Solubility and Emulsifying Properties of Caseins Chemically Modified by Covalent Attachment of L-Methionine and L-Valine

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L-Methionine and L-valine were covalently attached to the lysyl residues of casein by the N-carboxy anhydride method under polymerization and stepwise synthesis conditions. Influence of the extent of modification and length of the attached hydrophobic chains (two to six residues) on both solubility and emulsifying properties of modified caseins was then investigated. At acidic pH values, below the isoelectric point, polyvalyl derivatives were found to be more soluble and better emulsifiers than poly-methionylcaseins. By contrast, at alkaline pH, polyamino acid chain length and hydrophobicity became important controlling factors for solubility and emulsifying properties. However, the higher the number of attached residues the lower the solubility and the higher the emulsifying capacity. Attachment of long hydrophobic chains to about half of the lysyl residues of casein rather than shorter chains to almost all the ϵ-amino groups had a more pronounced effect on emulsifying properties. This suggests that the balance between hydrophile and lipophile plays an important part in the emulsifying ability of modified caseins.

A better knowledge of the physicochemical and functional properties of proteins containing a balanced composition in amino acids is certainly needed to enable a more accurate assessment of their potential use in formulated food systems. A number of molecular parameters such as mass, conformation, flexibility, net charge, and hydrophobicity of proteins as well as interactions with other food components have already been shown to play an important part in both their emulsifying and foaming properties (Kinsella, 1976; Keshavarz and Nakai, 1979; Nakai, 1983; Kato et al., 1985). During the past few years, several authors have tried to improve the functional properties of proteins by means of physical, chemical, and/or enzymatic procedures (Richardson, 1977; Cherry et al., 1979; Kinsella and Shetty, 1979; Phillips and Beuchat, 1981; Smith and Brekke, 1985). Beside the above-mentioned objective, useful information on the effect of protein modification on some if not all functional properties could therefore be expected. This is particularly true in the present investigation.

Among the wide range of reagents presently available for chemical modification of proteins, succinic anhydride (Groninger and Miller, 1975), citraconic anhydride (Brinegar and Kinsella, 1980), carbonyl compounds (Sen et al., 1981), and phosphoryl chloride (Woo et al., 1982; Matheis and Whitaker, 1984) have been thoroughly investigated with the aim of improving some if not all of the functional properties of proteins. Chemical methods involving the use of active esters of amino acids (Puigserver et al., 1978, 1979a,b) and N-carboxy α-amino acid anhydrides (Bjarnason-Baumann et al., 1977; Puigserver et al., 1982) have been successfully applied to food proteins in order to improve their nutritional value. The latter method has the additional advantage of polymerizing the amino acid derivative on proteins that may be expected to result in certain desirable specific properties (Gaertner and Puigserver, 1984, 1986).

The covalent attachment of hydrophobic groups to food proteins should have important and different effects on their conformation, solubility, and interfacial character-istics depending on both the nature and location of these groups on the polypeptide chain. The functional effects of covalently bound palmitoyl residues to α_{s1}-casein have recently been investigated (Haque and Kito, 1983) as well as the relationships between surface properties of food proteins and their hydrophobicity (Kato et al., 1983; Nakai, 1983; Townsend and Nakai, 1983; Shimizu et al., 1983; Voutsinas et al., 1983). Although functionality of proteins has generally been improved by solubility, contradictory results were reported with respect to emulsifying properties (Aoki et al., 1981; McWatters and Holmes, 1979; Wang and Kinsella, 1976).

The aim of this study has been to investigate the solubility and emulsifying properties of caseins containing polymethionyl and polyvalyl chains covalently attached to their lysyl residues through an isopeptide bond. Information on the influence of polyamino acid chain length on the functional properties has thus been obtained.

EXPERIMENTAL SECTION

Materials. Original alkali-soluble casein containing 80% protein (w/w) was obtained from Merck, FRG, and was freed from salts by extensive dialysis against water and finally freeze-dried before use. Both L-methionine and L-valine were from Sigma Chemical Co., St. Louis, MO, and the corresponding N-carboxy anhydrides were synthesized as already reported (Gaertner and Puigserver, 1984). Rapeseed oil (Sola) was from Astra-Calvé, Paris.

