Ability of Cyclodextrins To Inhibit Aggregation of β-Casein

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Cyclodextrins (CD) can form inclusion complexes with various guest molecules, causing significant changes in the properties of the latter. Virtually no information is available regarding the ability of CD to interact with proteins. This interaction is of interest because it could be used to improve the solubility of proteins or to stabilize unstable proteins against heat or freezing. β-Casein, a relatively hydrophobic globular protein that undergoes reversible aggregation when warmed in the presence of calcium, was considered a good choice for testing possible interaction between CD and proteins. CD and β-casein were mixed in aqueous solution at 3 °C (β-casein monomeric) and then held at 31 °C (causes aggregation). Following centrifugation, supernatant fluids were examined for protein content. In the presence of β-CD, aggregation of β-casein was inhibited significantly as compared to that in the control samples (no β-CD). On the basis of several indirect tests it was deemed probable that β-CD forms an inclusion complex with β-casein.

INTRODUCTION

Cyclodextrins (CD) are homogeneous, cyclic polymers composed of six (α-CD), seven (β-CD), or eight (γ-CD) α-D-glucopyranose units linked 1-4 (Griffith and Bender, 1973; Tabushi, 1982). These compounds are capable of forming inclusion complexes (Cramer et al., 1967; Schlenk and Sand, 1961) in which CD serves as the host and a guest molecule of suitable size, shape, and polarity occupies the hydrophobic cavity in the CD (Bergeron, 1977). Molecules such as fatty acids, amino acids, and some flavoring compounds can serve as guest molecules, and when they are complexed, improved stability or reduced volatility is often observed (Laakso, 1984; Lach and Chin, 1964; Lach and Cohen, 1963; Mifune and Shima, 1977; Schlenk et al., 1955; Szejtli, 1978, 1983; Szejtli et al., 1979; Szejtli and Bolla, 1980; Yamamoto et al., 1976).

Binding forces regarded as being important in stabilizing cyclodextrin inclusion complexes (CIC) include van der Waals interactions, hydrophobic associations, and hydrogen bonding (Griffith and Bender, 1973; Matsui et al., 1985). In addition, favorable free energy changes occur during formation of CIC because water in the CD cavity is replaced by a more compatible guest. This allows the removed water to assume a lower energy state and the CD to sometimes assume a less strained conformation (Bergeron and Meeley, 1976; Matsui et al., 1985). Although much is still unknown regarding the major contributions to energy changes during the formation of CIC, in many instances, hydrophobic associations and favorable conformational changes in CD are regarded as being of primary importance (Chacko and Saenger, 1981; Hingerty and Saenger, 1976; Klar et al., 1980; Lindner and Saenger, 1982; Matsui and Mochida, 1979).

Little attention has been given to possible interactions between CD and proteins, and it is conceivable that inclusion complexes involving the hydrophobic side chains of protein could form. If so, this could result in improved solubility for sparingly soluble proteins and improved stability for proteins during heating (e.g., avoidance of coagulation of egg white during pasteurization) and frozen storage (e.g., avoidance of gelation of egg yolk, avoidance of precipitation of casein in milk). Of interest in this study was the possible interaction between CD and β-casein. β-Casein, a globular protein with a random coil structure, is the most hydrophobic of the casein proteins, and it will aggregate reversibly when the temperature is raised in the presence of calcium (Andrews et al., 1979; Leslie et al., 1969; Payens and Van Markwijk, 1963; Pearce, 1975; Schmidt, 1980; Schmidt and Payens, 1972; Sullivan et al., 1955; Takase et al., 1980; Waugh et al., 1970; Zittle and Pepper, 1958). Aggregation of β-casein is believed to involve hydrophobic interactions between valine and penultimate isoleucines of the C-terminal amino acids (Bingham, 1971; Pearce, 1975; Thompson et al., 1967; Waugh, 1954).

