Interactions between Food Stabilizers and β-Lactoglobulin

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β-Lactoglobulin forms insoluble complexes with anionic polysaccharides under specified conditions of pH and ionic strength. No complexing with neutral polysaccharides was detected. For the weakly acidic carboxymethylcellulose (CMC), maximum interaction occurred at pH 4 provided the ionic strength was below 0.1. Under these conditions, the binding ratio was 45.3 ± 4.6 moles of carboxyl groups per mole of β-lactoglobulin. Further addition of CMC resulted in a peptization of the insoluble complex. For the strongly acidic carrageenan types and sulfated cellulose, similar interactions also occurred at low pH, but the complexes were not as readily dissociated by increasing the ionic concentration as were the complexes with CMC. These findings suggest that the complex formation is the result of ionic attractions between the positively charged protein and the negatively charged stabilizers. The presence of CMC during heating of β-lactoglobulin afforded some protection against denaturation of the protein at neutral pH, as measured by the depression in the amount of heat-exposed sulfhydryls.

The beneficial effect of stablilizers in foods is well known, but their mode of action in complex food systems, and their relative efficiency under varying conditions have not been sufficiently delineated. Much of the work concerned with the physical functions of stabilizers has been based on viscosity measurements, and evidence has been presented (Muck and Tobias, 1962) that their viscous behavior in milk may be related to their interaction with milk proteins. A specific interaction between sulfated hydrocolloids and calcium-sensitive caseins has been reported recently (Hansen, 1968; Lin and Hansen, 1968). Moving boundary electrophoresis as well as turbidimetric measurements (Asano, 1966) have suggested that carboxymethylcellulose (CMC) interacts with several milk proteins in acidified milks. Therefore, the milk protein reactivity of different hydrocolloids may be expected to exert an important role for the stability of processed dairy products (Gordon *et al.*, 1966). The purpose of this study has been to determine in model systems the factors that govern the interactions between β-lactoglobulin and food stabilizers.

EXPERIMENTAL

Materials. Sodium carboxymethylcellulose, with designated substitution ranges of 0.38 to 0.48 (4HP), 0.65 to 0.85 (7HP), and 0.85 to 0.95 (9HP), and sodium cellulose sulfate (Hercules Powder Co., Wilmington, Del.). Sodium salts of λ- and κ-carrageenan (Marine Colloids, Inc., Springfield, N. J.). Commercial grade guar gum; this sample contained a high proportion of insoluble material, which was removed by dispersing 10 grams of guar gum in 2000 ml. of distilled water and centrifuging the solution at 10,000 G for 20 minutes. The soluble material was recovered by adding an equal volume of absolute ethanol to the clear supernatant and extracting the precipitate first with absolute ethanol, then with anhydrous ethyl ether. Following removal of the solvent under vacuum, the purified gum was ground to a fine powder.

β-Lactoglobulin was isolated from milk by the method of Aschaffenburg and Drewry (1957).

Buffers. Stock acetate, chloride, and phosphate buffers were prepared from analytical reagents (Colowick and Kaplan, 1955). The selected ionic strengths were obtained by dilution or by the addition of sodium chloride.

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Sample Preparation. The stabilizers were dissolved separately in the selected buffers at 60° C. under rapid agitation. After cooling, these were mixed with solutions of β-lactoglobulin in the selected proportions.

Chemical Analysis. The CMC concentration was determined by the phenol–sulfuric acid method as reported by Hansen and Chang (1968). The protein concentration was determined spectrophotometrically at 280 mμ, using the specific absorbance value of 1.0. The measurements were, in each case, corrected for interference due to polysaccharides, as determined by individual standard curves.

Moving Boundary Electrophoresis. Electrophoresis was performed as previously described (Hidalgo and Hansen, 1968).

Analytical Ultracentrifugation. The sedimentation velocity experiments were performed at 20° C. in a Model E Spinco analytical ultracentrifuge.

Rate of Denaturation of β-Lactoglobulin. The rate of heat denaturation of β-lactoglobulin was determined by measuring the increase in exposed sulfhydryls with 5,5-dithiobis-(2-dinitrobenzoic acid) as reported by Koka *et al.* (1968).