Methods. *Preparation of Poly-L-valyl- and Poly-L-methionylcaseins.* Poly-L-valylcaseins V1 and V2 as well as poly-L-methionylcasein M2 were prepared by incubating a 5% solution of protein in a 0.1 M sodium citrate buffer (pH 6.5) with various amounts of the N-carboxy anhydride dissolved in the minimum volume of tetrahydrofuran (Gaertner and Puigserver, 1984). The molar ratios of reagent to protein amino groups were 1.8, 3.6, and 3.0 for samples V1, V2, and M2, respectively. The resulting reaction mixture was vigorously stirred for 15 min at 4 °C while the pH was kept constant by manual addition of 0.1 N NaOH. By contrast, poly-L-methionylcasein M1 was prepared by four successive additions of reagent to the 5% casein solution in 0.1 M bicarbonate at pH 10.2 (1.2:1.0 molar ratio of N-carboxy anhydride to amino groups each time) in order to modify all the protein lysyl residues. The reaction mixture was acidified with HCl to pH 3–4 and kept for 10 min at 20 °C between each coupling step. Modified proteins were then extensively dialyzed against distilled water and finally freeze-dried.

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Table I. Amino Acid Composition of Control and Modified Caseins (g/100 g of Protein)

	casein				
residue	polyvalyl V_2	polyvalyl V_1	poly-methionyl M_2	poly-methionyl M_1	control C
Asx	5.8	6.2	5.0	5.4	6.5
Thr	3.4	3.7	3.0	3.3	3.8
Ser	3.3	3.6	2.9	3.2	3.7
Glx	18.3	20.6	18.0	20.2	20.8
Pro	10.5	8.2	6.4	9.3	11.5
Gly	1.6	1.6	1.3	1.4	1.6
Ala	2.4	2.6	1.9	2.3	2.7
Val	17.3[a]	12.1[a]	4.7	5.3	6.1
Met	2.8	3.0	24.0[b]	15.0[b]	3.1
Ile	4.3	4.2	3.3	3.9	4.3
Leu	6.9	8.3	7.1	7.1	8.6
Tyr	4.8	5.3	4.1	4.7	5.4
Phe	4.3	4.7	5.0	4.2	4.8
Lys	8.1	8.9	7.5	7.9	9.1
His	2.3	2.5	2.5	2.8	3.2
Arg	3.3	3.6	2.8	3.2	3.7
Trp	0.8	0.9	0.7	0.8	0.9

[a] Highest value after 24, 72, and 96 h of hydrolysis in 5.7 N HCl at 110 °C. [b] As methionine sulfone.

Control casein was incubated in the citrate buffer containing 5% tetrahydrofuran (v/v) without the active anhydride prior to dialysis and freeze-drying. The amino acid composition of control and modified caseins (Table I) was determined with a Beckman Model 120 C autoanalyzer equipped with an ICAP 10 computer after hydrolysis of the proteins in distilled 5.6 N HCl at 110 °C for 24 h.

The citrate buffers were used throughout. Methionine was determined as methionine sulfone after performic acid oxidation of the protein while tryptophan was estimated according to Edelhoch (1967). The number of unreacted lysyl residues was determined as homoarginine following selective reaction of a 0.5% protein solution with 0.5 M O-methylisourea during 4 days at 4 °C, pH 10.5 (Kimmel, 1967). The reaction was stopped by adding an equal volume of 1 M phosphate buffer (pH 5.0). Homoarginine and lysine were then quantitatively determined with the amino acid analyzer after dialysis and acid hydrolysis of the resulting protein, as indicated above. The number of lysyl residues acylated by methionine, valine, and the corresponding polymers was assumed to be equal to the number of lysyl residues that were not guanidinated. The average chain length of covalently attached polymethionine and polyvaline could therefore be determined from the number of lysyl residues unavailable for chemical modification with O-methylisourea and the amount of methionine or valine covalently attached to casein (Table II).

Protein Solubility. Solubility of control and modified caseins (0.125% solution) in the range pH 1–12 was determined in either water or 0.1 M NaCl adjusted to the desired pH value with concentrated HCl or NaOH in order to limit dilution of protein samples. After a 20-min equilibration period in order to reach 20 or 50 °C, and an additional 30-min incubation under each condition, protein

suspensions were centrifuged at 50000g and 15 °C for 30 min in a Sorvall RC 5B. Protein in the supernatant was estimated by the Lowry method using a Jobin Yvon-Hitachi 100–80 A spectrophotometer. Results were expressed as percentage solubility.

