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Covalent Attachment of Amino Acids to Casein. 1. Chemical Modification and Rates of in Vitro Enzymatic Hydrolysis of Derivatives

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Active *N*-hydroxysuccinimide esters of various *tert*-butyloxycarbonyl-L-amino acids were used to covalently attach amino acids to casein through isopeptide bonds. Tryptophan was used to determine the best conditions for the reaction; glycine, alanine, methionine, *N*-acetylmethionine, aspartic acid, and asparagine were also covalently linked to casein. In vitro rate studies performed with bovine α-chymotrypsin, bovine pancreatin, and rat bile pancreatic juice indicated that hydrolysis of the modified casein derivatives were lower than that of unmodified protein. The rates of decreased hydrolyses did not result from changes in solubility properties but was rather due to steric hindrance as well as conformational changes of the modified protein as shown by fluorescence and absorption spectra. The facile covalent attachment of amino acids to proteins appears to be a promising method for improving the biological value of food proteins.

During the past 15 years, a great deal of scientific interest has focused on problems of world protein supply and, as a consequence, the number of studies related to food proteins has been constantly increasing. However, recently it has become more evident that a large number of food proteins should have their biological quality improved in order to better meet increasing world protein needs. Improvement of food proteins not only includes an increase in the nutritional value of the protein but also better functional properties adaptable to new uses.

There have been many investigations on functional properties of food proteins and a review of the extensive documentation available suggests that this field of interest is still highly promising (Ryan, 1977; Schoen, 1977). A variety of physical (Huang and Rha, 1974), chemical (Frazen and Kinsella, 1976; Miller and Groninger, 1976; Feeney, 1977a), microbial (Whitaker, 1978; Beuchat, 1978), and enzymatic methods (Richardson, 1977; Whitaker, 1977) have been used to change the functional properties of food proteins.

Nutritive value of a food protein depends to a large extent on its content of essential amino acids. As a consequence, proteins from plants, single cells and other less conventional sources have limited nutritional value because of their low content of one or more essential amino acids. Because of the economical, political, and nutritional importance of this problem, a number of important studies have been done on food proteins to improve their nutritional value. The potentials for improving protein quality in plants by genetic means are quite promising and a few investigators have already been successful in improving seed protein quality by genetic and breeding practices (Mertz et al., 1964; Munck, 1972; Johnson and Mattern, 1978). Fortifications of foods and feeds with essential amino acids (Benevenga and Cieslak, 1978), supplementation of cereal proteins by oilseed proteins (Sarwar et al., 1978), or enzymatic protein degradation and resynthesis for protein improvement (Yamashita et al., 1976; Fujimaki et al., 1977) have also attracted a lot of attention. However, although the usefulness of such studies for improving protein quality has been demonstrated, none of these techniques are entirely satisfactory.

The aim of this work has been to investigate the feasibility of covalently attaching amino acids to casein, chosen as a model of food protein, in order to study the effects of newly linked hydrophobic or hydrophilic groups

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on its physical, nutritional and digestibility properties. The rates of hydrolysis of the new derivatives by α-chymotrypsin, bovine pancreatin and rat bile pancreatic juice are also reported.

MATERIALS AND METHODS

Materials. Vitamin-free casein was obtained from Nutritional Biochemicals Corporation, Cleveland, OH; bovine α-chymotrypsin (twice crystallized) was from Worthington Biochemical Corporation, Freehold, NJ. Bovine serum albumin, bovine pancreatin, and *N*-acetyl-L-methionine were from Sigma Chemical Co., St. Louis, MO. Rat bile pancreatic juice was a gift of B. Schneeman of the Department of Nutrition, University of California, Davis. Dicyclohexylcarbodiimide and *N*-hydroxysuccinimide were from Aldrich Chemical Co., Milwaukee, WI, and ICN Pharmaceuticals, Inc., Irvine, CA, respectively. Bio-Gel P6, 100–200 mesh, was obtained from Bio-Rad Laboratories, Richmond, CA. *N*-Hydroxysuccinimide (OSu) esters of *tert*-butyloxycarbonyl (Boc) amino acids were from Bachem Fine Chemicals, Torrance, CA. The aspartic acid derivative was esterified at the α-carboxyl group only. All other reagents and chemicals were of analytical grade.

Methods. *N-Hydroxysuccinimide Ester of N-Acetyl-L-methionine.* The N-blocked amino acid was esterified according to an already described method (Anderson et al., 1964). Equimolar amounts of *N*-acetyl-L-methionine, *N*-hydroxysuccinimide, and dicyclohexylcarbodiimide in dimethylformamide were reacted for 16 h at 4 °C. The dicyclohexylurea formed was removed by filtration, the solvent was evaporated in vacuo, and the residue was crystallized from 2-propanol. The product, obtained in 75% yield, was chromatographically pure.

