Transglutaminase-Catalyzed Incorporation of Lysine Oligomers into Casein

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Transglutaminase was used as an alternative means of covalently attaching lysine oligomers up to pentalysine to some of the glutamine residues of casein in order to prepare branched polypeptide chain proteins. Under the most favorable experimental conditions, incorporation yield reached 30%. Prior protection of the lysyl residues of casein by either acetylation or guanidination to prevent ε-(γ-glutamyl)lysine cross-link formation was found to result in the overall incorporation of five to six lysine oligomers per mole of protein. However, some cross-linking already occurred due to the presence of new ε-amino groups in the modified protein. Incorporation of lysine oligomers containing more than four residues increased largely cross-link formation via intermolecular isopeptide bonds with a subsequent decrease in the reaction yield. These results indicate that introduction of polylysyl units at glutamines in casein by using transglutaminase may lead to a new type of cross-linked protein.

Guinea pig liver transglutaminase (EC 2.3.2.13) is capable of catalyzing the transfer of the γ-carboxamide group of peptide-bound glutamine residues to a variety of primary amines that serve as acyl acceptors. The enzyme has consequently been used to label various proteins (Dutton and Singer, 1975; Okumura and Jamieson, 1976; Gard and Lazarides, 1979) and more recently to prepare neoglycoproteins (Yan and Wold, 1984). Moreover, transglutaminase-catalyzed reaction may lead to the formation of both intra- and intermolecular isopeptide bonds between glutamine and lysine residues in proteins. The enzyme has therefore also been used in the cross-linking of food proteins with the aim of improving their nutritional and/or functional properties (Ikura et al., 1981; Motoki et al., 1984) as well as for constructing economically viable catalytic systems (Okumura et al., 1984).

Catalytic properties of transglutaminases have been extensively investigated (Folk and Finlayson, 1977; Folk, 1980) owing to their wide distribution in mammals and their involvement in important biological functions such as hemostasis or cellular endocytosis. It is worth stressing for instance that the acyl-donor ability of a peptide-bound glutamine residue has actually been found to depend on some structural features of either the peptide or the protein substrate for transglutaminase (Gorman and Folk, 1981).

A stereospecificity requirement for the attachment of peptide-bound lysine residues to the acyl–transglutaminase intermediate has also been reported, as well as the influence of any hydrophobic side chain of the amino acids directely adjacent to lysine (Gross et al., 1977; Schrode and Folk, 1979). However, when macromolecular substrates instead of rather short synthetic peptide substrates were used, the main controlling factor for the glutamine substrate specificity of transglutaminase remained poorly understood.

Chemical methods have been developed for the covalent attachment of amino acids, particularly methionine, and polyamino acids to proteins to improve their nutritional value (Puigserver et al., 1978; Gaertner and Puigserver, 1984) as well as of polylysine for enhancing their cellular uptake (Shen and Ryser, 1978). We felt it important to explore enzymatic methods for preparing protein derivatives containing a number of lysine oligomers covalently attached to some of the glutamine residues. It is the purpose of this paper to show that guinea pig liver transglutaminase can be used to efficiently incorporate lysine and lysine oligomers into casein.

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MATERIALS AND METHODS

Transglutaminase. The guinea pig enzyme was purified from fresh liver according to Brookhart et al. (1983) with minor modifications. In particular, the active fractions eluted from the QAE-Sephadex A 50 column were first absorbed on a Biogel HT hydroxylapatite column (4 × 25 cm), then eluted with a 5–200 mM KH_2PO_4 linear gradient, and finally further purified by filtration through a Sephadex G 150 column (2 × 180 cm). Transglutaminase activity was assayed by the colorimetric hydroxamate procedure of Folk and Cole (1966) whereas the method of Hartree (1972) was used to determine enzyme concentration. The purified enzyme gave a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). It was then stored as small aliquots at −20 °C in a 5 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 30% glycerol.

Protein Substrates. Alkali-soluble casein and α_{s1}-casein were from Merck (Darmstadt, FRG) and ICN Pharmaceuticals (Cleveland, OH), respectively. Protein amino groups were either acetylated according to Riordan and Vallee (1963) or guanidinated with *O*-methylisourea as described by Kimmel (1967).