Emulsifying Capacity. The emulsifying capacity of casein samples was determined according to Webb et al. (1970). In a typical experiment performed at room temperature, 187.5 mg of protein dissolved in either water or 0.1 M NaCl (150 mL of a 0.125% solution) was put into a jar equipped with two electrodes connected to a volt–ohm meter (Multimeter CdA 770) in order to detect the sudden increase in electrical resistance of the dispersion that occurs upon emulsion collapse. Each protein solution, the pH of which has previously been adjusted to the desired value in the range 1–12 with concentrated HCl or NaOH, was blended for 10 s at 17000 rpm. Rapeseed oil was then added while blending continuously at the rate of 51 g/min up to the point of oil inversion in the emulsion. Although the device was not refrigerated, temperature never exceeded 25 °C. Emulsification capacity was expressed as grams of emulsified oil per sample of casein (187.5 mg) and as percentage volume by dividing the oil phase volume required to reach the emulsion breakpoint by the total emulsion volume and multiplying by 100 as suggested by Acton and Saffle (1972).

Emulsifying Activity and Emulsion Stability. Control or modified casein (30 mL of a 0.125% solution in water or 0.1 M NaCl at the desired pH) and rapeseed oil (10 mL) were shaken vigorously and then blended for 30 s at 20000 rpm and 20 °C (Kinematica GmbH Polytron equipped with a Reco 20 T speed and time control system). The emulsifying activity index (EAI) was determined by the turbidimetric method of Pearce and Kinsella (1978) with slight modifications. One-milliliter duplicate aliquots of each emulsion were accurately mixed with 0.1 M NaCl containing 0.1% sodium dodecyl sulfate at pH 7.0 to a final dilution of 1:250 (v/v). Absorbance at 500 nm was then recorded with the aid of the Jobin Yvon/Hitachi 100-80 A spectrophotometer.

Emulsifying activity determinations in the absence of protein were also carried out to see whether protein was necessary for emulsion formation. Under such conditions, emulsions were rather unstable and turbidity was found to vary from one experiment to another. In a few cases, however, water-in-oil emulsions were formed and as little as 0.1% protein provoked a marked increase in both emulsion stability and reproducibility. All the data are uncorrected for turbidity in the absence of protein.

Emulsion stability after 24 h at room temperature, with or without an additional 30-min heating period at 80 °C, was assessed by the same procedure after adequate dilution and expressed as EAI(80 °C) and EAI(20 °C), respectively. In the latter case, turbidimetry measurements were also done at room temperature. Consequently, the emulsion stability was obtained from the difference between the highest EAI value [EAI(max)], which was found to be constant when the emulsion was kept for 24 h at room

Table II. Extent of Modification of Caseins

sample	amino acid content (no. residues/mol protein)					modified lysyl residues,[a] %	av length of polyamino acids (residues)
	Lys	Val	Met	inc in Val	inc in Met		
polyvalylcasein V_2	15	43	5	29		61	2.9
polyvalylcasein V_1	15	27	5	13		52	1.5
polymethionylcasein M_2	15	14	49		44	46	5.6
polymethionylcasein M_1	15	14	27		22	94	1.5

[a] Determined as those residues not transformed into homoarginine by reaction of caseins with O-methylisourea. Calculation was based on an average of 15 lysyl residues/mol of control casein (molecular weight 22000).
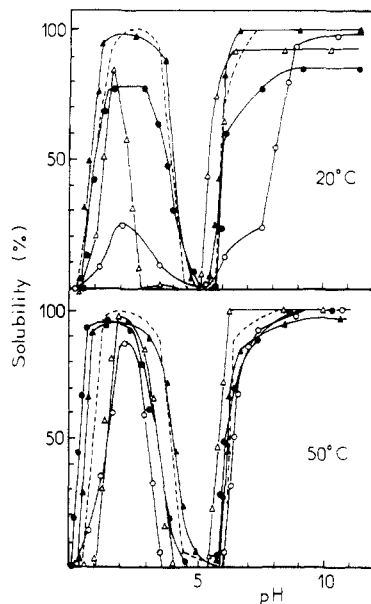
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Figure 1. Water solubility of control and modified caseins as a function of pH. Determinations were carried out after a 30-min incubation period and solubility was expressed as percent of total protein: control casein C (- - -); polyvalylcaseins V_1 (▲) and V_2 (●); polymethionylcaseins M_1 (△) and M_2 (○).

temperature, and EAI(80 °C) divided by EAI(max) and multiplied by 100.

All the data in the figures are means for four determinations, at least.

RESULTS

Solubility Properties. *Effect of Ionic Strength.* The water solubility curve of control casein at 20 °C as a function of pH was also obtained for polyvalylcaseins V1 and V2 (Figure 1). These modified caseins contained valine polymers of an average chain length of 1.5 and 2.9 residues, respectively, covalently attached to about half of their lysyl residues. By contrast, the almost completely modified casein derivative M1, characterized by the presence of polymethionine chains of an average of 1.5 residues per lysyl ε-amino group, as well as derivative M2 in which roughly 50% of the lysyl residues was acylated by longer polymers of about six residues, gave rise to different solubility curves.

