Effects of Various Heat Treatments on Structure and Solubility of Whey Proteins

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ABSTRACT

Structure and solubility of whey proteins are interrelated and affected by commonly used heat treatments. The relation between these characteristics, however, varies with the nature of the protein and the composition of the protein solution.

After a brief analysis of structure and properties of the major whey proteins in the native state, attention is given to effects of thermal treatments (up to 150°C) on structure and solubility of the different whey proteins. It is pointed out that mild heat treatments up to 60°C may affect reversibly the solubility and foaming properties of whey proteins. Conformational changes, as reflected by differential scanning calorimetry and observed above 60°C for α-lactalbumin and near 80 and 140°C for β-lactoglobulin, however, exert more serious effects on the functional properties of whey proteins. Modifications of cystine-residues in the polypeptide chain are detected by amino acid analysis upon heat treatments above 100°C under identical heating conditions as used for differential scanning calorimetry.

Special attention is given to the effect of changes of environmental conditions such as pH and the presence of lactose and calcium salts, to get more information on the complicated relation between structure and properties of proteins in whey.

INTRODUCTION

Heat treatments are important steps in both production of whey protein concentrates and processing of food products. The commonly used heat treatments such as preheating, pasteurization, and sterilization always affect the structure and properties of whey proteins, either reversibly or irreversibly.

Reversible changes of protein structure mostly occur in the temperature range up to 60°C and may affect the association or dissociation behavior of some whey proteins (13, 26). These changes often are ascribed to predenaturational transitions caused by a partial loss of the three-demensional protein structure (31) and changes of protein hydration (24).

Irreversible changes of protein structure may occur above the denaturation temperature of a protein and are influenced further by environmental conditions such as pH, ionic strength, and protein concentration (19). These irreversible denaturation effects mostly result in reduced protein solubility. Solubility, however, is an important characteristic for functional application of proteins in food systems, not only per se but also as a prerequisite for derived functional properties like emulsification, foaming, and gelation.

To arrive at understanding of the relationship between structure, solubility, and functionality of whey proteins, we attempted to analyze some reversible and some irreversible effects of heat treatments on whey proteins in simple aqueous solutions in three temperature regions, 4 to 60°C, 60 to 100°C, and above 100°C.

DISCUSSION

Characteristics of Whey Proteins

The major nitrogen compounds remaining in acid whey after coagulation of caseins consist of whey proteins: α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA), and the immunoglobulins (Ig) and the polypeptides proteose peptones (PP). Whey proteins are compact globular proteins that differ both in structure and in properties from each other as a result of differences in amino acid composition and sequence. Polypeptides, mainly proteolytic degradation products of

Received August 22, 1983.

casein(s), are mostly phosphorylated and do not occur in a compact globular structure.

In Table 1 some characteristics of major whey proteins and polypeptides are summarized. Protein concentration and number per molecule of reactive amino acids such as half-cystine (cys/2) and lysine-residues, as in Table 1, are important characteristics during heat treatments. These amino acids are mainly responsible for abilities of these proteins to interact during heating.

β-Lactoglobulin is the most abundant whey protein (Table 1). At room temperature and between pH 5 and 7 it occurs as a dimer consisting of two identical subunits, each with a molecular weight of 18,400. Above 40°C, the dimers start to dissociate into monomers, and this, in fact, is the most important species of β-lg during heat denaturation. The β-lg contains two disulphide bridges and one free thiol group per monomer. The thiol group is capable of interacting to form new disulphide bonds, and the rate of reaction increases sharply above pH 6.8 because of a conformational change of the molecule (11). In particular during heat treatments, this results in intramolecular or intermolecular disulfide exchange reactions with β-lg itself or with other thiol-containing proteins, which may affect solubility and functionality of these proteins.

α-Lactalbumin is the smallest and most heat-resistant whey protein and represents the quantitatively second most important protein of the whey. The cys/2 residues occur as four disulphide bridges per molecule and are mainly responsible for a reversible conformational change upon heat denaturation (25). The lysine residues are assumed to be exposed at the surface, as is typical of globular proteins (41). Recently α-la was described as an important Ca-binding protein (15). Certain carboxyl groups of the molecule are involved in binding of calcium ions, and loss of tightly bound calcium appears to be responsible for the conformational change of α-la around pH 4.0. This acid transition occurs as a reversible denaturation followed by a slow aggregation process (22).

Serum albumin represents the longest single polypeptide chain of all the whey proteins. It contains 17 cystine residues and 1 free thiol group per molecule, and consequently, all disulfide bridges in BSA are intrachain bonds. The BSA shows a heterogeneous electrophoretic behavior that might be ascribed to adsorption of fatty acids because BSA is a well-known transport protein for insoluble fatty acids in the blood circulatory system (35). Moreover, a recent calorimetric study revealed that fatty acids stabilize BSA against heat denaturation (14). On acidification to pH 4.0, the BSA molecule undergoes acid denaturation, which is ascribed to the mutual repulsion of positively charged amino acid residues (16).