Reaction of Casein with Boc-Amino Acid-OSu Esters. In a typical experiment performed at room temperature, casein either dissolved in a 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes) buffer (pH 8.0) or in a 0.1 M sodium borate buffer (pH 9.0) was incubated with a 10% (v/v) aqueous dimethylformamide solution containing the *N*-hydroxysuccinimide ester of the *tert*-butyloxycarbonyl amino acid (Boc-amino acid-OSu). After 2 h of incubation, the modified casein was separated from reaction byproducts either by dialysis against deionized water or by gel filtration on a Bio-Gel P6 column (1.3 × 17 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) at room temperature. The column was quite effective for purification of 1.0 mL of reaction mixture containing about 5 mg of protein.

Hydroxylaminolysis of Ester Linkages. Following derivatization, any ester bonds formed with the protein via reaction of the *N*-hydroxysuccinimide ester of the *tert*-butyloxycarbonyl amino acid with the phenolic group of tyrosine or the hydroxyl groups of serine or threonine were hydrolyzed with 0.5 M hydroxylamine in 0.1 M phosphate buffer, at pH 8.0. This reaction was complete within 2 h at room temperature. Hydrolysis could also be achieved at pH 10.0 but was complete only after 250 min at 25 °C.

Deblocking of Boc-Amino Acyl-Casein. Removal of the Boc group was generally achieved by treatment of lyophilized casein derivatives for 30 min with anhydrous trifluoroacetic acid (50–300 mg of protein/mL of TFA). The protein was then slowly dispersed with stirring in 0.5 M Tris-HCl buffer (pH 8.0–9.0) with continuous adjustment of pH with 10 N NaOH. Precipitation always occurred when the protein was changed from TFA to an aqueous medium; on further stirring, it dissolved. Following dialysis against water, the protein solutions were lyophilized.

Viscosity. A Cannon-Ubbelohde (semi-micro size 50) viscometer was used to measure the viscosity of 2% casein solutions in 0.1 M Tris-HCl buffer, pH 8.0, at 25 °C.

Protein Solubility. Samples containing 100 mg of protein in 1.0 mL of 0.1 M citrate buffer, pH 5.5, were incubated for 1 h at 60 °C and then for 48 h at 4 °C. Then, each suspension was centrifuged at 27000*g* for 1 h to separate soluble and insoluble protein fractions. The amount of protein in the supernatant liquid was measured by the biuret method.

Determination of Amino Groups, Tryptophyl Residues and Protein. The amount of free amino groups was quantitated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to the procedure of Fields (1972). For tryptophyl residues the method of Edelhoch (1967) was used. Protein concentrations were generally evaluated according to the method of Lowry et al. (1951) or the method of Gornall et al. (1949) when derivatization might interfere with the Lowry determination. A molecular weight of 23 000 was used for casein in determining molar concentrations of solution of the protein. Corrections were made for the molecular weights of casein derivatives according to the amount of added amino acid.

Fluorescence and Ultraviolet Absorption Spectra. Solutions of control casein (treated as in the modification except no active ester added) or modified casein at the same molar concentration were made in 0.05 M Tris-HCl buffer, pH 8.0. 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 spectrofluorimeter, Model 55. The absorbance of the solutions at 280 nm never exceeded 0.03. Ultraviolet absorption measurements were made at 20 °C in the same buffer as above but with higher protein concentrations with a Beckman double-beam spectrophotometer, Model Acta CIII.

Rates of in Vitro Hydrolysis. The initial rates of hydrolysis of casein and casein derivatives were determined by measuring the liberation of amino groups by the 2,-4,6-trinitrobenzenesulfonic acid method (Fields, 1972). Reaction mixtures containing 0.1% casein or casein derivative in 0.1 M phosphate buffer, pH 7.0, for pancreatin and pancreatic juice assays or in 0.02 M borate buffer, pH 8.2, for α-chymotrypsin assays were incubated at 38 °C with 1:60 (w/w; enzyme to protein) pancreatin, 1:3000 (v/v) pancreatic juice or 1:3000 (w/w) α-chymotrypsin. Aliquots were removed at frequent intervals for amino group determination.