Protein M1 was water insoluble in the range pH 3–5 and 90% soluble above pH 6 whereas protein M2 was only 15% and 25% soluble at pH 3 and 7, respectively. It should however be pointed out here that pH measurements in protein suspensions of low ionic strength are subject to some errors especially in the vicinity of the isoelectric point due to Donnan effects (Teppema and Brouwer, 1976). Changes in pH values (0.5–1.0 unit) were found to occur in the supernatant of centrifuged casein samples as compared to protein suspensions in the range pH 4.5–5.5.

Upon addition of 0.1 M NaCl, there was a slight decrease in solubility of both control and polyvalylcasein V1 below and above the isoelectric point (Figure 2). A marked decrease in the solubility of polyvalylcasein V2 was observed since the polyvaline chain length was increased from 1.5 to 2.9 residues. With respect to polymethionylcasein M1, the already observed insolubility in the range pH 3–5 was not extended down to pH 1. The same observation was made with casein derivative M2, containing longer polymethionine chains, in the range pH 1–8 and even at pH 10 where the solubility was hardly 20% that of control casein.

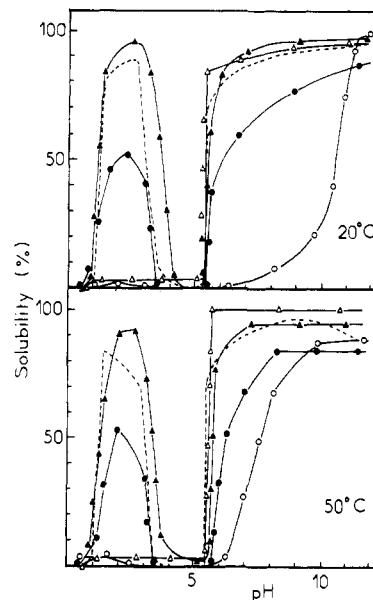*Effect of Thermal Processing.* There was no difference

Figure 2. Effect of ionic strength (0.1 M NaCl) on the solubility of control casein and casein derivatives. Symbols are those in Figure 1.
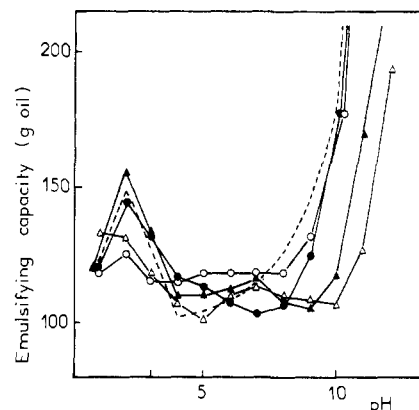

Figure 3. pH–emulsifying capacity curves of control and modified caseins. Results are expressed as grams of emulsified oil per 187.5 mg of protein in water. Symbols are as in Figure 1.

in water solubility between control and modified caseins after a 30-min heating period at 50 °C. A 15% and a 60% increase in the solubility of polyvalylcasein V2 and polymethionylcasein M2, respectively, was therefore ascertained. In the presence of 0.1 M NaCl, there was no significant effect of thermal processing below the isoelectric point, whereas above, the solubility of polymethionylcasein M2 was not as rapidly enhanced as in distilled water. Comparable conclusions were also reached when casein solutions were heated at 80 °C for 30 min (data not shown). Thus, somewhat unexpected results were obtained upon thermal processing of control and modified caseins (Figures 1 and 2) since the heat-induced unfolding of proteins usually results in the exposure of burried hydrophobic amino acids to the aqueous solvent with subsequent decrease in water solubility. A possible explanation to our finding could be that most of the added hydrophobic residues were internalized into the protein, making them unavailable and thus resulting in an increased water solubility of modified caseins at the higher temperatures.

Emulsifying Properties. *Emulsification Capacity.* The emulsifying capacity curves in water of modified caseins at different pHs were comparable to that of control casein in the range pH 1–8 (Figure 3). The high emulsifying capacity at high pH values as well as at the pH of

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Table III. Emulsifying Capacity (Oil Phase Volume Percent) of Caseins as a Function of pH according to Acton and Saffle (1972)

pH	control C		polyvalyl V_2		polyvalyl V_1		polymethionyl M_2		polymethionyl M_1:
	H_2O	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O
1	44	43	44	47	44	44	44	39	47
2	50	44	49	46	51	47	45	37	47
3	46	43	47	46	47	46	43	38	44
4	41	42	44	43	42	44	43	38	42
5	41	44	43	44	42	43	44	39	40
6	42	47	42	46	43	46	44	40	42
7	44	48	41	47	44	47	44	42	44
8	46	49	41	48	42	50	44	45	42
9	49	49	45	50	41	51	47	47	41
10	55	50	54	52	44	53	54	49	41
11	67[a]	57	75[a]	53	53	55	71[a]	51	46
12	71	57	68	57	65	56	63	54	56