TABLE 1. Some characteristics of the major whey proteins and polypeptides in whey and of β-casein.

Protein or polypeptide	Weight contribution	Molecular weight	Number contribution	Number of cys/2-res[1] per molecule	Number of lys-res per molecule
	(g/liter)	(X 10^8)/liter			
α-Lactalbumin	1.2	14,200	50	8	12
β-Lactoglobulin	3.3	18,400	100	5	15
Bovine serum albumin	.3	66,000	2.6	35	59
Immuno-globulin G	.5	160,000	1.9	64	180
β-Casein	...	24,000		0	11
Proteose peptone 8f		4,100		0	1
Proteose peptone 8s	.2	9,900	10[2]	0	6
Proteose peptone 5		12,300		0	7
Proteose peptone 3		22,000		0	15

[1] Cys/2 = half-cystine (cysteine plus reduced cystine); res = residues; lys = lysine.

[2] Based on equal weight distribution of all proteose peptones.

The Ig in milk and whey include IgG, IgA, and IgM, with IgG consisting up to 80% of the immunoglobulins. The IgG consists of four polypeptide chains, namely two light and two heavy chains joined by disulfide bridges. The immunoglobulins are very thermolabile whey proteins and have interesting, but thus far not well-understood, technological properties such as cold agglutination of fat globules in milk (27) and binding of fatty solutes and bacteria in desalted acidified whey (8).

Although β-casein is not a whey protein, it is included because it may be in sufficient quantities to affect functional properties of whey proteins. In particular, in whey obtained from cold milk by ultracentrifugal removal of casein micelles, outside the isoelectric point, β-casein is in significant amounts. Recently it was reported that in milk stored at 4°C, up to 40% of β-casein will dissociate from the casein micelles into the whey (34). Unlike whey proteins, β-casein contains a high proportion of proline residues, which may contribute to the randomness of the β-casein polypeptide chain. β-Casein is not susceptible to heat denaturation but is much more sensitive to enzymatic breakdown than are whey proteins.

The majority of the PP fractions in the whey have been identified as N-terminal segments of β-casein, produced by the proteolytic activity of indigenous plasmin (18). The plasmin-generated PP fractions, characterized as PP-5, PP-8f, and PP-8s are phospho-peptides that are thermostable and soluble at pH 4.6. The fractions PP-5 and PP-8s are typical soap-like molecules with a polar head and a stretched apolar tail. They are largely responsible for the good foaming properties of the proteose peptone fractions in whey (17). The PP-3 fraction, mentioned in Table 1, has been identified as a component of the milk fat globule membrane (20) and appears to be a glycoprotein.

Temperature-Induced Changes of Structure and Solubility of Whey Proteins in the Temperature Range Between 4 and 60°C

Mild temperature effects on structure and solubility of whey proteins are reversible and governed mainly by hydrophobic bonding (38). Hydrophobic bonding is enhanced when temperatures increase up to 60°C and is weakened as temperatures decrease. This implies that proteins with a relatively high amount of hydrophobic residues, such as β-la and β-casein, are sensitive to temperature-induced association or dissociation reactions (29). This interaction may be either intramolecular or intermolecular, depending on the folding ability of the polypeptide chain, and has consequences for protein solubility as a main functional property.

To illustrate just how sensitive protein solubility can be to the effects of temperature change and protein structure, we shall compare the solubility behavior of β-lg with that of isoelectric β-casein. Both proteins are soluble at 4°C, β-casein in an almost random structure and β-lg in a (partly) unfolded configuration as a result of cold denaturation (28). Heating of both protein solutions at pH 4.6 from 4 to 60°C results in intermolecular associations and insolubility for β-casein and intramolecular association (folding) without loss of solubility for β-lg. These significantly differing behaviors are ascribed mainly to the effect of mild heat treatments up to 60°C.

This does not mean that all native globular whey proteins retain good solubility upon heat treatments up to 60°C. For example, BSA is highly soluble (35% wt/wt) in pure water at 3°C but shows severe precipitation in the temperature range between 40 and 45°C (26). This changed solubility above 40°C parallels the reversible partial unfolding of BSA observed between 42 and 50°C by Lin and Koenig (23). Therefore, it is tempting to assume that more hydrophobic residues are exposed above 40°C, resulting in a higher tendency of BSA toward hydrophobic aggregation or association.

Mild heat treatments not only affect protein solubility but also other important functional properties, such as foaming ability. Peter and Bell (30) observed that a preheat treatment between 40 and 50°C appears to be responsible for significant improvement of whipping properties of whey protein concentrate (WPC) at 25°C. This improved foaming ability, which reversibly disappears after cooling WPC to 5°C, is caused primarily by fatty substances in the protein solution (2, 5). Lipids are more suface active than proteins and cause local gradients in surface tension during whipping, which results in rupture of foam lamellae and, thus, in destabilization of the foam.