RESULTS

Covalent Attachment of Amino Acids to Casein. *N*-Hydroxysuccinimide esters of Boc-amino acids, described already for chemical modification of proteins in aqueous solution (Blumberg and Vallee, 1975; Slotboom and de Haas, 1975) were used for attaching amino acids to amino groups of casein. The tryptophan derivative was used to determine the best conditions for the reaction.

As shown in Figure 1, under conditions used for the covalent attachment of amino acids to casein, Boc-tryptophan-OSu added to other functional groups in addition to the amino groups, namely the hydroxyl groups of tyrosyl, seryl, and threonyl residues as shown by treatment with 0.5 M hydroxylamine at pH 8.0. There was a linear relationship between the extent of modification and protein concentration up to 10–12 mg of casein/mL of buffer. Then, the extent of reaction reached a plateau because of limitation on available active ester as a result of base-catalyzed hydrolysis of the reagent and incomplete

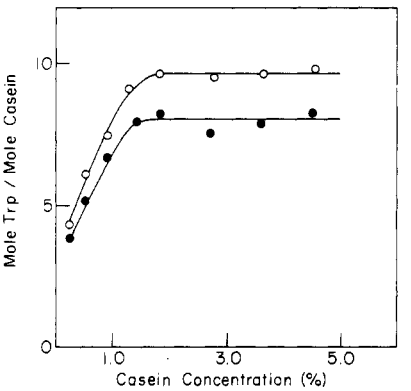

Figure 1. Effect of protein concentration on the extent of modification of casein with a molar ratio of Boc-Trp-OSu to protein amino groups at 25 °C (pH 8.0). Same conditions were used for the experiments shown in Figures 2 and 3. Effective removal of tryptophan linked to tyrosyl, seryl, and threonyl residues was achieved by a 2-h incubation of protein samples in 0.47 M hydroxylamine buffered at pH 8.0 and room temperature. Amount of tryptophan covalently bound was quantitated before (○) or after (●) hydroxylamine treatment.

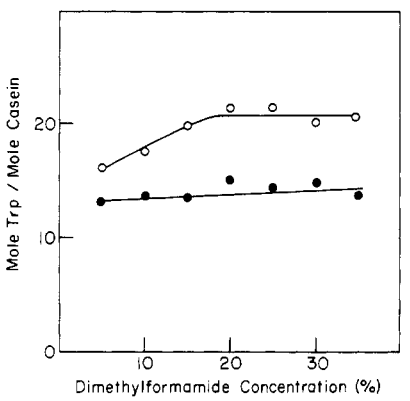

Figure 2. Influence of dimethylformamide concentration on the extent of reaction of Boc-Trp-OSu with casein. Protein concentration was 20 mg/mL; molar ratio reagent:amino groups was 1:1. Tryptophan was determined before (○) and after (●) incubation with hydroxylamine.

solubility of the active ester in the aqueous medium at higher protein concentration. Most of the amino groups of casein (80–85% of the total) were covalently modified. Increasing the reagent solubility with higher concentrations of dimethylformamide sharply increased ester formation but had a much smaller effect on amide formation (Figure 2). Under conditions where most of the reagent was soluble, ester bond formation was greater, suggesting that increasing the concentration of soluble reagent via increasing the concentration of dimethylformamide is not the method of choice for driving the modification of amino groups to completion. By contrast, the total amount (soluble and insoluble) of reagent used is very important. Complete modification of the ε-amino groups was achieved with about threefold molar excess of reagent over the amount of amino groups (Figure 3).

When reactions were performed in a pH 6.0–10.0 range, maximum modification occurred at pH 9.0 (Figure 4). The reaction was 15% more efficient at pH 9.0 than at pH 8.0. It should be pointed out that Boc-tryptophyl ester bonds formed with tyrosine were quite stable at pH 7.0 and 25 °C but were readily removed in the presence of 0.5 to 1.0 M hydroxylamine at pH 8.0 or at pH 10.0 (Puigserver et al., 1978). Nevertheless, the extent of formation and stability of ester bonds may be different with different amino acids (Blumberg and Vallee, 1975).

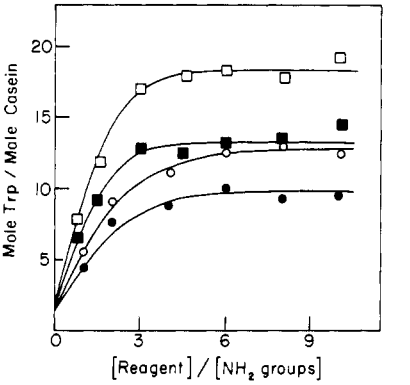

Figure 3. Modification of casein as influenced by Boc-Trp-OSu concentration. Protein concentrations were 5 mg/mL (○, ●) and 20 mg/mL (□, ■). (□, ○) and (■, ●) indicate determinations before and after hydroxylamine treatment.

