Enhancing the Gelation of β-Lactoglobulin

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The gelation characteristics of bovine β-lactoglobulin A (BLGA) have been enhanced by the selective introduction of cysteine substitutions to increase the free thiol content of the protein. A recombinant version of bovine β-lactoglobulin A (rBLG) has been modified by creating R40C (substitution of arginine at position 40 with cysteine), F82C (substitution of phenylalanine at position 82 with cysteine), and the double R40C/F82C variants. As expected, the number of free thiols increased correspondingly, suggesting that additional disulfide linkages are not formed. The strength of gels formed by heating at 70–90 °C was measured using a microscale penetration test. F82C and R40C/F82C displayed a gel strength equivalent to that of wild-type BLGA at a much lower concentration as compared to wild-type BLGA. R40C could not be brought to a sufficient concentration (>5%) without the formation of insoluble aggregates. Increasing the free thiol content also enhanced the ability of rBLG to form high molecular weight aggregates as observed during the heating of milk. An unexpected result was that the introduction of a free thiol also increased the ability of these rBLG variants to be cleaved by chymosin. Similarly, these variants were more susceptible to acid precipitation. These latter observations may be important for improving the performance of BLGA during the renneting process.

INTRODUCTION

The value of whey protein based ingredients is in part dependent upon their functional performance in various food products. Whey protein preparations are capable of forming thermoset gels, creating stable foams, and serving as emulsifiers (Morr and Foegeding, 1990). Unfortunately for a number of applications, the utility of whey proteins is not equivalent to that of other protein preparations (*i.e.*, soy protein) when compared on a cost basis.

β-Lactoglobulin (BLG) is a globular whey protein expressed in the glandular epithelium of the mammary gland of ruminants and several other species (Pervaiz and Brew, 1985; Hambling et al., 1992). The BLG variants isolated from bovine milk are among the most characterized forms of BLG. The protein exists as a dimer (molecular weight of 36 000) at physiological pH but dissociates into monomers below pH 3.0 (Tanford et al., 1959). Although the exact function *in vivo* is unclear, BLG is known to bind several small nonpolar ligands such as retinol, protoporphyrin IX, and free fatty acid (Futterman and Heller, 1972; Dufour et al., 1990; Puyol et al., 1991). A number of conformational changes in BLG are observed upon heating. On the basis of the known disulfide linkages between the C66/C160 and C106/C119 residues, the free thiol, C121, appears to mediate the intermolecular disulfide linkage formed upon heating (Creamer et al., 1983; Papiz et al., 1986). When BLG is heated, this free cysteine group, which is normally buried, is then exposed, leading to the formation of aggregates (Sawyer, 1968). It can react not only with itself but with other milk proteins, most notably κ-casein (McKenzie, 1971; Noh et al., 1989). This latter reaction appears to inhibit the renneting process, perhaps by occluding the site of hydrolysis in κ-casein (van Hooydonk et al., 1987). The naturally occurring BLG variants found in bovine milk can affect a number of properties including cheese yield and syneresis (Marziali and Ng-Kwai-Hang, 1986; Pearse et al., 1985; McLean and Schaar, 1989; Sweetsur and White, 1974). Preliminary data in our laboratory support the hypothesis that the C121 residue is responsible for thermal induced aggregation (S. Watkins and C. Batt, unpublished data). Furthermore, this intermolecular interaction appears to initiate gel network formation.

The ability of both whey proteins and purified BLG to form thermoset gels has been documented (Rector et al., 1989). A wide variety of gels can be formed, depending upon the protein concentration, pH, temperature, and ionic strength. BLG forms a translucent gel in the range 10–15% (w/v) at 70–80 °C (pH 8.0), while whey protein isolate forms a slightly turbid gel. Although some physical properties have been studied, including gel strength and optical transparency, little is known about the matrix cross-linking and interstitial space volume. It does, however, appear that the network linkages can be modified, yielding gels of different strengths. A number of thiol reductants and modifying agents including *N*-ethylmaleimide, dithiothreitol, and β-mercaptoethanol appear to affect gel strength (Mulvihill et al., 1991). In addition, calcium and sodium ions can be added to alter the properties of BLG (Mulvihill and Kinsella, 1988) and whey protein gels (Shimada and Cheftel, 1988; Mangino, 1992). Furthermore, these gel modifying agents can, as expected, alter the reversibility of the whey protein gel.