Cooney (2) postulates that this foam-depressing action of lipids is reduced strongly by formation of protein-lipid-phospholipid complexes during heat treatments prior to whipping. We remarked in the previous section that particularly BSA adsorbs fatty acids. These protein-lipid complexes may dissociate during cooling to 4°C, which results again in impaired foaming properties of the WPC, caused by free lipids. This postulate is supported by the results of De Wit (5), which show completely defatted whey has excellent whipping properties irrespective of the preheating temperature between 4 and 60°C. In this way mild heat treatments are responsible for a number of features such as changes of solubility and functionality of whey proteins in the temperature range between 4 and 60°C.

Temperature-Induced Changes in Structure and Solubility of Whey Proteins Caused by Heat Denaturation Between 60 and 100°C

With respect to solubility of whey proteins in this temperature range, it is important to consider protein denaturation as a two-step process: an unfolding step that may be either reversible or irreversible and an aggregation step that mostly follows irreversible unfolding.

Unfolding of globular proteins is accompanied by an endothermal heat effect (heat uptake). This effect may be observed by differential scanning calorimetry (DSC), a technique by which the difference in heat flow between a sample and a reference sample is measured as a function of temperature or time. For reviews on details of DSC of globular proteins, we refer to (32, 42).

Typical thermograms of the major whey proteins as well as of the WPC from which they were isolated are in Figure 1. The heating rate used in these thermograms was 21.4° K/min, a rate close to that in the heater of industrial pasteurizers.

Figure 1 shows that the various whey proteins unfold at (slightly) different temperatures, ranging from an apparent transition temperature (T_{tr}) of 68°C for α-la to T_{tr} 89°C for the Ig (Table 2, column 3). The apparent T_{tr} of proteins is used generally as a measure their denaturation temperature when environmental effects, such as pH and salts, are studied. However, the apparent T_{tr} of the various whey proteins are affected differently by heating rate.

To determine the denaturation temperature (T_d), we eliminated the effect of heating rate on T_{tr} by extrapolation to zero heating rate (10). The T_d is a well-defined protein character in a specific solvent. The T_d for the major whey proteins, determined in phosphate buffer at pH 6.0, are summarized in Table 2 (column 4) and reveal that α-la (62°C) has the lowest and β-lg (78°C) the highest T_d. Consequently, α-la appears to be the least thermostable whey protein, and this conclusion differs from the general view based on denaturation determined by protein solubility measurements at pH 4.6.

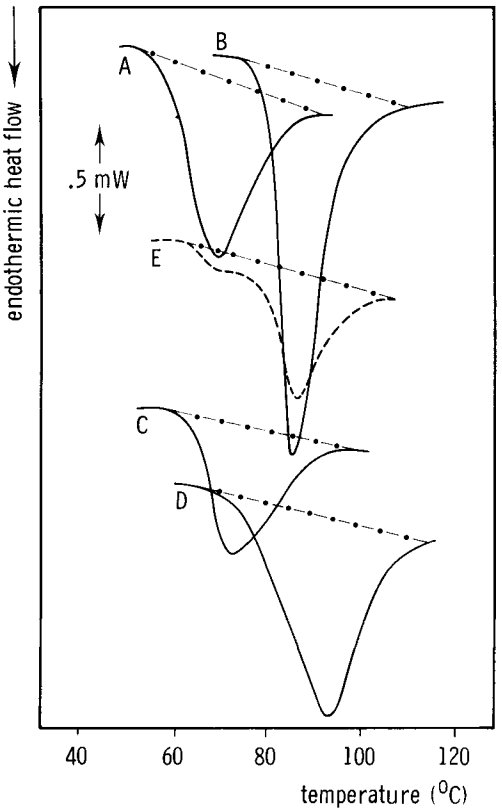

Figure 1. Differential scanning calorimetry curves of the heat denaturation of α-lactalbumin (A), β-lactoglobulin (B), defatted bovine serum albumin (C), immunoglobulin G (D), and defatted ultrafiltrate-whey protein concentrate (E) as a function of temperature in .07 *M* phosphate buffer at pH 6.0. Protein concentration 8 to 10%. Heating rate 21.4° K/min.

TABLE 2. Thermodynamic characteristics of heat denaturation of some whey proteins and of whey protein concentrate in .7 *M* phosphate buffer at pH 6.0. Reference: heat-denatured whey protein concentrate. Heating rate (H_r) 21.4° K/min.

Whey protein	Molecular weight	T_{tr}[1] (± .5°C) at H_r 21.4° K/min	T_d (± .5°C) at H_r 0° K/min	ΔH (J/g)	ΔH (kJ/mol)	SE
α-Lactalbumin	14,200	68	62	17.8	253	17
β-Lactoglobulin	18,400	83	78	16.9	311	15
Immunoglobulin (G)	36,000[2]	89	72	13.9	500	15
Bovine serum albumin[3]	66,000	70	64	12.2	803	14
Whey protein concentrate	...	68/83	62/78	11.5		

[1] T_{tr} = Transition temperature; T_d = denaturation temperature; ΔH = endothermal heat effect.