[a] Results obtained by using 120 mL of a 0.125% protein solution instead of 150 mL. All data are mean values of duplicates. NaCl concentration was 0.1 M.
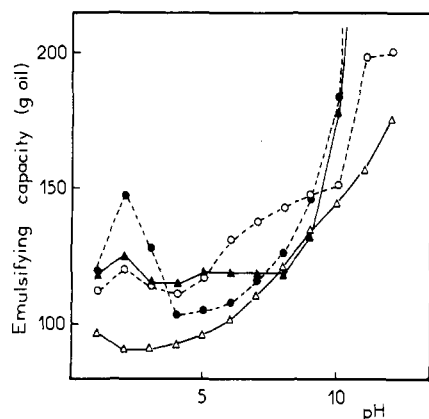

Figure 4. Emulsifying capacity of two casein samples as influenced by 0.1 M NaCl: control casein in water (●) or 0.1 M NaCl solution (O); polymethionylcasein M_2 in water (▲) or 0.1 M NaCl solution (△).

Figure 5. pH–turbidity curves as compared to pH–solubility curves of control and two modified caseins. Control casein C, polyvalylcasein V_2, and polymethionylcasein M_2 were in either water (▲) or 0.1 M NaCl solution (O). Solubility (%) in water with and without 0.1 M NaCl is indicated as a dashed line and a solid line, respectively.

minimum solubility might suggest that fatty acids or other surfactants in the oil are contributing to emulsion. Near the isoelectric point, addition of as much as 100–120 g of oil was necessary to reach the point of oil inversion in the emulsion, characterized by an infinite resistance. By contrast, at alkaline pH, polyvalylcasein V1 and polymethionylcasein M1 were significantly less effective (Table III). Compared to that of control casein and of the two other modified proteins V2 and M2 (>450 g of oil/187.5 mg of protein at pH 11), the emulsification capacities of caseins V1 and M1, under the same experimental conditions, were 170 and 128 g, respectively. By using 120 mL of a 0.125% protein solution instead of 150 mL, values of 244, 363, and 300 g of oil were obtained for control casein and modified caseins V2 and M2, respectively. Such a high value for unmodified casein has already been reported (Pearson et al., 1965).

Upon addition of 0.1 M NaCl to the medium, the emulsifying capacity of control casein was found to be slightly decreased at pH 2, as compared to that in water, and considerably decreased at pH 11–12. By contrast, significantly higher capacity values were obtained in the range pH 5–8 corresponding to the minimum emulsifying capacity in water (Figure 4). The shape of the curves was unchanged for both polyvalylcaseins V1 and V2 (not shown) but was somewhat different for polymethionylcasein M2 since the emulsifying capacity was lower except at pH 8–9 where it was comparable to that in water. The NaCl-induced increase in the emulsifying capacity in some pH ranges has already been observed with meat proteins (Carpenter and Saffle, 1965), leaf protein concentrates
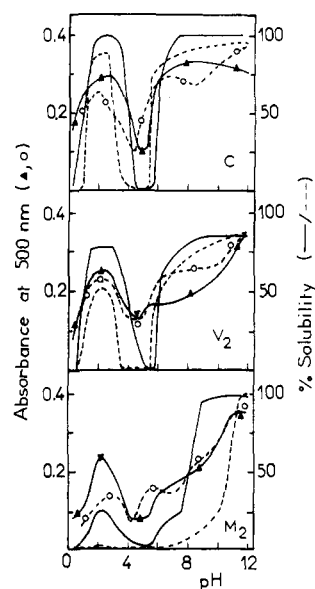
(Wang and Kinsella, 1976), and bovine serum albumin (Waniska et al., 1981).

Although the water solubility of polymethionylcasein M2 at pH 2.0–3.0 was improved from 25% to 100% upon heating, the emulsifying capacity was not. There was also no change in the presence of 0.1 M NaCl. Since protein hydrophobicity increases upon heating, this observation might suggest that conformational changes of the polypeptide chain occurred, thus preventing some of the covalently linked polymethionine chains to interact with lipids.