As an alternative to chemical or enzymatic modification of the milk protein (Lakkis and Villota, 1992), the prospects of improving BLG's functionality via site-specific structural modifications are dependent upon the ability to rationally design and then alter and produce variant forms of the protein (Kang and Richardson, 1985). These requisite components are now available in the form of a 2.7-Å-resolution structure which has been determined by X-ray analysis of the orthorhombic crystal form of BLGA (Papiz et al., 1986) in addition to an efficient method for producing modified forms of the protein (Jamieson et al., 1987; Batt et al., 1990). We describe improvements made to BLG which enhance its gelation properties and increase its sensitivity to chymosin and acid.

MATERIALS AND METHODS

Strains and Plasmids. *Escherichia coli* JM105 (Δ*lac pro, thi, strA, endA, sbcB*15, *hspR*4, [F′*traD*36, *proAB*⁺, *lacI*ᵠ,

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ZΔM15]) was used for the production of rBLG. *E. coli* JM2r⁻ (*hsdR*17, *mcrAB*, *recA*1, Δ*lac pro*, [F'*traD*36, *proAB*⁺, *lacI*ᵠ, ZΔM15]) was used as a recipient for site-directed mutagenesis reactions. pTTQ18 (Amersham, Arlington Heights, IL) was used as an expression vector; it carries a *tac* promoter, which is regulated by plasmid-borne *lacI*ᵠ gene. Bacteriophage M13mp19 was used for mutagenesis and sequencing.

DNA Manipulations. Restriction digests, isolation of DNA fragments, ligation reactions, and transformations were performed as described by Sambrook et al. (1989). All restriction and other DNA-modifying enzymes were purchased from United States Biochemicals (Cleveland, OH) or New England Biolabs (Beverly, MA) and used according to their instructions.

Site-Directed Mutagenesis. The R40 and F82 codons of the rBLG gene (*blgA*) cloned into M13mp19 (Jamieson et al., 1987) were targeted for oligonucleotide-mediated site-directed mutagenesis. Two oligonucleotides used to introduce mutations were

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R40C        3'-CGGGGGGACACGCACATACACG-5'
                  A  P  L  C  V  Y  V
F82C        3'-GGACGCCACACGTTCTAGCTAG-5'
                  P  A  V  C  K  I  D
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[Underlined nucleotides or amino acids indicate changes from wild-type rBLG. The oligonucleotide primer sequences which are derived from the antisense strand are displayed 3′ → 5′ to allow the logical presentation (N → C) of the amino acid sequence.] Selection of mutants was facilitated by enzymatic removal of the parent strand as described by Vandeyar et al. (1988).

PCR. Two primers were constructed to create an *Eco*RI restriction site, 23 bp upstream of the start codon, and a *Xba*I site at the stop codon. The 5′ PCR primer was 33 nt (5′-AGCTATGACCATGATGAATTCAAGCTTAGGAGG-3′; *Eco*RI site underlined). The 3′ PCR primer was 23 nt (5′-CTCGGTACCCGGGATCCTCTAGA-3′; *Xba*I site underlined) and amplified the flanking MCS of M13mp19. A 100-μL reaction mixture was prepared in a 0.5-mL microfuge tube as follows (Innis et al., 1990): 100 ng of M13mp19::*blgA*, 1 μL (50 pmol) of each primer, 5 μL of 1 M KCl, 0.67 μL of 50 mM Tris-HCl (pH 8.8), 1.5 μL of 0.1 M $MgCl_2$, 1 μL of BSA (New England Biolabs, 10 μg/μL), 5 μL of each 1 mM dNTP, and 2 units of *Taq* DNA polymerase (Promega Co., Madison, WI). After 75 μL of mineral oil was added on top of the reaction mixture, a total of 32 cycles were performed with a Hybaid thermal reactor (Hybaid Limited, Middlesex, England) using the following conditions: denaturation at 95 °C for 60 s, annealing at 60 °C for 60 s, extension at 70 °C for 60 s. After amplification, the PCR product was extracted with phenol/chloroform, ethanol precipitated, and restricted with *Eco*RI and *Xba*I. The *blgA* was ligated into pTTQ18, and the ligation mixture was then digested with *Bam*HI to enrich for recombinant colonies. Insertion of the *blgA* results in the loss of the *Bam*HI site in the multiple cloning site of pTTQ18. Therefore, restriction with *Bam*HI serves to linearize any nonrecombinant molecules which in their linear form do not efficiently transform *E. coli*.

