Enzymatic Modification of Proteins: Effects of Transglutaminase Cross-Linking on Some Physical Properties of β-Lactoglobulin

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Transglutaminase was isolated from guinea pig liver and used to cross-link β-lactoglobulin (and casein) via γ-carboxyl (glutamine)–ε-amino (lysine) isopeptide bond formation. The optimum reaction conditions (19 μmol of bond formation/min per mg of protein) was at pH 8 and required calcium (4 mM). Both casein and β-lactoglobulin were facilely cross-linked to form a range of oligomers and polymers. Cross-linked β-Lg molecules were separated by ultrafiltration into polymers ranging in molecular mass from (20–99) × 10^3 and >100 × 10^3 Da. These were readily dispersible and had reduced viscosities of 3.0 and 5.3 compared to 2.9 mL/g for native β-Lg. Dispersions of β-Lg polymers were heat stable, remaining viscous at 100 °C. Extensive polymerization of β-Lg resulted in the formation of weak gels.

Many proteins lack appropriate functional properties for specific applications because of their particular structure or conformational state. Modification of proteins provides a practical approach for improving some functional properties (Feeney and Whitaker, 1977, 1982; Feeney, 1980). While chemical modification has been extensively explored for improving functional properties of food proteins as functional ingredients (Feeney and Whitaker, 1982; Kinsella and Shetty, 1979), concerns about safety and nutritional effects have prevented their adoption. The use of enzymes to manipulate the functional properties of proteins warrants more systematic study because enzymatic modification provides specificity and control not available with most chemical methods. More importantly perhaps is the fact that enzymatic methods of modification may circumvent concerns about safety. Furthermore, with the burgeoning developments in biotechnology and recombinant DNA techniques the availability of a wide range of enzymes suitable for modifying proteins should provide new opportunities in food protein chemistry.

To date, with the exception of chymosin-induced coagulation of casein, enzymatic modification of functional proteins has involved mostly hydrolysis to improve the solubility and whipping properties of proteins. However, the possibility of using enzymes to improve or modify rheological properties of proteins under controlled conditions deserves systematic study.

The enzyme transglutaminase catalyzes acyl-transfer reactions in which the γ-carboxamide group of glutamine is the acyl donor and usually a nucleophilic reagent such as the ε-amino group of lysine or water represents the acyl acceptor with the formation of a new isopeptide bond and the concomitant release of ammonia. Transglutaminase (TGase) is widespread in animal tissue and is involved in polymerization of proteins in the epidermis and the formation of clots and coagula from proteins (Betty and Wold, 1984; Gorman and Folk, 1980).

There have been a number of reports concerning the activity of transglutaminases in modifying the cross-linking proteins of biological interest (Gorman and Folk, 1980; Betty and Wold, 1984). A number of papers concerning the transglutaminase-induced polymerization and gelation of caseins (Motaki et al., 1984; Nio et al., 1985, 1986) and TGase-mediated incorporation of amino acids into proteins (Ikura et al., 1980, 1981) have recently been published.

Transglutaminase has potential useful applications for the manipulation of the rheological properties of food proteins. However, research is currently limited by the scarcity of transglutaminase. In the present study the activity of transglutaminase isolated from guinea pig liver on β-lactoglobulin was studied in terms of its effects on solubility, viscosity, and gelling behavior.

MATERIALS AND METHODS

Materials. (Benzyloxycarbonyl)-L-glutaminylglycine, β-lactoglobulin (3× crystallized), and calmodulin (bovine brain) were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent-grade urea was obtained from Pierce Chemical Co. (Rockford, IL). All other chemicals used in this study were reagent grade, and doubly distilled, deionized water was used.

Purification of Transglutaminase. Transglutaminase was purified 80-fold from fresh guinea pig livers by the method of Connellan et al. (1971) as improved by Brookhart et al. (1983).

