Immobilization of Biotinylated Transglutaminase by Bioselective Adsorption to Immobilized Avidin and Characterization of the Immobilized Activity†

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Transglutaminase was immobilized on a porous glass support by biotinylation followed by adsorption to avidin that had been immobilized by adsorption to the biotinylated aminopropyl glass. Thus, avidin served as a protein spacer between the support and the enzyme. Both the biotinylation of enzyme amino groups and the association of the biotinylated enzyme with soluble avidin caused some loss of enzyme activity. This loss could account for the 3-fold reduction in the specific activity of the immobilized enzyme with carbobenzoxyglutaminylglycine and hydroxylamine as substrates. However, with α_s-casein as a substrate, a 24-fold reduction in the k_{cat} value was observed, implying that the rate of reaction with this large substrate molecule was limited by mass transfer. The pH optima and temperature dependences of enzyme catalysis were similar for both the soluble and immobilized enzyme, although the slight differences observed for the immobilized form were also indicative of mass transfer effects. A bimodal pH activity profile with optima at pH 6.5 and 7.5 was obtained with both enzyme forms. Treatment of α_s-casein with immobilized enzyme caused a rapid disappearance of the monomeric protein with concomitant appearance of dimers and higher cross-linked polymers.

Keywords: *Immobilized transglutaminase; biotinylated transglutaminase; bioselective adsorption; immobilized avidin; cross-linked α_s-casein*

INTRODUCTION

Proteins can be modified enzymatically to improve their nutritional and functional properties. As opposed to chemical modifications, enzyme-catalyzed changes in protein structure provide greater specificity and opportunity for a better control of the extent of reaction, and, from a food safety viewpoint, the products are less likely to be antinutritional or biologically hazardous.

Hydrolytic enzymes, especially proteinases, are currently used extensively in the food industry (Whitaker, 1977; Fox and Grufferty, 1991). Recently, nonhydrolytic modifications of protein by transglutaminase-catalyzed cross-linking (Ikura et al., 1980a,b; Motoki and Nio, 1983; Motoki et al., 1984; Kurth and Rogers, 1984; Nio et al., 1985, 1986; Motoki et al., 1987a,b; Tanimoto and Kinsella, 1988; Aboumahmoud and Savello, 1990; Ikura et al., 1992) and by addition of amino acid or carbohydrate moieties (Yan and Wold, 1984; Ikura et al., 1985) have been investigated. Guinea pig liver transglutaminase (R-glutaminyl-peptide:amine γ-glutamyl-transferase; EC 2.3.2.13) is a calcium-dependent transferase that catalyzes post-translational modification of proteins via acyl transfer reactions of the γ-carboxamide group of glutaminyl residues with the ϵ-amino group of lysyl residues as the usual reacting nucleophile (Folk, 1980; Lorand and Conrad, 1984; Greenberg et al., 1991). However, other primary amines, and also water, can serve as the reacting nucleophile (Lorand et al., 1979; Motoki et al., 1986), allowing for the deamidation or attachment of various moieties.

Although dramatic changes in protein functionality have been demonstrated in laboratory studies, large-scale modification of proteins by transglutaminase-catalyzed reactions has been limited by the availability of enzyme. This problem is exacerbated by the low turnover number of the enzyme; thus, large amounts are required to achieve high levels of cross-linking. Furthermore, because protein functionality is so dramatically affected by cross-linking, in many potential applications a limited extent of reaction would be desirable. Hence, a downstream inactivation step would be required which could result in a loss of the desired functionality that was created by the cross-linking.

Immobilization of the enzyme potentially can overcome these limitations by greatly increasing productivity by allowing reuse of the enzyme for extended periods of time and by eliminating the requirement for a downstream inactivation step (Swaisgood, 1991). Recently, we reported the characteristics of transglutaminase that was immobilized by covalent attachment to polylysyl-α_s-casein bonded on porous controlled-pore glass (CPG) (Oh et al., 1993). The importance of a protein spacer between the surface and the enzyme for high enzyme activity was demonstrated. In this study, a novel method for immobilization of transglutaminase by bioselective adsorption, using avidin–biotin technology with avidin as the protein spacer, is described. The kinetic characteristics of the immobilized biocatalyst were determined, and a fluidized-bed bioreactor was operated with α_s-casein as the substrate.

MATERIALS AND METHODS

All chemicals were of reagent grade. *o*-Phthalaldehyde (OPA) (fluorescent grade) and α-casein (85% α_s-casein) were obtained from Sigma Chemical Co. (St. Louis, MO).