Expression and Purification of rBLG. *E. coli* JM105 carrying pTTQ18BLG7.7.1 was grown in LB medium with ampicillin (50 μg/mL) at 37 °C and induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) at OD_{600} = 0.5 (Batt et al., 1990). pTTQ18BLG7.7.1 is a pTTQ18 derivative that carries the cDNA for BLGA cloned into the multiple cloning site. Its construction is described in detail in Batt et al. (1990). When cultures of JM105 carrying pTTQ18BLG7.7.1 are induced by the addition of IPTG, they accumulate mature wild-type BLGA as intracellular inclusion bodies. After 4 h at 37 °C, the cells were harvested by centrifugation and lysates were prepared in 100 mM Tris-HCl (pH 8.0) 2.0 mM EDTA by sonication. Insoluble proteins were collected from the lysate by centrifugation at 7000*g* for 10 min. The pellet was washed once with 50 mM Tris-HCl (pH 7.5) and dissolved in 0.1 M Tris-HCl (pH 8.0) containing 8 M urea and 2 mM dithiothreitol. After centrifugation to remove any insoluble material, the supernatant was then diluted 32-fold with 0.1 M Tris-HCl (pH 7.5) containing 0.25 mM oxidized glutathione, 0.5 mM reduced glutathione, and 0.2 mM EDTA and held at 0 °C. After 10 min of incubation at 0 °C, the temperature was raised to 22 °C and the solution stirred for 16 h. The pH of the solution was adjusted to 2.8 with acetic acid, and the resulting precipitate was removed by centrifugation at 9000*g* for 10 min. Finally, the acid-soluble material was applied to Sephadex G-50 in a 2.8 cm × 90 cm column equilibrated with 50 mM citric acid–sodium phosphate buffer (pH 2.8), and fractions containing renatured rBLG (as determined by PAGE) were collected. The protein concentration was estimated using the molecular absorption coefficient ϵ_{278} = 17 600 M^{-1} cm^{-1}.

Gelation Measurements. Gels were prepared with various concentrations of either wild-type or mutant rBLG in 50 mM Tris-HCl (pH 8.0) in a total volume of 20–50 μL using a 1.27 mm (i.d.) glass capillary tube. The tube was heat sealed at one end and the solution centrifuged at 2000*g* to remove any air bubbles. The tubes were heated at the prescribed temperature, and the strength of the resulting gel was determined. Gel strength was measured on 20-μL samples using a microscale penetrometer as described (Lee and Batt, 1993). The weight necessary to penetrate the sample to a depth of 33% of the original height was recorded.

Other Techniques. The number of free thiol groups was measured according to the method of Ellman (1959). The purified protein (162.5 μg) dissolved in 250 μL of 50 mM Tris-HCl (pH 8.0) was added to 875 μL of 100 mM Tris-HCl (pH 8.0) or 875 μL of 8 M urea/100 mM Tris-HCl (pH 8.0), and then 10 μL of 1% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added. After 10 min, the absorbance was measured at 412 nm.

Chymosin sensitivity was carried out by a modification of the method of Reddy et al. (1988). BLGA and rBLG were pretreated by heating at 90 °C for 5 min or reduced with 6 M urea 2.8 mM DTT before chymosin cleavage. A 1.0-mL solution of 0.04% BLG was prepared in 0.1 M sodium phosphate–citrate buffer (pH 5.5). Chymosin (1.5 units; Sigma Chemical Co., St. Louis, MO) was added, and digestion carried out at 35 °C for 1 h. The reaction was terminated by adjusting the pH to 2.5 with acetic acid, and the products were analyzed by 12.5% SDS–PAGE.

Acid stability was determined by the addition of trichloroacetic acid (TCA). A 1.0-mL solution of 0.05% rBLG was prepared in 50 mM glycine–HCl (pH 2.8), and then 1.0 mL of 3% TCA was slowly added. The solution was allowed to stand at room temperature for 5 min and then centrifuged at 12 000 rpm for 5 min. The supernatant was dialyzed against 50 mM glycine–HCl (pH 2.8) using a Centricon 10 (Pharmacia, Piscataway, NJ). Both the pellet and the supernatant were analyzed using 12.5% SDS–PAGE.

