Characteristics of an Immobilized Form of Transglutaminase: A Possible Increase in Substrate Specificity by Selective Interaction with a Protein Spacer†

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Transglutaminase was covalently immobilized on poly(lysyl)-α_s-casein which was covalently attached to 3-aminopropyl porous glass. Attachment of polylysine to the protein spacer, using soluble transglutaminase as a catalyst, greatly increased the concentration of immobilization sites, resulting in a 74% yield of immobilized protein. Catalytic efficiencies, k_{cat}/K_m, for the immobilized form were 12–13% of that for the soluble enzyme with either α_s-casein or β-lactoglobulin as substrate. Also, the catalytic efficiency was 5-fold greater with α_s-casein than with β-lactoglobulin using either soluble or immobilized enzyme. Selective interaction of the protein substrate with the protein spacer resulted in selective catalysis of isopeptide bond formation in α_s-casein due to increased local concentrations of this protein in the immobilized enzyme matrix. When a mixture of α_s-casein and β-lactoglobulin was used as substrate, preferential cross-linking of α_s-casein occurred with the immobilized enzyme as compared with the soluble enzyme.

INTRODUCTION

Transglutaminases (R-glutaminyl-peptide:amine-γ-glutamyl-yltransferase; EC 2.3.2.13) are calcium-dependent acyl transferases that catalyze formation of amide bonds between the γ-carboxyl group of glutaminyl residues and a primary amino group, usually the ϵ-amino group of a lysyl residue (Folk, 1980; Folk and Finlayson, 1977; Lorand and Conrad, 1984; Ichinose et al., 1990; Greenberg et al., 1991). Some specificity is observed for the glutaminyl residue, and thus, not all glutaminyl residues are reactive (Yan and Wold, 1984); however, a broad specificity is observed for the nucleophilic acceptor (Lorand et al., 1979). In the catalytic mechanism, a thiolester intermediate is formed by acylation of the active site Cys 276 of the guinea pig liver enzyme (Ikura et al., 1988; Ichinose et al., 1990). If a primary amine is not available, water will also function as the nucleophilic acceptor, resulting in deamidation (Motoki et al., 1986). The primary structure of the guinea pig liver enzyme has been determined (Ikura et al., 1988) from analysis of the cDNA, and a molecular weight of 76 620 was indicated.

Intra- and intermolecular cross-linking as well as the addition of peptide or other moieties to protein has the potential for desirable changes in functionality (Whitaker, 1977). For example, transglutaminase has been used to form gels (Nio et al., 1985, 1986), to form cross-linked protein films with α_{s1}-casein for enzyme immobilization (Motoki et al., 1987b), to form cross-linked hompolymers or heteropolymers of proteins (Motoki and Nio, 1983; Ikura et al., 1980a,b; Motoki et al., 1984, 1987a,c; Kurth and Rogers, 1984; Tanimoto and Kinsella, 1988; Aboumahmoud and Savello, 1990), and to introduce other moieties into the protein structure (Ikura et al., 1985; Yan and Wold, 1984). Dramatic changes in functionality have been demonstrated in studies with milk, muscle, and soy proteins.

Recently, we have proposed that novel functionalities may be designed by limited cross-linking of different structural domains released from a variety of proteins by limited proteolysis (Swaisgood et al., 1993). In this study, we examine the possibility of using an immobilized form of transglutaminase for cross-linking of proteins. To our knowledge, this represents the first study of a covalently immobilized form of this enzyme. α_s-Casein was chosen as a protein spacer on which to attach the enzyme because of its open flexible structure and also to test the possibility of altering substrate specificity by affinity interaction of soluble substrate with the spacer linking the enzyme to the surface. Some preliminary results of this study were previously published in a symposium proceedings (Swaisgood et al., 1993).