Emulsifying Activity. Figure 5 shows the pH–emulsifying activity curves in water and 0.1 M NaCl solution of control casein, as well as polyvalylcasein V2 and polymethionylcasein M2 as examples of highly modified proteins. All the curves, including those for polyvalylcasein V1 and polymethionylcasein M1 (not shown), were found to follow the corresponding pH–solubility curves. Comparable results were also obtained when emulsions were kept at room temperature for 24 h and even after an additional 30-min heating period at 80 °C. As already stressed for solubility, polyvalylcaseins were found to be better emulsifiers than polymethionylcaseins at pH values

Table IV. Emulsion Stability of Control and Modified Caseins after 24 h at 20 °C and 30 min at 80 °C[a]

	control C		polyvalyl V_2		polyvalyl V_1		polymethionyl M_2		polymethionyl M_1	
pH	H_2O	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O	NaCl	H_2O	NaCl
2	7	8	12	13	11	4	9	nd	12	nd
3	10	12	17	13	14	7	17	21	16	nd
6	17	13	35	17	52	17	nd	nd	9	4
9	3	10	27	7	6	2	14	13	8	2
10	0	6	17	7	4	1	2	2	8	5

[a] Results are expressed as percent of the difference between EAI(max) and that after thermal processing (see the Experimental Section). nd = not determined because of emulsion collapse.
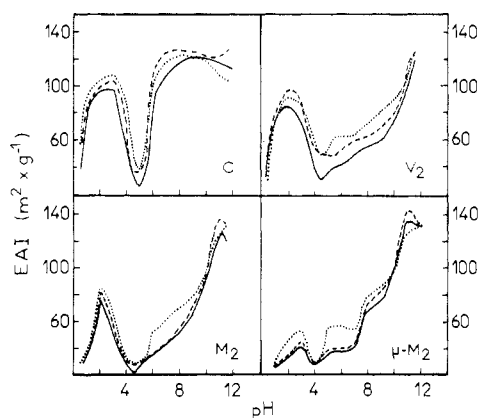

Figure 6. Effect of thermal processing on the emulsifying stability of caseins. Experiments were carried out in water for control casein C, polyvalylcasein V_2, and polymethionylcasein M_2 and also in the presence of 0.1 M NaCl in the latter case (μ-M_2). The emulsifying activity index (EAI) was determined immediately after formation of the emulsion (···) or after 24 h at 20 °C (- - -) or with an additional heating at 80 °C for 30 min (—).

below the isoelectric point. By contrast, the chain length of covalently attached polymers became predominant above the isoelectric point. Moreover, when poly-methionylcasein M2 was submitted to a thermal processing before emulsion was formed, a slight increase in the emulsifying activity was observed.

Emulsifying Stability. The emulsifying activity index, which is related to the interfacial area of the emulsion, was determined either right after the emulsion was performed or following an additional 24-h period at 20 °C without or with subsequent heating at 80 °C for 30 min. Figure 6 shows that the maximum stability of the emulsion in the case of control csein, polyvalylcasein V2, and poly-methionylcasein M2 was observed at highly acidic and alkaline pH values (1–2 and 9–11, respectively). Comparable curves were also obtained with the two other modified caseins.

Upon addition of NaCl to the medium, the emulsifying stability of polyvalylcaseins was almost unchanged whereas that of polymethionylcaseins considerably decreased at acidic pH values. Table IV summarizes the emulsion stability data in water and NaCl solution at a few selected pH values. When a stable emulsion was obtained, the emulsifying stability was higher in distilled water for pH values below the isoelectric point and in 0.1 M NaCl solution above the isoelectric point.

As for emulsifying activity, the emulsifying stability of polyvalylcaseins was found to be higher than that of po-lymethionylcaseins for pH values below the isoelectric point, whereas above, the shorter the length of the cova-lently attached polymers, the better the stability. These results are in complete agreement with those regarding protein solubility. After heating and subsequent centri-fugation, emulsions were stable in water provided the pH was below the isoelectric point. Near the isoelectric point, emulsions collapsed independently of the protein involved

whereas above this pH emulsions were quite stable. Comparable results were also observed upon addition of NaCl to the medium.