Transglutaminase Reaction. Enzyme-catalyzed incorporation of amino acids into proteins (5 mg/mL) was carried out in a 0.1 M Tris-HCl buffer, pH 7.6, containing 10 mM $CaCl_2$, 20 mM dithiothreitol (DTT), 50 mM amino acid, and transglutaminase (50 μg/mL). Control experiments without transglutaminase were also performed to prove beyond doubt that covalent incorporation of amino acids did take place under the experimental conditions used throughout. After incubation of the reaction mixture at 37 °C, reaction was stopped by adding ethylenediaminetetraacetate (EDTA) at a final concentration of 40 mM. The resulting solution was then extensively dialyzed against distilled water and finally freeze-dried.

SDS–Polyacrylamide Gel Electrophoresis. Slab gels containing 12.5% polyacrylamide with a stacking gel of 3% were used. Gel electrophoresis in the presence of SDS (0.1%) was performed according to Laemmli (1970).

Amino Acid Analysis. Proteins were hydrolyzed under vacuum with distilled 5.6 N HCl at 110 °C for 24 h. Their amino acid composition was subsequently determined with a Beckman Model 120 C autoanalyzer equipped with an ICAP 10 computer.

Chemicals. L-Methionine ethyl ester hydrochloride and L-lysine hydrochloride were obtained from Fluka AG

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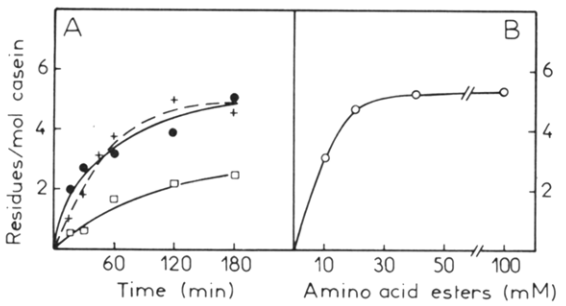

Figure 1. Transglutaminase-catalyzed incorporation of amino acid ethyl esters into acetylated casein as a function of time (A) and concentration of the amino acid derivatives (B). Reaction was carried out as indicated in the Materials and Methods by using 50 mM amino acid ethyl ester in (A) and a 4-h incubation period in (B). Key: L-lysine ethyl ester (+); L-methionine ethyl ester (●); L-phenylalanine ethyl ester (□); average value for lysine and methionine derivatives (○).

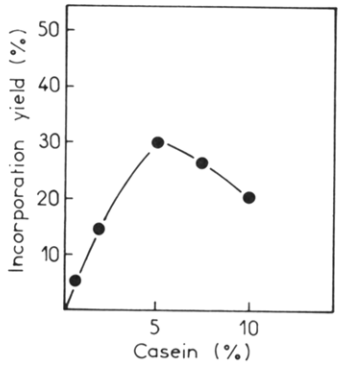

Figure 2. Influence of protein concentration on methionine incorporation into acetylated casein by transglutaminase. Reaction conditions are those in Figure 1 with 20 mM methionine ethyl ester.

(Buchs, Switzerland). *N*-Carbobenzoxy-L-glutaminylglycine and lysine oligomers were from Bachem Fine Chemicals (Bubendorf, Switzerland). Poly-L-lysine (4–15 kDa) and L-phenylalanine ethyl ester hydrochloride were from Sigma Co. (St. Louis, MO). All other reagents and chemicals were of analytical grade.

RESULTS

The kinetic study of lysine incorporation into alkali-soluble casein was compared with that of both methionine and phenylalanine to get better insight into the specificity of transglutaminase regarding amine substrate attachment. However, since the α-carboxyl group of amino acids is known to severely decrease the acyl-acceptor ability of the α-amino group in the enzyme-catalyzed incorporation of amino acids into proteins, ester derivatives rather than free amino acids were used throughout. Moreover, ϵ-amino groups of the lysyl residues of casein were chemically protected prior to transglutaminase reaction when protein cross-linking was not wanted.