MATERIALS AND METHODS

Materials. Transglutaminase from guinea pig liver, α_s-casein (α_s-CN), dephosphorylated α_s-casein (80% dephosphorylated), β-lactoglobulin, trypsin, poly(L-lysine) (MW 10 200), ribonuclease, glutaraldehyde (25%), *o*-phthalaldehyde (OPA), β-mercaptoethanol (β-ME), sodium dodecyl sulfate (SDS), sodium tetraborate, trinitrobenzenesulfonic acid (TNBS), EDTA (3-aminopropyl)triethoxysilane, and imidazole were purchased from Sigma Chemical Co. (St. Louis, MO). Controlled-pore glass beads (CPG 3000, 300-nm pore diameter) were obtained from Electro-Nucleonic Corp. (Fairfield, NJ). Quinine sulfate and $CaCl_2 \cdot 2H_2O$ were from Fisher Scientific (Raleigh, NC), ethanol was from Aaper Alcohol and Chemical Co. (Shelbyville, KY), and dithiothreitol (DTT) was from Schwarz/Mann Biotech (Cleveland, OH).

Methods. *Immobilization of Transglutaminase.* (*a*) *Silanization of CPG Beads.* γ-Aminopropyl glass beads were prepared similarly to the nonaqueous silanization procedure described by DuVal et al. (1984). Acid-cleaned dry beads (5 g) were reacted with 4 volumes of 10% (3-aminopropyl)triethoxysilane in toluene for 4 h at 76 °C. The derivatized beads were rinsed with actone and then deionized water and dried. The presence of amino groups was confirmed by reaction with TNBS (Taylor, 1979).

(*b*) *Attachment of the α_s-Casein Spacer.* The γ-aminopropyl glass beads were mixed with deionized water, degassed, collected on a filter, and activated by reaction with 5 volumes of 10% glutaraldehyde for 1 h at room temperature (product **II**, Figure 1). α_s-Casein (100 mg in 20 mL of 0.1 M sodium phosphate, pH

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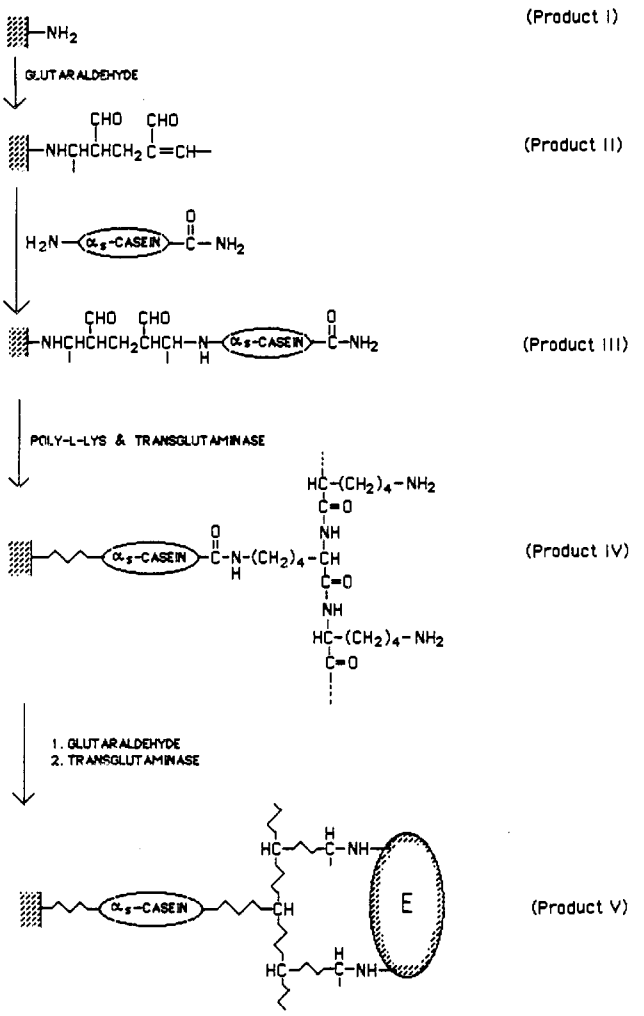

Figure 1. Schematic diagram illustrating the preparation of immobilized transglutaminase.

7.0) was added to 3 g of activated beads and allowed to react overnight in a cold room (product III, Figure 1). Remaining aldehyde groups were blocked with glycine by reaction with 5 volumes of 0.1 M glycine in the phosphate buffer for 1 h at room temperature. The α_s-casein derivatized beads were thoroughly washed with deionized water.