RESULTS

Preliminary electrophoretic experiments on solutions prepared by mixing β-lactoglobulin and CMC at pH 4.6, ionic strength 0.1, as described by Asano (1966), were met with difficulties, because of the formation of insoluble complexes which gave rise to excessive turbidity in the sample solutions. This turbidity could, however, be removed by increasing the salt concentration of the system above 0.1 ionic strength, but under these conditions electrophoretic analysis did not show evidence of the interactions reported by Asano (1966). These observations suggested that the formation of insoluble complexes was dependent upon other factors than pH alone. Therefore, the approach followed in this study was to determine in model systems the conditions that favored the complexing between β-lactoglobulin and individual food stabilizers. Initial experiments were conducted on mixtures of β-lactoglobulin and CMC (7HP) and the extent of interaction was determined by measuring the amount of protein remaining in the clear supernatants after centrifugation of the solutions at 5000 G for 30 minutes. Figure 1 illustrates the effect of pH on the amount of protein precipitated by CMC at the ionic strength of 0.05. Maximum complexing occurred

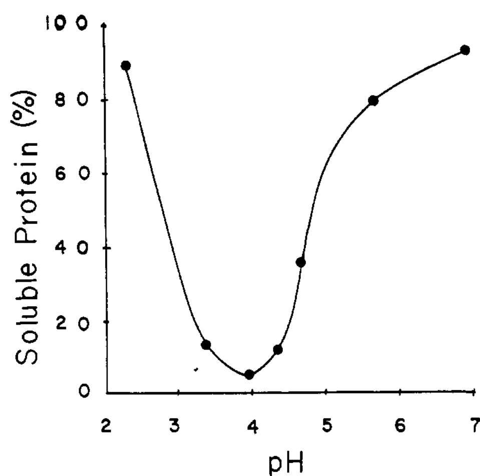

Figure 1. Effect of pH on precipitation of β-lactoglobulin by CMC

Protein 0.6%, stabilizer 0.2%, NaCl/HCl buffer, ionic strength 0.05

at pH 4, whereas no protein was precipitated at pH below 2.5 or above 7.

To determine the effect of ionic strength on the complex formation, solutions containing β-lactoglobulin and CMC in buffers of increasing ionic strength were prepared at the pH of maximum interaction—(i.e., pH 4). Figure 2 shows that the amount of protein precipitated by CMC decreased as the salt concentration was increased. At ionic strengths above 0.2, no protein sediment was observed.

The proportions of the constituents in the insoluble complex were estimated by adding increasing amounts of different types of CMC to a solution of β-lactoglobulin at pH 4, ionic strength 0.05. The solubility curves are presented in Figure 3 and show that the interaction depended, in a characteristic manner, upon the relative concentration of CMC. As the CMC concentration was increased there was initially a sharp drop in the amount of soluble protein leading to a minimum in the curves. At this point, the protein was nearly quantitatively precipitated and analysis of the supernatant showed

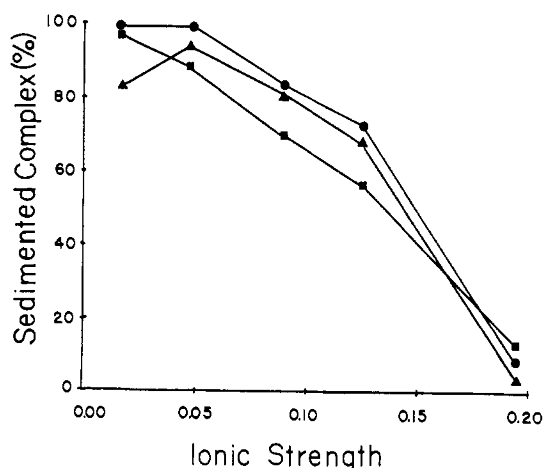

Figure 2. Effect of ionic strength on precipitation of β-lactoglobulin by CMC-7HP at pH 4

Protein 0.27%, sodium acetate buffer, ionic strength 0.05
▲—CMC 0.1%
●—CMC 0.06%
■—CMC 0.03%