Figure 1A shows that the rate of transglutaminase-catalyzed incorporation of L-lysine into casein was quite comparable to that of methionine but significantly higher than that of phenylalanine. The side-chain bulkiness of the latter amino acid is probably responsible for its lower acyl-acceptor ability. As indicated in Figure 1B, the optimal nucleophile concentration was estimated to be about 20 mM, whether lysine or methionine was considered. Under such experimental conditions, five to six amino acid residues were incorporated per mole of acetylated casein whereas the reaction yield did not exceed 5%. However, when casein concentration was increased up to 5% in the presence of 20 mM methionyl ethyl ester, the reaction yield was also increased up to 30% (Figure 2). At a higher acetyl casein concentration, both protein solubility and methionine incorporation were found to be significantly decreased. Extrapolation of these data to lysine seems now quite reasonable since there was no difference in transglutaminase-catalyzed incorporation of these two amino acid derivatives.

Table I. Incorporation of L-Lysine and L-Lysine Oligomers into Guanidinated Casein by Transglutaminase

amine substr	incorporation, mol/mol of casein: Lys[a]	incorporation, mol/mol of casein: Lys peptide[b]	amine substr	incorporation, mol/mol of casein: Lys[a]	incorporation, mol/mol of casein: Lys peptide[b]
Lys	5.0		Lys_4	22.5	5.6
Lys_2	9.6	4.8	Lys_5	18.0	3.6
Lys_3	15.9	5.3			

[a] Determined by amino acid analysis. [b] Calculated by dividing the lysine figure by the chain length of lysine oligomers.

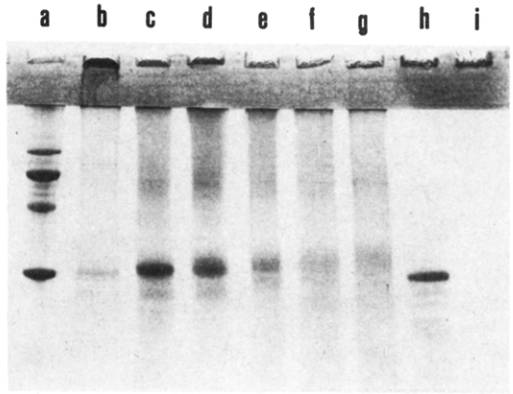

Figure 3. SDS–polyacrylamide gel electrophoresis of α_{s1}-casein modified by transglutaminase. The protein was incubated for 4 h with either lysine or lysine peptides (20 mM) as indicated under Materials and Methods: (a) standard proteins, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B, the M_r of which are 29, 45, 68, and 97 kDa, respectively; (b) unmodified casein; (c–g, i) guanidinated casein in the presence of Lys, Lys_2, Lys_3, Lys_4, Lys_5, and high molecular weight commercial polylysine, respectively; (h) control reation for (i) with guanidinated casein in the presence of polylysine and absence of transglutaminase.

L-Lysine oligomers could also be enzymatically incorporated into guanidinated casein under the same experimental conditions. However, the presence of a free ϵ-amino group in lysine oligomers resulted in the formation of a number of intermolecular cross-links (see below). As indicated in Table I, the protein cross-linking that occurred with di-, tri-, and tetralysine led to comparable incorporations of these peptides into casein since the lysine figure was n times the lysine peptide figure when the substrate is Lys_n. By contrast, incorporation of pentalysine was severly decreased (3.6 mol/mol of protein instead of 5.2 ± 0.4 for di- to tetralysine), probably as a result of increased cross-link formation. Since all the above-mentioned results were derived from amino acid analyses, the complete removal of lysyl species in excess by dialysis before acid hydrolysis of the modified protein and subsequent amino acid analysis was of prime importance. This was ascertained by running a control experiment without transglutaminase.

Figure 3 shows that the formation of intermolecular cross-links and consequently changes in the molecular weight of protein derivatives upon translglutaminase re-

action could indirectly be detected by means of SDS–polyacrylamide gel electrophoresis. When protein cross-linking was pronounced enough, casein derivatives were able to enter neither the stacking gel nor the separating polyacrylamide gel. Unmodified α_{s1}-casein was found to be highly polymerized since it became unable to penetrate into the gel (slot b). By contrast, no polymerization occurred when guanidinated casein instead of unmodified casein was used under the same experimental conditions (data not shown).