Male guinea pigs (Hartley strain 450–500 g) were killed, and livers were perfused with saline, excised, and homogenized with 50 mL of cold (4 °C) sucrose (0.25 M) for 1 min with a Polytron PT10 homogenizer (Brinkman) at intermediate speed. The homogenate was centrifuged for 1 h at 105000*g* in a Sorvall ultracentrifuge (Model OT-D-65B, Sorvall Instruments) using a type SW27 rotor.

The supernatant was filtered through four layers of cheesecloth and put on a DEAE–cellulose column (2.1 × 20 cm) previously equilibrated with 5 mM Tris-HCl, pH 7.5, containing 2 mM EDTA. After the column was washed with 75 mL of the equilibrating buffer, the supernatant protein was applied and eluted from the column with a 480-mL linear gradient of 0–1.0 M NaCl in the same buffer. The fractions eluted between 0.25 and 0.4 M NaCl containing TGase were combined and applied to a column (1.2 × 25 cm) of hydroxyapatite Bio-gel HT with 10% Celite (Bio-Rad) previously equilibrated with 5 mM potassium (K) phosphate, pH 7.2, containing 0.15 M KCl, 2 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM ATP. The protein was eluted from the column with a 120-mL linear gradient of 5–200 mM potassium phosphate in the same buffer. Aliquots (3 mL) were collected, and the fractions containing TGase were combined. This preparation could be further purified by agarose gel filtration (Folk, 1971). For all experiments described in this study the solution was dialyzed for 48 h against 5 mM Tris-HCl, pH 7.5, containing 2 mM EDTA. The dialyzed TGase solution was frozen as discrete drops in liquid nitrogen and stored at −80 °C.

Measurement of Transglutaminase Activity. Transglutaminase activity was measured by the colorimetric hydroxamate procedure of Folk (1971) using 30 mM (benzyloxycarbonyl)-L-glutaminylglycine, 1 mM EDTA, 5 mM $CaCl_2$, and 0.1 M hydroxylamine in 0.1 M Tris–

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acetate, pH 6.0 and 37 °C. A unit of activity was defined as the amount of enzyme that catalyzes formation of 1 μmol of hydroxamate/min (Folk, 1971) and specific activity as activity/mg of protein. Protein concentration was determined by the Biuret method.

Enzymatic Reaction. The enzymatic reaction was carried out at 37 °C in 0.1 M Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$, 10 mM dithiothreitol (0.05 unit/mL), 10 μM (phenylmethyl)sulfonyl fluoride protein substrate (10 mg/mL), and transglutaminase (0.05 unit/mL) at 37 °C. The reaction was stopped by adding 0.2 M EDTA to chelate the calcium. The degree of cross-linking was monitored by measuring the decrease of amino groups by the trinitrobenzenesulfonate method (Wang et al., 1976).

The effects of the concentration of calmodulin and calcium on the transglutaminase activity were examined at different calmodulin and calcium concentrations by the colorimetric hydroxamate procedure under assay conditions as described above.

SDS–Polyacrylamide Gel Electrophoresis and Urea Gel Electrophoresis. Polyacrylamide gel electrophoresis in SDS and urea was carried out on disc gels (3.5% stacking gel, 7.5% separating gel) by the method of Laemmli (1970). The gel was stained with 0.0125% Coumassie brilliant blue R-250 in methanol–acetic acid–water (10:10:80) and destained in the same solution (Utsumi and Kinsella, 1985b).

Separation of Cross-Linked β-Lactoglobulin. The cross-linked proteins, i.e. β-lactoglobulin polymers, were separated by ultrafiltration. After an appropriate period of enzymatic reaction (2 h), 2 mL of 0.4 M EDTA and 20 mL of water were added to stop the reaction. The solution was filtered using a membrane (Y100), Amicon) to recover cross-linked β-lactoglobulin, i.e. protein polymers of molecular mass greater than 100 000 Da. The volume of the retentate was reduced to 5 L, and this solution, denoted cross-linked C100, was dialyzed against water and lyophilized. The filtrate was further ultrafiltered using membrane (UM20, Amicon), the volume reduced to 6 mL, and this solution dialyzed and lyophilized. The proteins in this fraction ranged from 20 to 99 Da and were denoted cross-linked 20–99 (C20+).