[2] Average molecular weight of light chain (M_r 23,400) and heavy chain (M_r 48,500) polypeptides.

[3] Defatted, according to the procedure of Chen (1).

[4] Defatted, showing the transition temperatures of α-lactalbumin and β-lactoglobulin.

This discrepancy is explained by high reversibility in denaturation (unfolding) of α-la as will be discussed later.

The endothermal heat effect (ΔH) that accompanies protein unfolding during denaturation is derived from the peak area and is independent of heating rate. The ΔH from the different whey proteins at pH 6.0 are in Table 2 (columns 5 and 6), expressed on weight and molar basis. These ΔH are thermodynamic quantities, which are related to protein denaturation determined by protein solubility only when protein unfolding is reversible process.

This can be verified by repeating the run after cooling of the same DSC sample. Results of such experiments reveal that α-la is the only reversible heat denatured whey protein (reversibility >90%), whereas all the other whey proteins and also WPC no longer show endothermal heat effects of unfolding in a repeated run. These results demonstrate that α-la, although it is the whey protein with the lowest T_d, appears to be (at pH 6.0) most thermostable against protein aggregation because of its high capability of renaturation on cooling. This renaturation effect of α-la is not observed in a repeated run of WPC, which might be due to heat-induced interactions with β-lg and BSA.

Effects of pH on Changes of Structure and Properties of Whey Proteins Beyond the Denaturation Temperature

Environmental conditions strongly affect folding ability and solubility of whey proteins during and after denaturation. Because pH affects conformation of major whey proteins, this has consequences for solubility and other functional properties of whey proteins. Elsewhere (9) we have shown that WPC has different properties when heat treated at pH 3.0, 6.0, or 7.5.

For that reason, we studied thermal behavior of major whey proteins by DSC at pH 3.0, 6.0, and 7.5. Results (Table 3) were obtained from nonbuffered protein solutions at a heating rate of 5.4° K/min. The T_{tr} and ΔH at pH 6.0 appear to be lower than those in Table 2, which should be ascribed both to absence of buffer (for T_{tr} and ΔH) and to lower heating rate (resulting in lower T_{tr}). The two transition temperatures and the higher ΔH for BSA were due to protein fractions with differing amounts of adsorbed fatty acids in the sample, as shown by Gumpen et al. (14). The ΔH at pH 3.0 show that both α-la and BSA already are unfolded prior to heat treatment (ΔH = 0), and these observations confirm the generally accepted conclusion that these proteins are subject

TABLE 3. Transition temperatures (T_{tr}) and denaturation heat (ΔH) of some whey proteins and whey protein concentrate in nonbuffered solutions at a heating rate of 5.4° K/min. Reference: heat-denatured whey protein concentrate.

Whey protein	pH = 3.0			pH = 6.0			pH = 7.5		
	T_{tr}	H		T_{tr}	ΔH		T_{tr}	ΔH	
	(°C)	(J/g)	SE	(± .5°C)	(J/g)	SE	(± .5°C)	(J/g)	SE
α-la	. . .		0	63	13.8	.4	62	13.8	.4
β-la	82	16.6	.4	78	15.9	.4	71	11.2	.7
IgG[2]	53	3.3	.4	78	13.9	.4	77	14.3	.4
BSA[3]	. . .		0	68/74	16.0	.3	66/68	11.6	.2
WPC	83	10.9	.4	74	10.9	.4	70	10.9	.4

[1] α-la = α-Lactalbumin; β-lg = β-lactoglobulin; IgG = immunoglobulin G; BSA = bovine serum albumin; WPC =whey protein concentrate.

[2] Crystallized bovine serum albumin (BDH-chemicals), containing some fatty acids.

[3] Defatted, showing one transition temperature in a nonbuffered system.

to acid denaturation. Probably the same explanation holds for some of the polypeptide chains of IgG, as indicated by the much lower ΔH at pH 3.0 compared with those at pH 6.0 and 7.5.

The DSC results of β-lg show, in contrast to those of α-la, increased thermostability at pH 3.0 and decreased stability at pH 7.5 as compared to that at pH 6.0, both observed as ΔH and T_{tr}. The last feature also is observed for BSA and parallels the increased reactivity of the free thiol group in these proteins above pH 6.0. The reactivity of the thiol group will increase by more than two orders of magnitude both for β-lg (11) and for BSA (37) when pH is increased from 6.0 to 7.5. This will result in increased disulfide interchange catalyzed by proton dissociated thiol groups at alkaline pH. Creighton (3) has argued that at sufficiently high activity of thiol groups, the formation of intramolecular disulfide bonds will, at low protein concentration, prevail over intermolecular disulfide interaction. Therefore, partial protein unfolding (denaturation) and protein solubility may coincide to some extent, as we discussed in (6), and good solubility of both β-lg and WPC after denaturation under slightly alkaline conditions is theoretically expected and actually observed. Moreover the WPC solutions modified thermally in this way appear to have casein-like solubility properties observed as reversible precipitation upon acidification to pH 4.6 and subsequent pH-increase to pH 6.5 (36).