In the presence of lysine oligomers (slots c–g), however, some protein cross-linking occurred since lysine peptides were now playing the part of bi- or multifunctional reagents. Cross-linking of guanidinated casein was found to be enhanced as the length of the lysine oligomer incorporated into the original protein was increased. There was only a small amount of free casein left without any cross-linking or no free protein at all when trilysine (slot f) and pentalysine (slot g) were used as acyl acceptors. At least two of the amino groups of each lysine oligomer were expected to be involved in the formation of an isopeptide bond with two glutamine residues from distinct polypeptide chains.

Comparable observations were also made with a high molecular weight poly-L-lysylguanidinated casein sample (slot i), whereas guanidinated casein freed from noncovalently attached polylysine was easily identified under denaturing conditions, in the presence of SDS, when no transglutaminase was added to the reaction mixture (slot h). In this case, the material present in the stacking gel should be polylysine, which was found to easily precipitate with SDS. The presence of some contaminating precipitated casein could not however be ruled out. By contrast, under nondenaturating conditions, it has never been possible to directly visualize any transgluaminase-catalyzed cross-link formation between 4–15-kDa poly-L-lysine and casein since the protein and lysine polymer are tightly associated through ionic interactions to form a noncovalent complex. Moreover, polylysine could be stained neither with Coomassie Blue nor with Amido Black in the absence of SDS for still unknown reasons.

DISCUSSION

The present study shows that guinea pig liver transglutaminase was able to catalyze the incorporation of lysine oligomers into casein since as much as 5 mol of di- to tetralysine was conjugated to the protein. It is worth stressing here that under the same experimental conditions neither lysozyme nor ovalbumin and serum albumin were found to be acyl-donor substrates for the enzyme. However, with respect to albumin it has been reported that a number of peptide-bound glutamine residues might function as acyl donors after chemical modification or heat denaturation. Both procedures are known to result in conformational changes of serum albumin polypeptide chain (Ikura et al., 1984).

The highest level of amino acid incorporation was obtained with acylated casein in which 5–6 glutamines out of 15 were modified. Comparable extents of modification have been reported for transglutaminase-catalyzed incorporation of methionine and dansylcadaverine into maleylated and succinylated β-casein, respectively (Ikura et al., 1984; Yan and Wold, 1984) as well as lysine containing dipeptides into α_{s1}-casein (Ikura et al., 1985). The fact that less than half of casein glutamine residues may function as γ-carboxamide group donors was clearly consistent with transglutaminase specificity, which has already been shown to depend on the primary structure surrounding each glutamine residue (Gorman and Folk, 1980). Other structural features of glutamine substrates for transglutaminase may also be involved since our attempts to improve the acyl-donor ability of casein, ovalbumin, and β-lactoglobulin were not successful. For instance, amidation of these proteins by the method of Lewis and Shafer (1973), which resulted in a twofold increase in the total number of both glutamine and asparagine residues, did not significantly improve the transglutaminase-catalyzed incorporation of the primary amine substrates. However, the rather poor solubility of the modified proteins may at least partly explain this observation. Our kinetic study on the incorporation of lysine, methionine, and phenylalanine esters to casein may also shed light on transglutaminase specificity regarding particularly the reaction of amine substrate attachment to the acyl intermediate (Gross et al., 1977). A marked preference has been observed with branched-chain aliphatic amines containing a methylene chain, the length of which was equal to that of lysine side chain. Comparable observations were made in this study with the α-amino group of amino acid esters when side-chain bulkiness was considered. Thus, the aromatic amino acid phenylalanine was found to have a lower reactivity than the hydrophobic amino acid methionine that possesses a side methylene chain.

It is noteworthy that incorporation of lysine oligomers into proteins as compared to free lysine resulted in a considerable increase of the amount of covalently bound amino acid since the longer the lysine oligomer the higher the amount. Although some protein cross-linking occurred when lysine oligomers were used as acyl acceptor, the formation of inter- as well as intramolecular cross-links did not lead to any decrease in the number of accessible glutamines as long as the chain length of the lysine oligomer was below four residues. With respect to the covalent attachment of high molecular weight polylysine to guanidinated casein, the absence of any free protein on the slab gels indicated that the protein was probably linked with polylysine.

Although globular proteins are known to be rather poor substrates for transglutaminase, it was worth trying to covalently attach high molecular weight polylysine to a number of selected enzymes. Such a modification may have practical interest in enzyme technology by making economically viable catalytic systems as well as by enhancing cellular penetration of macromolecules or decreasing their immunogenicity as compared to their native counterparts. Unfortunately, our attempts to conjugate positively charged polylysine to chymotrypsin, ribonuclease, or catalase were not as successful as expected. Thus, much more work is needed to improve the transglutaminase reaction in that case.