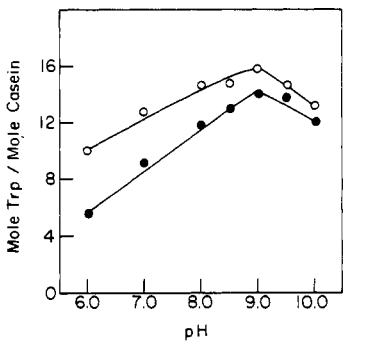

Figure 4. pH dependence of covalent attachment of tryptophan to casein. Same conditions as in Figure 3. ○ and ● indicate before and after hydroxylamine treatment. The following buffers were used: sodium citrate, pH 6.0; potassium phosphate, pH 7.0; Hepes, pH 8.0; sodium borate, pH 9.0; and sodium bicarbonate, pH 10.0. The experiment was done at room temperature.

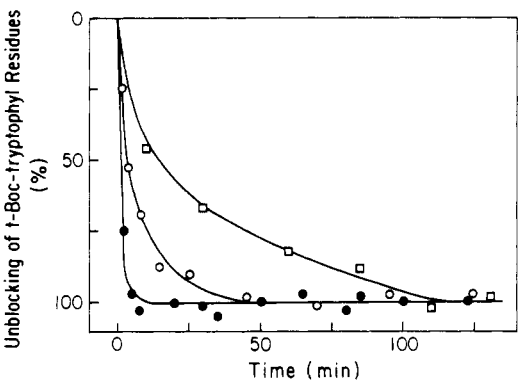

Figure 5. Time course of removal of amino protecting groups in trifluoroacetic acid. Boc-Trp-casein (250 mg/mL) was incubated in pure TFA at 0 °C (□), 25 °C (●), or in a mixture TFA/H_2O, 70:30 (v/v) at 25 °C (○). The amount of unblocking was estimated by amino group determination on aliquots of sample dissolved in a 0.1 M sodium borate (pH 9.0) after gel filtration on a Bio-Gel P6 column (1.3 × 17 cm).

Stability of Peptide and Isopeptide Bonds under Conditions Used for Deblocking of Boc Derivatives. Removal of Boc groups was readily achieved by treatment of proteins with anhydrous trifluoroacetic acid (TFA) under conditions where peptide and isopeptide bonds are expected to be stable. As shown in Figure 5, Boc group removal occurred very rapidly when a 60-fold molar excess of anhydrous TFA over Boc-tryptophyl groups of casein was used at room temperature (250 mg of protein/mL of TFA). The reaction still occurred at 0 °C or when an aqueous solution of the acid was used (70% TFA, v/v)

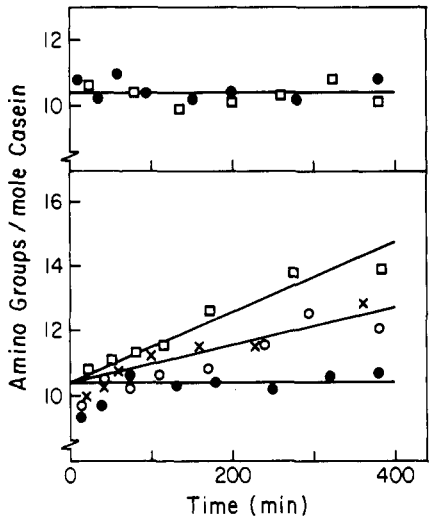

Figure 6. Peptide bond stability of unmodified casein in trifluoroacetic acid at 25 °C (upper part) and 50 °C (lower part). Twenty-five milligrams of protein was incubated in pure TFA (●) or in TFA/water mixtures: 10% water (×), 25% water (□), and 50% water (○).

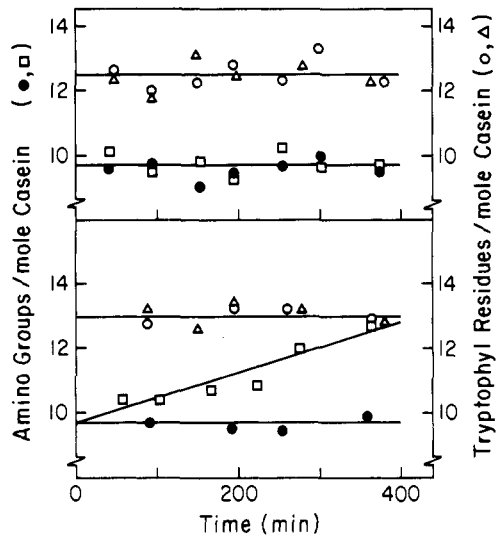

Figure 7. Isopeptide bond stability of Boc-Trp-casein in TFA at 25 °C (upper part) and 50 °C (lower part). Twenty-five milligrams of casein derivative incubated in pure TFA (●, ○) or in a mixture TFA/H_2O, 75:25 (v/v) (□, △). In both conditions, tryptophan was determined after dialysis of each sample against water.

