Thermodynamic Compatibility of Substrate Proteins Affects Their Cross-Linking by Transglutaminase

Xiao-Qing Han and Srinivasan Damodaran*

Department of Food Science, University of Wisconsin—Madison, 1605 Linden Drive, Madison, Wisconsin 53706

The formation of heterologous dimers and polymers between different proteins by transglutaminase reaction was studied. Although β-casein and β-lactoglobulin formed homologous polymers in single-substrate systems, only β-casein was polymerized; β-lactoglobulin became a nonreactive substrate in a β-casein/β-lactoglobulin binary system. In contrast, succinylated β-casein formed heterologous dimers, trimers, and oligomers with β-lactoglobulin. Also, β-casein did not form a heterologous dimer or polymer with bovine serum albumin (BSA), whereas α-lactalbumin formed a heterologous dimer and polymer with BSA. Analyses of reaction products of other binary systems, such as α-lactalbumin/β-casein, β-lactoglobulin/ovalbumin, and α-lactalbumin/ovalbumin, suggested that heterologous cross-linking between two proteins by transglutaminase probably depends on the thermodynamic compatibility of mixing of the substrate proteins at the enzyme's active site. Thus, cross-linking between dissimilar proteins, such as caseins and albumins, is not possible, whereas cross-linking between similar proteins, e.g., albumins, globulins, and caseins, can occur.

Keywords: *Transglutaminase; protein cross-linking; thermodynamic compatibility*

INTRODUCTION

Transglutaminase (EC 2.3.2.13) catalyzes an acyl transfer reaction between the γ-carboxamide group of glutamine residues and the ε-amino group of lysyl residues of peptide chains. In addition to this cross-linking reaction, it can also catalyze aminolysis of the γ-carboxamide group of peptide-bound glutamine residues, hydrolysis of the γ-carboxamide group of glutamine residues when the level of amine substrate is low or absent, and hydrolysis and aminolysis of certain aliphatic amides (Folk, 1983). Because of these multiple reactions catalyzed by transglutaminase, it has been difficult to develop a mechanistic model for its catalysis of protein cross-linking reaction. In particular, how two macromolecular substrate proteins come close against steric hindrance at the active site of the enzyme and form an ε-(γ-glutamyl)lysyl cross-link is still unclear.

The transglutaminase-catalyzed cross-linking of proteins often leads to polymerization and, in some cases, nonthermal gelation of several proteins (Kang et al., 1994; Nio et al., 1985, 1986; Motoki et al., 1987; Sakamoto et al., 1994; Nonaka et al., 1992; Tanaka et al., 1990). In addition, this reaction has also been shown to be useful for incorporating essential amino acids, such as L-lysine and L-methionine, into food proteins (Ikura et al., 1981, 1985). Perhaps the most important application of the transglutaminase reaction is in the synthesis of heterologous protein conjugates with novel functional properties. For instance, it is well recognized that proteins that possess good foaming and emulsifying capacity often do not possess the ability to stabilize foams and emulsions, whereas proteins that exhibit poor foaming and emulsifying capacity often display the ability to stabilize the dispersed phases. If this is the case, then a logical approach to improving the foaming and emulsifying properties of proteins is to synthesize protein conjugates in which one of the components imparts emulsifying and foaming capacity and the other imparts stability.

Several studies have shown that transglutaminase can catalyze the homologous polymerization of several proteins, whereas its ability to catalyze the heterologous polymerization or dimerization of proteins has not been experimentally demonstrated. Motoki and Nio (1983) reported that the transglutaminase reaction produced high molecular weight polymers when acetylated α_{S1}-casein was incubated with several proteins, including β-lactoglobulin and soy 11S and 7S globulins. Similarly, it has been claimed that transglutaminase catalyzed cross-linking of myosin to soy globulins, casein, and gluten (Kruth, 1983; Kruth and Rogers, 1984). However, no direct evidence indicating formation of heterologous polymers between native proteins has been shown in the above studies. Because the polymers formed in all of these investigations could not penetrate the separating gel of SDS—PAGE, it was not possible to distinguish whether the polymers were a mixture of homologous polymers of each protein species or heterologous polymers of different protein species.

Generally, protein substrates of transglutaminase are classified into four groups: (1) Gln-Lys-type, in which both Gln and Lys residues are available for cross-linking; (2) Gln-type, in which only the Gln residue is available for reaction; (3) Lys-type, in which only Lys residues are available; and (4) a nonreactive type, in which both Glu and Lys residues are unavailable for reaction (Ikura et al., 1984). This classification is mainly based on the accessibility of Lys and Gln residues located on the protein's surface.

According to the above classification, a mixture of two Gln-Lys-type substrate proteins or a mixture of Gln-type and Lys-type substrate proteins should be able to form heteroconjugates in transglutaminase-catalyzed reaction. However, in addition to the availability of Lys and Gln residues, another factor that could potentially affect cross-linking of two different macromolecular protein substrates by transglutaminase is the thermo-

* Author to whom correspondence should be addressed [telephone (608) 263-2012; fax (608) 262-6872; e-mail sdamodar@facstaff.wisc.edu].

