text{-}\{\text{GP}\}_m$$

where n = 2, 3, 4, and 5 and m = 10 and 26.

At alkaline pH

$$\beta\text{-LG} + m\text{GP} \rightleftharpoons \beta\text{-LG-}\{\text{GP}\}_m$$

where m = 10 and 26.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Analysis of cross-linking reaction kinetics between β-LG and GP at pH 6.75 and 10.5 (Tables S1 and S2), UV−vis absorption spectra for GP (Figure S1), cross-linking reaction kinetics between β-LG and GP (Figure S2), topographical AFM images of β-LG with and without GP at pH 6.75 and 10.5 (Figures S3 and S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (215) 233-6438. Fax: (215) 233-6795. E-mail: phoebe.qi@ars.usda.gov.

Notes

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The USDA is an equal opportunity provider and employer.

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge Winnie Yee for performing and Dr. Peter H. Cooke, ERRC, ARS, USDA, for helping with the AFM experiments discussed in this paper.

■ REFERENCES

(1) Endo, T.; Taguchi, H. The constituents of *Gardenia jasminoides* geniposide and genipin-gentiobioside. *Chem. Pharm. Bull.* **1973**, *21* (12), 2684−2688.

(2) Djerassi, C.; Gray, J.; Kincl, F. Naturally occurring oxygen heterocyclics. IX. Isolation and characterization of genipin. *J. Org. Chem.* **1960**, *25* (12), 2174−2177.

(3) Koo, H.; Lim, K.; Jung, H.; Park, E. Anti-inflammatory evaluation of *Gardenia* extract, geniposide and genipin. *J. Ethnopharmacol.* **2006**, *103* (3), 496−500.

(4) Nam, K.; Choi, Y.; Jung, H.; Park, G.; Park, J.; Moon, S.; Cho, K.; Kang, C.; Kang, I.; Oh, M.; Lee, E. Genipin inhibits the inflammatory response of rat brain microglial cells. *Int. Immunopharmacol.* **2010**, *10* (4), 493−499.

(5) Fujikawa, S.; Nakamura, S.; Koga, K. Genipin, a new type of protein crosslinking reagent from *Gardenia* fruits. *Agric. Biol. Chem.* **1988**, *52* (3), 869−870.

(6) Butler, M.; Ng, Y.-F.; Pudney, P. Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41* (24), 3941−3953.

(7) Aramwit, P.; Siritientong, T.; Kanokpanont, S.; Srichana, T. Formulation and characterization of silk sericin-PVA scaffold cross-linked with genipin. *Int. J. Biol. Macromol.* **2010**, *47* (5), 668−675.

(8) Sung, H.; Huang, R.; Huang, L.; Tsai, C.; Chiu, C. Feasibility study of a natural crosslinking reagent for biological tissue fixation. *J. Biomed. Mater. Res.* **1998**, *42* (4), 560−567.

(9) Liang, H.; Chang, W.; Lin, K.; Sung, H. Genipin-crosslinked gelatin microspheres as a drug carrier for intramuscular administration: In vitro and in vivo studies. *J. Biomed. Mater. Res., Part A* **2003**, *65* (2), 271−282.

(10) Chang, Y.; Tsai, C.; Liang, H.; Sung, H. In vivo evaluation of cellular and acellular bovine pericardia fixed with a naturally occurring crosslinking agent (genipin). *Biomaterials* **2002**, *23* (12), 2447−2457.

(11) Chang, C. Effects of nerve growth factor from genipin-crosslinked gelatin in polycaprolactone conduit on peripheral nerve regeneration—In vitro and in vivo. *J. Biomed. Mater. Res., Part A* **2009**, *91* (2), 586−596.

(12) Chen, Y.; Chang, J.; Cheng, C.; Tsai, F.; Yao, C.; Liu, B. An in vivo evaluation of a biodegradable genipin-cross-linked gelatin peripheral nerve guide conduit material. *Biomaterials* **2005**, *26* (18), 3911−3918.