instead of pure TFA. No additional amino groups could be detected on incubation of unmodified casein (25 mg of protein/mL) up to 2 h at 25 °C (Figure 6). The hydrolysis of casein peptide bonds started when the temperature reached 50 °C in the presence of TFA containing 10 and 25% water only. Maximum velocity occurred when TFA contained 25% of water. Under all these conditions, there is a 50-fold molar excess of TFA over peptide bonds and a 600-fold molar excess over isopeptide bonds, when a modified casein was used instead of the unmodified protein. Finally, it should be emphasized that in the complete absence of water, even at 50 °C, the stability of peptide bonds to TFA is very high whereas removal of Boc groups took place within a few minutes.

As shown in Figure 7, isopeptide bonds were more stable to TFA than peptide bonds. Under conditions where hydrolysis of peptide bonds occurred the number of tryptophyl residues linked to casein via an isopeptide bond remained unchanged.

Table I. Some Physical Properties of Modified Caseins

protein	% modification[a]	viscosity,[b] cSt (−) TFA	viscosity,[b] cSt (+) TFA[c]	solubility, % (−) TFA	solubility, % (+) TFA[c]
control casein	0	1.10	1.11	57	53
alanylcasein	88	1.11	1.12	53	53
tryptophylcasein	92	1.08	1.10	26	25
aspartylcasein[d]	83	1.09	1.18	50	45

[a] Determined by amino group estimation with TNBS and amino acid analysis following hydrolysis of proteins in 6 N HCl at 110 °C for 24 h. [b] Each value ($\eta = t_s \times k_{cSt \cdot s^{-1}}$ with $k = 0.004$) is the average of ten determinations. [c] Removal of Boc group by incubation at room temperature for 15 min of samples containing 50 mg of protein/mL of TFA. The samples were then treated as described in the text. Symbols (−) and (+) stand for before and after TFA treatment. Viscosity and solubility values are corrected for moisture content. [d] The isopeptide bond involves the α-carboxyl group of aspartic acid.

Table II. In Vitro Digestion Studies of Modified Caseins

protein	modification,[a] %	rel initial rate, %[b] (−) TFA	rel initial rate, %[b] (+) TFA
control casein	0	100	100
alanylcasein	44	49	43
	79	18	43
	88	10, 34[c]	43, 68[c]
tryptophylcasein	54	0	0
	87	0	0
	95	0	0
	97	0	0
aspartylcasein[e]	36	18	65
	65	2	39
	83	2, 15[c]	18, 57[c]
glycylcasein	91	50[d]	73[d]
methionylcasein	89	32[d]	46[d]

[a] Determined as in Table I. Performed with [b] bovine α-chymotrypsin, [c] bovine pancreatin, or [d] rat bile pancreatic juice. Reaction mixtures containing 0.1% protein in 0.1 M phosphate buffer, pH 7.0, for pancreatin and pancreatic juice assays or in 0.02 M borate buffer, pH 8.2, for α-chymotrypsin assays, were incubated at 38 °C with 1:60 (w/w; enzyme to protein) pancreatin, 1:3000 (v/v) pancreatic juice or 1:3000 (w/w) α-chymotrypsin. [e] The isopeptide bond involves the α-carboxyl group of aspartic acid.

Properties of Modified Caseins. Viscosity of 2% solutions of modified caseins was not changed when a large number of hydrophobic groups (for example, ten Boc-alanyl residues/mole of protein corresponding to an 8% weight increase in hydrophobic residues) was linked to the ε-amino groups of lysyl residues of the protein (Table I). This was true even with bulky groups (Boc-Trp) or negatively charged hydrophilic groups (Boc-Asp). When the amino group of the newly attached amino acid was freed of the Boc group, the aspartylcasein (the isopeptide linkage involves the α-carboxyl group of aspartic acid) derivative was the only modified protein with a significantly increased viscosity. This increase could be the result of conformational changes caused by electrostatic repulsions as shown below. Changes in solubility were not appreciable except for Boc-tryptophylcasein and tryptophylcasein; tryptophan is known for its poor solubility in aqueous medium.