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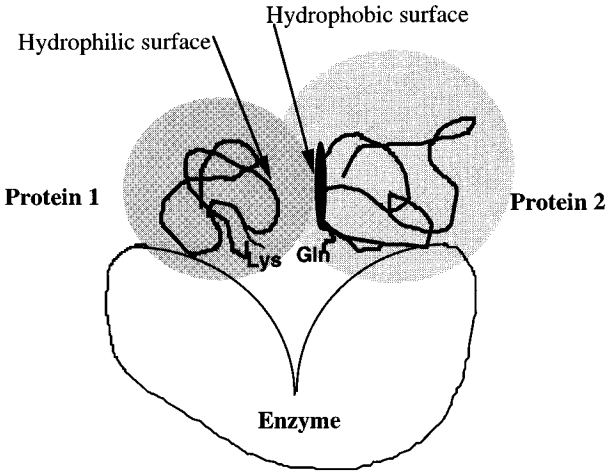
Scheme 1



dynamic compatibility of mixing of the protein substrates at the enzyme's active site.

The thermodynamic compatibility is related to the nature and the *intensity* of interaction between two macromolecules as they approach each other. As stated by Flory (1986), two polymers are mutually compatible with one another only if their free energy of interaction is favorable, i.e., negative. In a majority of cases, the interaction energy, ΔG_{ij}, where the subscripts i and j refer to polymers 1 and 2, respectively, is usually positive, and thus incompatibility of dissimilar polymers is the rule and compatibility is the exception (Flory, 1986). Proteins belonging to different classes have been shown to exhibit limited thermodynamic compatibility, and an aqueous mixture of binary protein solution separates into two distinct phases. In proteins, limited thermodynamic incompatibility arises as a result of interaction between polar and apolar surfaces. Because of this mutual repulsion between polar and apolar surfaces, two dissimilar proteins will approach each other only up to a distance at which the free energy of the interaction is zero. Beyond this closest distance of approach, a positive free energy of interaction will develop. Because of this mutual repulsion, i.e., limited thermodynamic compatibility, each protein molecule will possess an excluded volume which cannot be accessed by other proteins. (It should be remembered that the *excluded volume* referred to here is not the same as that resulting from the spatial requirements of polymer molecules. In this respect, the excluded volume referred to here will be the sum of those arising from spatial requirements and from the positive free energy of polar−nonpolar repulsive interactions.) Because of this excluded volume effect, thermodynamically incompatible proteins may not be able to mix at the active site of transglutaminase, as shown in Scheme 1. The inability to come close together at the active site and form a transient enzyme−substrate complex may preclude formation of heterologous dimers and polymers.

In this paper we provide evidence to show that heteroconjugation does not occur in some mixtures of Gln-Lys-type substrate proteins and formation of heteroconjugates between two proteins may fundamentally depend on their thermodynamic compatibility of mixing at the enzyme's active site.

MATERIALS AND METHODS

Pig liver transglutaminase, bovine serum albumin (BSA), β-casein, ovalbumin, α-lactalbumin, β-lactoglobulin, CBZ-L-glutaminylglycine, and hydroxylamine were obtained from Sigma Chemical Co. (St. Louis, MO). The enzyme and other proteins were used as such without further purification. All other chemicals and reagents used in this study were of analytical grade.

Assay of Transglutaminase Activity. Transglutaminase activity was measured using the colorimetric hydroxamate assay method described by Folk and Cole (1965). The final reaction mixture contained 0.1 M Tris−acetate buffer, pH 6.0, 30 mM CBZ-L-glutaminylglycine, 100 mM hydroxylamine, 10 mM glutathione (GSH), and 5 mM $CaCl_2$. After 10 min of incubation with enzyme solution at 37 °C, ferric chloride−trichloroacetic acid reagent was added to stop the reaction. The precipitate was removed by centrifugation, and the resulting red color was measured at 525 nm. L-Glutamic acid−γ-monohydroxamic acid was used as a standard for calibration. One unit of transglutaminase activity was defined as the amount of enzyme needed to produce 1 μmol of hydroxamic acid/min.

Protein Cross-Linking Mediated by Transglutaminase. Substrate proteins were incubated with transglutaminase in 0.1 M Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, and 1.0 mM glutathione. A standard reaction system contained 1% total protein substrates at pH 7.5 and an enzyme level of 10 units/g of protein. The reaction mixtures were incubated at 37 °C, and the reaction was terminated by adding SDS−polyacrylamide electrophoresis (SDS−PAGE) sample buffer containing 2% SDS and 5% β-mercaptoethanol. The reaction products were analyzed by SDS−PAGE.