Gel Filtration of Cross-Linked Protein. The molecular weight patterns of TGase cross-linked and ultrafiltered β-lactoglobulins were estimated by gel filtration on Bio-Gel A-0.5m (100–200 mesh) (Bio-Rad) in Tris-HCl, pH 8.0, containing 20 mM mercaptoethanol and 4 M urea. Sample proteins were applied to the column (1.2 × 50 cm) and eluted with the same buffer, and the proteins in eluate fractions were quantified by absorbance at 280 nm.

Solubility of Cross-Linked Protein. Native β-lactoglobulin and separated cross-linked β-lactoglobulins were dissolved in water at a concentration of 0.1% (w/v) in 0.2 M NaOH (or HCl to adjust pH, and the turbidity of the solutions was measured by spectrophotometry (Cary 219, Varian) at 500 nm.

Heat Stability of Cross-Linked Protein. The heat stability of cross-linked β-Lg was examined by measuring the turbidity of the cross-linked protein at different temperatures. Native β-lactoglobulin, cross-linked β-Lg C100, and cross-linked β-Lg C20+ were dissolved in 0.05 M sodium phosphate buffer, pH 7.5, at a concentration of 1.5% (w/v) and heated at different temperatures for 30 min. The solutions were cooled to 25 °C and then centrifuged at 10000*g* for 30 min, and the protein concentration in the supernatant was determined by the Biuret method.

Viscosity of Cross-Linked Protein. Intrinsic viscosities of native β-lactoglobulin, cross-linked β-Lg C100, and cross-linked β-Lg C20+ were measured on a Cannon-Fenske capillary viscometer. Each protein was dissolved in 0.05 M sodium phosphate buffer, pH 7.0, and the viscosities were measured at 25 ± 0.02 °C. The reduced viscosity (η_r) was calculated from

$$\eta_r = (t - t_0)/t_0 \times g/L$$

where t_0 = time of water flow, t = time of protein solution flow, g/L = concentration of protein.

Gelation of Cross-Linked Protein. Gelation of protein samples was achieved by heat treatment and/or directly by transglutaminase cross-linking. The method of Utsumi and Kinsella (1985) was used to assess heat-induced gelation. Aliquots (30 μL) of the protein solutions (10% β-lactoglobulin, 5% cross-linked β-Lg C100, 10% cross-linked β-Lg C20+) in 30 mM Tris-HCl buffer, pH 8.0, were transferred by syringe to capillary tubes sealed at one end with poly(vinylidene chloride) film and then centrifuged at low speed (500*g*) to remove air bubbles. The sealed tubes were heated at specified temperatures up to 99 °C for 30 min, cooled by immersing in water at 15 °C, and held at 4 °C for 20 h to allow complete gelation.

In the cross-linking gelation method, native β-lactoglobulin at concentrations of 1–10% (w/v) was dissolved in the assay Tris-HCl buffer as described above. The concentrations of the enzyme ranged from 0.125 to 1.25 units/mL.

Table I. Isolation and Yield of Crude Transglutaminase from Guinea Pig Liver

fraction	protein,[a] mg	sp act.,[b] units/mL	purifcn factor
supernatant	6720.0	0.03	
DEAE–cellulose	378.0	0.50	17
hydroxylapatite	58.5	2.50	83

[a] Based on 50.6 g of liver. [b] Micromoles of hydroxamate formation/milligram of protein per minute.

RESULTS AND DISCUSSION

Transglutaminase was enriched over 80-fold and obtained in reasonable yields from fresh guinea pig livers (Table I). The hydroxyapatite fraction was kept at −80 °C and thawed prior to use in the various experiments. There was no loss in activity during storage. The specific activity was similar to that reported by Folk and Chaung (1973) using comparable methods of isolation. Further purification using protamine and agarose gel filtration was possible. The crude hydroxyapatite fraction was used in the cross-linking experiments.