Purification of Transglutaminase. Transglutaminase was isolated and purified from guinea pig liver using a

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modification of the method of Brookhart et al. (1983). Fresh guinea pig liver, obtained from the College of Veterinary Medicine, NCSU, was perfused with 0.15 M NaCl at 4 °C. A 20-g portion of the tissue was homogenized with 100 mL of 0.25 M sucrose, 3 mM EDTA, and 2 mM dithiothreitol (DTT) for 2 min with a cycle blender (Oster Co., Milwaukee, WI) at liquefying speed. The homogenate was centrifuged at 105000*g* for 30 min. The supernatant was filtered through four layers of cheesecloth, and solid NaCl was added to achieve a final concentration of 0.20 M. The resulting solution was loaded on a DEAE-Sepharose (Sigma) column (2.6 × 20 cm), previously equilibrated with 5 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 0.1 mM DTT, and 0.20 M NaCl. Proteins were eluted from the column with a step gradient of 5 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 0.1 mM DTT, and 0.2–0.6 M NaCl. The fractions containing transglutaminase activity were pooled and applied to a column of hydroxyapatite (Bio-Gel HT with 10% Celite; Bio-Rad Laboratories, Hercules, CA) (2.5 × 20 cm) previously equilibrated with 5 mM potassium phosphate at pH 7.2, 0.15 M KCl, 2 mM EDTA, 0.1 mM DTT, and 0.1 mM ATP. The protein was eluted from the column with a step gradient of 5–75 mM potassium phosphate in the same buffer. The fractions containing transglutaminase activity were combined, dialyzed against distilled–deionized water, concentrated by ultrafiltration, and lyophilized. The transglutaminase lyophilized powder was stored in a desiccator at −20 °C until use.

Effect of Biotinylation and Association with Avidin on Transglutaminase Activity. The transglutaminase was biotinylated with sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC Biotin, Pierce, Rockford, IL) in 50 mM bicarbonate buffer, pH 8.5, at molar ratios of biotin/transglutaminase ranging from 0 to 150. NHS-LC Biotin reagent solution was added to transglutaminase solution and incubated at 4 °C for 2 h. Unreacted NHS-LC Biotin was removed by dialyzing with a Centricon 30 microconcentrator and washing twice with 50 mM sodium phosphate, pH 6.0, containing 0.02% NaN_3. Aliquots of biotinylated transglutaminase were assayed for activity according to the method of Folk (1971) using carbobenzoxy-L-glutaminylglycine (CBZ-GlnGly) and hydroxylamine as substrate. The extent of biotinylation per mole of transglutaminase was estimated according to the spectrophotometric method of Green (1970) using the dye 4-hydroxyazobenzene-2′-carboxylic acid (HABA).

The biotinylated transglutaminase solution was mixed with avidin solution in the phosphate buffer, pH 6.0, to give a final mole ratio ranging from 0 to 16 of avidin to biotinylated transglutaminase. After incubation at 4 °C for 20 min, transglutaminase activity of the reaction mixture was assayed according to the method of Folk (1971). The excess of biotin-binding avidin subunits in the reaction mixture was determined according to the method of Green (1970).

Measurement of Transglutaminase Activity. Activity of soluble transglutaminase was determined according to the method of Folk (1971) using 30 mM CBZ-GlnGly, 1 mM EDTA, 5 mM $CaCl_2$, and 100 mM hydroxylamine in 0.1 M Tris–acetate buffer, pH 6.0, at 37 °C. A unit of activity was defined as the formation of 1 μmol of hydroxamate/min. Protein concentration was estimated with the Bio-Rad protein assay kit (Bio-Rad Laboratories) using bovine serum albumin (BSA, Sigma) as the standard.

The activity of immobilized transglutaminase was estimated by recirculating the substrate solution (CBZ-GlnGly and hydroxylamine) through a microcirculation reactor that allowed initial rates to be determined on small quantities of beads with negligible external diffusion limitation (Taylor and Swaisgood, 1980). Activity was assayed according to the method of Folk (1971).

Immobilization of Transglutaminase. Transglutaminase was immobilized on glass beads by bioselective adsorption using avidin–biotin technology. The general scheme for immobilization is illustrated in Figure 1.