In Vitro Enzyme Digestion Studies. The initial rates of hydrolysis of casein modified through covalent attachment of hydrophobic and hydrophilic amino acids are reported in Table II. Liberated amino groups, determined by TNBS analysis (Fields, 1972), were used as a measure

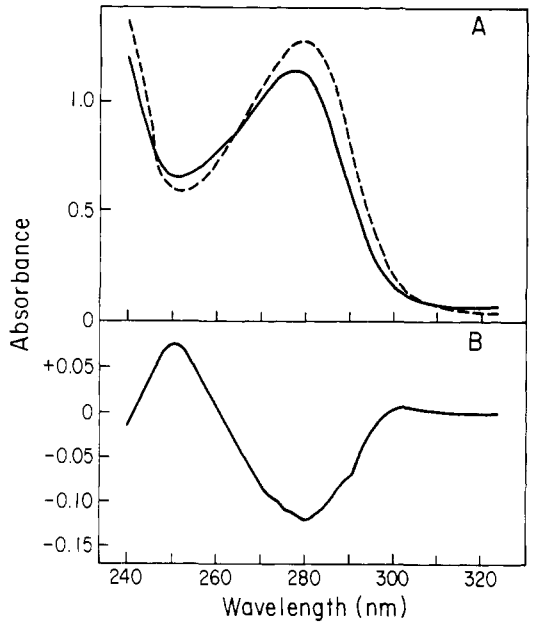

Figure 8. Ultraviolet absorption spectra (A) of unmodified (--) and aspartylcasein (——) at the same concentration (about 1.5 mg/mL). Isomolar solutions were prepared by measuring the absorbance at 280 nm of completely denatured protein (guanidinium hydrochloride and sodium dodecyl sulfate). For the difference spectrum (B) control casein was used as reference.

of the rates of enzymatic digestion. When the amino protecting group was still present, the decrease in initial rates of digestion by α-chymotrypsin may be correlated with the extent of modification.

Conformational Changes of Casein Derivatives. Casein has very little tertiary structure. However, a conformational change seems to occur when amino acids are covalently linked to ϵ-amino groups of casein. A difference absorption spectrum was clearly demonstrated for aspartylcasein (Figure 8) and *N*-acetylmethionylcasein (not shown). These two amino acids have the same effect on casein which is to neutralize the positive charge of ϵ-amino groups. With other amino acids such as alanine, methionine, and asparagine, a difference spectrum was obtained but its general shape was different. This last group of amino acids did not modify the electrical charge of casein. Thus, a difference was noted between those amino acids which modify the charge of casein and those which do not and their ability to produce a conformational change of the protein.

This observation was in fact confirmed by fluorescence measurements on the same derivatives (Figure 9). Amino acids which did not modify the charge of casein had no effect on tryptophan fluorescence. In contrast, those changing the charge gave rise to a different spectrum. The fluorescence spectra of Boc-tryptophylcasein and tryptophylcasein were different (Figure 10). Especially noteworthy is the 10-nm shift toward shorter wavelengths of the maximal emission of Boc-tryptophylcasein compared to that yielded by the unprotected form (345 nm), or by free tryptophan (350 nm). This shift is indicative of the presence of tryptophan residues in an apolar environment (Teale, 1960; Cowgill, 1967; Nieto et al., 1973) and may explain the increase of initial rates of digestion of casein by chymotrypsin upon removal of the amino protecting group.

DISCUSSION

Chemical methods for attaching amino acids to proteins are directly derived from procedures for synthesis of

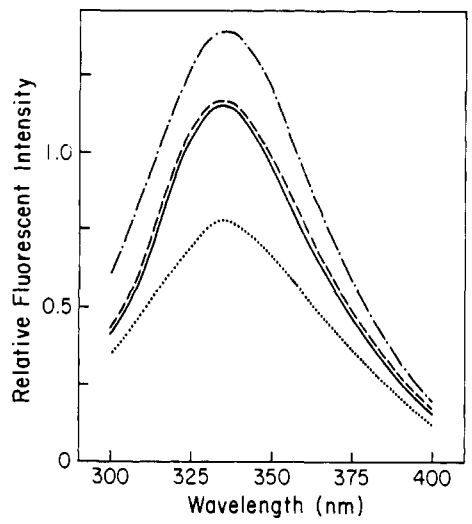

Figure 9. Fluorescence spectra of equimolar concentrations (8.9 $\times 10^{-7}$ M) of control casein (——), alanyl- or methionylcasein (- -), aspartylcasein (···), and *N*-acetylmethionylcasein (-·-).