The pH–activity curve for TGase showed a bell-shaped curve in the range pH 7.5–8.5 with an optimum at pH 8. Clarke et al. (1959) reported best activity between pH 7 and 8 for the incorporation of amine substrates into proteins. Folk (1971) observed that optimum TGase activity, using a synthetic substrate, was pH 6. Kurth and Rogers (1984) and Kurth (1983), using immobilized enzyme, found reasonable activities at pH 5.5–7.0. However, in studies examining the polymerization of food proteins optimum reaction rates were observed at pH 7.5 (Ikura et al., 1980; Motoki et al., 1984).

Transglutaminase requires calcium, with optimum activity being observed around 4 mM (Table II). Comparable results were obtained with either synthetic substrate or β-lactoglobulin. Calmodulin increased TGase activity slightly at all levels of calcium, and bovine brain calmodulin was more effective than that from heart tissue (data not shown).

Folk and Chaung (1973) studied the calcium requirement of transglutaminase and showed that the dissociation

Table II. Effects of Calcium and Calmodulin Concentration on Transglutaminase Activity

	transglutaminase act. at calmodulin concn, μg/mL		
Ca^{2+} concn, mM	0	1.75	8.75
0.01	0	0.9	2.8
1.00	6.0	6.6	6.9
4.00	17.2		19.0

[a] Micromoles of hydroxamate formed/milliliter per minute.

Table III. Rate of Cross-Linking of β-Lactoglobulin and α_s-Casein by Transglutaminase[a]

extent of cross-linking expressed as dec in free amino gps	reaction time, min			
	15	30	60	120
β-Lactoglobulin				
percent decrease	6.6	10.0	21.0	28.0
content, M^3	0.5	0.8	1.7	1.8
mol/mol protein	1.0	1.5	3.1	3.3
α_s-Casein				
percent decrease	12.0	16.0	30.0	34.0
content, M^{-3}	0.7	0.9	1.8	2.0
decrease in 10^{-3} M, mol/mol protein	1.7	2.2	4.3	4.8

[a] Reaction conditions as in Materials and Methods using 1 mg/mL of protein and enzyme concentrations 20 μg or 0.05 unit/mL.

constant of TGase is $<10 \times 10^{-3}$ M for calcium. The transglutaminase from human platelets and chicken gizzard apparently requires both calcium and calmodulin (Cheung, 1982), whereas plasma transglutaminase is insensitive to calmodulin (Puszkin and Raghuraman, 1985). We observed that calmodulin enhanced TGase activity at high calcium concentrations (10–100 mM). However, it is unlikely that this concentration is of physiological significance. Calmodulin was not used in subsequent experiments.

The rate of cross-linking of roteins, e.g. β-Lg, and of α-casein by TGase was linear with time (Table III). The extent of cross-linking of α-casein was more than that of β-Lg even though both proteins with comparable molecular weights have similar lysine contents while the numbers of glutamine residues are 9 and 14 for β-Lg and casein, respectively. It is conceivable that the predominantly random structure of the casein, in contrast to the more globular structure of β-Lg, provides more facile access of the enzyme to reactive groups in the casein. The rate of TGase activity was linear with time up to 60 min, and the rates of activity increased with substrate β-lactoglobulin concentration (Figure 1).

On the basis of these observations, a β-lactoglobulin (or casein) concentration of 1% and an enzyme concentration of 0.12 unit/mL were chosen, and incubations were carried out for 2 h for the preparation of cross-linked proteins. In the presence of TGase approximately 35% of the β-lactoglobulin was converted to polymers larger than 100 kDa (C100) and >40% to polymers ranging from 20 to 99 kDa (C20–99), respectively. The elution behavior of these products on Bio-gel chromatography is shown (Figure 2).

SDS–polyacrylamide gel electrophoresis revealed that most of the polymerized protein in fraction C100 did not enter the gel, whereas with fraction C20–99 less of the protein failed to penetrate the gel but several bands were observed in addition to β-Lg in both samples. The patterns reflected the formation of populations of β-Lg, oligomers, and polymers of varying molecular sizes.