Derivatization of Glass Beads. Controlled-pore glass beads (CPG 2000–200 mesh, 200 nm mean pore diameter, Sigma) were cleaned, silanized, and derivatized following the procedures of Janolino and Swaisgood (1982). The resulting aminopropyl glass beads (0.5 g) were biotinylated with sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC Biotin, Pierce) in 50 mM bicarbonate buffer, pH 8.5, by recirculating the solution through the beads at 4 °C for 24 h. The beads were washed three times with 50 mM sodium phosphate, pH 6.0, containing 0.02% NaN_3. Four milliliters of avidin solution (2.5 mg/mL in 50 mM phosphate, pH 6.0, containing 0.9% NaCl and 0.02% NaN_3) was added to the biotinylated glass beads and recirculated at 4 °C for 24 h. The beads were then washed with 50 mM sodium phosphate, pH 7.0, containing 0.02% NaN_3 until avidin could not be detected in the washing. The avidin–biotin glass beads were stored in the phosphate buffer at 4 °C until use.

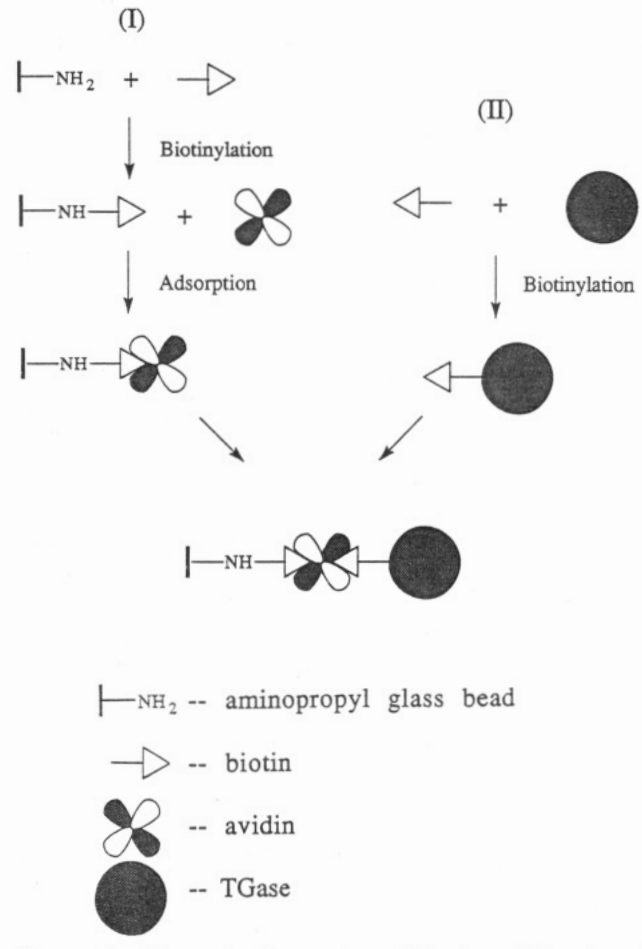

Figure 1. Schematic illustration of the immobilization of transglutaminase on controlled-pore glass via the interaction of avidin with biotin.

Biotinylation of Transglutaminase. Purified transglutaminase powder (6.1 mg) was dissolved in 50 mM bicarbonate buffer, pH 8.5, at a concentration of 3 mg/mL. The enzyme was biotinylated with NHS-LC Biotin at a ratio of 16 mol of biotin/mol of transglutaminase by incubating the solution at 4 °C for 2 h. The reaction mixture was dialyzed and washed with 50 mM sodium phosphate, pH 7.0 (containing 0.02% NaN_3), three times using a Centricon 30 microconcentrator (Bio-Rad). The biotinylated transglutaminase solution was then recirculated over the avidin–biotin beads at 4 °C for 2 h. The immobilized enzyme was extensively washed with 50 mM Tris-HCl, pH 7.0, containing 2 mM DTT and 3 mM EDTA and stored in this buffer at 4 °C.

Activity of the Biocatalyst on α_s-Casein. Intermolecular cross-linking of α_s-casein catalyzed by immobilized transglutaminase was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the method of Laemmli (1970). Five milliliters of α_s-casein solution (5 mg/mL in 50 mM imidazole buffer, pH 7.5, containing 5 mM $CaCl_2$ and 10 mM DTT) was recirculated through a fluidized-bed bioreactor with 0.5 mL of immobilized transglutaminase beads

at 37 °C. Samples were withdrawn at various time intervals from the reaction mixture and were electrophoresed on a LKB 2050 Midget electrophoresis unit (LKB Produktor AB, Bromma, Sweden) with 4% stacking gel and 10% separating gel under denaturing conditions (2% SDS, 5% 2-mercaptoethanol).