(c) *Attachment of Poly(L-lysine) to the α_s-Casein Spacer.* The α_s-casein–CPG beads were washed and equilibrated with 25 mM imidazole, pH 7.5, containing 5 mM $CaCl_2$ and 10 mM DTT (buffer A). Wet beads were collected by decantation of supernatant liquid, and 5 mL of a solution containing 50 mg of poly(L-lysine) and 3.6 mg of transglutaminase in buffer A was added. The resulting slurry was incubated at room temperature for 24 h. Following reaction, the beads were removed by filtration and washed with deionized water (product IV, Figure 1).

(d) *Attachment of Transglutaminase.* The ϵ-amino groups of poly(L-lysyl)-α_s-casein–CPG were activated by reaction of 2 g of beads with 10 volumes of 10% glutaraldehyde for 1 h at room temperature. The activated beads were removed by filtration and washed with water and then 0.1 M sodium phosphate, pH 7. The resulting wet beads were added to 5.0 mL of transglutaminase solution, 0.589 mg/mL protein as measured spectrophotometrically. The slurry was incubated at room temperature for 30 min and then allowed to stand at 7 °C for an additional 20 h. Aliquots were removed from the supernatant liquid and the protein concentrations determined spectrophotometrically after 1 and 20 h of reactions at 7 °C. The amount of protein immobilized was calculated from the difference in protein concentrations of the original solution and the solution after immobilization as determined spectrophotometrically. Unreacted glutaraldehyde moieties were blocked by addition of the wet beads to 0.1 M glycine in the phosphate buffer for 1 h at room temperature. The transglutaminase beads were washed with water and stored in buffer A at 4 °C (product V, Figure 1).

Assay of Transglutaminase Activity. (a) *Quantitation of Amino Groups in the Substrate Solution.* Primary amino groups were quantitated following the procedure described by Goodno et al. (1981). Fresh OPA reagent was prepared daily by dissolving 40 mg of OPA in 1.0 mL of 95% ethanol and mixed with 25 mL of 0.1 sodium tetraborate, 2.5 mL of 20% (w/w) SDS, and 0.1 mL of mercaptoethanol. This mixture was then diluted to 50 mL with deionized water to make the OPA reagent solution. Amino groups in protein substrate were assayed by adding 50 μL of the protein solution to 3.00 mL of OPA reagent, mixing vigorously for 30 s, and, after reaction for an additional 90 s, reading the fluorescence intensity (scanning spectrofluorometer, Optical Technology Devices, Inc., Elmsford, NY) at 456 nm using an excitation of 346 nm. The fluorescence intensity of 0.1 μg/mL quinine sulfate solution was also measured, and the fluorescence intensity of protein samples is recorded relative to that of this solution. Relative fluorescence was related to amino group concentrations by preparing a standard curve with various concentrations of ribonuclease (linear fit correlation was 0.996). To prevent possible errors due to batch-to-batch variation or deterioration during the day, calibration was performed using a standard ribonuclease solution with each OPA reagent solution before and after its use.

(b) *Assay of Enzymic Activity with Protein Substrate.* Enzyme activity was determined by measuring the decrease in primary amino group concentrations due to isopeptide bond formation using the fluorescent OPA technique and various concentrations of α_s-casein or β-lactoglobulin as substrate. Immobilized enzyme activity was assayed in 5-mL vials by adding 0.2 mL of beads (0.26 g), removing excess liquid, and adding 1.00 mL of substrate in buffer A. The reaction was stopped by adding 106 μL of 400 mM EDTA, giving a final EDTA concentration of roughly 33–35 mM, which inactivates the enzyme. Controls were prepared by adding the EDTA solution immediately after the substrate solution. Mixing during incubation was achieved by inverting the vials by fixation to the head of a peristaltic pump. All incubations were at room temperature. Assay vials were rotated an additional 15 min following the reaction termination, and then 200-μL aliquots were taken from the liquid portions of both sample and control vials and the amino group concentrations assayed with the OPA method. To eliminate the possibility of anomalous rate measurements due to catalyzed cross-linking of protein in solution to protein on the matrix, immobilized enzyme was first incubated with protein prior to its use in these measurements.