(13) Lee, W.; Lee, S. Effect of gelatin on the swelling behavior of organic hybrid gels based on *N*-isopropylacrylamide and gelatin. *J. Appl. Polym. Sci.* **2005**, *98* (3), 1092−1099.
(14) Solorio, L.; Zwolinski, C.; Lund, A.; Farrell, M.; Stegemann, J. Gelatin microspheres crosslinked with genipin for local delivery of growth factors. *J. Tissue Eng. Regener. Med.* **2010**, *4* (7), 514−523.
(15) Bi, L.; Cao, Z.; Hu, Y.; Song, Y.; Yu, L.; Yang, B.; Mu, J.; Huang, Z.; Han, Y. Effects of different cross-linking conditions on the properties of genipin-cross-linked chitosan/collagen scaffolds for cartilage tissue engineering. *J. Mater. Sci. Mater. Med.* **2011**, *22* (1), 51−62.
(16) Kosaraju, S.; Puvanenthiran, A.; Lillford, P. Naturally crosslinked gelatin gels with modified material properties. *Food Res. Int.* **2010**, *43* (10), 2385−2389.
(17) Muzzarelli, R. Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydr. Polym.* **2009**, *77* (1), 1−9.
(18) Touyama, R.; Inoue, K.; Takeda, Y.; Yatsuzuka, M.; Ikumoto, T.; Moritome, N.; Shingu, T.; Yokoi, T.; Inouye, H. Studies on the blue pigments produced from genipin and methylamine. II. On the formation mechanisms of brownish-red intermediates leading to the blue pigment formation. *Chem. Pharm. Bull.* **1994**, *42*, 1571−1578.
(19) Lee, S.; Lim, J.; Bhoo, S.; Paik, Y.; Hahn, T. Colorimetric determination of amino acids using genipin from *Gardenia jasminoides*. *Anal. Chim. Acta* **2003**, *480* (2), 267−274.
(20) Lee, J.; Hahn, T.; Paik, Y. Physicochemical characteristics for the transformation of blue pigments from genipin of *Gardenia jasminoides* with amino acids. *Agric. Chem. Biotechnol.* **1998**, *41* (5), 399−404.
(21) Paik, Y.; Lee, C.; Cho, M.; Hahn, T. Physical stability of the blue pigments formed from geniposide of *Gardenia* fruits: Effects of pH, temperature, and light. *J. Agric. Food Chem.* **2001**, *49* (1), 430−432.
(22) Farrell, H. M., Jr.; Jimenez-Flores, R.; Bleck, G. T.; Brown, E. M.; Butler, J. E.; Creamer, L. K.; Hicks, C. L.; Hollar, C. M.; Ng-Kwai-Hang, K. F.; Swaisgood, H. E. Nomenclature of the proteins of cows' milk—Sixth revision. *J. Dairy Sci.* **2004**, *87* (6), 1641−1674.
(23) Sawyer, L.; Kontopidis, G. The core lipocalin, bovine β-lactoglobulin. *Biochim. Biophys. Acta* **2000**, *1482* (1−2), 136−148.
(24) Sakai, K.; Sakurai, K.; Sakai, M.; Hoshino, M.; Goto, Y. Conformation and stability of thiol-modified bovine β-lactoglobulin. *Protein Sci.* **2000**, *9* (9), 1719−1729.
(25) Hoffmann, M. A. M.; van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: Role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* **1997**, *45* (8), 2942−2948.
(26) Creamer, L. K.; Bienvenue, A.; Nilsson, H.; Paulsson, M.; van Wanroij, M.; Lowe, E. K.; Anema, S. G.; Boland, M. J.; Jimenez-Flores, R. Heat-induced redistribution of disulfide bonds in milk proteins. 1. Bovine β-lactoglobulin. *J. Agric. Food Chem.* **2004**, *52* (25), 7660−7668.
(27) Tanford, C.; De, P. K. The unfolding of β-lactoglobulin at pH 3 by urea, formamide, and other organic substances. *J. Biol. Chem.* **1961**, *236* (6), 1711−1715.
(28) Bell, K.; McKenzie, H. A. β-Lactoglobulins. *Nature* **1964**, *204* (4965), 1275−1279.
(29) Timasheff, S. N.; Susi, H. Infrared investigation of the secondary structure of β-lactoglobulins. *J. Biol. Chem.* **1966**, *241* (1), 249−251.
(30) Tanford, C.; Bunville, L. G.; Nozaki, Y. The reversible transformation of β-lactoglobulin at pH 7.51. *J. Am. Chem. Soc.* **1959**, *81* (15), 4032−4036.
(31) Yagi, M.; Sakurai, K.; Kalidas, C.; Batt, C. A.; Goto, Y. Reversible unfolding of bovine β-lactoglobulin mutants without a free thiol group. *J. Biol. Chem.* **2003**, *278* (47), 47009−47015.
(32) Sakurai, K.; Goto, Y. Dynamics and mechanism of the Tanford transition of bovine β-lactoglobulin studied using heteronuclear NMR spectroscopy. *J. Mol. Biol.* **2006**, *356* (2), 483−496.
(33) Taulier, N.; Chalikian, T. V. Characterization of pH-induced transitions of β-lactoglobulin: Ultrasonic, densimetric, and spectroscopic studies. *J. Mol. Biol.* **2001**, *314* (4), 873−889.
(34) Yagi, M.; Kameda, A.; Sakurai, K.; Nishimura, C.; Goto, Y. Disulfide-linked bovine β-lactoglobulin dimers fold slowly, navigating a glassy folding landscape. *Biochemistry* **2008**, *47* (22), 5996−6006.