SDS−PAGE. SDS−PAGE was performed on a 5−20% gradient slab gel using a discontinuous buffer system as described by Laemmli (1971). Protein products of the transglutaminase reaction were mixed directly with SDS−PAGE sample buffer solution (0.02 M phosphate, pH 7.2, containing 5% β-mercaptoethanol, 2% SDS, 5% glycerol, and 0.05% bromphenol blue). In most of the experiments, about 70 μg of protein was loaded per well. The gel electrophoresis was carried out at a constant voltage. The gel was stained with 0.1% Coomassie Brilliant Blue-R 250. Molecular weight marker proteins were run along with test proteins, and a standard curve obtained by plotting the logrithm of molecular weight against electrophoretic mobility (i.e., the distance from the top of the separating gel to the position of the protein) was used to determine the molecular weights of protein species formed in the transglutaminase reaction.

Succinylation. To 10 mL of 2% protein solution adjusted to pH 9.0 was added a total of 0.4 g of succinic anhydride in incremental amounts over a period of 2 h. During the reaction, the pH of the reaction solution was maintained at 9.0 by adding 1 M NaOH using a pH-stat (Model 450, Fisher Scientific Instruments). At the end of the reaction, the solution was exhaustively dialyzed against distilled water and freeze-dried.

RESULTS AND DISCUSSION

***β*-Casein/*β*-Lactoglobulin Binary System.** Figure 1A shows polymerization of β-casein by transglutaminase in a single-substrate system. The protein was completely polymerized within 60 min of reaction time into high molecular weight polymers that could not penetrate the stacking and separating gels, indicating that β-casein behaved as a Gln-Lys-type substrate for transglutaminase.

Figure 1B shows the results of transglutaminase reaction between β-casein and β-lactoglobulin. The SDS−PAGE (5−20% gradient gel) patterns of an untreated mixture of β-casein and β-lactoglobulin suggest that the apparent molecular weights of these proteins based on their mobility in this gel are about 18 000 and 30 900, respectively (lane 2). When a mixture of β-casein and β-lactoglobulin was incubated with transglutaminase at an enzyme to substrate ratio of 2 units/g of substrate, the concentration of monomeric β-casein decreased and several new bands of high molecular

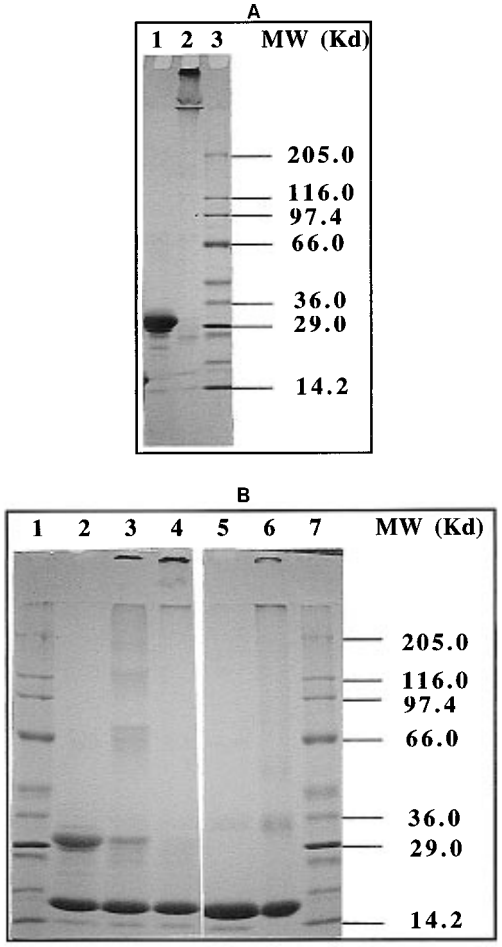


Figure 1. SDS−PAGE profiles of transglutaminase-mediated polymers formed between β-casein and β-lactoglobulin. (A) Polymerization of β-casein alone: lane 1, β-casein control; lane 2, β-casein treated with transglutaminase under "standard" conditions (see Materials and Methods for details); lane 3, protein markers. (B) Polymerization in β-casein/β-lactoglobulin binary system: lane 1, protein markers; lane 2, β-casein + β-lactoglobulin control; lane 3, β-casein + β-lactoglobulin treated with transglutaminase (a 1:1 mixture containing 5% total substrate protein was treated with the enzyme at an enzyme to substrate ratio of 2 units/g of substrate); lane 4, β-casein + β-lactoglobulin (1:1) treated with transglutaminase at 1% total substrate protein and at an enzyme to substrate ratio of 10 units/g of substrate; lane 5, β-lactoglobulin control; lane 6, β-lactoglobulin alone treated with transglutaminase under "standard" conditions; lane 7, protein markers.

