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Covalent Attachment of Poly(L-methionine) to Food Proteins for Nutritional and Functional Improvement

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Methionine, the first limiting essential amino acid of many plant proteins, has been covalently attached to casein and β-lactoglobulin by using the N-carboxyanhydride method. Introduction of as much amino acid as 30% of protein weight led to the formation of polymethionine chains linked to the lysyl residues through isopeptide bonds. Under polymerization conditions at pH 6.5, attachment efficiency was close to 80% and about 40% of the lysyl residues were acylated. In contrast, successive addition of reagent at pH 10.2 reduced the efficiency to 25–30% while all the ϵ-amino groups were modified. Important changes in solubility properties were observed with polymethionylcasein and to a lesser extent with methionylcasein. "In vitro" digestion studies, performed with bovine α-chymotrypsin and trypsin at pH 8.2 and 38 °C, resulted in a significant decrease of hydrolysis of casein derivatives that was correlated with chain length and distribution of methionine polymers as well as conformational changes as indicated by fluorescence studies. Polymerization of amino acids onto food proteins may be a valuable procedure for improving their nutritional and functional properties.

Supplementation of foods and feeds with free essential amino acids is attractive since it represents a simple and effective means to improve protein quality. It has nevertheless some well-known disadvantages such as deteriorative reactions resulting in the modification of sensory properties and in decreased biological utilization of the supplemented free amino acids as compared to peptides (Puigserver et al., 1982). During the past few years, a number of authors have investigated the feasibility of covalently attaching limiting amino acids to food and feed proteins in order to improve their nutritional and/or functional properties.

Papain-catalyzed incorporation of methionine into soy proteins through a single-step process has been successfully used to increase their content in this limiting amino acid (Yamashita et al., 1979b; Arai et al., 1979). The enzymatic method has the obvious advantage of incorporating exclusively L-methionine with a yield as high as 80% when the racemic amino acid ethyl ester is used (Yamashita et al., 1979a). Chemical methods already available for covalently attaching amino acids to proteins include those used to modify carboxyl and amino groups. The carbodiimide condensation reaction, which requires carboxyl

group activation, has recently been used to covalently link methionine, tryptophan, or lysine to soy protein isolates and wheat gluten (Li-Chan et al., 1979; Voutsinas and Nakai, 1979). Although peptide and isopeptide bonds are formed in this case, it has nevertheless been shown that α-carboxyl groups reacted more rapidly than β- or γ-carboxyl groups and that the resulting covalently linked amino acids were readily released by a pepsin–pancreatin mixture. When the ϵ-amino group of lysyl residues was modified by use of N-hydroxysuccinimide esters (Puigserver et al., 1978, 1979b) or N-carboxyanhydrides of limiting amino acids (Bjarnason-Baumann et al., 1977), isopeptide bonds only are formed. However, it should be mentioned that no polypeptidyl derivatives were obtained under experimental conditions of the latter study. Amino acids covalently attached to the ϵ-amino group of lysyl residues were as readily available as the free forms (Puigserver et al., 1979a). Intestinal membrane-bound aminopeptidase appears to be the unique enzymatic activity able to hydrolyze the isopeptide bond effectively.

Since the first methionyl residue attached to each lysine of casein by an isopeptide bond was biologically available in the rat, it may therefore be expected that subsequent attachment of additional residues through a polymerization reaction will also lead to methionine available in vivo. Protein amino groups will act as initiators of the polymerization reaction of N-carboxymethionine anhydride yielding polymethionine chains of different length. By use

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of N-carboxy α-amino acid anhydrides, a number of polypeptidyl proteins or polypeptidyl enzymes have been prepared for structure–function relationship studies and immunological investigations (Glazer et al., 1962; Wellner et al., 1963; Uren and Ragin, 1979). In contrast, little attention has been paid to polymerizing essential amino acids onto food proteins for nutritional improvement though synthetic poly-L-lysine, known to be readily hydrolyzed by trypsin, has already been used in lysine-deficient protein diets (Newman et al., 1980).