Characterization of Immobilized Transglutaminase. Amino acid analysis was used to quantitate the total amount of protein immobilized and to determine the ratio of avidin to transglutaminase immobilized on the glass beads. The amino acid analysis was performed according to the method of Bidlingmeyer et al. (1984) as modified by Walsh and Swaisgood (1993). Protein samples were hydrolyzed at 110 °C for 24 h, dried under nitrogen, and stored frozen until assayed. The protein hydrolysates were derivatized with phenyl isothocyanate (PITC) to form the phenylthiohydantoin derivatives of amino acids for detection at 254 nm. The HPLC system used in the analysis consisted of a Model 510 pump, a Model UK6 injector, an automated gradient controller system, and a Model 990 photodiode array detector (Waters, Milford, MA). The detector was equipped with an APC IV series computer (NEC Information System, Inc., Foxborough, MA) for data acquisition and spectral analysis. Analysis was performed using a Beckman C_{18} (25 cm × 4.6 mm, 5 micron particle size) column (Beckman Instruments, Fullerton, CA) with a Hamilton (Reno, NV) C_{18} guard column. The column temperature was maintained at 45 °C. The solvent system consisted of two eluents: (A) 0.014 M sodium acetate, pH 4.8, containing 0.125 mL of triethylamine/liter and (B) 65% acetonitrile, 35% water. Standard curves were constructed from analysis of amino acid standards (Beckman).

The percent avidin immobilized on the glass beads was estimated from the solution of simultaneous equations for the total amounts of Thr and Arg determined by amino acid analysis (Walsh and Swaisgood, 1993). Analysis of the avidin and transglutaminase preparations used in the immobilization indicated concentrations of 0.542 and 0.264 mmol/g, respectively, for Arg and 1.235 and 0.296 mmol/g, respectively for Thr. These two amino acids were selected for development of the equations because their compositions in the two preparations were substantially different and the accuracy of their determination by the procedure used was judged to be better than that of other residues.

Determination of Kinetic Constants. The kinetic constants, K_m and V_{max}, were determined from initial rate measurements with both soluble and immobilized transglutaminase, using α_s-casein as a substrate. The substrate was dissolved in 50 mM imidazole buffer, pH 7.5, containing 5 mM $CaCl_2$ and 10 mM DTT. The reaction was performed by recirculating the substrate solution (varied from 0.1 to 0.5 mg/mL) through a microcirculation reactor with 0.1 mL of immobilized transglutaminase beads at 37 °C. Remaining primary amino groups in the casein molecule were determined according to the method of Goodno et al. (1981). The fluorescence intensity (scanning spectrofluorometer, Optical Technology Devices, Inc., Elmsford, NY) was measured at 456 nm with an excitation of 346 nm (10−20-nm slits), and 0.1 μg/mL quinine sulfate solution was used as a reference for calculation of the relative fluorescence intensities. Relative fluorescence was converted to amino group concentration using ribonuclease as a standard ($Y = 0.1889x + 0.1071$, $r = 0.999$). Disappearance of amino groups during the reaction was calculated using the lysine fluorescence value of 2.28×10^8/mol of lysyl residue in ribonuclease (Goodno et al., 1981). The K_m and V_{max} values were estimated by using direct linear plots (Eisenthal and Cornish-Bowden, 1974).

Determination of pH Optimum and Temperature Optimum. The effect of pH on the activity of soluble and immobilized transglutaminase was examined using α_s-casein (5 mg/mL) as a substrate in 50 mM imidazole buffer, containing 5 mM $CaCl_2$ and 10 mM DTT with pH ranges from 6.0 to 8.0, adjusted with NaOH. The reaction was performed in the microcirculation reactor containing 0.1 mL of immobilized enzyme beads at 37 °C. The enzyme activity was determined according to the OPA method (Goodno et al., 1981) as described previously.

Table 1. Purification of Transglutaminase from Guinea Pig Liver

purification step	total protein[a] (mg)	sp act.[b] (U/mg)	total act. (U)	yield (%)	fold purification
supernatant	1975	0.03	60	100	1
DEAE-Sepharose	36	1.34	47.5	79	45
hydroxyapatite	6.3	3.74	23.6	39	125
commercial TGase[c]		3.39			

[a] Based on 20 g of liver. [b] One unit (U) of enzyme activity is 1.0 μmol of hydroxamate formed/min. [c] Purchased from Sigma.