The same immobilized enzyme beads were repeatedly used in all of these experiments, and no loss of activity was detectable when they were reassayed under standard conditions. Between periods of use, the beads were stored in buffer A at 4 °C.

A stock solution of soluble transglutaminase was prepared in buffer A at a concentration of 0.60 mg of protein/mL. Activity of the soluble enzyme was assayed by mixing 1.60 mL of stock substrate dissolved in buffer A with 40 μL of stock enzyme to give a final enzyme concentration of 14.6 μg of protein/mL. The reaction was stopped by adding EDTA and the amino group concentration determined as described for the immobilized enzyme assay.

(c) *Assay of Enzymic Activity with Dipeptide and Hydroxylamine Substrate.* Activity was measured spectrophotometrically by determination of the hydroxamate formed from carbobenzoxy (CBZ)-L-glutaminylglycine and hydroxylamine (Folk, 1971). Briefly, 0.1 mL of immobilized transglutaminase beads was added to a 1.0-mL solution containing 4.5 mmol of hydroxylamine and 1.5 mmol of CBZ-L-GluGly in 25 mM imidazole, pH 7.5, with 5 mM $CaCl_2$, 10 mM DTT, and the specific NaCl concentration. After incubation for 1 h with mixing as described above, an aliquot was removed. Following the addition of Fe^{3+} as described by Folk (1971), the absorbance was read at 525 nm.

Effect of Ionic Strength and Dephosphorylation of α_s-Casein Substrate on Enzyme Activity. (a) *Effect of Ionic Strength.* Substrate solutions containing 0.1 mg/mL α_s-casein and 0.1 mg/mL dephosphorylated α_s-casein were prepared in buffer A containing 0, 0.05, 0.10, 0.15, or 0.20 M NaCl. Duplicate vials,

one serving as the control, were prepared with 0.1 mL of immobilized enzyme and equilibrated with the appropriate ionic strength buffer prior to addition of substrate. The assay procedure was as previously described, and the extent of reaction, determined from the decrease in amino group concentration, was measured after a 1-h incubation at room temperature.

Effects of ionic strength on the soluble enzyme activity were also determined by incubation of 2.0 mL of the above substrate solutions mixed with 5 μL of the stock soluble enzyme (0.60 mg/mL) for 20 min at room temperature. The reaction was stopped by addition of 197 μL of 400 mM EDTA. Other assay procedures were as previously described.

(b) Effect of Dephosphorylation of α_s-Casein. Stock solutions containing 0.20 mg/mL α_s-casein or dephosphorylated α_s-casein were prepared in buffer A. Substrate solutions were prepared by mixing these stock substrate solutions in various specified volume ratios. Samples and controls were treated as described above, and the extent of reaction was determined following a 1-h incubation at room temperature.

Cross-Linking Reactions with Mixtures of α_s-Casein and β-Lactoglobulin. A stock solution containing 0.10 mg/mL α_s-casein and 0.30 mg/mL β-lactoglobulin was prepared in buffer A. The cross-linking reactions were carried out as previously described except that 2.0 mL of substrate solution was added. Cross-linking catalyzed by the soluble enzyme was performed similarly by mixing 15 mL of the substrate solution with 0.40 mL of enzyme solution (0.60 mg/mL). As with all experiments, controls were performed simultaneously by addition of EDTA immediately after the substrate.

After completion, 1 mL of liquid aliquots was removed from both reactions and 50 μL used for the OPA assay to determine the amount of cross-linking. The remaining portion was heated at 92 °C for 10 min to inactivate the enzyme. An 800-μL aliquot of the heat-treated solution was fractionated by precipitation of all α_s-casein with Ca^{2+} at a final concentration of 103 mM Ca^{2+}. All α_s-caseins are precipitated at Ca^{2+} concentrations greater than 8 mM (Toma and Nakai, 1973; Aoki et al., 1985). After Ca^{2+}-precipitated protein was removed by centrifugation, the amino groups remaining in the supernatant should represent that in β-lactoglobulin and cross-linked β-lactoglobulin, whereas that precipitated should represent α_s-casein, cross-linked α_s-casein, and cross-linked α_s-casein–β-lactoglobulin, assuming that the latter is also precipitated. These amino group concentrations were also determined by OPA assay.