RESULTS

Placement of Free Thiols. The general strategy was to introduce free thiols into regions that were predicted to be hydrophobic and were spatially distant from the free thiol at C121. A hydrophobicity plot revealed a number of potential areas, and two codons were chosen where minimal changes to the nucleotide sequence would result in a cysteine substitution (Figure 1). In the original BLG X-ray structure, the α-carbons for the mutations R40C and F82C were 13.08 and 16.75 Å from the α-carbon of the C121 (Papiz et al., 1986).

The mutations were introduced using oligonucleotides specifying the desired changes (see Materials and Methods). Selection of mutants in the population of M13mp19::*blgA* was facilitated by enzymatic removal of the parental template using procedures as described by Vandeyar et al. (1988). Mutant rBLGs were then produced by subcloning the *blgA* into pTTQ18 and inducing their expression with IPTG. The R40C, F82C, and R40C/F82C expressed well, and the proteins could be purified in a manner similar to that used for the wild-type rBLG. Typical yields after purification were approximately 25 mg/L of original fermentation broth. The number of free thiol residues in both the wild-type and mutant rBLGs

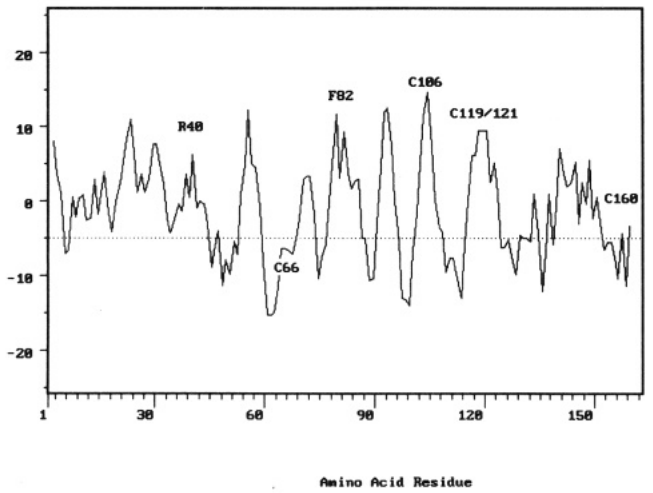

Figure 1. Hydrophobicity plot of BLG. Plot was prepared using the SOAP program in the PCGENE program suite. The *Y* axis is an arbitrary scale that defined the relative hydrophobic and hydrophilic inclination for an amino acid side chain. The region above the dashed line is hydrophobic, while the region below the dashed line is hydrophilic. Selected amino acid residues are noted.

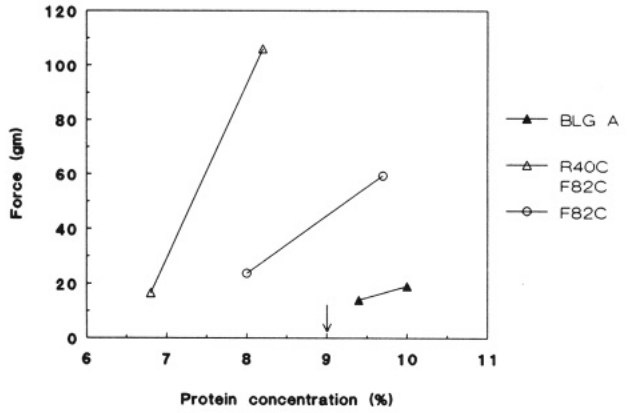

Figure 2. Effect of BLG concentration on the strength of gels produced from BLGA, F82C, and R40C/F82C rBLGs. Samples were heated at 90 °C for 15 min. The gel strength was measured by microscale penetrometry (Lee and Batt, 1993). Vertical arrow (↓) denotes the minimum concentration where wild-type BLG will form a gel under the conditions described under Materials and Methods.

was determined according to Ellman's (1959) method. As expected, a total of two free thiols were detected in both the R40C and the F82C rBLGs when subjected to denaturation with urea. The double mutant R40C/F82C when denatured had a total of three free thiols as measured by DTNB.