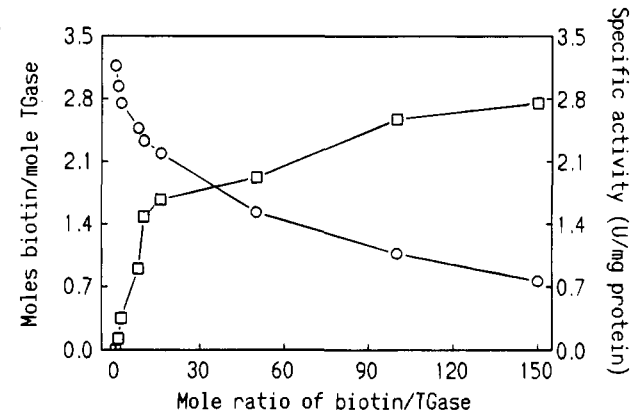

Figure 2. Relationship between the biotinylation reagent to transglutaminase mole ratio and the degree of biotinylation and activity of the enzyme. Reaction was in 50 mM bicarbonate buffer, pH 8.5, for 2 h at 4 °C. The activity was assayed after removal of unreacted biotinylation reagent. (□) Incorporation of biotinyl group (mol/mol of transglutaminase; (○) specific activity of transglutaminase [μmol of hydroxamate formed min^{-1} (mg of protein)$^{-1}$].

The effect of temperature on the transglutaminase activity was determined using α_s-casein (5 mg/mL in the imidazole buffer, pH 7.5) as a substrate. The reaction was performed under the conditions described previously except that the temperature was varied.

RESULTS

Immobilization of Transglutaminase. The specific activity of the transglutaminase preparation used for immobilization was increased 125-fold over that of the supernatant from guinea pig liver homogenate by chromatography with DEAE-Sepharose and hydroxyapatite (Table 1). The specific activity of the preparation was comparable to that of a commercially available enzyme and to that reported by Tanimoto and Kinsella (1988) for a similar preparation.

Biotinylation of the soluble enzyme caused some loss of activity. The relationship between the mole ratio of biotin to transglutaminase and both the extent of enzyme biotinylation and specific activity is shown in Figure 2. Both the extent of biotinylation and the specific activity changed rapidly with increasing mole ratios up to 16. On further increasing mole ratios, the extent of biotinylation and the specific activity appeared to approach a plateau, indicating completion of the reaction with available reactive primary amino groups. Because of the inverse relationship between the extent of biotinylation and specific activity, conditions were selected to ensure that most of the enzyme molecules would be modified by a single biotinyl group to minimize activity loss. Thus, a biotin reagent/transglutaminase mole ratio of 16 was used which resulted in an average biotinyl incorporation of 1.5 mol/mol of enzyme, with 70% of the initial activity remaining.

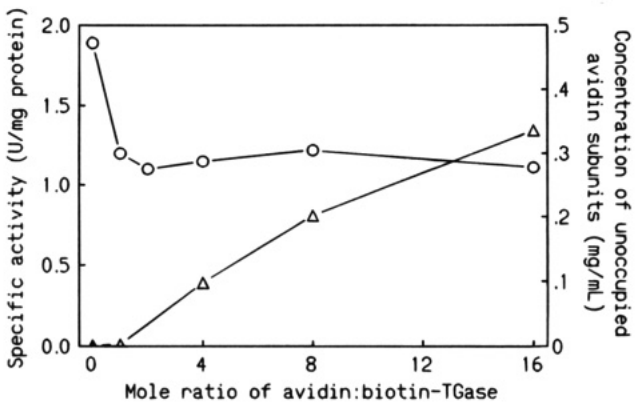

Figure 3. Effect of association of biotinylated transglutaminase with soluble avidin on the enzyme activity. Association reactions were in 50 mM sodium phosphate buffer, pH 6.0, for 20 min at 4 °C. (△) Concentration of unoccupied avidin subunits; (○) specific activity [μmol of hydroxamate formed min^{-1} (mg of protein)$^{-1}$].

Table 2. Characteristics of Immobilized Transglutaminase

item	
total protein immobilized[a]	25.6 μg/mg of beads
amount of avidin	10.0 μg/mg of beads
amount of TGase	15.6 μg/mg of beads
molar ratio of avidin/TGase[b]	1.2
sp act.[c]	5.5 U/mL of beads or 1.1 U/mg of TGase

[a] Total immobilized protein, avidin, and transglutaminase were determined according to the method of Walsh and Swaisgood (1993), using amino acid analysis. [b] The molecular weights of avidin and transglutaminase used in calculation are 67 000 (Green, 1975) and 80 000 (Folk, 1980), respectively. [c] One unit (U) of enzyme activity is 1.0 μmol of hydroxamate formed/min.