DISCUSSION

In the present study, we were able to show that covalent attachment of valine and methionine to the lysyl residues of casein resulted in different effects on the water solubility of modified caseins. For pH values below the isoelectric point, polyvalylcaseins were found to be more soluble than polymethionylcaseins although in both cases the higher the number of attached residues, the lower the water solubility of modified caseins. Above the isoelectric point, solubility of casein derivatives was increased as M1 > V1 > V2 > M2, suggesting that the average length of the covalently attached polymers became a determinant factor inde-pendently of the amino acid. Moreover, the possible ex-istence of intramolecular hydrophobic interactions between polymethionine or polyvaline chains covalently attached to casein may induce conformational changes resulting in the stabilization of more soluble protein structures over a wide pH range. When the ionic strength of the medium was increased, a much more important negative effect on the solubility of methionylcaseins was observed at pH values below the isoelectric point. Neutralization of pos-itive and/or negative charges in polymethionylcaseins by chloride and/or sodium ions certainly favored hydrophobic interactions between polyamino acid chains, thus leading to protein aggregation with subsequent decreased solu-bility. Hydrophobic interaction has also been found to occur when β-casein was reacted with acyl anhydrides. The association of the acyl derivatives of β-casein increased as the length of the n-alkyl chain substituent increased probably due to enhanced hydrophobic interactions (Evans et al., 1971).

The shape of the pH–emulsifying capacity curves of casein derivatives was not quite comparable to that of solubility. However, the more water-soluble polyvalyl-caseins V1 and V2 at acidic pH, as compared to poly-methionylcasein M2, were also better emulsifiers. Such a positive correlation between solubility and emulsifying capacity of proteins has already been reported (Crenwelge et al., 1974; Volkert and Klein, 1979). It is nevertheless worth stressing that, in a number of other examples, a poor correlation was observed (Wang and Kinsella, 1976; McWatters and Cherry, 1977; McWatters and Holmes, 1979; Aoki et al., 1980). This was also the case for poly-valylcasein V1 in water at alkaline pH.

If only soluble proteins were able to spread around surface of oil droplets, one would expect an increase in the emulsifying ability of all modified proteins as they become more soluble. Since it was not possible to find such a correlation, this therefore implies the involvement of other factors such as protein hydrophobicity (Kato et al., 1981; Nakai, 1983; Voutsinas et al., 1983). Although a good correlation has often been observed between the emulsi-fying capacity and hydrophobicity of proteins, it should be pointed out that the emulsifiying ability of proteins

could also depend on the distribution of hydrophobic and hydrophilic residues in the polypeptide chain. Hydrophobicity should undoubtedly be the main factor responsible for the very high emulsifying capacity of control casein and related derivatives V2 and M2 at alkaline pH. These two modified proteins contained polyvaline and polymethionine chains of an average length of 2.9 and 5.6 residues covalently attached to about 50% of the lysyl residues, respectively.

Valine is more hydrophobic than methionine (Rekker, 1977; Kyte and Doolittle, 1982), and consequently, the casein derivative V2 was expected to have a higher emulsifying capacity in water than M2. That was not the case in the range pH 1–10, probably because of intramolecular as well as intermolecular interactions pushing some of the covalently attached polyvaline chains into the interior of the molecule with subsequent protein folding and masking of few hydrophobic polymers. By contrast, as a result of protein unfolding at pH 11, the attached polyvalyl chains became an important factor controlling the formation of oil-in-water emulsions. Emulsifying capacity values of 363, 300, and 244 g/187.5 mg of protein in water solution at pH 11 were obtained for polyvalylcasein V2, polymethionylcasein M2, and control casein, respectively. Polyvalylcasein V1 and polymethionylcasein M1, which contained rather short hydrophobic amino acid chains linked to some or to all of the lysyl residues, were found to have a lower emulsifying capacity in water at alkaline pH than control casein. Since maximum water solubility of both proteins was reached at alkaline pH, such an unexpectedly low emulsifying capacity might result either from the presence of hydrophobic residues still involved in protein–protein interactions, and consequently unavailable to interact with oil, or from an unfavorable balance between the hydrophile and lipophile in modified caseins as compared to control casein. Thus, hydrophobicity became the major controlling factor for emulsification with highly modified proteins.

However, determination of the hydrophobicity of valine and methionine from their contribution to the retention time of a number of peptides in high-performance liquid chromatography has been found to differ from that derived from octanol–water partition as reported by Rekker (1977). Methionine has a positive contribution to retention twice as high as valine (Meek, 1980; Sasagawa et al., 1982). These results suggested a higher probability for polymethionine rather than polyvaline to move into the hydrophobic interior of the modified proteins as already observed (Gaertner and Puigserver, 1984), thus leading to possible contradictory conclusions. The emulsifying activity of caseins chemically modified by covalent attachment of polyamino acids could not exclusively be explained in terms of solubility and hydrophobicity. The distribution of hydrophile and lipophile into and around the polypeptide chain is also an important controlling factor. For instance, the presence of short hydrophobic chains attached to casein has been shown not to induce conformational changes (Gaertner and Puigserver, 1984) but may change the balance between hydrophilic and hydrophobic regions at the surface of the protein and lead to significantly decreased emulsifying properties.