Properties of Cross-Linked Lactoglobulin. *Dispersibility.* All proteins were rapidly hydrated and easily dispersed in aqueous solution above pH 6. However, below

Table IV. Viscosity of Native and Transglutaminase-Polymerized β-Lactoglobulin (β-Lg) as Affected by Protein Concentration

	reduced viscosities, mL g^{-1}		
protein concn, g/mL	native β-Lg	polymerized β-Lg C20–99	polymerized β-Lg C100
1.0	2.97	3.00	5.60
2.0	3.05	3.20	6.30
3.0	3.15	3.30	6.80
6.0	3.25	3.75	8.00
8.0	3.37		

Table V. Heat Stability of Native and Cross-Linked β-Lactoglobulin Polymer Mixtures Generated by Transglutaminase Cross-Linking

	solubility, %		
temp, °C	β-Lg	cross-linked 100	cross-linked 20–99
40	100.0	100	100
55	100.0	100	100
65	100.0	100	100
75	96.2		
80	71.6	100	99
90	64.1	100	100
99	57.2	100	100

Table VI. Heat Stability of Increasing Concentrations of Native and β-Lactoglobulin Polymerized by Transglutaminase after Heatng at 90 °C for 30 min

	solubility, %		
protein concn, %	native β-Lg	cross-linked 20–99	cross-linked 100
1.0	57.2	100	100
2.0	soft gellike	100	100
5.0	precipitate	soft gellike	90

pH 6 both the C100 and C20–100 polymerized β-Lg fractions became turbid reaching a peak, i.e. minimum solubility around pH 4.6 (Figure 3). The point of maximum turbidity between pH 4.5 and 5 was lower than the isoelectric point of native β-Lg at pH 5.2 (Timasheff and Townsend, 1968). The acidic shift in minimum solubility of the polymerized β-Lg may reflect the decreased number of free amino groups of lysine and possibly some deamination of glutamine by TGase. Motoki et al. (1984) and Gill and Headon (1985) also reported that the solubility of glycinin, whey protein, and casein decreased following transglutaminase-mediated polymerization. The observation that the solubility of polymerized β-Lg was 100% above pH 6 is of significance in terms of uses in food products.

The viscosity of β-Lg polymers progressively increased as the concentration of the cross-linked proteins increased, particularly in the case of the highly polymerized C100 preparation (Table IV). The apparent reduced viscosities showed values of 2.9, 2.6, 3.0, and 5.3 for the native, cross-linked C20+ and cross-linked C100 preparations, respectively. These latter values are less than those obtained for completely denatured β-Lg (Tanford, 1968), suggesting that the cross-linking did not involve extensive unfolding of β-Lg molecules.

The cross-linked β-Lg showed marked heat stability at pH 7, i.e. the polymerized β-Lg remained in dispersion even when 1% solutions were held at 100 °C for 30 min (Table V). This is in contrast to native β-Lg, which rapidly denatured at temperatures above 70 °C. The polymerized β-Lg retained solubility at 100 °C at concentrations up to 5% (Table VI), , whereas β-Lg formed gels or precipitates under similar conditions. From these data, it is not known whether the increased thermal stability of polymerized

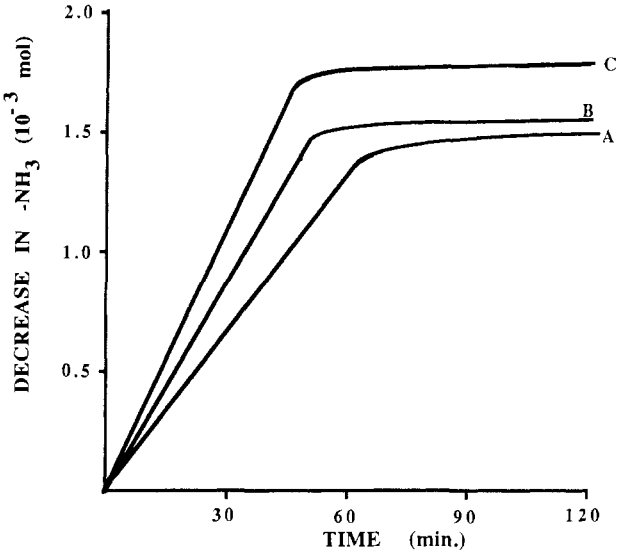

Figure 1. Rate of cross-linking of β-lactoglobulin by transglutaminase as a function of time and substrate concentration. Concentration of β-lactoglobulin: A, 0.5%; B, 1.08%; C, 2.0%.