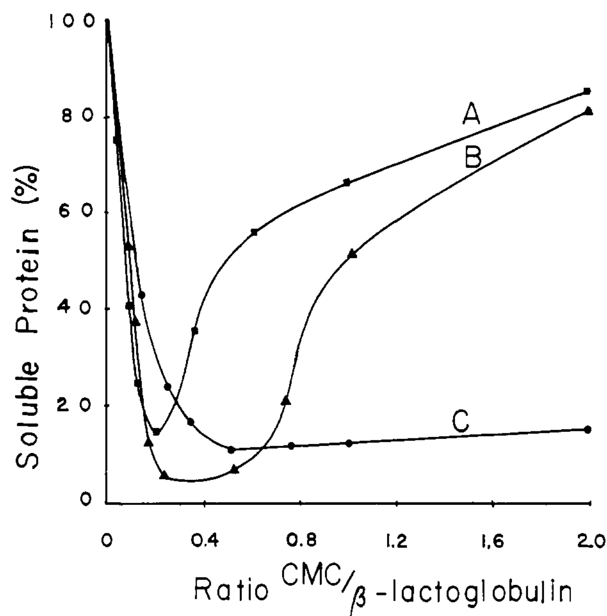

Figure 3. Effect of CMC concentration on precipitation of β-lactoglobulin at pH 4

Sodium acetate buffer, ionic strength 0.05
A. CMC-9HP
B. CMC-7HP
C. CMC-4HP

that virtually all (93%) of the CMC was bound to the protein in the precipitate. Values determined from the corresponding proportions of CMC and protein in the complex indicated an apparent binding ratio of 0.25 for type 9HP, 0.35 for type 7HP, and 0.50 for type 4HP. Assuming an average degree of substitution of 0.90, 0.75, and 0.43 mole of carboxyl, respectively, for each subunit in the three types, these binding ratios corresponded to 45.3 ± 4.6 moles of carboxyl groups bound per mole of β-lactoglobulin (42,000 mol. wt.). Thus, the constancy of the number of carboxyl groups present in the complex confirmed the ionic nature of the interaction. Furthermore, the results suggested that all of the 47 cationic groups of the protein were involved in the binding.

Inspection of the curves in Figure 3 revealed that further addition of CMC beyond the concentration of maximum interaction resulted in a peptization of the insoluble complex. This effect was pronounced for CMC with the highest content of carboxyl (9HP and 7HP), but was virtually absent for the more weakly carboxylated material (4HP). In the peptized solutions, only slight turbidity was apparent and no material was sedimented at 5000 G. The ultracentrifuge pattern of such a mixture is shown in Figure 4 for CMC-7HP and revealed the presence of fast-moving molecular complexes, with no evidence of any free β-lactoglobulin. Therefore, the complex appears to have been solubilized without undergoing dissociation.

The complex sedimented under the conditions for maximum interaction (pH 4, ionic strength 0.05) was redissolved in buffer by increasing the ionic strength to 0.25. The electrophoretic pattern of the solution obtained is shown in Figure 5. The presence of two independently migrating peaks with mobilities corresponding to the single constituents demonstrated that at this ionic strength the complex was dissociated, and that the complex formation was of ionic nature.

The previous results suggested that the complex formation at pH 4 was initially due to a stoichiometric, ionic binding between the positively charged protein and the negatively

Table I. Properties of Complexes of β-Lactoglobulin with Sulfated Stabilizers

Stabilizer	pH: Maximum complexing	pH: No complexing	Ratio Stab/β-Lact.: Maximum precipitation	Ratio Stab/β-Lact.: Peptization	Ionic Strength for Dissociation
κ-Carrageenan	Below 2.5	Above 6	0.3	None	0.20
λ-Carrageenan	Below 4.0	Above 5	0.2	None	0.65
Cellulose sulfate	Below 5.0	Above 6	0.2	0.4	0.36

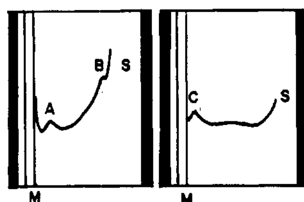

Figure 4. Ultracentrifuge pattern of the peptized β-lactoglobulin–CMC complex at pH 4

Sodium acetate buffer, ionic strength 0.02
Left: after 2 minutes. Right: after 70 minutes
Speed 52,000 r.p.m.