weight polymers appeared (Figure 1B, lane 3). The intensity of β-lactoglobulin monomer band remained the same as that of the untreated control (lane 2), indicating that β-lactoglobulin did not take part in the transglutaminase-induced polymerization reaction. Thus, the bands corresponding to molecular weights of 64 000−70 000, 120 000, and the high molecular weight polymers that appeared at the top of the stacking and separating gels must be homologous polymers of β-casein. At an enzyme to substrate ratio of 10 units/g of protein substrate, monomeric β-casein disappeared completely, whereas no reduction occurred in the band intensity of monomeric β-lactoglobulin (Figure 1, lane 4). This indicated that even at this high enzyme to substrate ratio, the high molecular weight polymers found at the top of the separating and stacking gels (lane 4) were only homologous polymers of β-casein. These results clearly showed that in the β-casein/β-lactoglobulin binary system, only β-casein was polymerized by transglutaminase and β-lactoglobulin was left untouched.

The above results tentatively suggested that β-lactoglobulin was not a good substrate for transglutaminase. To verify if this was true, β-lactoglobulin alone was treated with transglutaminase at an enzyme to substrate ratio of 10 units/g of substrate. Figure 1B (lanes 5 and 6) shows SDS−PAGE profiles of untreated and transglutaminase-treated β-lactoglobulin. A significant decrease in β-lactoglobulin monomer occurred, and bands corresponding to β-lactoglobulin dimer (∼36 000 molecular weight) and polymers that could not penetrate the stacking and separating gels appeared in the SDS−PAGE gel (lane 6). It should be noted that the band corresponding to α-lactalbumin contaminant (MW 14 200) also has disappeared in the enzyme-treated sample, whereas it did not do so in the β-casein/β-lactoglobulin binary system (lanes 3 and 4).

These results unequivocally indicate that, in a single-protein system, β-lactoglobulin (and α-lactalbumin as well) acts as a Gln-Lys-type substrate for transglutaminase and forms homologous dimers and polymers, whereas in the presence of β-casein it is not utilized by the enzyme. Although both β-casein and β-lactoglobulin are Gln-Lys-type substrates for transglutaminase, they are unable to form a heterologous dimer or polymer. This seemingly strange behavior ought to be related to their inability to come together simultaneously at the active site of the enzyme and form a transient enzyme−β-casein−β-lactoglobulin complex. It seems that whereas two protein molecules of the same kind, e.g., β-casein−β-casein or β-lactoglobulin−β-lactoglobulin, can come together at the active site of the enzyme, two dissimilar molecules, i.e., β-casein and β-lactoglobulin, cannot simultaneously approach the active site of the enzyme. This phenomenon must be related in part to thermodynamic incompatibility of mixing of these two dissimilar proteins at the enzyme's active site. The thermodynamic incompatibility arises from repulsive interaction between the hydrophobic β-casein and the hydrophilic β-lactoglobulin molecules as they approach the enzyme's active site. β-Casein ranks among the most hydrophobic proteins known. Its average hydrophobicity is 5.56 kJ mol^{-1} $residue^{-1}$, and its nonpolarity index (the fraction of nonpolar residues) is 0.47. The entire net charge of the protein (−12) at pH 7.0 resides at the N-terminal 1−42 segment. All five phosphoserine residues are present in this segment. The rest of the protein chain, i.e., four-fifths of the molecule, is extremely hydrophobic and electrically neutral. The average hydrophobicity increases from 3.5 kJ mol^{-1} $residue^{-1}$ at the N-terminal end to about 8.3 kJ mol^{-1} $residue^{-1}$ toward the C-terminal end. In contrast, the surface of β-lactoglobulin is so hydrophilic that it is soluble even at its isoelectric pH (Zhu and Damodaran, 1994). Because of this large difference in the hydrophilic/hydrophobic characteristics, repulsive interactions between these dissimilar proteins would increase their excluded voumes (Polyakov et al., 1986), which in turn would increase the closest distance of approach. Thus, the inability of β-lactoglobulin and β-casein to come closer together at the active site of the enzyme seems to preclude the possibility of heterologous dimer or polymer formation.

Although the inability to form heterologous polymers can be explained using the thermodynamic incompatibility concept, it is not clear as to why homologous β-lactoglobulin dimer or polymer is not formed in the presence of β-casein. A probable reason for this is differences in reaction rates. At similar reaction condi-

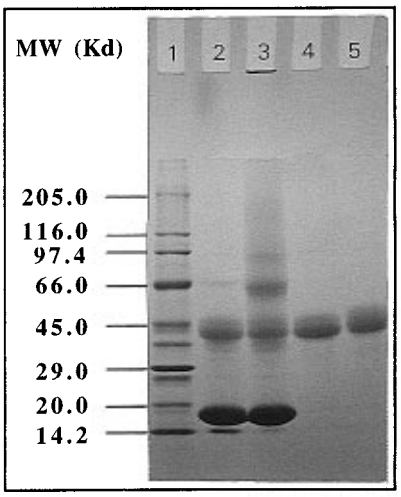


Figure 2. SDS−PAGE profiles of transglutaminase-catalyzed polymerization of succinylated β-casein and β-lactoglobulin. Lane 1, protein markers; lane 2, succinylated β-casein + β-lactoglobulin (1:1, 1% total protein) control; lane 3, succinylated β-casein + β-lactoglobulin (1:1, 1% total protein) treated with transglutaminase; lane 4, succinylated β-casein control; lane 5, succinylated β-casein alone treated with transglutaminase.