The use of lysine oligomers with a variable chain length in the transglutaminase-catalyzed modification of food proteins may lead to a new type of cross-linked protein with interesting functional and/or nutritional properties. High molecular weight cross-linked proteins with meshes of different size may represent a rather unexplored way of entrapping a number of active principles.

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Registry No. Lys, 56-87-1; Lys_2, 13184-13-9; Lys_3, 13184-14-0; Lys_4, 997-20-6; Lys_5, 19431-21-1; transglutaminase, 80146-85-6; L-lysine ethyl ester, 4117-33-3; L-methionine ethyl ester, 3082-77-7;

L-phenylalanine ethyl ester, 3081-24-1.

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α-Amylase Assay and Action Pattern Determination Using Radioactive Substrate, HPLC, and a Radioactive Flow Detector

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A new assay system is presented for the analysis of α-amylase. The disappearance of ^{14}C-labeled starch substrate and the appearance of its radioactive degradation products were monitored by HPLC and a radioactive flow detector/integrator. The hydrolysis of radioactive substrate was proportional to enzyme concentration when two commercially available α-amylase preparations of *Bacillus subtilis* origin were studied. The method demonstrated an average recovery of 101.7 ± 6.5% when modified food starch was spiked with amylase and analyzed. In addition, the method was shown to be useful for predicting detailed action patterns of various types of amylases.

α-Amylases (1,4-α-D-glucan glucanohydrolase) hydrolyze starch, glycogen, and their degradation products by splitting central 1,4-α-glucosidic linkages, causing rapid liquefaction and slower saccharification. α-Amylases have been detected in plants, in microorganisms, and in the pancreas, blood, urine, and saliva of animals. The presence of α-amylases has been confirmed in milk (Guy and Jenness, 1958), whey protein powder (Thomas et al., 1984), fungal protease preparations that are commonly used as substitutes for calf rennets in cheese making (Thomas et al., 1984), and other food ingredients.

Adding α-amylase-contaminated ingredients to food products containing starch-thickening agents can cause spoilage in the form of a dramatic loss of viscosity or thinning. For example, in some instances ultrahigh-temperature- (UHT-) sterilized puddings have undergone dramatic viscosity losses within a few days to several months after manufacture (Anderson et al., 1983). Although they were not able to identify the exact source of the enzyme, Barefoot and Adams (1980) concluded that the cause of thinning of sterile UHT-treated pudding was an extremely heat-stable amylase—probably a bacterial α-amylase that tends to be more heat stable than those from other sources. They recommended that manufacturers of puddings and similar products containing starch as their primary thickening agent take steps to control α-amylase—either by keeping the enzyme out of the product or by inactivating the enzyme in the product.

Numerous assay procedures have been applied to monitor α-amylase activity (Allen and Spradlin, 1971). Most of these methods measure either the increase in reducing groups liberated as starch hydrolysis proceeds or the decrease in the starch iodine color that occurs as the starch substrate is degraded.

Malacinski (1971) developed a microassay for α-amylase based on the solubilization of ^{14}C-labeled starch. With this assay technique, [^{14}C]starch was incubated with enzyme for 30 min at 37 °C. An aliquot of the reaction mixture was then precipitated with 95% ethanol and carrier nonradioactive starch. The rate at which radioactive starch was hydrolyzed was determined by measuring the accumulation of radioactivity in the supernatant fluid. Malacinski demonstrated that this radioactive substrate approach offered at least two significant advantages over commonly used colorimetric procedures: It was more sensitive and applicable to more complex sample matrices (e.g., crude tissue extracts). Malacinski's radiochemical procedure was approximately 15-fold more sensitive than the starch–iodine assay and 2.5-fold more sensitive than the dinitrosalicylate assay.

Rather than using alcohol precipitation as a means of fractionating [^{14}C]starch from its hydrolysis products, Thomas et al. (1984) used paper chromatography and resolved ^{14}C-labeled maltotriose (dp 3), maltose (dp 2), and glucose (dp 1) degradation products. By using more so-

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