Susceptibilities of Native and Cross-Linked α_s-Casein to Proteolysis by Trypsin. (a) Preparation of Cross-Linked α_s-Casein. A 0.50 mg/mL substrate solution of α_s-casein was prepared in buffer A containing 0.02% sodium azide. Prior to use in the cross-linking reaction, immobilized transglutaminase was pretreated with the substrate solution for 30 min at room temperature. Following pretreatment and thorough rinsing with buffer A, beads were added to the substrate solution and incubated for 10 min, after which an aliquot of the supernatant was removed and the enzyme inactivated by addition of EDTA. Cross-linking was allowed to proceed in the remaining mixture for an additional 22 h at room temperature. Following reaction, another aliquot was removed, EDTA was added, and the amino group concentration was determined by OPA analysis. Results indicate that 37% of the original amino groups had undergone cross-linking during the 22-h period. After a satisfactory extent of cross-linking was ensured, EDTA was added to the remaining mixture and its liquid portion was decanted and stored at 7 °C.

(b) Proteolysis with Trypsin. Both untreated and cross-linked α_s-caseins (5.0 mL) were incubated with 0.25 mg of trypsin dissolved in 25 mM imidazole, pH 7.5. The ratio of α_s-casein to trypsin was 10:1. Immediately after mixing, a 0.30-mL aliquot was removed and frozen. The remainder was incubated with constant stirring at 37 °C for 1–9 h during which 0.30-mL aliquots were removed at various times and frozen. A control containing buffer A and trypsin was treated similarly. Proteolysis was measured, after thawing, by OPA analysis of total amino groups in a 50-μL aliquot (Church et al., 1985) using the method described previously.

Table I. Comparison of the Kinetic Parameters for the Enzyme-Catalyzed Cross-Linking of α_{s1}-Casein and β-Lactoglobulin[a,b]

	β-lactoglobulin			α_{s1}-casein		
enzyme	k_{CAT}, min^{-1}	K_M, mg/mL	k_{CAT}/K_M, mL/min·mg	k_{CAT}, min^{-1}	K_M, mg/mL	k_{CAT}/K_M, mL/min·mg
soluble	4.89	0.58	8.4	4.86	0.14	34.7
immobilized	0.26	0.27	0.98	1.99	0.44	4.6

[a] Activities were measured using a fluorescent OPA technique for measuring disappearance of primary amino groups. Enzyme catalysis was observed in 25 mM imidazole buffer, at pH 7.5, containing 5 mM $CaCl_2$ and 10 mM DTT at room temperature. [b] Preliminary results for these studies were published in a symposium paper (Swaisgood, et al., 1993).

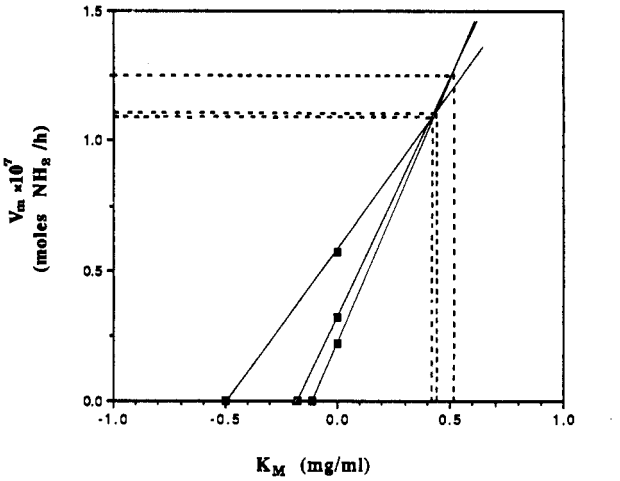

Figure 2. Direct linear plot of the initial rate kinetic data for immobilized transglutaminase with α_s-casein as substrate. Activity was measured at pH 7.5 and room temperature by determination of the decrease in primary amino group concentration using the fluorescent OPA method.