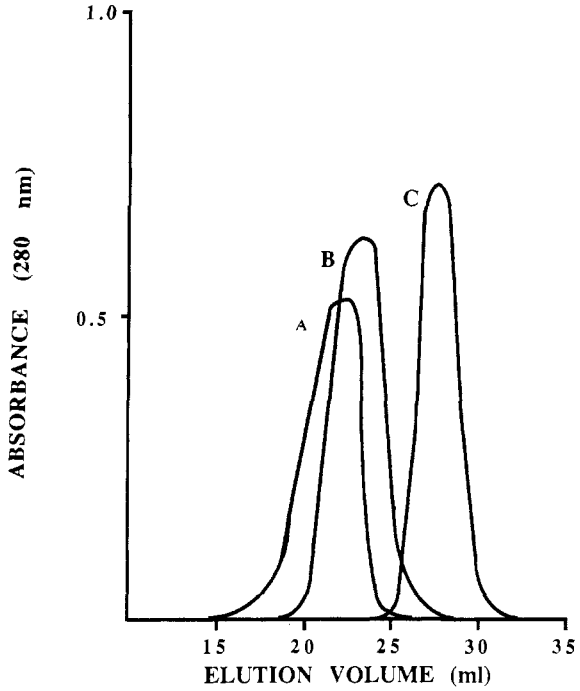

Figure 2. Retention behavior of native and transglutaminase cross-linked β-lactoglobulin separated on Bio-Gel A chromatography as described in Materials and Methods: A, native β-Lg; B, cross-linked β-Lg ranging from 20 to 99 kDa; C, polymers of β-Lg polymer >100 kDa.

β-Lg is caused by the increased number of intramolecular crosslinks between glutamyl and lysine residues or intermolecular cross-linking or a combination of both. This observation warrants further examination as an approach for stabilizing food proteins to high temperatures.

In contrast to native β-Lg, which formed firm, hard clear gels upon heating concentrations of 10% at 95 °C for 30 min, the polymerized β-Lg failed to form gels (Table VII). In fact the cross-linked polymer C20–99 formed a loose soft coagulum upon heating under similar conditions. The extensively polymerized fraction C100 remained in solution and failed to form any gellike structure upon heating 5% solutions at 95 °C. Unfortunately, insufficient protein was available to study higher concentrations. These data suggest that the cross-linked proteins contained intramolecular bonds that impeded thermally induced unfolding of the molecules, thereby limiting the network formation that apparently occurred with native β-lactoglobulin.

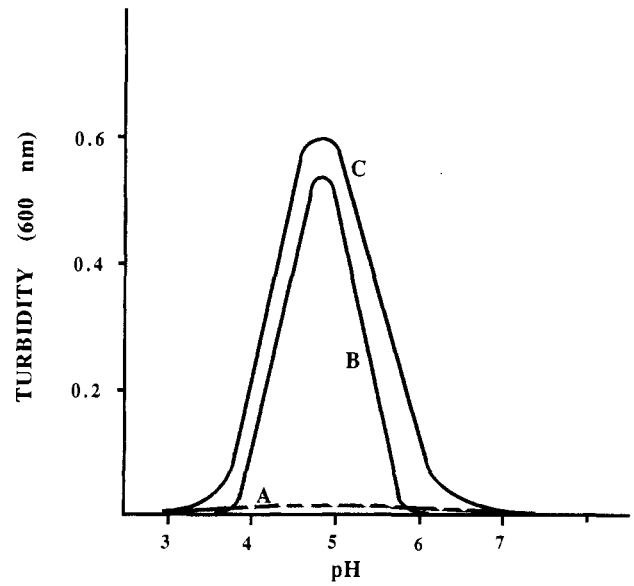

Figure 3. pH insolubility (turbidity) profiles of native and transglutaminase cross-linked β-lactoglobulin: A, native β-lactoglobulin; B, cross-linked β-Lg C20–99; C, cross-linked β-Lg C100.