The purpose of the present work has been to polymerize N-carboxy α-amino acid anhydrides onto food proteins and evaluate the influence of covalently attached poly(amino acids) on physical and digestibility properties of the modified proteins. Casein and β-lactoglobulin were used as model proteins, but the modification has also been extended to several methionine-deficient proteins.

MATERIALS AND METHODS

Materials. Phosgene was from L'Air Liquide, France, while casein was obtained from Merck, FRG. Methionine, β-lactoglobulin, and the standard proteins, bovine serum albumin and ovalbumin, used for molecular weight determinations were from Sigma Chemical Co., St.-Louis, MO. Chymotrypsinogen, chymotrypsin, and trypsin were from Worthington Biochemical Corp., Freehold, NJ. Soy protein isolate was from Purina Co., France; pea and bean protein isolates were from INRA, Clermont-Ferrand, France. α-tert-Butyloxycarbonyl-L-methionine N-hydroxysuccinimide ester was from Bachem Fine Chemicals, Switzerland. All other reagents and chemicals were of analytical grade.

Methods. *Synthesis of N-Carboxy-L-methionine Anhydride (NCA).* Synthesis of N-carboxy-L-methionine anhydride was carried out in a hood with good draft at 45 °C according to previously described methods (Bloom et al., 1962; Hirschmann et al., 1971). L-Methionine (25 g), suspended with stirring in anhydrous tetrahydrofuran (1 L), was solubilized by phosgene bubbling, and then anhydrous nitrogen was passed through the reaction mixture for 2 h to remove any excess of phosgene. After successive evaporations of tetrahydrofuran, the resulting oily product (31 g) could be stored at −20 °C for several months and used for protein modification without further purification. Attempts to crystallize N-carboxy-L-methionine anhydride in ethyl acetate-hexane mixtures were unsuccessful. Quantitative estimation of NCA in the oily product was routinely performed according to the titrimetric method of Patchornik and Shalitin (1961), in which the carbon dioxide evolved upon treatment with dilute acid was absorbed by a mixture containing benzylamine, ethanol, and dioxane (1:3:3 v/v) and then titrated with sodium methylate by using thymol blue as an indicator. N-Carboxy-L-methionine anhydride (29 g) was recovered in 90% yield.

Reaction of Proteins with N-Carboxy-L-methionine Anhydride. Proteins, either dissolved in a 0.1 M sodium citrate buffer, pH 6.5, or a bicarbonate buffer, pH 10.2, were incubated for 15 min at 4 °C with a tetrahydrofuran solution containing the NCA. The organic solvent never exceeded 10% (v/v) of the aqueous solution. Under polymerization conditions, an excess of amino acid anhydride over protein amino groups was added with vigorous stirring while the pH was kept constant at 6.5 by addition of 0.1 N NaOH. In stepwise synthesis, where the coupling step occurred at pH 10.2, the reaction mixture was acidified with hydrochloric acid to pH 3–4 and kept for 10 min at 20 °C before the next addition of reagent at the alkaline pH. Modified proteins were separated from reaction byproducts by dialysis against water and finally freeze-dried.

The overall yield of this procedure was about 80% under polymerization conditions and about 40% in stepwise synthesis.

Amino Acid Analysis and Amino Group Determination. Polymethionyl proteins were assayed for the amount of covalently attached amino acid and number of unreacted ε-amino groups of the lysyl residues. The amino acid composition of control or modified protein samples was determined with a Beckman Model 120 C autoanalyzer equipped with an ICAP 10 computer, following hydrolysis of the proteins with distilled 5.6 N HCl at 110 °C for 24 h. Methionine was determined as methionine sulfone after performic acid oxidation of the protein (Hirs, 1956; Moore, 1963). Free amino groups were estimated as homoarginine following selective reaction of the lysyl residues with O-methylisourea during 4 days at 4 °C, pH 10.5, using a 0.5% protein solution and 0.5 M reagent (Kimmel, 1967). Then, reaction was stopped by adding an equal volume of 1 M phosphate buffer (pH 5.0). After dialysis of the resulting protein against water and hydrolysis as indicated above, homoarginine was quantitated with the amino acid analyzer as a peak emerging shortly after arginine. The number of lysyl residues acylated by methionine or polymethionine chains was assumed to be equal to the number of lysyl residues that were not guanidinated.