Association of biotinylated transglutaminase with avidin caused a further loss (approximately 37%) of specific activity. Results of the titration of 13 μmol of biotinylated transglutaminase with avidin on the specific activity are shown in Figure 3. Addition of avidin to unmodified transglutaminase did not affect its activity. The concentrations of unoccupied biotin binding sites are also shown. It appears that all of the biotinylated enzyme was bound to avidin at an avidin/biotinylated enzyme mole ratio of approximately unity.

The amounts of protein immobilized as determined by amino acid analysis and the specific activity of the biocatalyst are given in Table 2. Results of amino acid analyses were also used to estimate the amounts of transglutaminase and avidin immobilized. The values obtained are listed in the table and indicate an avidin/transglutaminase mole ratio of 1.2. The specific activity of immobilized transglutaminase was 1.1 U/mg of enzyme as assayed with the small dipeptide substrate, CBZ-GlnGly. Comparison with the specific activity of the soluble preparation (Table 1) indicates that 30% of the original activity was observed. Another preparation of immobilized enzyme prepared in a similar manner yielded a specific activity of 5.3 U/mL of beads as compared to a value of 5.5 U/mL for the preparation described in Table 2.

Characteristics of the Immobilized Enzyme Activity. The kinetic parameters, K_m and k_{cat}, determined using α_s-casein as a substrate, are listed in Table 3. In these studies, the protein served both as an acyl donor and as an acyl acceptor. The Michaelis constant thus determined represents an overall apparent value. This value was 3-fold higher for the immobilized biocatalyst as compared to the soluble enzyme, while k_{cat} was reduced by 24-fold with the protein substrate and the catalytic efficiency, k_{cat}/K_m, was 84-fold lower.

Table 3. Apparent Kinetic Constants of Soluble and Immobilized Transglutaminase[a]

enzyme	K_m (μM)	k_{cat} $(min)^{-1}$	k_{cat}/K_m (mM min^{-1})
soluble	4	3.15	855.2
immobilized	13	0.13	10.2

[a] Initial rates were measured using a fluorsecent OPA method for determination of the disappearance of primary amino groups. K_m and V_{max} were estimated from direct linear plots (Eisenthal and Cornish-Bowden, 1974) of data from duplicate measurements using four substrate concentrations ranging from 4.1 to 16.4 μM. The reactions were performed at 37 °C in 50 mM imidazole buffer, pH 7.5, containing 5 mM $CaCl_2$ and 10 mM DTT, and by using α_s-casein (M_r = 24 420) as substrate.

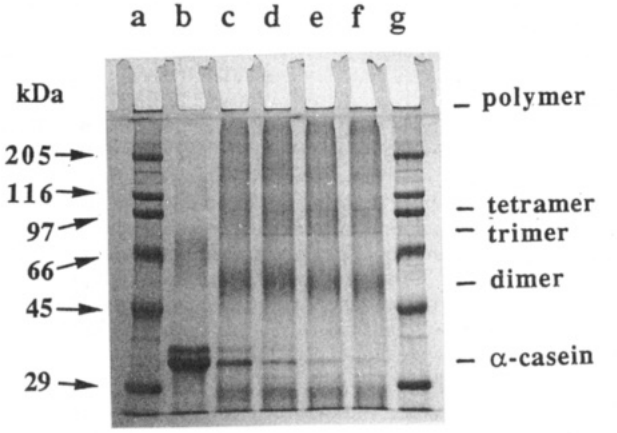

Figure 4. SDS–PAGE analysis of α_s-casein subjected to enzyme-catalyzed cross-linking with immobilized transglutaminase. Lanes a and g contain standard molecular weight markers: (1) myosin (205 000), (2) β-galactosidase (116 000), (3) phosphorylase (97 400), (4) bovine serum albumin (66 000), (5) ovalbumin (45 000), and (6) carbonic anhydrase (29 000). Lanes b–f: α_s-casein solutions treated with immobilized transglutaminase for 0, 5, 10, 20, and 40 min., respectively. The positions marked dimer, trimer, and tetramer are those calculated from a fit of the R_f values for the standard marker proteins and the expected molecular weights based on a value of 24 420 for monomer.