tions, the rate of cross-linking of β-lactoglobulin in a single-protein system is much slower than that of β-casein. For instance, within 60 min of reaction time, almost all β-casein monomer disappears (Figure 1B, lane 4), whereas only a fraction of β-lactoglobulin monomer is polymerized (Figure 1B, lane 6). Because of this difference in rates, the active site of the enzyme might be continually occupied by β-casein, resulting in lack of polymerization of β-lactoglobulin.

To elucidate if the inability of β-casein and β-lactoglobulin to form heterologous dimer or polymer is indeed due to the thermodynamic incompatibility of these proteins, the transglutaminase reaction between succinylated β-casein and β-lactoglobulin was studied. The rationale for this is that when β-casein is succinylated, the electrically neutral segment 43−209 of the molecule will become highly negatively charged. On the basis of the amino acid sequence, the net charge of this segment at pH 7.0 should increase from 0 to −20. This should result in an increase in the hydrophilicity of this segment as well as that of the entire molecule. This may greatly decrease the repulsive hydrophobic−hydrophilic interaction between (succinylated) β-casein and β-lactoglobulin and enable them to become at least partially thermodynamically compatible.

Figure 2 shows SDS−PAGE profiles of the reaction products formed between succinylated β-casein and β-lactoglobulin. It should be noted that the apparent molecular weight of succinylated β-casein in the SDS−PAGE was about 40 000 (Figure 2, lanes 2−5) compared to 30 900 for the unmodified β-casein (Figure 1A). Succinylated β-casein treated with transglutaminase showed no high molecular weight bands (lanes 4 and 5), confirming that all lysyl groups in β-casein have been modified and the protein was no longer a Gln-Lys-type substrate for transglutaminase. The β-lactoglobulin/succinylated β-casein mixture treated with transglutaminase contained protein bands corresponding to molecular weights of 64 000 and 91 000, as well as high molecular weight polymers that could not penetrate the stacking gel (lane 3). Since no such molecular weight bands appeared when β-lactoglobulin alone was incubated with transglutaminase (Figure 1B, lane 6), it must be concluded that these protein species must be heterologous dimers, trimers, and polymers of β-lactoglobulin and succinylated β-casein. The band corresponding to an apparent molecular weight of 64 000 appears to be a heterologous dimer of β-lactoglobulin and succinylated β-casein, and the band corresponding to an apparent molecular weight of 91 000 appears to be a trimer made up of two β-lactoglobulin chains and one succinylated β-casein chain (lane 3). It must be pointed out that, on the basis of the apparent molecular weights of β-lactoglobulin and succinylated β-casein, the molecular weight of β-lactoglobulin/succinylated β-casein dimer must be only about 58 000 (i.e., 40 000 + 18 000) and that of the β-lactoglobulin−β-lactoglobulin−succinylated β-casein trimer must be only about 76 000 (i.e., 18 000 + 18 000 + 40 000); however, the apparent molecular weights of these species in SDS−PAGE (i.e., 64 000 and 91 000) appear to be larger than the above values. These higher than expected molecular weights must be due to abnormal mobility of these species (similar to the abnormal mobilities of native and succinylated β-caseins) in SDS−PAGE. The results indicate that, in the succinylated β-casein/β-lactoglobulin binary system, succinylated β-casein acts as the Gln-type substrate and is able to cross-link to a limited extent with the Gln-Lys-type β-lactoglobulin. That is, unlike β-lactoglobulin and native β-casein, β-lactoglobulin and succinylated β-casein seem to exhibit limited thermodynamic compatibility of mixing at the enzyme's active site.

One could argue that the ability of succinylated β-casein to cross-link with β-lactoglobulin and the inability of native β-casein to cross-link with β-lactoglobulin are related to conformational differences between native and succinylated β-caseins. However, this reasoning is questionable, because, even in the native state, β-casein is predominantly in an unordered, flexible conformation (Swaisgood, 1986). Thus, Gln residues must be readily available in both the native and succinylated β-caseins for cross-linking with β-lactoglobulin. The differences in the abilities of native and succinylated β-caseins with β-lactoglobulin therefore ought to be related to differences in their thermodynamic compatibility of mixing with β-lactoglobulin at the active site of the enzyme.