Protein Solubility. Samples containing 50 mg of control casein or modified casein in 2.0 mL of a 0.1 M citrate buffer, pH 6.5, were successively incubated for 1 h at 60 °C and 24 h at 4 °C. After 1-h centrifugation at 27000g and 4 °C, the amount of soluble protein was determined by the biuret method.

Fluorescence Spectra. After excitation at 278 nm, the fluorescence emission was recorded at 20 °C between 300 and 400 nm with the aid of a Fica double-beam spectrofluorometer, Model 55. Equimolar solutions of control or modified proteins, never exceeding 0.04 absorbance unit at 280 nm, were prepared in a 0.05 M Tris-HCl buffer, pH 8.0.

Polyacrylamide Gel Electrophoresis. The electrophoretic mobility of proteins was determined under standard conditions with a Tris–glycine buffer, pH 8.1, and 7.5% polyacrylamide gels. Reduced and alkylated proteins were also separated according to their apparent molecular weights by 10–18% polyacrylamide gradient gel electrophoresis in sodium dodecyl sulfate as previously described by Maizel (1969). The reference proteins used were bovine serum albumin (69 000), ovalbumin (43 000), bovine chymotrypsinogen (25 000), β-lactoglobulin (18 000), and cytochrome c (13 000). Proteins were stained with amido black while Coomassie brilliant blue was used for alkylated proteins.

In Vitro Enzyme Digestion. The initial rates of hydrolysis of casein and modified caseins were determined by measuring the liberation of amino groups by the 2,4,6-trinitrobenzenesulfonic acid method (Fields, 1972). Equimolar concentrations of casein or casein derivatives (about 0.1% solutions) in a 0.02 M sodium borate buffer, pH 8.2, were incubated at 38 °C with 1:2000 (w/w) α-chymotrypsin or trypsin to protein substrate.

RESULTS

Polymerization of L-Methionine on Casein Amino Groups. Quite different experimental conditions should be used for single addition or polymerization of methionine per amino group of protein (Puigserver et al., 1982). As shown in Figure 1, a marked effect of pH on the amount of methionine grafted onto casein and extent of lysine modification was observed. Attaching an average of seven to eight methionyl residues on each of the seven modified
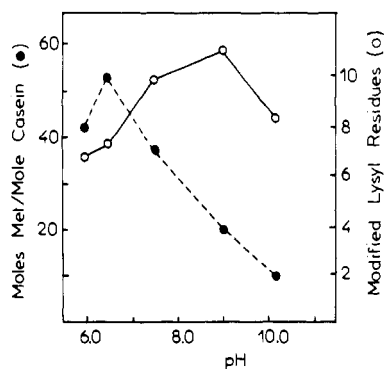
Figure 1. pH dependence of reaction of casein with *N*-carboxy-L-methionine anhydride at 4 °C. Protein concentration was 40 mg/mL; molar ratio of reagent: amino groups was 5:1. The following buffers were used: sodium citrate, pH 6.0 and 6.5; sodium phosphate, pH 7.5 and 9.0; sodium bicarbonate, pH 10.2. Casein samples were supposed to have an average molecular weight of 23 000 and contain about 12 mol of lysine/mol of protein.
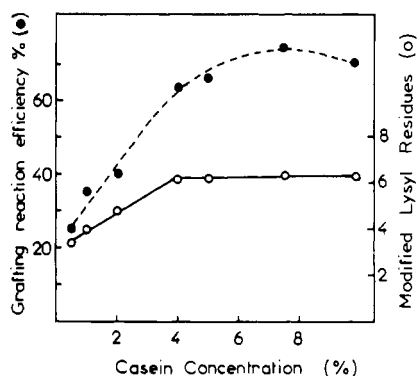