RESULTS

Qualitative analysis of surface amino groups with TNBS yielded colorless derivatives of product **III** while product **IV** was deep yellow, indicating that poly(L-lysyl)-α_s-casein–CPG exhibited a high concentration of amino groups. Because of the high cost of the enzyme, a low concentration (0.6 mg/mL) and limited amount (2.95 mg) were offered. However, the high concentration of surface amino groups resulted in a high percentage of immobilization (74% or 2.18 mg) of the enzyme offered. Furthermore, the rate of immobilization was very rapid, with 90% of the reaction being completed within 90 min. When putrescine was employed in place of poly(L-Lys), no immobilization of the enzyme was observed. This suggests that multiple amino groups per glutaminyl residue must be present for efficient immobilization.

Kinetics of the enzyme-catalyzed cross-linking using both α_s-casein and β-lactoglobulin as substrate were determined from initial rate measurements with both soluble and immobilized transglutaminase. Analysis of these data by direct linear plots (Eisenthal and Cornish-Bowden, 1974; Cornish-Bowden, 1976) yielded the kinetic parameters listed in Table I. A typical direct linear plot is shown in Figure 2. These values represent an overall K_m for α_s-casein or β-lactoglobulin as the only substrate. Using dimethylcasein as the acyl donor and putrescine as the acceptor, Wong et al. (1990) obtained a K_m of 0.057 mM for dimethylcasein with the rat liver enzyme. By comparison, with α_s-casein as the only substrate, our value with the soluble enzyme is 0.006 mM (0.14 mg/mL) and with the immobilized form, 0.019 mM (0.44 mg/mL). The

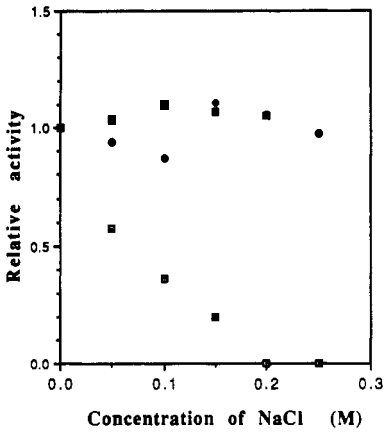

Figure 3. Effect of NaCl concentrations on the activity of soluble (◆) or immobilized (⊡) transglutaminase with a 1:1 mixture of 0.1 mg/mL solutions of α_s-casein and dephosphorylated α_s-casein as substrate. Effect of NaCl concentration on the activity of immobilized transglutaminase with CBZ-L-glutaminylglycine and hydroxylamine as substrate (■). Assay conditions were pH 7.5 and room temperature.

catalytic efficiency, k_{cat}/K_m, is 4.1- and 4.7-fold greater with α_s-casein as substrate for soluble and immobilized enzyme, respectively. However, with the soluble enzyme, the K_m changes, whereas with the immobilized enzyme the major difference between the two substrates is with k_{cat}. With both substrates, the catalytic efficiency is reduced roughly 8-fold by immobilization.

Possible effects on the rate due to attraction of soluble α_s-casein to the surface as a result of Ca^{2+}-induced association with attached α_s-casein were examined. Results indicate that dephosphorylation of α_s-casein had little effect on the enzyme activity. However, a surprising and dramatic effect of NaCl on the activity of immobilized transglutaminase with α_s-casein was observed (Figure 3). This effect was not observed with a small dipeptide substrate for the immobilized enzyme (Figure 3), nor was it observed with the soluble enzyme and α_s-casein (Figure 3).