α-Lactalbumin/BSA Binary System. Figure 3 shows SDS−PAGE profiles of the transglutaminase reaction products formed in the α-lactalbumin/BSA binary system. In the single-substrate system, no reduction in the concentration of BSA monomer occurred after the transglutaminase reaction (Figure 3A, lanes 1 and 2), indicating that BSA was not a Gln-Lys-type substrate. However, in the α-lactalbumin/BSA binary system, a reduction in the concentrations of both α-lactalbumin and BSA monomers occurred compared to the untreated control (Figure 3B, lanes 1 and 2), showing that BSA acted as either a Gln-type or Lys-type substrate in the presence of α-lactalbumin, which is a Gln-Lys-type substrate. Conversely, native BSA cannot form a homologous dimer or polymer, and it can only be able to form a heterologous dimer or polymer with a Gln-Lys-type substrate. The protein band corresponding to a molecular weight of 28 000 appeared to be a homologous dimer of α-lactalbumin, and the band corresponding to a molecular weight of 78 000 appeared to be a heterologous dimer of BSA and α-lactalbumin (Figure 3B, lane 1). The polymers at the top of the stacking and separating gels must be a mixture of homologous polymers of α-lactalbumin and

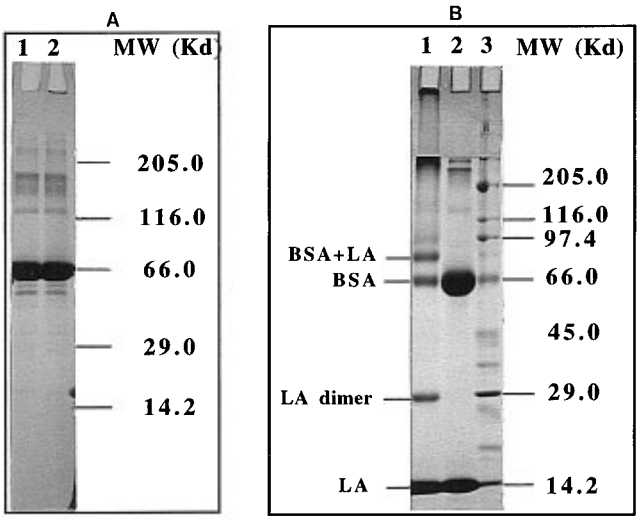


Figure 3. SDS−PAGE profiles of transglutaminase-catalyzed polymerization between α-lactalbumin (LA) and BSA. (A) Polymerization of BSA in a single-substrate system: lane 1, BSA control; lane 2, BSA alone treated with transglutaminase. (B) Polymerization in α-lactalbumin/BSA binary system: lane 1, LA + BSA (1:1, 1% total substrate) treated with transglutaminase; lane 2, LA + BSA (1:1, 1% total) control; lane 3, protein markers. Cross-linking reactions were performed under "standard" conditions.

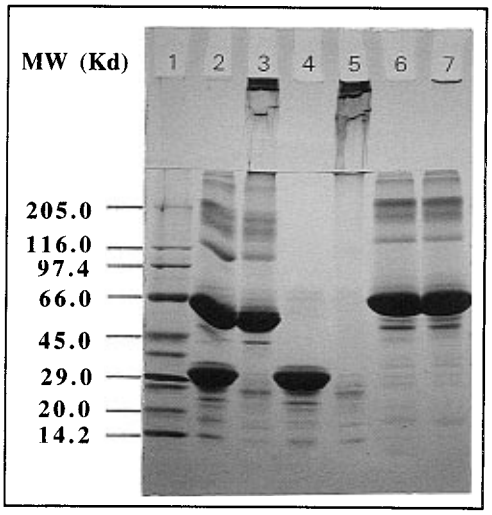


Figure 4. SDS−PAGE profiles of transglutaminase-catalyzed polymerization between β-casein and BSA. Lane 1, protein markers; lane 2, β-casein + BSA (1:1) control; lane 3, β-casein + BSA (1:1, 1% total substrate) treated with transglutaminase; lane 4, β-casein control; lane 5, β-casein alone treated with transglutaminase; lane 6, BSA control; lane 7, BSA alone treated with transglutaminase. All cross-linking reactions were performed under "standard" conditions.

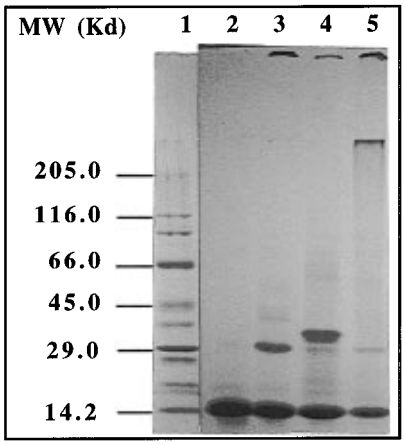


Figure 5. SDS−PAGE profile of transglutaminase-catalyzed polymerization between β-casein and α-lactalbumin. Lane 1, protein markers; lane 2, α-lactalbumin control; lane 3, α-lactalbumin alone treated with transglutaminase; lane 4, α-lactalbumin + β-casein (1:1) control; lane 5, α-lactalbumin + β-casein (1:1, 1% total substrate) treated with transglutaminase. All cross-linking reactions were performed under "standard" conditions.

heterologous polymers of BSA and α-lactalbumin (Figure 3B, lane 1). The results indicate that BSA and α-lactalbumin are compatible substrates for transglutaminase and it is possible to synthesize heterologous dimer and polymers of these two albumin-type proteins.