Temperature-Induced Changed of Structure and Properties of Whey Proteins Between 100 and 150°C

Heat treatments above 100°C are common in processing and preservation of food, but much remains to be elucidated as to their effects on structure and properties of whey proteins. Of particular interest are the temperature-induced changes of β-lg, which strongly affect heat stability of milk and milk products at temperatures above 100°C (33). Fox (12) observed that β-lg is mainly responsible for the rather unusual pH effect between pH 6.0 and 7.0 on the stability of milk proteins during heat treatments at 140°C.

The DSC results in Figure 2 show that in addition to the denaturation at 80°C β-lg shows a distinct thermal effect near 140°C, which is small at pH 6.0 (curve B) but increases significantly at pH 7.5. Apparently this increase occurs at the expense of denaturation near 80°C, as can be inferred from reduction of ΔH at pH 7.5 in comparison with those at pH 6.0 (Table 3). These high-temperature effects are not observed in a solution of α-la (Figure 2, curve A), indicating that the endothermal heat effect above 100°C is not caused by chemical

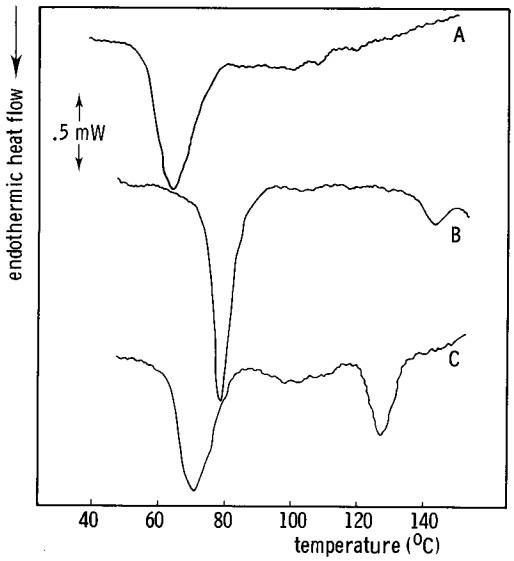

Figure 2. Differential scanning calorimetry curves of the heat denaturation of α-lactalbumin at pH 6.0 A, β-lactoglobulin at pH 6.0 (B), and pH 7.5 (C). Protein concentration 8 to 10%; heating rate 5.4° K/min.

breakdown of amino acid residues, as verified (7).

As discussed in (7), we conclude that a partial stabilization of the β-lg structure occurs during denaturation near 80°C, due to disulfide interchange, followed by destabilization of the residual protein structure near 140°C. We postulate that destabilization of residual structure of β-lg at 140°C is induced by a breakdown of disulfide bonds, as observed by Watanabe and Klostermeyer (40).

To verify these chemical degradation effects under conditions of a DSC-run, we heated three separate β-lg solutions at pH 3.0, 6.0, and 7.5 to a temperature of 155°C at a rate of 5.4° K/min. The heat-treated protein solutions subsequently were hydrolyzed and analyzed for amino acid composition (39). Results, in Table 4 demonstrate that, apart from the cys-residues, none of the amino acids appears to be damaged significantly by heating at these three pH. Degradation of cys-residues clearly increases at higher pH. Klostermeyer et al. (21) reported that dehydro-alanine (DHA) is produced predominantly.

TABLE 4. Effect of heat treatments[1] up to 155°C on the amino acid composition of a 1% β-lactoglobulin solution in water at different pH in moles/18,400.

Amino acid[2]	Without heat treatment	Heated at H 3.0	Heated at pH 6.0	Heated at pH 7.5
Trp	1.6	1.6	1.6	1.6
Ile	8.4	8.3	8.4	8.3
Tyr	3.8	3.8	3.7	3.7
Phe	3.7	3.8	3.8	3.8
Pro	8.2	8.4	8.1	8.2
Leu	22	22	22	22
Val	8.8	8.6	8.7	8.5
Lys	15.0	15.0	14.8	15.0
Met	3.9	4.0	3.9	3.9
Cys/2	4.8	4.7	4.4	4.2
Ala	13.9	13.8	13.8	13.9
Arg	3.1	3.0	3.0	3.0
His	1.8	2.1	2.0	1.9
Thr	8.0	7.9	7.8	7.9
Ser	7.0	6.9	6.8	6.9
Gly	3.4	3.4	3.5	3.4
Asx	15.0	15.0	14.9	14.9
Glu	25.0	25.1	25.0	24.9
Lal[3]	0	0	.031	.050

[1] Heating rate: 5° K/min.