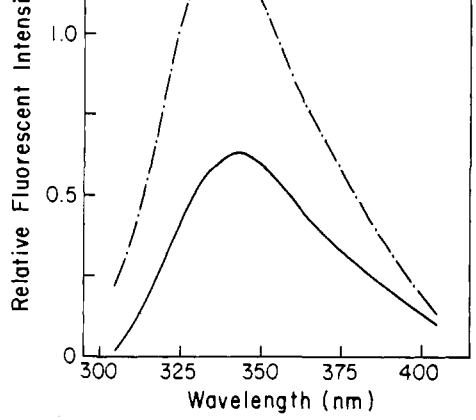

Figure 10. Fluorescence spectra of isomolar concentrations (8.9 $\times 10^{-7}$ M) of Boc-tryptophylcasein (-·-) and tryptophylcasein (——).

peptides in homogeneous solution or solid phase peptide synthesis (Merrifield, 1969; Fridkin and Patchornik, 1974; Katsoyannis and Schwartz, 1977; Doscher, 1977). Among these are two different methods in which the amino group responsible for the nucleophilic attack of an activated carboxyl group is the amino group of a protein. Active *N*-hydroxysuccinimide esters of N-protected amino acids and α-amino acid *N*-carboxyanhydrides (Leuchs' anhydrides) involved in these methods are generally prepared by esterification of the N-blocked amino acid (Anderson et al., 1964) or by direct treatment of the unprotected amino acid with phosgene (Hirschmann et al., 1971), respectively. Depending on whether the amino group is an α-amino group or an ϵ-amino group of lysine, the resulting amide linkage is either a peptide or an isopeptide bond, respectively.

As shown in the present work, a series of *N*-hydroxysuccinimide esters of *tert*-butyloxycarbonyl-L-amino acids have been quite effective in obtaining covalent addition of hydrophobic (Gly, Ala, Met, *N*-acetyl-Met) and hydrophilic (Asn, Asp) groups into casein via peptide and isopeptide bonds. Although a complete modification of casein amino groups (>99%) occurred with about a threefold molar excess of reagent over the amount of amino

groups at pH 8.0 (Figure 3), a degree of modification as high as 90–95% was generally achieved with a 1.3-fold molar excess of reagent at pH 9.0. Some modifying reagent is lost by competitive hydrolysis of the reagent by water or by hydroxyl ions. It was also interesting to note that, in contrast to Leuchs' anhydrides which often gave rise to multiple addition of the added amino acid to the same protein amino group (Hirschmann et al., 1967), the reagent used in the present study only yielded peptidyl protein and no polypeptidyl derivatives. Moreover, covalent attachment of amino acids through amide bond formation could be obtained exclusively since ester bonds, resulting from addition of the reagent to functional groups other than amino groups, were easily removed at alkaline pH or in the presence of hydroxylamine (Puigserver et al., 1978). The amount of dimethylformamide (15%) used to solubilize the reagent might be decreased or completely eliminated since increasing the concentration of soluble reagent did not increase the degree of modification of amino groups (Figure 2). *N*-Hydroxysuccinimide esters of Boc-amino acids have been widely used previously to study the effect of polypeptide chain elongation on biological activity of various proteins such as ε-guanidinated trypsin (Robinson et al., 1973), thermolysin (Blumberg and Vallee, 1975), pancreatic phospolipase A_2 (Slotboom and de Haas, 1975), soybean trypsin inhibitor (Kowalski and Laskowski, Jr., 1976), and subunit III of bovine procarboxypeptidase A (Puigserver, 1976).

Although catalytic hydrogenation is the most elegant way to remove carbobenzoxy groups, this amino protecting group could not be used for casein which contains sulfur amino acids. Thus, the Boc group, which is readily removed with anhydrous trifluoroacetic acid, was used throughout this work. Use of this urethane-type protecting group has the advantage that racemization is precluded because formation of an oxazolone intermediate is not possible (Katsoyannis and Ginos, 1969). If deblocking is not a required step, acetyl is one of the best protectors for amino groups as it occurs naturally in biological systems.

The higher stability of the isopeptide bond as compared to the peptide bond under conditions generally used for deblocking of Boc derivatives is noteworthy (Figure 7). In strong acidic conditions both peptide and isopeptide bonds were considerably more stable than Boc groups so no hydrolysis of the protein moiety occurred during the deblocking step. This was quite important with regard to in vitro digestion studies.