Cross-linking of α_s-casein was substantiated by SDS–PAGE analysis. Results, shown in Figure 4, indicate that after a 5-min reaction, 60% of the casein monomer had disappeared with the concomitant appearance of dimer, tetramer, and higher polymers, some of which accumulated at the stacking gel interface. The commercial α_s-casein used as substrate contained both α_{s1}- and α_{s2}-caseins and minor amounts of unknown proteins. Increased spreading of the band corresponding to dimer may have resulted from this heterogeneity. The source of minor bands of lower apparent molecular weight is not known but could be caused by a contaminating proteolytic activity in the transglutaminase preparation. After 40 min, all of the monomeric casein had disappeared and a large quantity of the polymers could not enter the stacking gel.

The effect of pH on the activity of soluble and immobilized transglutaminase is shown in Figure 5. A bimodal pH optimum was observed for both forms of the enzyme with α_s-casein as substrate. The optimum activity observed at pH 6.5 was slightly greater than that at pH 7.5. The bimodal pH optimum was also observed with soluble enzyme using CBZ-GlnGly and hydroxylamine as substrates; however, the lower pH optimum was at 6.0, in agreement with the results of

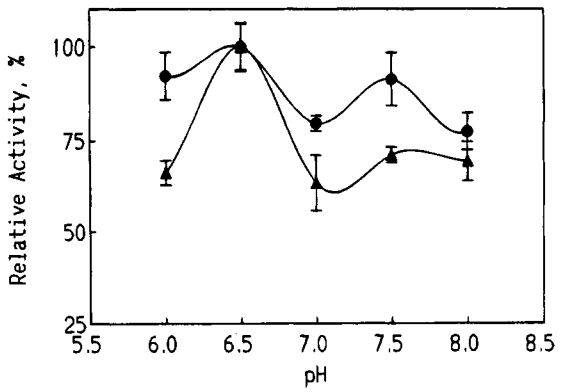

Figure 5. Effect of pH on the reaction rates as measured by disappearance of protein amino groups for soluble (▲) and immobilized (●) transglutaminase-catalyzed cross-linking. The reaction was at 37 °C in 50 mM imidazole buffer, containing 5 mM $CaCl_2$ and 10 mM DTT. The substrate was 5 mg/mL α_s-casein, and 0.1 mL of immobilized transglutaminase beads was used in the bioreactor. Each data point represents the mean of three separate determinations.

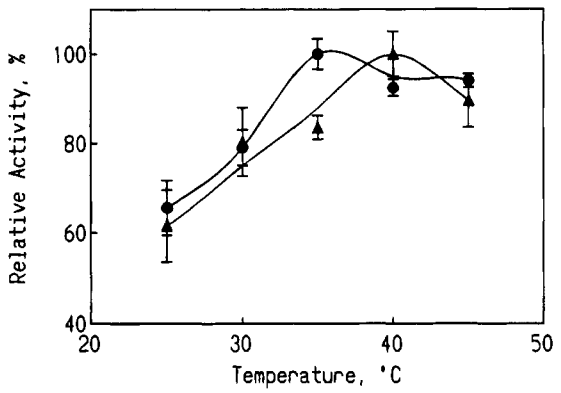

Figure 6. Effect of temperature on the reaction rate as measured by disappearance of protein amino groups for soluble (▲) and immobilized (●) transglutaminase-catalyzed cross-linking. The reaction was in 0.2 M Tris–acetate buffer, pH 7.5, containing 5 mM $CaCl_2$ and 1 mM EDTA. The substrate was 5 mg/mL α_s-casein. The bioreactor contained 0.2 mL of immobilized transglutaminase beads. Each data point represents the mean of three separate determinations.

Folk (1971). Data shown in Figure 5 also suggest that immobilized transglutaminase activity was less sensitive to pH change, especially around the pH 6.5 optimum.

The effect of temperature on the activity of soluble and immobilized transglutaminase (Figure 6) indicated that the activity of the immobilized form was less sensitive to temperature changes in the range of 35–45 °C. The soluble enzyme exhibited a distinct optimum at 40 °C; for example, at 45 °C the activity of the soluble enzyme decreases 12%, while that of the immobilized form was only 6% lower than at 40 °C.

DISCUSSION

Biotinylation of transglutaminase appears to plateau at about 3 mol/mol of enzyme, which is substantially less than the 29 lysyl residues present in the protein molecule (Ikura et al., 1988). Thus, a large fraction of these residues are apparently not accessible to the biotinylation reagent. This factor is probably responsible for making the enzyme less susceptible to autocatalyzed cross-linking. Loss of some activity, due to biotinylation, may be caused by a subtle conformational change of the enzyme or by steric hindrance of the active site. Although the sites of biotinylation were not identified, it should be noted that a lysyl residue is only four residues removed from the active site cysteinyl residue (Ikura et al., 1988).