[2] Trp = Tryptophan; Ile = isoleucine; Tyr = tyrosine; Phe = phenylalanine; Pro = proline; Leu = leucine; Val = valine; Lys = lysine; Met = methionine; Cys/2 = half-cystine; Ala = alanine; Arg = arginine; His = histidine; Thr = threonine; Ser = serine; Gly = glycine; Asx = aspartic acid; Glu = glutamic acid.

[3] Determined according to the method described in reference (4).

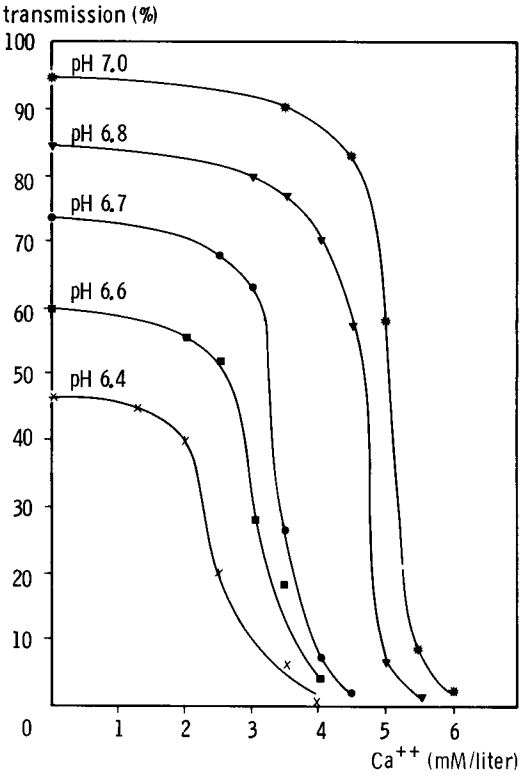

Figure 3 Effect of Ca^{++} concentration on the turbidity (as transmission at 600 nm) after a 15-min heat treatment at 120°C of a 1% β-lactoglobulin solution at different pH. Temperature 25°C; $CaCl_2$ added after cooling for 20 h at 5°C.

It appears then that these heat treatments of β-lg solutions near 140°C will reduce the number of covalent cross links, which might have consequences for protein unfolding and solubility.

If, however, 5% lactose were in the β-lg solution during the mentioned high-heat treatments at pH 7.5, over 60% of the lys-residues appeared to be destroyed. Moreover, reduced solubility, a brownish color, and an unpleasant taste, probably caused by Maillard reactions, made these severely heat-treated protein solution unsuitable for application in human food products.

Effect of Calcium on Solubility of β-Lactoglobulin Above 100°C

Figure 3 shows results of the effect of calcium ions on aggregation of β-lg after a heat treatment for 15 min at 120°C. The intersection points with the ordinate indicate that, upon this heat treatment and in the absence of calcium, the protein solubility increases at higher pH. Moreover, it appears from Figure 3 that stability of denatured β-lg against calcium flocculation increases slightly with increasing pH but decreases rather sharply with increasing calcium concentration at a fixed pH.

From the sigmoidal curves in Figure 3, we define calcium flocculation as the calcium concentration at which light transmittance is reduced to 50% of its initial value. The flocculations obtained in this way are plotted as a function of pH in Figure 4 (curve C). For comparison these results are plotted together with identical curves obtained by milder heat treatments of β-lg solutions.

Curve A in Figure 4 shows the pH-dependence of flocculation of β-lg solutions heated for 10 min at 80°C in the presence of calcium ions. Elsewhere (6) we have shown that the Ca^{++}-equivalents given by curve A just compensate the pH-induced charge increase of β-lg, indicating isoelectric β-lg precipitation by calcium.

In the same pH-range concentrations of less than 2 m*M* $CaCl_2$, during heat treatment of

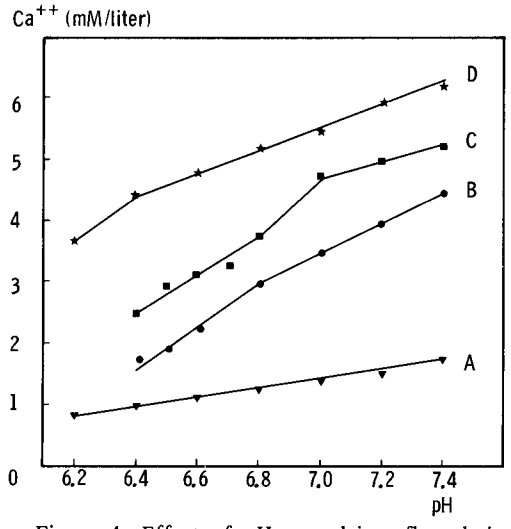

Figure 4. Effect of pH on calcium flocculation of a β-lactoglobulin solution heated: 1) for 10 min at 80°C in the presence of Ca^{++} during heat treatment (A); 2) for 30 min at 95°C (B), 15 min at 120°C (C) and 10 min at 80°C, (D) when Ca^{++} is added after heat treatment.