BSA/β-Casein Binary System. The results of the BSA/α-lactalbumin binary system suggest that because native BSA is either a Gln-type or Lys-type substrate, it cannot form a homologous dimer or polymer, but it can form a heterologous dimer or polymer with a Gln-Lys-type substrate such as α-lactalbumin. If this is the case then, since β-casein also is a Gln-Lys-type substrate, BSA should be able to form a heterologous dimer or polymer with β-casein, provided they are thermodynamically compatible. In Figure 4, lanes 2 and 3 show SDS−PAGE profiles of untreated and transglutaminase-treated BSA/β-casein binary solutions. (It should be pointed out that when mixed with β-casein, BSA exhibited an abnormal mobility in SDS−PAGE.) In the enzyme-treated sample (lane 3), β-casein monomer disappeared, whereas the concentration of BSA monomer remained the same as that of the untreated control (lane 2), indicating that only β-casein was polymerized. The results show that even though BSA was either a Gln-type or Lys-type substrate, it could not form a heterologous dimer or polymer with the Gln-Lys-type β-casein as it did with α-lactalbumin (Figure 3). This dichotomic behavior of BSA must be related to differences in its thermodynamic compatibility of mixing with β-casein and α-lactalbumin. The thermodynamic incompatibility between β-casein and BSA precludes the simultaneous approach of these proteins at the enzyme's active site, whereas compatibility between BSA and α-lactalbumin allows mixing of these proteins at the enzyme's active site and formation of heterologous dimers and polymers.

α-Lactalbumin/β-Casein Binary System. To elucidate if native β-casein interfered with polymerization of α-lactalbumin as it did with that of β-lactoglobulin (Figure 1B), the transglutaminase reaction products of the β-casein/α-lactalbumin binary system were analyzed. In Figure 5, lanes 2 and 3 show SDS−PAGE profiles of control α-lactalbumin and transglutaminase-treated α-lactalbumin, respectively. In this single-protein system, α-lactalbumin formed homologous dimer, trimer, and polymer when treated with transglutaminase. Lanes 4 and 5 show the profiles of control and transglutaminase-treated mixtures, respectively, of α-lactalbumin and β-casein. In the enzyme-treated sample (lane 5), β-casein monomer disappeared completely and a substantial decrease in the concentration of α-lactalbumin monomer also occurred. Only one band corresponding to α-lactalbumin dimer (MW ~28 000) appeared, and no band corresponding to a heterodimer of β-casein/α-lactalbumin was found. The data suggest that both β-casein and α-lactalbumin are polymerized

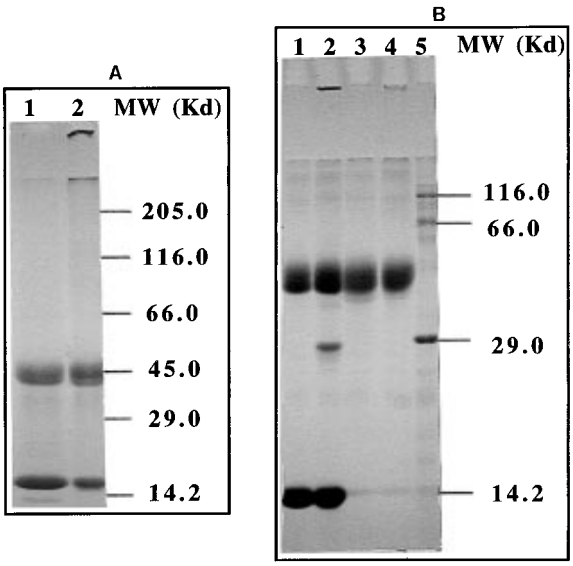


Figure 6. (A) SDS−PAGE profiles of polymers formed in transglutaminase-catalyzed reaction between ovalbumin and β-lactoglobulin. Lane 1, ovalbumin + β-casein control; lane 2, ovalbumin + β-lactoglobulin (1:1, 1% total substrate) treated with transglutaminase. (B) SDS−PAGE (12.5% gel) profiles of polymers formed between ovalbumin and α-lactalbumin. Lane 1, ovalbumin + α-lactalbumin control; lane 2, ovalbumin + α-lactalbumin (1:1, 1% total substrate) treated with transglutaminase; lane 3, ovalbumin control; lane 4, ovalbumin alone treated with transglutaminase; lane 5, protein markers. All cross-linking reactions were performed under "standard" conditions.

and β-casein did not interfere with the accessibility of α-lactalbumin to the enzyme's active site as it did with β-lactoglobulin (Figure 1B). Comparison of the concentrations of α-lactalbumin monomer in the enzyme-treated single-substrate (lane 3) and binary (lane 5) systems indicated that more α-lactalbumin monomer was consumed in the binary system than in the single-substrate system. It is not clear whether the polymers formed in the binary system were only homologous polymers of β-casein and α-lactalbumin or both homologous and heterologous polymers of the proteins.