Figure 2. Influence of casein concentration on the reaction yield and extent of modification of lysyl residues. Reaction was carried out at 4 °C in a 0.1 M sodium phosphate buffer, pH 7.0, with a molar ratio of reagent: amino groups of 3:1.

lysyl residues, out of the twelve present in the protein, was achieved at pH 6.5. At higher pH values, polymerization of methionine was severely decreased while more lysyl residues became acylated. This resulted from an increased base-catalyzed hydrolysis of the reagent and a higher number of amino groups available for nucleophilic attack of the anhydride at alkaline pH, respectively. Thus, polymerization of methionine occurred at pH 6.5-7.0 whereas single addition of the amino acid per amino group of protein will be favored at pH 10.0. Increasing the protein concentration up to 4% (w/v) sharply increased the yield of methionine polymerization and degree of modification of lysyl residues (Figure 2). Then, both values reached a plateau since the reaction mixture tended to gelatinize, favoring hydrolysis of *N*-carboxy-L-methionine anhydride as compared to attachment to casein.

The successive addition at a ratio of 1.5:1.0 of *N*-carboxy-L-methionine anhydride to the amino groups of casein at pH 10.2 led to complete modification of the lysyl residues and consequently to the formation of methionine polymers of a shorter average chain length (Kircher et al., 1980; Puigserver et al., 1982). The efficiency of stepwise attachment of methionine to casein was about 40% in contrast to the one of polymerization, which reached 80%. Morever, as indicated in Figure 3, the coupling between ε-amino groups and methionine was sharply enhanced by increasing the *N*-carboxyanhydride concentration. In the most extensively modified casein samples, up to 75 methionyl residues were linked via both the α- and ε-amino groups of the protein, leading to an average chain length
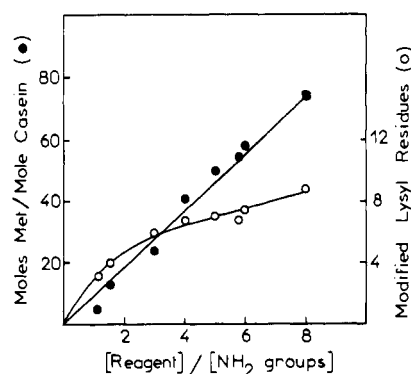
Figure 3. Modification of casein under polymerization conditions as influenced by *N*-carboxy-L-methionine anhydride concentration. Reaction was performed at 4 °C in a 0.1 M sodium citrate buffer, pH 6.5; protein concentration was 40 mg/mL.

Table I. Extent of Modification of Several Food Proteins with *N*-Carboxy-L-methionine Anhydride

protein	amino acid content[a]		modified lysyl residues, %[b]	efficiency of grafting reaction, %
	Lys	Met		
control casein	9.0	3.0	0	
polymethionylcasein[c]		22.0	43	70
polymethionylcasein[d]		14.0	>95	23
control β-lactoglobulin	12.0	3.2	0	
polymethionyl-β-lactoglobulin[c]		32	42	80
polymethionyl-β-lactoglobulin[d]		24	>95	26
control pea protein	6.1	0.4	0	
polymethionyl pea protein[c]		18.0	40	73
polymethionyl pea protein[d]		13.9	>95	29
control bean protein	6.0	0.4	0	
polymethionyl bean protein[c]		19.0	41	75
polymethionyl bean protein[d]		18.0	>95	37
control soy protein	6.3	1.3	0	
polymethionyl soy protein[c]		21.0	40	68

[a] Content in g/100 g of protein. [b] Determined as those residues that were not transformed into homoarginine by reaction of proteins with *O*-methylisourea. Calculation based on an average of 12 and 14 lysyl residues per mol of control casein (M_r 23 000) and of β-lactoglobulin (M_r 18 000), respectively. [c] Modified by a single addition of *N*-carboxy-L-methionine anhydride at pH 6.5. Molar ratio of reagent:protein amino groups was 4:1. [d] Modified by four successive additions of *N*-carboxy-L-methionine anhydride at pH 10.2 with 1.5:1.0 molar ratio of reagent:amino groups each time.

of poly(L-methionine) side chains of seven to eight residues.