Further evidence for an interaction between the matrix spacer and soluble α_s-casein was obtained by comparison of the rates of cross-linking of a mixture of α_s-casein and β-lactoglobulin by soluble and immobilized enzyme. In these experiments, α_s-casein and its cross-linked product were removed by precipitation with Ca^{2+} and the decrease in OPA-reactive amino groups in supernatant β-lactoglobulin was compared to the total decrease in OPA-reactive amino groups prior to α_s-casein precipitation. Plots of the value for the supernatant vs the total decrease are shown in Figure 4 for immobilized and soluble enzyme. A lesser slope is indicative of more cross-linking of α_s-casein than of β-lactoglobulin alone and/or cross-linking of α_s-casein to β-lactoglobulin. The slope for immobilized transglutaminase is roughly 1.3, whereas that for the soluble enzyme is 4.9.

Evidence for association of α_s-casein with the matrix even in the absence of free Ca^{2+} was also obtained. Incubation of the immobilized enzyme, inactivated by adjustment to 46 mM EDTA, with a solution of α_s-casein indicated adsorption of roughly 2.7 mg/g.

Both the reduction of chain flexibility and the formation of isopeptide bonds involving lysyl residues as a result of cross-linking should reduce the tryptic digestibility. Progress curves for the digestion of native and cross-linked α_s-casein by trypsin are shown in Figure 5. Results indicate the rate of hydrolysis is lower for the cross-linked protein.

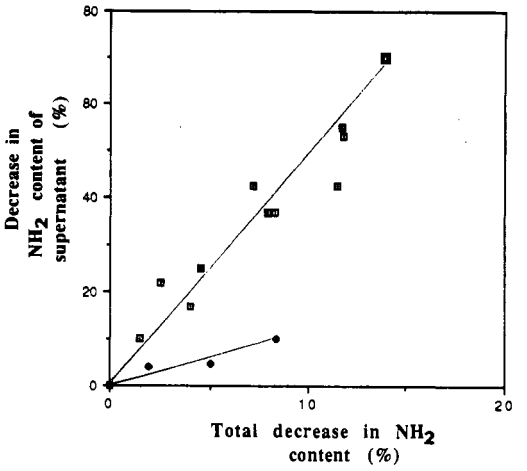

Figure 4. Cross-linking of a solution containing 0.1 mg/mL α_s-casein and 0.3 mg/mL β-lactoglobulin as catalyzed by immobilized transglutaminase (◆) or soluble transglutaminase (⊡). After reaction, the mixture was fractionated by adjustment to 0.1 M Ca^{2+} to precipitate all forms of α_s-casein. The percent decrease in amino group concentration relative to zero-time controls for both the complete mixture and the calcium-fortified supernatant was measured. Decrease of amino group concentration in the supernatant reflects isopeptide bond formation in β-lactoglobulin and, possibly, removal of β-lactoglobulin by cross-linking with α_s-casein causing precipitation.

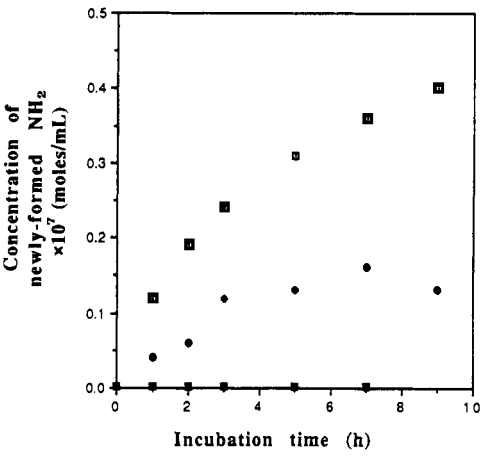

Figure 5. Progress curves for the digestion of α_s-casein (⊡) and cross-linked α_s-casein (◆) with trypsin. Digestion was at pH 7.5 and 37 °C with a 10:1 (w/w) substrate/enzyme ratio. Primary amino group concentrations were measured by the fluorescent OPA method. An enzyme blank was also analyzed (■).

DISCUSSION

A preliminary comparison of enzyme immobilized directly to aminopropyl beads via glutaraldehyde with that immobilized to poly(lysyl)-α_s-casein beads indicated that greater activity was achieved with the proteinaceous spacer. Furthermore, the yield of immobilized protein (74%) was remarkably high considering the low concentration that was offered. This value is well within the range of good binding yields generally obtained (50–90%) (Buchholtz and Klein, 1987). For example, using various tresylated matrices for immobilization of hexokinase or trypsin, binding yields of 53–80% were obtained (Nilsson and Mosbach, 1987).