Covalent attachment of hydrophobic or hydrophilic amino acids to casein, whether these groups were bulky, uncharged or negatively charged, did not change appreciably the viscosity and solubility properties of casein. With proteins lacking ordered tertiary structure such as casein (Taborsky, 1974) only extensive conformational changes will be expected to induce modification of the physical properties studied. Although several globular proteins have accessible hydrophobic groups (Klotz, 1970; Hofstee, 1975), small conformational changes might be expected when hydrophobic groups are attached to the side chain of lysyl residues because they would tend to move into the hydrophobic interior of these proteins.

Another point of interest is related to the in vitro enzyme digestion of casein derivatives. There is a direct relationship between the increase in size of modifying amino acids (Boc-Gly ≤ Boc-Ala < Boc-Met < Boc-Asp) and the decrease in initial rates of hydrolysis as determined with pancreatic enzyme mixtures. However, the large decrease of the initial rate in the case of aspartylcasein might also be correlated to a conformational change of the protein (Figures 8 and 9). On the other hand, one cannot rule out the effect of the negatively charged side chains as indicated by the higher extent of digestion of alanylcasein by chymotrypsin compared to aspartylcasein. The different environment of the lysyl residues and consequently of other peptide bonds of the protein, as influenced by conformational changes caused by the removal of Boc groups (Figure 10), may also be involved in the increase of in vitro enzyme digestion. Reductive alkylation of protein amino groups (Means and Feeney, 1968; Means, 1977) has established that in vitro digestibility of various alkylated proteins are significantly lowered over that of the unmodified protein (Lin et al., 1969; Galembeck et al., 1977; Lee et al., 1978; Sen et al., unpublished data).

Although fortification of food with free amino acids (Altschul, 1974) is widely used in formulating animal diets, it has several disadvantages which include loss of free amino acid in food preparation, taste, and difference in absorption than when ingested in a protein (Crampton, 1972; Matthews, 1972). The covalent attachment of amino acids to proteins in aqueous solution should therefore be of interest to the food industry. The covalent attachment of limiting essential amino acids to proteins is an attractive approach for improving the nutritional value of food proteins. The amino acid is biologically available (Bjarnason-Baumann et al., 1977; Puigserver et al., 1978; Puigserver et al., 1979). It cannot be lost during processing. Moreover, the physical properties of proteins may be improved via the modification. The odor developed by addition of free methionine to foods, resulting from methional formation (Ballance, 1961), is essentially eliminated when the amino acid is covalently bound to casein. By use of amino acids with protected amino groups, deteriorative reactions involving lysyl residues of protein (Carpenter and Booth, 1973; Feeney et al., 1975; Feeney, 1977b; Cheftel, 1977) may be essentially eliminated during processing and storage. Moreover, it is becoming more and more evident that, although similar, the metabolism of ingested free amino acids is not quite the same as when they are ingested covalently attached in proteins. This difference is primarily at the absorption level (Crampton, 1972; Matthews, 1972). The rate of hydrolysis of isopeptide bonds appears to be largely dependent upon the nature of the bound amino acid as studied with ε-lysyl-isodipeptides as a model (Finot et al., 1978). The site-location of the enzymatic activity responsible for the in vivo hydrolysis of the methionyl ε-lysyl isopeptide bond will be reported shortly (Puigserver et al., 1979).

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Nonenzymic Formation of Dimethylamine in Dried Fishery Products

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Dimethylamine (DMA) forms very rapidly in heat-dried and freeze-dried fish muscle. The DMA forms regardless of species. The rate of DMA formation was related to the water activity (Aw) of the product with minimum amounts forming at Aw <1 and Aw 4. Evidence was developed to show that the formation of DMA in these products did not result from enzymic activity. In vitro studies show that several ionic constituents such as Fe^{2+}, Sn^{2+}, and SO_2 induce the degradation of trimethylamine oxide (TMAO) to DMA. Metal chelators such as EDTA and phytic acid in the presence of Fe^{2+} and Sn^{2+} rapidly accelerate the formation of DMA.

Until a few years ago, only passing attention was paid to the formation of dimethylamine (DMA) in fishery products. It was well known, for example, that when fresh fish spoils, it is trimethylamine (TMA) that forms and not DMA. The presence of DMA in fish flesh was deemed somewhat of a nuisance because when present in significant quantities, it interfered with the analysis of TMA (Tozawa et al., 1970). It was also fairly well recognized that DMA was rather species dependent, forming in significant quantities only in gadoid species such as hake, pollock, and cod, but not in other commercially important species. Later, food scientists began to show some concern because

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