The two ways of covalently attaching methionine to casein, polymerization at pH 6.5 or stepwise synthesis at pH 10.2, have been applied to a number of food proteins by using approximately a 1:1 molar ratio of reagent over the acylatable amino groups. As shown in Table I, with 4 mol of *N*-carboxy-L-methionine anhydride/amino group, an average of 40% of the lysyl residues of each protein were modified at pH 6.5 with an attachment efficiency higher than 70%. A 5-15-fold increase of the methionine content of proteins was observed under polymerization conditions, resulting in polymethionyl proteins containing as much as 20% methionine (w/w). By contrast, four successive additions at pH 10.2 of 1.5 mol of reagent/amino group allowed lower attachment efficiencies, in the range of 25-35%, though all the lysyl residues were modified. A 4-8-fold increase of the methionine content of proteins was obtained under stepwise synthesis conditions.

Electrophoretic Characterization of Modified Proteins. Control casein as well as modified caseins gave two bands on 7.5% polyacrylamide gels whose electrophoretic mobilities increased with the extent of acylation of the lysyl residues (Table II). This findings was

Table II. Electrophoretic Mobility of Native and Modified Caseins[a]

modified ε-amino groups, %	R_{f_1}	R_{f_2}
0	0.42	0.77
23	0.45	0.77
33	0.48	0.80
65	0.51	0.81
82	0.55	0.83
100	0.59	0.84

[a] Electrophoresis was performed at pH 8.0 on 7.5% polyacrylamide gels. Modification of lysyl residues was determined as in Table I.
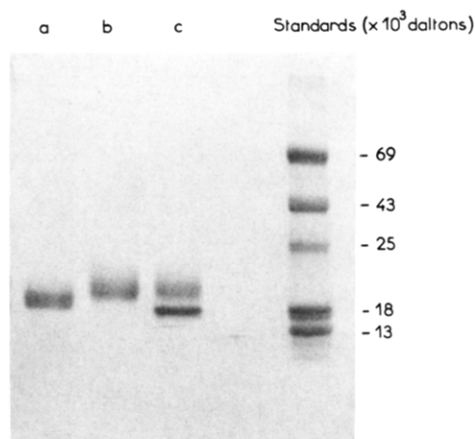

Figure 4. Polyacrylamide gel electrophoresis of modified β-lactoglobulin in sodium dodecyl sulfate. (a) Polymethionyl-β-lactoglobulin with 25 residues linked and all the lysyl residues acylated. (b) Polymethionyl-β-lactoglobulin with 50 residues linked. (c) Native β-lactoglobulin (lower band) and modified protein as in (b).

somewhat expected since attachment of methionine or polymethionine to casein resulted in the blocking of some of the ε-amino groups of lysyl residues ($pK = 10.5$) and their replacement by α-amino groups ($pK = 9.2$), increasing therefore the overall negative charge of polymethionyl caseins at pH 8.1. The fact that electrophoretic mobility of the fast moving band (R_{f_2}) was less affected than that of the slower one (1.1-fold instead of 1.4-fold) was consistent with a lower number of lysyl residues in the former.

In contrast, polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the modified β-lactoglobulin samples gave a single wide band as indicated in Figure 4 (slots a and b). This was noteworthy since standard β-lactoglobulin and, to a lesser extent, control β-lactoglobulin (slot c, lower band) were found to contain the well-known polypeptide chains A and B of slightly different molecular weights. The estimated molecular weights of the two modified samples (19 000 and 21 000) were not quite consistent with the introduction of 25 and 50 methionyl residues, respectively. Such a discrepancy might be due to some unusual behavior of the polypeptidyl protein under our experimental conditions resulting probably from the branched structure of the modified protein.