Gelation Characteristics. The wild-type BLGA, R40C, and F82C rBLGs were capable of forming gels when heated; however, the minimum protein and gelation temperature in addition to the strength of the resulting gels varied significantly. The strength of the gels was measured by the microscale penetrometry which permitted the use of small sample volumes, an important factor given the limited supply of rBLG (Lee and Batt, 1993). Wild-type BLGA when heated at 90 °C for 15 min formed gels with strengths of 14–19 g over a concentration range of 9.4–10% (Figure 2). It did not, however, form a gel when the protein concentration was reduced to below 9%. In contrast, the F82C rBLG formed a gel at concentrations down to 8% with a gel strength of 23.7 g. Gels of the R40C/F82C rBLG were formed at concentrations as low as 6.8% and had a gel strength of 16.5 g. The F82C and R40C/F82C rBLGs formed transparent gels; however, the

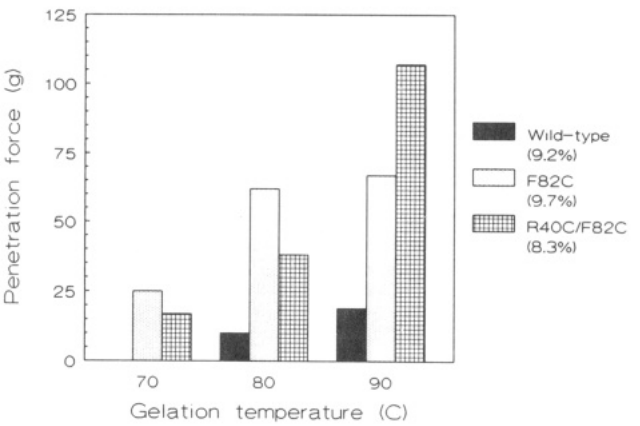

Figure 3. Effect of gelation temperature on the strength of gels prepared from wild-type, F82C, and R40C/F82C BLGs. Solutions at the concentrations noted were heated at 70, 80, or 90 °C for 15 min. The gel strength was measured by microscale penetrometry (Lee and Batt, 1993).

R40C rBLG formed a coagulum gel, and therefore its gel strength could not be accurately measured. It was not possible to concentrate any of the mutant rBLGs above 10% as they began to form aggregates during the concentration process even when the samples were held at 4 °C during concentration.

The effect of temperature on the strength of the gel was measured for the wild-type BLGA and mutant rBLGs. Figure 3 presents the strength of gels using wild-type BLGA, F82C, and R40C/F82C rBLGs when the samples were heated at 70–90 °C. Wild-type BLGA at a concentration of 9.2% does not form a gel when heated at 70 °C, which is in contrast to F82C where, at a 9.7% concentration, a gel with a strength of 25 g is formed. The F82C gel formed at 80 °C is 6.2-fold stronger than the wild type, and this difference in gel strength is similar for gels formed at 90 °C. The double mutant R40C/F82C formed a slightly weaker gel at both 70 and 80 °C as compared to the F82C rBLG. When heated at 90 °C, however, the strength of the R40C/F82C was greater than that of either the wild type or F82C.

Chymosin and Acid Sensitivities. The enhanced gelation characteristics of the mutant rBLG are most likely a function of the increased number of free thiols and perhaps the exposure of these additional free thiols on the surface of the protein. The exposure of the free thiols would most likely result in a less compact structure which would be predicted to be more sensitive to chymosin and acid. BLG is known to be resistant to chymosin, in part due to the absence of the preferred cleavage site, F–M, in addition to its compact structure. The wild-type BLGA and F82C and R40C rBLGs were tested for their susceptibility to cleavage by chymosin. In addition, another mutant rBLG, H146R, was subjected to the same analysis to evaluate the effect of presumably irrelevant amino acid substitutions. This latter change targeted the H146 residue, which appears to reside on the dimer interface and may be responsible for the pH-dependent dimerization process (Tanford et al., 1959). The common band in all samples to which chymosin was added corresponds to chymosin. Wild-type BLGA is not cleaved by chymosin except to a limited extent when the protein is heated at 90 °C for 15 min (Figure 4). Similarly, the H146R rBLG is relatively resistant to chymosin, except again when the protein is heated prior to digestion. H146R is very sensitive when the protein is reduced, presumably due to the unfolding upon reduction of the disulfide linkages. In contrast, the R40C rBLG is more sensitive when heated