Comparison of the catalytic efficiencies, k_{cat}/K_m, for soluble and immobilized enzyme indicates that the immobilized form is 12–13% as effective as the soluble enzyme with either substrate. However, the k_{cat} for the immobilized form is 41% of the soluble with α_s-casein but only 5% with β-lactoglobulin. Activity yields for other

immobilized enzymes, usually based on a standard assay, have varied greatly but typically are in the range 25–80% (Buchholtz and Klein, 1987). For example, hexokinase immobilization to tresylated agarose gave an activity yield of 25% (Nilsson and Mosbach, 1987). It is not unusual for proteinases to exhibit low activity yields with large substrates; for example, immobilized trypsin exhibited a 15% activity yield with casein as substrate (Bar-Eli and Katchalski, 1963).

Although we have no direct evidence that soluble α_s-casein was not cross-linked to α_s-casein on the matrix, thus giving artifactually high activities, we do not believe this occurred for the following reasons. First, all of the accessible and reactive glutaminyl residues in the matrix α_s-casein should have reacted with poly(lysine) in the presence of transglutaminase. Second, all of the accessible and reactive lysyl residues that did not react with transglutaminase in the presence of glutaraldehyde most likely would have reacted with glycine in the presence of glutaraldehyde. Thus, there should be no accessible glutaminyl or lysyl residues on the matrix. Finally, in the unlikely event that such residues were present, the immobilized enzyme was first incubated with substrate α_s-casein prior to any of the studies. Also, there appeared to be no loss of activity upon repeated use of the immobilized enzyme.

For the case of β-lactoglobulin as substrate, it appears that mass transfer rates did not affect the kinetics since the apparent K_m of the immobilized form is similar to that of the soluble enzyme. The same appears to be true for α_s-casein, although the apparent K_m is slightly larger with immobilized enzyme. This result is quite surprising since α_s-casein is known to extensively self-associate in the presence of 5 mM Ca^{2+} (Dosako et al., 1980), and in fact, we observed that these solutions were quite turbid. Hence, mass transfer rates with such large complexes would be expected to limit the reaction rate even for the low level of enzyme loading on the matrix obtained in this study. However, the apparent K_m is also affected by substrate attraction to the matrix, but in the opposite direction from mass transfer effects. Thus, we examined this system for possible α_s-casein interactions between the matrix and solution that would result in a higher concentration of the substrate protein in the matrix than in the bulk solution.

With immobilized transglutaminase, a large decrease in cross-linking rates for α_s-casein, but not for the dipeptide substrate, was observed with increasing NaCl concentrations. Increasing salt concentrations are known to decrease the interaction between Ca^{2+} and α_s-casein (Parker and Dalgleish, 1981; Dalgleish and Parker, 1980; Jang and Swaisgood, 1990). Therefore, we suggest that increasing salt concentration decreases Ca^{2+} binding and, consequently, binding of α_s-casein in solution to matrix α_s-casein through Ca^{2+} bridging between anionic phosphate clusters. Loss of this interaction lowers the α_s-casein concentration in the matrix, and mass transfer rate limitations would further lower the reaction rate.

Further evidence for a specific interaction between solution α_s-casein and the matrix was obtained by comparison of the relative cross-linking rates of α_s-casein and β-lactoglobulin mixtures by soluble and immobilized enzyme. Results clearly show increased rates of α_s-casein cross-linking with β-lactoglobulin using the soluble enzyme relative to that observed with the immobilized form.

These results suggest that the use of an affinity matrix could provide additional specificity for an immobilized enzyme. By selective interaction with a particular substrate, the matrix concentration of that substrate is increased, thus lowering its apparent K_m and increasing the rate of reaction in the first-order region. Bayer et al. (1990) first demonstrated the principle of "targeted catalysis" by showing selective cleavage of biotinylated transferrin mixtures of other proteins with biotinylated proteinases in the presence of avidin. In principle, incorporation of an enzyme into an affinity matrix should provide additional selectivity for that substrate attracted to the matrix.

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