***β*-Lactoglobulin/Ovalbumin and α-Lactalbumin/Ovalbumin Binary Systems.** Figure 6A shows SDS−PAGE profiles of the transglutaminase reaction products formed in the β-lactoglobulin/ovalbumin binary system. The concentration of β-lactoglobulin monomer in the transglutaminase-treated sample (lane 2) was lower compared to that of the control (lane 1), whereas the concentration of ovalbumin monomer remained unchanged, suggesting that the polymers that appeared at the top of the stacking and separating gels are mainly homologous polymers of β-lactoglobulin. In this binary system, β-lactoglobulin behaves as a Gln-Lys-type substrate, whereas ovalbumin behaves as a nonreactive substrate for transglutaminase. It should be noted that ovalbumin behaved as a nonreactive type substrate when treated alone with transglutaminase (Figure 6B, lanes 3 and 4), showing that nonreactivity of ovalbumin in the ovalbumin/β-lactoglobulin binary system was not due to the presence of β-lactoglobulin. In other words, in this binary system, the absence of heterologous polymer formation between β-lactoglobulin and ovalbumin was not related to the thermodynamic incompatibility of mixing at the enzyme's active site but was due mainly to the nonreactivity of ovalbumin. This interpretation is reasonable because, being globular proteins, both β-lactoglobulin and ovalbumin should be reasonably thermodynamically compatible.

Figure 6B shows the transglutaminase reaction products formed in the α-lactalbumin/ovalbumin binary system. In this system, α-lactalbumin acted as the Gln-Lys-type substrate and ovalbumin acted as a nonreactive substrate (lanes 1 and 2), as it did in the single-protein system (lanes 3 and 4). In addition to α-lactalbumin polymers at the top of the stacking gel, a band corresponding to α-lactalbumin dimer (MW ∼28 000) also appeared in the reaction product (lane 2).

The results presented here clearly show that not all Gln-Lys-type substrate proteins have the ability to form heterologous polymers in the transglutaminase-catalyzed reaction. Heterologous dimer or polymer formation seems to be dependent on the thermodynamic compatibility of the substrate proteins. Table 1 summarizes the properties of the protein substrates used and their ability/inability to form heterologous dimers and polymers with each other. Dissimilar proteins such as caseins and albumins cannot form heterologous dimers. This is primarily because of the inability of these proteins to overlap one another at the enzyme's catalytic sites. The thermodynamic incompatibility of mixing arises because of repulsive interactions between the hydrophobic and hydrophilic proteins. On the other hand, proteins of similar kind, i.e., globular proteins such as albumins and globulins, can form dimers and polymers in the transglutaminase reaction because of their thermodynamic compatibility of mixing. Two incompatible proteins, e.g., β-casein and β-lactoglobulin, can be made to be compatible either by converting the hydrophobic protein into a hydrophilic protein or by converting the hydrophilic protein into a hydrophobic protein through chemical modification. Thus, succinylation increases the hydrophilicity of β-casein and converts it to be partially compatible with β-lactoglobulin and facilitates the formation of heterologous dimers and polymers. In addition to thermodynamic compatibility, the structural state and disposition of lysyl and glutamine residues in proteins also seem to affect formation of heterologous polymers. Thus, although ovalbumin and α-lactalbumin are compatible proteins, they do not form heterologous polymers because both lysyl and glutamine residues of ovalbumin are unavailable for the transglutaminase reaction.

Table 1. Summary of the Properties of the Proteins Used and Their Ability or Inability To Form Heterologous Dimers and Polymers with Each Other

		cross-linking with						thermodynamic compatibility with					
protein	type of substrate	CN	suCN	LA	LG	BSA	OV	CN	suCN	LA	LG	BSA	OV
β-casein (CN)	Gln-Lys	+	+	+/−	−	+	−	+	+	+/−	−	−	−
succinylated β-casein (suCN)	Gln	+	−	+/−	+			+	+	+/−	+		
α-lactalbumin (LA)	Gln-Lys	+/−	+	+		+	−	+/−	+	+	+	+/−	
β-lactoglobulin (LG)	Gln-Lys	−	+	+	+		−	−	+		+		−
BSA	Gln or Lys	−	+	+	−	−	−	−	+	+	−	+	
ovalbumin (OV)	Cln or Lys	−	+	−	−	−	−	−	+/−	−	−	−	

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