Some Physical Properties of Modified Caseins. As shown in Figure 5, an overall decrease in solubility of casein related to the degree of polymerization of methionine on the lysyl residues was observed. Solubility rapidly decreased when more than 25 methionyl residues were linked to casein, while complete insolubility was reached with a derivative containing 50–60 residues of methionine covalently attached to the protein. In the latter case, about 60–70% of the lysyl residues were acylated, leading to polymethionine chain lengths of 8–10 residues. Stepwise
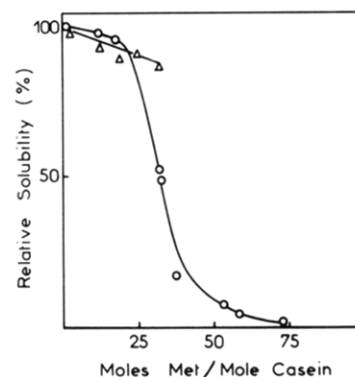

Figure 5. Solubility of casein as influenced by the covalent attachment of methionine on lysyl residues under polymerization conditions at pH 6.5 (O) or stepwise peptide synthesis at pH 10.2 (Δ).
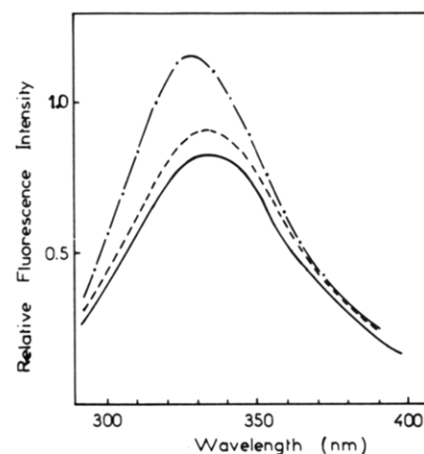

Figure 6. Fluorescence spectra of equimolar concentrations (5.4×10^{-6} M) of casein (—), polymethionylcasein with an average chain length of five methionyl residues on 50% of the lysyl residues (–·–), and polymethionylcasein with all the lysyl residues modified and an average of three methioninyl residues per amino group (- - -).

addition of up to 30 methionyl residues to casein at pH 10.2 (2–3 mol of methionine/lysyl residue) had only a small effect on protein solubility (10% decrease instead of 50%). This has already been observed with other casein derivatives (Puigserver et al., 1978). In contrast, comparable modification of casein at pH 6.5 or pH 10.2 (16 g of methionine/100 g of protein) led to about the same increase in viscosity (1.45 instead of 1.30 cSt·s⁻¹, data not shown). It was surprising indeed that attaching polymethionyl chains of quite different length, inducing probably different conformational changes of the modified proteins, led to the same change in viscosity. It should therefore be suggested that the severe decrease in solubility of polymethionyl casein as compared to that of methionyl casein would be the result of hydrophobic interactions between long enough polymethionyl side chains rather than of some conformational transition.

Although amino acids that did not modify the charge of casein were found to have no effect on tryptophan fluorescence (Puigserver et al., 1979b), attachment of polymethionine chains to casein resulted in both an increase of intrinsic fluorescence and a 5–10-nm shift toward shorter wavelengths of the maximal emission of casein derivatives as compared to control casein (Figure 6). This shift, known to be consistent with an apolar environment of the tryptophan residues, has already been observed with other casein derivatives (Puigserver et al., 1979b). Such a conformational change of the protein did not occur when less
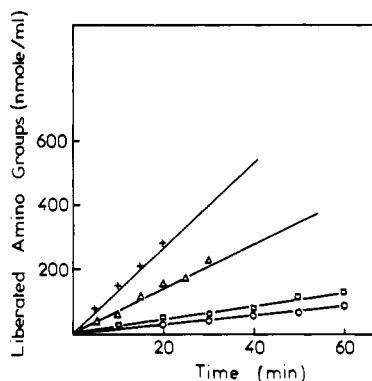
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Figure 7. Initial rates of hydrolysis of casein derivatives by chymotrypsin. Native casein (+), methionylcasein containing 12 residues linked with one methionine per amino group (△) prepared as described by Puigserver et al. (1979b), and polymethioninyl-casein containing either 37 residues of methionine covalently attached (□) or only 25 residues attached but all the lysyl residues acylated (○) were used.