(35) Townend, R.; Winterbottom, R. J.; Timasheff, S. N. Molecular interactions in β-lactoglobulin. II. Ultracentrifugal and electrophoretic studies of the association of β-lactoglobulin below its isoelectric point. *J. Am. Chem. Soc.* **1960**, *82* (12), 3161−3168.
(36) Townend, R.; Weinberger, L.; Timasheff, S. N. Molecular interactions in β-lactoglobulin. IV. The dissociation of β-lactoglobulin below pH 3.52. *J. Am. Chem. Soc.* **1960**, *82* (12), 3175−3179.
(37) de Wit, J. N.; Swinkels, G. A. A differential scanning calorimetric study of the thermal denaturation of bovine β-lactoglobulin. Thermal behaviour at temperatures up to 100 °C. *Biochim. Biophys. Acta* **1980**, *624* (1), 40−50.
(38) Wada, R.; Fujita, Y.; Kitabatake, N. Effects of heating at neutral and acid pH on the structure of β-lactoglobulin A revealed by differential scanning calorimetry and circular dichroism spectroscopy. *Biochim. Biophys. Acta* **2006**, *1760* (6), 841−847.
(39) Qi, X. L.; Holt, C.; McNulty, D.; Clarke, D. T.; Brownlow, S.; Jones, G. R. Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochem. J.* **1997**, *324* (Part 3), 341−346.
(40) Aymard, P.; Durand, D.; Nicolai, T. The effect of temperature and ionic strength on the dimerisation of β-lactoglobulin. *Int. J. Biol. Macromol.* **1996**, *19* (3), 213−221.
(41) Partanen, R.; Torkkeli, M.; Hellman, M.; Permi, P.; Serimaa, R.; Buchert, J.; Mattinen, M.-L. Loosening of globular structure under alkaline pH affects accessibility of β-lactoglobulin to tyrosinase-induced oxidation and subsequent cross-linking. *Enzyme Microb. Technol.* **2011**, *49* (2), 131−138.
(42) Coussons, P. J.; Price, N. C.; Kelly, S. M.; Smith, B.; Sawyer, L. Transglutaminase catalyses the modification of glutamine side chains in the C-terminal region of bovine β-lactoglobulin. *Biochem. J.* **1992**, *283* (Part 3), 803−806.
(43) Nieuwenhuizen, W. F.; Dekker, H. L.; Groneveld, T.; De Koster, C. G.; De Jong, G. A. Transglutaminase-mediated modification of glutamine and lysine residues in native bovine β-lactoglobulin. *Biotechnol. Bioeng.* **2004**, *85* (3), 248−258.
(44) Baez, G. D.; Moro, A.; Ballerinia, G. A.; Busti, P. A.; Delorenzi, N. J. Comparison between structural changes of heat-treated and transglutaminase cross-linked β-lactoglobulin and their effects on foaming properties. *Food Hydrocolloids* **2011**, *25* (7), 1758−1765.
(45) Eissa, A. S.; Puhl, C.; Kadla, J. F.; Khan, S. A. Enzymatic cross-linking of β-lactoglobulin: Conformational properties using FTIR spectroscopy. *Biomacromolecules* **2006**, *7* (6), 1707−1713.
(46) Buchert, J.; Ercili Cura, D.; Ma, H.; Gasparetti, C.; Monogioudi, E.; Faccio, G.; Mattinen, M.; Boer, H.; Partanen, R.; Selinheimo, E.; Lantto, R.; Kruus, K. Crosslinking food proteins for improved functionality. *Annu. Rev. Food Sci. Technol.* **2010**, *1* (1), 113−138.
(47) Qi, P. X.; Onwulata, C. I. Physical properties, molecular structures and protein quality of texturized whey protein isolate (WPI): Effect of extrusion temperature. *J. Agric. Food Chem.* **2011**, *59* (9), 4668−4675.
(48) Whittaker, A. G.; Mount, A. R.; Heal, M. R. *Physical Chemistry*; Taylor and Francis Group: London, U.K., 2000; p 296.
(49) Park, J.; Lee, J.; Kim, H.; Hahn, T.; Paik, Y. Isolation and characterization of water-soluble intermediates of blue pigments transformed from geniposide of *Gardenia jasminoides*. *J. Agric. Food Chem.* **2002**, *50* (22), 6511−6514.
(50) Starcher, B. A ninhydrin-based assay to quantitate the total protein content of tissue samples. *Anal. Biochem.* **2001**, *292* (1), 125−129.
(51) Helfferich, F. G. *Kinetics of Multistep Reactions*, 2nd ed.; Elsevier, Ltd.: Amsterdam, The Netherlands, 2004; Vol. *40*, p 488.
(52) Mi, F.; Shyu, S.; Peng, C. Characterization of ring-opening polymerization of genipin and pH-dependent cross-linking reactions between chitosan and genipin. *J. Polym. Sci., Part A: Polym. Chem.* **2005**, *43* (10), 1985−2000.

(53) Slusarewicz, P.; Zhu, K.; Hedman, T. Kinetic analysis of genipin degradation in aqueous solution. *Nat. Prod. Commun.* **2010**, *5* (12), 1853−1858.
(54) Brown, E. M.; Pfeffer, P. E.; Kumosinski, T. F.; Greenberg, R. Accessibility and mobility of lysine residues in *β*-lactoglobulin. *Biochemistry* **1988**, *27* (15), 5601−5610.
(55) Imafidon, G. I.; Ng-Kwai-Hang, K. F.; Harwalkar, V. R.; Ma, C. Y. Differential scanning calorimetric study of different genetic variants of *β*-lactoglobulin. *J. Dairy Sci.* **1991**, *74* (8), 2416−2422.