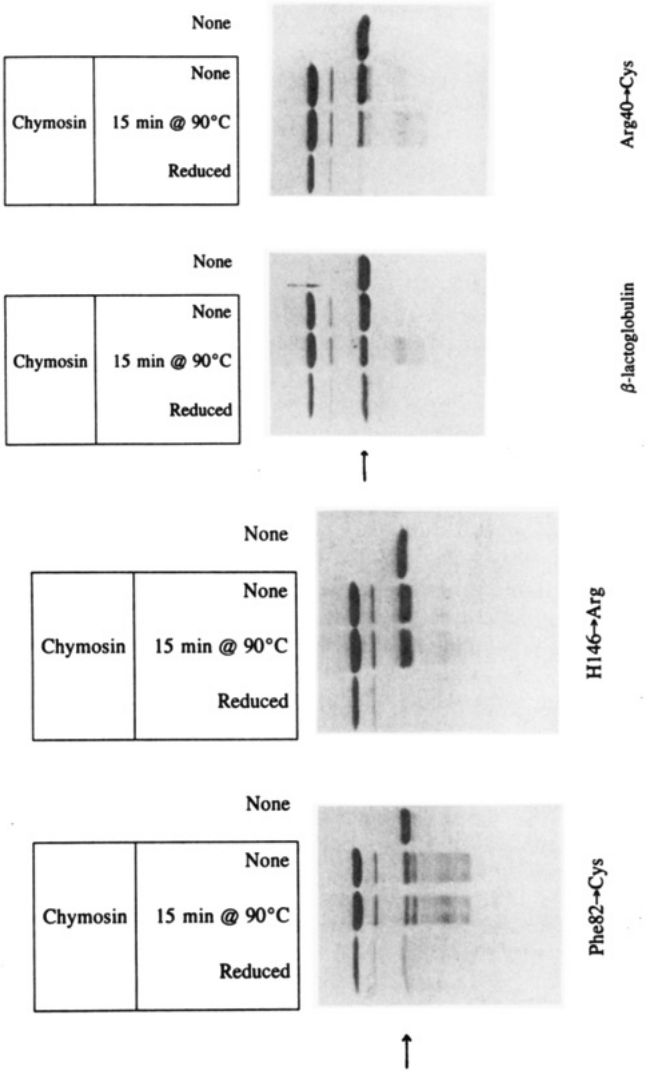

Figure 4. Cleavage of wild-type, H146R, R40C, and F82C rBLGs by chymosin. BLG solutions were digested with 1.5 units of chymosin for 1 h at 35 °C. The digestion products were then separated on a 12.5% SDS–PAGE and visualized by staining with Coomassie blue. The upper band in enzyme-digested lanes is chymosin. The direction of migration is left to right with the anode (+) at the right of the gel.

and is completely digested when it is reduced prior to chymosin treatment. The F82C appears to be the most sensitive and is digested even in the absence of heating or reduction prior to treatment. The double mutant R40C/F82C has not been examined to date.

A second predicted difference would be the sensitivity of the mutant rBLGs to precipitation by acid. Although all mutants were able to remain in solution at pH 2.8 (a part of the normal purification process), they differ in their ability to be precipitated by the addition of 1.5% trichloroacetic acid (Figure 5). Neither the wild-type nor the H146R rBLG was precipitated by the addition of trichloroacetic acid, and the majority of the protein remained in solution. In contrast, approximately 70–80% of the R40C rBLG was precipitated by the addition of trichloroacetic acid with the remainder found in solution. As for the chymosin sensitivity, the F82C appears to be the most sensitive to trichloroacetic acid as evidenced by the significant fraction which precipitated out of solution.

DISCUSSION

BLG in addition to other proteins including ovalbumin, bovine serum albumin, and soybean glycinin forms a

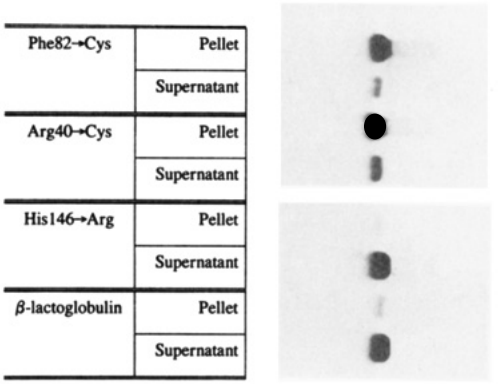

Figure 5. Effect of trichloroacetic acid on the preparation of wild-type, H146R, R40C, and F82C rBLGs. BLG solutions were prepared, and TCA was added to a final concentration of 1.5%. After 5 min at room temperature, the samples were centrifuged at 12 000 rpm for 5 min. The supernatant was removed and the pellet resuspended in its original volume using 25 mM glycine–HCl (pH 2.8). The pellet and the supernatant were analyzed by a 12.5% SDS–PAGE and the BLG visualized by staining with Coomassie blue. The direction of migration is left to right with the anode (+) at the right of the gel.