Table III. Enzymatic Digestion of Casein Derivatives As Influenced by Extent of Modification

protein	Met bound	amino group modifica-tion, %	initial rates, %[a]	
			α-chymo-trypsin	trypsin
control casein	0	0	100	100
methionylcasein	12	95	52	53
polymethionylcasein	11	35	32	34
polymethionylcasein	37	60	18	14
polymethionylcasein	25	95	8	11

[a] Reaction mixtures containing equimolar concentrations of proteins (about 0.1%) in a 0.02 M borate buffer, pH 8.2, were incubated at 38 °C with 1:2000 (w/w) α-chymotrypsin or trypsin.

than three methionyl residues were linked per amino group of casein or in the case of β-lactoglobulin even if the protein was extensively modified (50 mol of methionine/mol of protein, data not shown).

In Vitro Enzyme Digestion of Casein Derivatives. The initial rates of hydrolysis of control and modified caseins by bovine α-chymotrypsin are shown in Figure 7. Covalent attachment of methionine or polymethionine chains to casein resulted in a decrease of the rates of enzymatic digestion, a marked effect being observed in the latter case. Moreover, as summarized in Table III, the decrease in initial rates of digestion by α-chymotrypsin and trypsin were drastically influenced by the degree of acylation of the lysyl residues as well as by the chain length of polymers.

DISCUSSION

Polymerization of N-carboxy-α-amino acid anhydrides, also known as Leuchs' anhydrides, and their stepwise addition to amino acids or peptides in organic solvents have been intensively reviewed (Katchalski and Sela, 1958; Sela and Katchalski, 1959; Swarc, 1965; Bamford and Block, 1972). However, in spite of their recent use in stepwise peptide synthesis in aqueous solution (Hirschmann et al., 1971; Pfaender et al., 1976; Kircher et al., 1980), their ability to covalently attach essential amino acids to food or feed proteins has not still been widely investigated (Pieniazek et al., 1975; Bjarnason-Baumann et al., 1977; Puigserver et al., 1982). Since protein amino groups may act as initiators of the polymerization reaction, it is therefore reasonable to expect the formation of poly-peptidyl proteins in a good yield, under mild experimental conditions. This will obviously be of considerable interest

with proteins containing limiting levels of lysine as, for instance, the cereals. It is also interesting to note that, in contrast to the active ester method, which requires protection of the amino acid prior to its covalent attachment to proteins, the N-carboxyanhydride method avoids treating the modified proteins to remove protecting groups, except for lysine, aspartic acid, and glutamic acid (Hirschmann et al., 1971; Bamford and Block, 1972; Puigserver et al., 1978). Another important feature of this method is certainly the retention of the L configuration of amino acids during synthesis of N-carboxy α-amino acid anhydrides as well as under addition conditions (Katchalski and Sela, 1958).

As shown in the present work, depending on experimental conditions, covalent attachment of methionine to food proteins gave rise to two distinct types of derivatives. At pH 6.5–7.0 and high protein concentration, polymerization of methionine on some of the protein amino groups occurred with an efficiency higher than 70%. Even with a high molar ratio of N-carboxyanhydride to amino groups, all the lysyl residues were not acylated, suggesting that some of them were chemically less reactive or accessible to the reagent than others. In the polymethionyl proteins prepared throughout this study, the average chain length of grafted polymers was estimated to be in the range of five to six residues. However, the polymethionyl chain covalently attached to the protein α-amino group should be somewhat longer than those linked to the ε-amino group of lysyl residues since a more rapid initiation of polymerization is expected with the former on account of its lower pK.