M. Meniscus
S. Sedimented complex
A. Fast moving sedimenting complex (S_{20}:40)
B. Fast moving sedimenting complex (S_{20}:380)
C. Excess of free CMC (S_{20}:1.4)

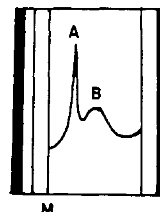

Figure 6. Ultracentrifuge pattern of the dissociated β-lactoglobulin – CMC complex at pH 7

Speed 52,000 r.p.m., time 280 minutes
Sodium acetate buffer, ionic strength 0.02
A. CMC (S_{20}:-1.1)
B. β-lactoglobulin (S_{20}:2.1)

charged stabilizers. At higher stabilizer concentration this reaction was superseded by a peptization of the complex. An experiment was conducted to determine if this secondary interaction occurred at neutral pH, in the absence of the initial pH-dependent interaction. Ultracentrifugation of solutions containing β-lactoglobulin and CMC at pH 7, as shown in Figure 6, disclosed that no interaction occurred at this pH, because the two colloids sedimented independently.

A further experiment was designed to determine if at pH 7 the presence of CMC caused changes in the quaternary and tertiary structure of the protein. This was accomplished by measuring the amount of exposed sulfhydryl groups in the protein in the presence and in the absence of CMC. The results revealed that the amount of exposed sulfhydryls of β-lactoglobulin was not affected by CMC; thus the native

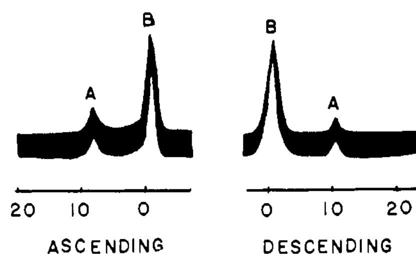

Figure 5. Moving boundary electrophoretic pattern of β-lactoglobulin and CMC at pH 4, ionic strength 0.25

Protein 0.6%, stabilizer 0.2%, sodium acetate buffer. Mobility scale: −cm. $volt^{-1} sec.^{-1} \times 10^5$
A. CMC
B. β-lactoglobulin

configuration of the protein was presumably not altered by the stabilizer. However, Figure 7 shows that in water solutions CMC depressed the rate of heat-induced release of sulfhydryls of β-lactoglobulin, thus exerting some protective effect against heat denaturation of the protein. This protective effect was not observed in buffer solutions of ionic strengths above 0.02.

Further studies have shown that the observed complex formation with β-lactoglobulin is limited to anionic polysaccharides because guar gum and locust bean gum, which are neutral poylsaccharides, did not complex with the protein under any of the conditions explored in the pH range from 2 to 7. Table I shows the properties of the protein complexes formed by polysaccharides containing other types of functional groups. The strongly sulfated carrageenan and cellulose sulfate both interacted with β-lactoglobulin at acidic pH, and the properties of the interaction products were similar to those of the β-lactoglobulin–CMC complex. The observation was made, however, that the binding between β-lactoglobulin and these colloids was not as readily dissociated by increasing the salt concentration as in the case of complexes formed with CMC.

DISCUSSION

The basic concept for the present study has been that hydrophilic stabilizers are capable of interacting with specific food components under different environmental conditions and that these reactions may contribute to their functional properties. The validity of this concept has been firmly established by the findings that anionic food stabilizers and β-lactoglobulin are capable of interaction under specified conditions of pH, ionic strength, and ratio of stabilizer to

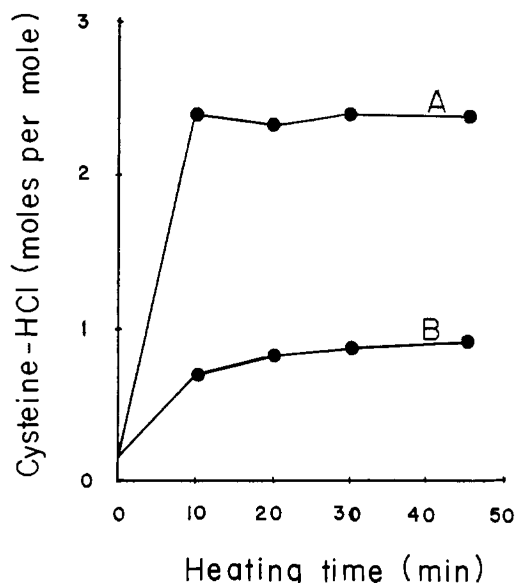

Figure 7. Effect of CMC on heat-induced release of sulfhydryl groups of β-lactoglobulin at neutral pH