Table VII. Gelation Behavior of Cross-Linked β-Lactoglobulin upon Heating at 95 °C for 30 min

β-lactoglobulin	concn, % (w/w)	gel	gel characteristic
native β-Lg	10	yes	hard
cross-linked β-Lg 20–99	10	coagulum	soft
cross-linked β-Lg 100	5	no	soln

Table VIII. Changes Induced in Increasing Concentrations of β-Lactoglobulin Solutions following Exposure to Transglutaminase for 2 h

enzyme concn, units/mL	β-Lg concn (w/v)	description
0.25	1	no gel
0.62	1	no gel
0.12	2	turbid
1.25	5	white turbid precipitate
1.25	10	white coagulum

The possibility of using transglutaminase to induce the formation of gels in protein solution at ambient temperatures (25–30 °C) by progressive covalent cross-linking to yield a firm network was studied. Exposure of increasing concentrations of β-Lg to transglutaminase resulted in the formation of network structures and weak gels particularly at higher protein concentrations (Table VIII). The coagula or gels formed at 10% β-Lg were firm and retained moisture. These experiments indicated that TGase might provide an enzymatic method for producing soft custard-like structures useful in food product formulation and a controllable method for producing coagula with varying rheological properties. Recently, Nio et al. (1986) reported that gels can be formed by cross-linking of casein with transglutaminase.

These studies suggest that TGase may have potential application for the controlled modification of proteins to improve their functional properties. Transglutaminase demonstrates broad specificity in terms of protein substrates requiring only peptide-linked glutamine as acyl donor, and it can effectively utilize a broad range of amines

as acyl acceptors (Folk, 1971; Nio et al., 1986; Ikura et al., 1981). The potential for modifying proteins in a rational manner warrants more extensive studies and the development of practical sources of transglutaminase.

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Comparison of Yeast Biomass Production in Five Wood Aqueous Extracts

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Hot water aqueous extracts of the woods of five trees—mimosa (*Albizia julibrissin*), mulberry (*Morus rubra*), mesquite (*Prosopis glandulosa*), pecan (*Carya illinoensis*), and live oak (*Quercus virginiana*)—were compared for composition and ability to support yeast growth. The mesquite extract yielded the highest biomass, whereas the oak extract yielded the lowest. In the aerobic growth study, *Rhodotorula rubra* biomass production correlated with the amount of reducing sugar consumed but not with the amount of D-glucose or total carbohydrate present.

In a semiarid ecosystem, trees are often utilized in ways that differ from those common in wetter habitats. Their foliage and fruit may be important sources of nutrients for domestic livestock (Simpson and Solbrig, 1977; Bhatia and Ratan, 1983) or could serve as feedstock for industrial ethanol production (Felker et al., 1980). Likewise, while wood from trees is primarily used for fuel or as a building material, the wood of mesquite trees has been used as a substrate for the production of both bacterial (Thayer, 1976; Thayer and Murray, 1977) and yeast (Wilson and Thayer, 1978, 1982; Stanlake, 1986) biomass. The bacterial or yeast biomass could then be included as a nitrogen source in an animal ration. This report extends this line of study by comparing yeast biomass production in aqueous mesquite extract to that in aqueous extracts of four other hardwoods.

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MATERIALS AND METHODS

Wood samples of mimosa (*Albizia julibrissin*), mulberry (*Morus rubra*), mesquite (*Prosopis glandulosa*), pecan (*Carya illinoensis*), and live oak (*Quercus virginiana*) were collected as green stems measuring 1–4 cm in diameter

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