After single addition of a slight excess of N-carboxy-L-methionine anhydride over the acylatable amino groups at pH 10.2, about 75% of the lysyl residues of casein were modified while two more additions of the reagent resulted in a completely acylated protein. However, under stepwise peptide synthesis conditions, the efficiency of covalent attachment of methionine dropped to 30–40% and the average chain length of polymers never exceeded two to three residues. An increased formation of reaction by-products such as hydantoic acids (Hirschmann et al., 1967) may also occur at alkaline pH. It therefore appears that N-carboxy α-amino acid anhydrides are more suited for polymerization reactions than stepwise peptide synthesis. The fact that a variety of food proteins were modified to comparable extents and with about the same yields, in this work, supported the idea that polymerization of amino acids on proteins may be widely used and have interesting practical implications. In contrast, the efficiency of enzyme-catalyzed incorporation of amino acids into these proteins was found to give variable results since it is directly related to the ability of the polypeptide chain to be hydrolyzed by the enzyme (data not shown).

It is noteworthy that drastic changes in solubility of modified caseins was directly related to covalent attachment of long-chain polymers of methionine. Therefore, insolubility of highly modified polymethionylcasein was probably due to the presence of a relatively large number of additional hydrophobic side chains exposed to the aqueous solvent. Although casein is known to lack ordered tertiary structure (Slattery, 1976), the fact that conformational changes may lead to lowered solubility cannot be ruled out. Interactions between methionine polymers attached on distinct polypeptide chains were supposed to occur in polymethionyl β-lactoglobulins, leading to some aggregation and subsequent precipitation of the modified protein. External hydrophobic groups, present on β-lactoglobulins prior to covalent attachment of methionine

polymers (Nishikawa et al., 1968; Hofstee, 1975), will probably interact with polymethionine chains, preventing them from moving into the hydrophobic interior of the protein. The lack of any conformational change of β-lactoglobulins following covalent attachment of methionyl residues is certainly in favor of this hypothesis.

Another point of interest is related to the important decrease of initial rates of hydrolysis of modified caseins by chymotrypsin, indicating that conformational changes resulted in a lowered accessibility of some of the peptide bonds to the digestive enzyme through steric hindrance due to the presence of long polymethionyl chains might also be involved. It is nevertheless surprising that trypsin gave about the same results as chymotrypsin since chemical modification of the lysyl residues is certainly an additional reason to severely decrease tryptic digestion of the modified polypeptide chain. The lower "in vitro" digestibility of comparably acylated polymethionyl- and methionylcaseins has already been observed with alkylated proteins (Sen et al., 1981).

Polymerization of methionine on proteins should be useful for improving the nutritional value of food proteins as well as to protect lysyl residues against deteriorative reactions. Such reactions are known to occur during food processing and storage (Cheftel, 1977; Feeney, 1977). The amino acid or its related polymer will be covalently attached to the ϵ-amino groups of lysyl residues through an isopeptide bond that has already been shown to be readily hydrolyzed by intestinal aminopeptidase. It is therefore expected that polymers will be as available biologically as the released free amino acid although this still needs to be clearly established. Since the limiting amino acid covalently linked to the protein will be in large excess, as compared to usual nutritional requirements, it will then be necessary to dilute polymethionyl proteins with other unmodified proteins in order to get the most efficient protein food. Because of important changes in physical properties, especially solubility, such modified proteins may have potential applications for improving methionine utilization by ruminants. Chemical derivatization of amino acids and proteins has already been shown to lower ruminal degradation (Broderick, 1975). Rumen proteolysis studies "in vivo" and "in vitro", as well as bioavailability studies in the rat, are in progress. The functional properties of polymethionyl proteins and kinetic studies on enzymatic release of methionine in vitro will be reported shortly.

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