Protein 0.6%, CMC 0.2%, heating temperature 80° C., ionic strength below 0.02
A. Control
B. With added CMC-7HP

protein. In general, the complex formation occurs through two separate reactions, a primary interaction which leads to the formation of insoluble complexes, and a secondary reaction which solubilizes the initial product.

The close pH dependence of the primary reaction and its sensitivity to variations in buffer salt concentrations suggest that the interaction would be of ionic nature. The inertness of the neutral polysaccharide, guar gum, further supports the requirement for ionic groups in the interacting polysaccharide.

β-Lactoglobulin was completely precipitated by CMC at stabilizer concentrations which were linearly related to the degree of carboxylation of the CMC. The nearly constant ratio of 45 moles of carboxyl bound per mole of β-lactoglobulin corresponded to all of the available cationic groups in β-lactoglobulin (Brand *et al.*, 1945). Assuming a molecular weight of 300,000 for these types of CMC the minimum particle weight for the insoluble complex would be in the order of one million, presumably with the molecules of β-lactoglobulin surrounding a central core of CMC. This molecular arrangement would inhibit the hydrophilic properties of the CMC, thus contributing to the insolubility of the complex.

The results have shown that the insoluble complexes are peptized upon further addition of stabilizer. This secondary solubilization phenomenon may be attributed to the redistribution of protein molecules on the total available CMC, causing a reduction in particle size and a partial exposure of CMC to hydration. The evidence obtained demonstrated that solubilization of the complex due to excessive stabilizer was not similar to the solubilization caused by changes in pH or ionic strength. In the case of the peptization process, the protein remained bound to CMC whereas under the influence of increasing salt concentrations the complex dissociated into individual components. Since the hydrophilic properties of CMC increase with the degree of carboxylation, the peptization phenomenon may be expected to be more pronounced for CMC with a high degree of substitution. This effect may explain why CMC-9HP and -7HP both were capable of peptizing the complex, whereas the relatively weakly carboxylated CMC-4HP was not.

The complexes between β-lactoglobulin and sulfated stabilizers were not as readily dissociated by increasing the ionic strength as in the case of the complexes with CMC, possibly because the ester sulfate is a stronger acidic group than the carboxyl. However, other factors are also important in determining the properties of the complexes, since their resistance to high ionic strength and their pH of maximum interaction varied with the type of sulfated stabilizers. Presumably, the number and location of the sulfate groups, as well as the chemical structure of the hydrocolloids, strongly affect the forces that govern their interaction with proteins.

Although it was not possible to detect by ultracentrifugation or by electrophoresis any interaction between β-lactoglobulin and CMC at neutral pH, CMC was capable of depressing the rate of heat denaturation of the protein when the ionic strength was less than 0.02. A similar depression in the rate of protein denaturation has been claimed for the enzyme, firefly luciferase, when heated in the presence of molecular sieve materials (Chapelle *et al.*, 1967). These authors considered that the presence of a physical barrier between individual protein molecules may prevent random protein-protein interactions, presumed to be important for heat denaturation.

The interactions between anionic stabilizers and protein which have been reported in this paper must be viewed as a separate behavior from the specific interactions by which a number of sulfated polysaccharides induce stable micelles in calcium-sensitive caseins (Hansen, 1968; Lin and Hansen, 1968). The ionic interaction appears to be of a general type and operational whenever anionic stabilizers are added to soluble protein. It is still not known if these interactions are beneficial or detrimental to the quality of the particular food product in which they are used. However, the results of this investigation suggest that by careful control of pH, ionic strength, and stabilizer concentration, it is possible to regulate the reactions leading to complex formation.

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