It is now well accepted that hydrophobicity and hydrophobic interactions are important controlling factors for the functional properties of food proteins (Kinsella, 1979; Kato et al., 1981). Significant correlations were found to exist between surface hydrophobicity and both interfacial tension and emulsifying activity of proteins (Keshavarz and Nakai, 1979; Kato and Nakai, 1980). It is nevertheless worth stressing that a higher correlation was found between the emulsifying property and hydrophobicity rather than solubility. With respect to caseins, Sen et al. (1981) have shown that highly alkylated casein derivatives have a better emulsifying activity than the unmodified protein whereas Aoki et al. (1981) and Voutsinas et al. (1983) showed that increasing the protein hydrophobicity may have in many instances adverse effects on emulsifying properties. The covalent attachment of palmitoyl chains to α_{s1}-casein has been reported to improve both the formation and stabilization of emulsions (Haque and Kito, 1983). By using meat proteins, Li-Chan et al. (1984) have found that surface hydrophobicity was most important for predicting the emulsifying property of proteins with high solubility whereas solubility parameters became determinant with weakly soluble proteins.

The covalent attachment of hydrophobic amino acids to casein decreased water solubility and slightly improved emulsifying properties. It is therefore evident that besides hydrophobicity and the balance of hydrophile and lipophile, a number of other factors including molecular mass and flexibility of proteins may also be involved in the emulsifying properties of proteins. Since solubility is also an important controlling factor for diffusion and concentration of proteins at the oil–water interface, the important decrease in protein solubility observed after covalent attachment of a large number of hydrophobic residues could not therefore lead to really improved emulsifying properties. Although quite interesting from the nutritional point of view, the covalent attachment of methionine to food proteins should not exceed a certain level in order to improve both solubility and emulsifying properties.

Since the major stated objective of this study was to gain understanding of the effects of hydrophobic polyamino acids covalently attached to the lysyl residues of casein on both protein solubility and emulsifying properties, extremes of pH were also investigated as were low ionic strengths. It is nevertheless beyond doubt that trying to get a better insight into modified protein functionality does not necessarily mean direct applications to formulated food systems.

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Interaction between κ-Casein and β-Lactoglobulin: Possible Mechanism

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κ-Casein (κ-C) and β-lactoglobulin (β-Lg) interact to form a κ-C/β-Lg complex (A₄) apparently composed of three molecules of β-Lg and one molecule of κ-C. In a freshly dispersed equimolar mixture of native κ-C and β-Lg maintained at 25 °C in 20 mM imidazole at pH 6.8, approximately 14% occurred as a κ-C/β-Lg A₄ complex which increased to about 40% when 20 mM ethylene glycol bis(2-aminoethoxy)-N,N,N',N'-tetraacetic acid (EGTA) was added. The EGTA apparently caused a structural change that resulted in a β-Lg complex (A₃) that was highly reactive with monomeric κ-C. This reactivity was quenched in the presence of 2.3 M urea. With time, this A₄ complex became stabilized by covalent bonds. Heating at 70 °C increased the rate of formation of the covalently bonded κ-C/β-Lg complex. A possible mechanism for the interaction of κ-C with β-Lg involves the formation of a β-Lg homotrimer (A₃) complex via hydrophobic interactions that interacts with a κ-C molecule.

Nonfat dry milk (NDM) is a significant source of functional protein for the food industry (Kinsella, 1985). The cheese industry represents a large potential market for NDM, if the NDM could be used for the manufacture of rennet curds and cheeses. However, the heat treatment used in the manufacture of NDM reduces the susceptibility of the κ-casein to the normal action of chymosin because of complex formation between κ-C/β-Lg (Sawyer, 1968; McKenzie, 1971). This is because in the routine manufacture of NDM milk rceives a prior heat treatment to cause interaction of κ-C with β-Lg and eliminate the

loaf-depressing effect in NDM intended for use in the baking industry (Guy, 1970). Unfortunately, the same heating conditions may be used for the preparation of NDM for other than baking applications, thereby limiting its potential use in rennetted cheese/curd type products.

Though many of the heat-induced interactions occurring in milk have been elucidated, not all the interactions occurring within and between protein components are understood. Heat treatment may cause denaturation particularly of whey proteins, resulting in adsorption to caseins and to possible interaction between β-lactoglobulin (β-Lg) and κ-casein (κ-C) via hydrophobic interactions (Doi et al., 1983), disulfide/sulfhydryl interchange reaction, and/or via thiol oxidation (Purkayastha et al., 1967), resulting in

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