Association of the biotinylated enzyme with avidin caused a further loss of activity, again suggesting that one of the biotinylation sites must be very close to the active site region. The combined effects of biotinylation and association with avidin accounted for all of the reduction of activity with the small dipeptide substrate for the immobilized enzyme in comparison with the soluble form. However, with the protein substrate α_s-casein, k_{cat} and the catalytic efficiency of the immobilized form were reduced by 24- and 84-fold, respectively. The observed K_m value (4 μM) of the soluble enzyme is in agreement with that reported by Ikura et al. (1990) for acetyl-α_{s1}-casein (3.19 μM) with histamine as the acyl acceptor and by Oh et al. (1993) for a similar α_s-casein substrate (6 μM). The 3-fold increase in K_m for α_s-casein with the immobilized enzyme together with the large reduction in the apparent k_{cat} strongly argues for mass transfer limitations with this large substrate (Swaisgood, 1991). α_{s1}-Casein undergoes extensive self-association in the presence of 5 mM Ca^{2+} (Dosako et al., 1980), and these substrate solutions exhibited considerable turbidity. Hence, limited diffusibility of these molecules, even in the wide-pore (200-nm pore diameter) matrix used for immobilization, is not surprising. Because of extensive self-association, the highest substrate concentration used was about 5 K_m, which is considerably less than the 100K_m value recommended to approach the true V_{max} of an immobilized enzyme (Buchholtz and Klein, 1987; Swaisgood, 1991). Therefore, we conclude that the 3.3-fold reduction in specific activity measured with the dipeptide substrate was a better index of loss in active site integrity and that loss of true catalytic activity by the immobilized enzyme is caused by biotinylation and association with avidin.

The pH optimum for catalytic activity results from a complex interplay of the ionization of prototrophic groups in the enzyme catalytic and substrate binding sites and the ionization state of the groups in the acyl donating and accepting substrate molecules. For a protein substrate, the structural stability that reflects the chain flexibility around the susceptible glutaminyl and lysyl residues presents an additional complicating parameter. Previous studies of acyl transfer with CBZ-GlnGly and hydroxylamine as substrates (Folk, 1971) or of hydrolysis with *p*-nitrophenyl acetate as substrate (Folk et al., 1967) indicated an optimum at pH 6 and probably reflects the acylation step of the enzyme mechanism. Using proteins as the acyl donor and various amines as the acyl acceptor, pH optima ranging from about 7.5 to 9.0 have been reported (Clarke et al., 1959; Wong et al., 1990). The optima in these studies were dependent on the nucleophilicity of the attacking nitrogen atom and thus probably reflect the second step of the enzyme mechanism. In our studies with protein as the only substrate, the pH–activity profile appeared to be bimodal for both soluble and immobilized enzyme. Furthermore, the maximum activity was observed at pH 6.5, which is close to the maximum at pH 6.0 obtained with CBZ-GlnGly and hydroxylamine as substrates (Folk, 1971; this study). These results suggest that many of the previous studies of protein cross-linking may not have been performed at the optimum pH for catalysis.

Less sensitivity of the immobilized enzyme to variation of pH is consistent with the previous conclusion that with protein as substrate the reaction rate is mass transfer limited. Thus, reduction of the true reaction rate by a shift from the pH optimum alleviates some of the diffusion limitation with a resulting increase in the effectiveness factor (Swaisgood, 1991), and consequently, the observed rate is not reduced proportionately.

Temperature stability observed with the soluble enzyme is in agreement with previous literature which indicates that activity is rapidly lost at temperatures above 40–45 °C (Clarke et al., 1959; Wong et al., 1990; Nury and Meunier, 1990). Results were similar for both forms, although activity of the immobilized form appears to plateau at the higher temperatures. Such a plateauing effect is consistent with the rate of reaction being limited by the rate of mass transfer.

Avidin serves a bifunctional role in the immobilization by strongly binding the biotinylated enzyme to the matrix [the avidin–biotin interaction has a dissociation constant of 10^{-15} M (Green, 1975)] and by providing a protein spacer between the surface and the enzyme. Previous studies in our laboratory have shown that a protein spacer is required for immobilized transglutaminase activity (Oh et al., 1993). Furthermore, although an effort was not made to maximize the amount of transglutaminase immobilized, attachment of additional enzyme to such porous matrices would not be likely to yield higher activities because the rate of reaction already appeared to be limited by mass transfer rates with protein substrates.

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