macromolecular matrix when heated above the appropriate concentration and temperature. Two basic types of protein gels can be formed: a coagulant, which is probably an aggregation of denatured proteins, and a transparent gel, which represents a slightly more ordered structure with both intermolecular disulfide linkages and electrostatic and hydrophobic interactions (Hashizume and Sato, 1988; Arntfield et al., 1991; Mangino, 1992; Shimada and Cheftel, 1988, 1989). Soluble egg white aggregates are polymerized upon standing through a mechanism which involves sulfhydryl–disulfide interchange and sulfhydryl oxidation (Mine, 1992). Schmidt et al. (1979) reported that increasing the sulfhydryl content resulted in an increase in the strength and texture of whey protein gels. For BLG, the mechanism by which a gel is formed is not clear, but it is probably initiated by a disulfide interchange. This interchange is most likely mediated by the free thiol at C121 which attacks the C119–106 disulfide. Blocking the free thiol with *N*-ethylmaleimide drastically reduces the ability of bovine BLG to form a thermoset gel, as does the addition of a reducing agent, *i.e.* 2-mercaptoethanol (Hashizume and Sato, 1988; Mulvihill et al., 1991). In addition, naturally occurring BLG variants which lack a free thiol (*i.e.*, porcine BLG) do not form gels (S. Watkins and C. A. Batt, unpublished results). Finally, introduction of a third disulfide linkage by site-directed mutagenesis to create either an A132C or a L104C rBLG results in a protein which cannot form a gel (Cho et al., 1992).

The role of the free thiol in initiating and then forming a gel suggests that additional free thiols might promote a stronger gel. This was elegantly demonstrated by the chemical thiolation of BLG which resulted in a gel that could be chemically polymerized (Kim et al., 1990a). These experiments involved the chemical modification of one or more of the accessible lysine residues to introduce additional free thiols. Chemical oxidation could then be used as an alternative to heating to induce gel formation. A correlation was observed between the polymerization of BLG and the number of free thiol groups added. A minimum of eight free thiols were necessary to achieve gelation at a concentration of 5%, and no overt control could be instituted on the placement of the thiol groups. We have chosen to extend these efforts and employ a site-specific placement of additional free thiols. Although we have not exhaustively examined the effect of either thiol location or number on the gelation properties of BLG, we

have achieved stable gels at concentrations as low as 6.8% with only two additional free thiols.

The resistance of BLG to chymosin cleavage and precipitation by trichloroacetic acid has been reported (Harper et al., 1989; Fox et al., 1967). Heating of milk causes BLG to partially aggregate on the casein micelles through a thiol interaction with κ-casein (Sawyer, 1969; Singh and Fox, 1987). The aggregation of BLG on the casein micelles reduces the effectiveness of chymosin (Sawyer, 1968); however, up to a certain point, an increase in curd yield may be realized. Overheating can, however, significantly inhibit chymosin cleavage and results in a failure of the curd to properly form. Therefore, the proper control of BLG–κ-casein interactions is essential.

The mutations, F82C and R40C, which we have made to BLGA increase susceptibility to chymosin cleavage. This combined with their enhanced sensitivity to acid precipitation may render them less likely to remain in the whey after the renneting and fermentation process. It is difficult, however, to estimate at the present time their susceptibility after aggregation on the casein micelles, a process that should be enhanced due to their increased free thiol content. It will be interesting to test the performance properties of these modified rBLGs in a milk substrate background.

The ability to modify proteins in a selective manner through site-directed mutagenesis and then to test the resultant effect of these modifications on their functional properties will hopefully enhance our knowledge on the structure–function relationships mediating these economically valuable characteristics. A limited number of studies have been conducted as exemplified by the improvements reported in the functional properties of soybean glycinin (Kim et al., 1990b). Glycinin derivatives could be produced which had enhanced gelation properties by inserting series of methionine residues and thus increasing the hydrophobicity of selected regions. The results of our study clearly indicate the potential benefit of increasing the gel strength by the addition of free thiols. Further modifications to alter the charge density of the surface and the exposure of hydrophobic regions may have similar benefits on the gelation properties of BLGA.

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