desalted whey cause abundant protein aggregation (6). This dramatic decrease of protein solubility has not been observed during heat treatments of normal whey above pH 6.5 (36), containing 2.5 m*M* Ca^{++} on average. Important differences in this respect between desalted whey and normal whey are the presence of phosphate and of citrate. In particular, phosphate causes, upon heating, an increasing amount of Ca-phosphate with increasing pH. In normal whey the phosphate obviously competes with whey proteins for calcium, resulting in an improved solubility of whey proteins (β-lg included.) This is important information for the functional application of WPC.

Much higher flocculations are when calcium ions are not present during heat treatment but added to the heat-denatured protein solution after cooling. Curves B and D in Figure 4 show that flocculations are reduced upon higher preheat treatments of the β-lg solution. However, the points plotted in curve C show that a heat treatment at 120°C results again in increased stability of β-lg against calcium. It appears from these observations that the reduced sensitivity of denatured β-lg to calcium flocculation parallels the breakdown of disulfide bonds as discussed in the previous section. This implies that thiol-disulfide interchange reactions affect not only structure of whey proteins but also sensitivity of the latter to flocculation by calcium; it confirms our results obtained by blocking thiol groups (6).

We conclude, therefore, that structure and solubility of whey proteins are governed by effects of temperature, pH, and composition. Up to 150°C we distinguish three temperature ranges for protein behavior: 1) between 4 and 60°C, causes reversible physicochemical changes such as hydrophobic association and partial unfolding, mainly observed as changes of solubility and foamability; 2) between 60 and 100°C, causes mainly irreversible physicochemical changes induced by protein denaturation, resulting in modified properties during heat treatment after small pH variations; 3) between 100 and 150°C, causes mainly irreversible chemical changes such as Maillard reactions and cys-breakdown, with still unknown functional consequences. More knowledge of the mechanism of these effects will improve our understanding of the functional behavior of whey proteins in complex food systems.

ACKNOWLEDGMENTS

The authors are much indebted to P. J. van Rooijen for amino acid analyses and determinations of lysino-alanine.

REFERENCES

1 Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem. 242:173.
2 Cooney, C. M. 1974. A study of foam formation by whey proteins. Ph.D. Thesis, Univ. Minnesota.
3 Creighton, T. E. 1978. Experimental studies of protein folding and unfolding. Prog. Biophys. Mol. Biol. 32:231.
4 De Koning, P. J., and P. J. van Rooijen. 1982. Aspects of the formation of lysinoalanine in milk and milk products. J. Dairy Res. 49:725.
5 De Wit, J. N. 1975. Foaming properties of whey protein concentrates. Zuivelzicht 67:228.
6 De Wit, J. N. 1981. Structure and functional behavior of whey proteins. Neth. Milk Dairy J. 35:47.
7 De Wit, J. N., and G. Klarenbeek. 1981. A differential scanning calorimetric study of the thermal behaviour of bovine β-lactoglobulin at temperatures up to 160°C. J. Dairy Res. 48:293.
8 De Wit, J. N., G. Klarenbeek, and R. de Boer. 1978. A simple method for the clarification of whey. Page 919 *in* Proc. 20th Int. Dairy Congr., Paris.
9 De Wit, J. N., G. Klarenbeek, and E. Hontelez-Backx, 1980. Use of modified of whey protein concentrates. Zuivelzicht 72:959.
10 De Wit, J. N., and G.A.M. Swinkels. 1980. A differential scanning calorimetric study of the thermal denaturation of bovine β-lactoglobulin. Thermal behaviour at temperatures up to 100°C. Biochim. Biophys. Acta 624:40.
11 Dunnill, P., and D. W. Green. 1966. Sulphydryl groups and the $N \rightleftarrows R$ conformational change in β-lactoglobulin. J. Mol. Biol. 15:147.
12 Fox, P. F., and M.C.T. Hoynes. 1975. Heat stability of milk; influence of colloidal calcium phosphate and β-lactoglobulin. J. Dairy Res. 42:427.
13 George, C., S. Guinand, and J. Tonnelat. 1962. Etude thermodynamique dela dissociation reversible de la β-lactoglobulin B pour des pH superieurs a 5.5. Biochim. Biophys. Acta 59:739.
14 Gumpen, S., P. O. Hegg, and M. Martens. 1979. Thermal stability of fatty acid serum albumin complexes studied by differential scanning calorimetry. Biochim. Biophys. Acta 574:189.
15 Hiraoka, Y., T. Segawa, K. Kuwajima, S. Sugai, and N. Murai. 1980. α-lactalbumin: a calcium metalloprotein. Biochim. Biophys. Res. Commun. 95:1098.
16 Haurowitz, B. 1963. Albumins, globulins and other soluble proteins. The chemistry and function of proteins. Ch. 8. F. Haurowitz, ed. Academic Press, New York and London.
17 Jelen, P. 1974. Utilization of cheese whey components in human foods. IV. Int. Congr. Food Sci. Technol. 5c:12.
18 Jenness, R. 1979. Comparative aspects of milk

proteins. J. Dairy Res. 46:197.
19 Joly, M. 1965. A physico-chemical approach to the denaturation of proteins. Molecular biology, an international series of monographs and text books. Vol. 6. B. Horecker and N. O. Kaplan, ed. Academic Press, London and New York.
20 Kester, J. J., and J. R. Brunner. 1982. Milk fat globule membrane as possible origin of proteose-pepton glycoproteins. J. Dairy Sci. 65:2241.
21 Klostermeyer, H., and E. H. Reimerdes. 1977. Heat induced crosslinks in milk proteins and consequences for the milk system. Protein crosslinking. Ch. 7. M. Friedman, ed. Plenum Press, New York, NY.
22 Kronman, M. J., R. E. Andreotti, and R. Vitols, 1964. Inter- and intra-molecular interactions of α-lactalbumin. II. Aggregation reactions at acid pH. Biochemistry 3:1152.
23 Lin, V.J.C., and J. L. Koenig. 1976. Raman studies of bovine serum albumin. Biopolymers 15:203.
24 Lobos, Z. J., and L. E. St.-Pierre. 1975. Water structure: Anomalies in interfacial tension with temperature for bovine serum albumin. J. Colloid Interface Sci. 51:196.
25 Lyster, R.L.J. 1979. Milk and dairy products. in: Effects of heating on food stuffs. Ch. 11. R. J. Priestley, ed. Appl. Sci. Publ. London.
26 Macritchie, F. 1973. Effects of temperature on dissolution and precipitation of proteins and polyamino acids. J. Colloid Interface Sci. 45:235.
27 Mulder, H., and P. Walstra. 1974. Creaming and separation. The milkfat globule. Ch. 8. Commonw. Agric. Bur., Bucks.
28 Pace, C. N., and C. Tanford. 1968. Thermodynamics of the unfolding of β-lactoglobulin A in aqueous urea solutions between 5 and 55°C. Biochemistry 7:198.
29 Payens, T.A.J., and H. J. Vreeman. 1982. Casein micelles and micelles of κ- and β-casein. Vol. 1. Page 543 *in* Solution behavior of surfactants K. L. Mital and E. J. Fendler, ed. Plenum Press, New York, NY.
30 Peter, P. N., and R. W. Bell. 1930. Normal and modified foaming properties of whey-protein and egg-albumin solutions. Ind. Eng. Chem. 22:112.
31 Pfeil, W. 1981. Thermodynamics of α-lactalbumin unfolding. Biophys. Chem. 13:181.
32 Privalov, P. L., and N. N. Khechinashvili. 1974. A thermodynamic approach to the problem of stabilization of globular protein structure: A calorimetric study. J. Mol Biol. 86:665.
33 Rose, D. 1963. Heat stability of bovine milk: A review. Dairy Sci. Abstr. 25:45.
34 Schmutz, M., and Z. Puhan. 1981. Chemisch-physikalische Veranderungen wahrend der Tiefkuhl-lagerung von Milch. Dtsch. Molkerei Z. 17:552.
35 Spector, A. A. 1975. Fatty acid binding to plasma albumin. J. Lipid Res. 16:165.
36 Stichting Bedrijven van het NIZO. 1980. A method of preparing modified whey proteins. Dutch Pat. Appl. 80:06237.
37 Svenson, A., and J. Carlsson 1975. The thiol group of bovine serum albumin. High reactivity at acidic pH as measured by the reaction with 2,2′-dipyridyl disulphide. Biochim. Biophys. 400:433.
38 Veldkamp, W. B., and J. R. Votano. 1980. Temperature dependence of macromolecular interactions in dilute and concentrated hemoglobin solutions. Biopolymers 19:111.
39 Visser, S., P. J. van Rooijen, C. Schattenkerk, and K.E.T. Kerling. 1976. Peptide substrates for chymosin (rennin). Biochim. Biophys. Acta 438: 265.
40 Watanabe, K., and H. Klostermeyer. 1976. Heat-induced changes in sulphydryl and disulphide levels of β-lactoglobulin A and the formation of polymers. J. Dairy Res. 43:411.
41 Wertz, D. H., and H. A. Scheraga. 1978. Influence of water on protein structure. An analysis of the preferences of amino acid residues for the inside or outside and for specific conformations in a protein molecule. Macromolecules 11:9.
42 Wright, D. J. 1982. Application of scanning calorimetry to the study of protein behaviour in foods. *in* Development in Food Proteins–1. Ch. 2. B.J.F. Hudson, ed. Appl. Sci. Publ., London, NJ.