The purpose of this study was to ascertain whether CD and β-casein can interact to inhibit aggregation of β-casein at 31 °C and, if so, to explore the nature of this interaction.

MATERIALS AND METHODS

Chemicals. CDs (α, β, and γ) and modified β-CD (hydroxyethyl-β-CD; degree of polymerization 3.6) were obtained from American Maize-Products Co., Hammond, IN, and β-casein was obtained from Sigma Chemical Co., St. Louis, MO. These chemicals were used as received. All other chemicals were of reagent grade.

Methods. To prepare the CD solution, an accurately weighed amount of CD (α, β, or γ) was mixed with sufficient 50 mM dimethylarsenic acid (DMAA) buffer to produce the desired concentration, and the solution was stirred at moderate speed for 20 min at room temperature by using a 2.5-cm Teflon stir bar. In some studies, glucose was used instead of β-CD.

A β-casein solution (1% w/v) was prepared by stirring the sample at moderate speed (no foaming) for 3 h at 3 °C. Sufficient $CaCl_2$ was added at 3 °C to bring the solution to 0.03 M. This concentration of $CaCl_2$ was used in all experiments, except those involving equilibrium dialysis. After thorough mixing of β-casein and $CaCl_2$ by gentle stirring for an additional 1 h at 3 °C, the solution was centrifuged for 10 min at 3 °C and 9000*g* (RC-5 Superspeed refrigerated centrifuge, Sorvell, Du Pont Instruments, Newtown, CT). The pH was then adjusted to 6.0 by using 1.0 N NaOH (7-9 drops) and the supernatant fluid was quantitatively transferred to a 125-mL volumetric flask. This solution was stored at 3 ± 1 °C until used (no more than 20 h). Preparation of all β-casein solutions was done in a cold room (3 ± 1 °C) after pipets, beakers, test tubes, and all solutions had equilibrated to the cold room temperature. This procedure was followed to assure that the β-casein-Ca solution never warmed above 4 °C (maintained β-casein in monomeric state).

To prepare the CD-β-casein-Ca solution, 10 mL each of the CD and β-casein-Ca solutions were combined and adjusted to pH 9.0 (1.0 N NaOH) and then sonified (power supply Model LS-75; converter Model S-75, Branson Instruments, Inc., Stram-

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Table I. Composition of Standard Samples

component	concn
dimethylarsenic acid (DMAA) buffer	50 mm
β-casein	4.170×10^{-4} or 2.085×10^{-4} M
β-cyclodextrin	3.340×10^{-3} or 3.128×10^{-3} M
$CaCl_2$	0.03 M

ford, CT) for 10 s at the highest power level. The sample beaker was immersed in ice water during sonification. The adjustment to pH 9.0 was done to facilitate interaction between CD and β-casein. This was followed by 20 min of gentle stirring at 3 °C. The sonification–stirring procedure was repeated six times. The solution was then centrifuged at 9000*g* for 10 min at 3 °C, and absorbance of the supernatant fluid was measured at 280 nm. The supernatant fluid was adjusted to pH 6.0 with 1.0 N HCl and then placed in a shaking water bath for 2 h at 31 °C (to promote aggregation). A temperature of 31 °C was sufficiently high to produce aggregation at a reasonable rate. The sample was again centrifuged at 9000*g* for 10 min at 25 °C, and absorbance of the supernatant fluid at 280 nm was measured. DMAA buffer was used as a blank. The procedure described to this point is referred to as the "standard procedure" under Results and Discussion. The concentration of β-casein in the supernatant fluid was determined from a standard curve (either 31 or 3 °C as appropriate). The composition of the standard sample is shown in Table I. Control samples were devoid of CD.

For the equilibrium dialysis study at 3 °C, a 10-mL volume of β-casein (0.1% w/v, 4.17×10^{-5} M) in 50 mM phosphate buffer, pH 8.0, was sealed in a dialysis tube (Spectral/Por, Spectrum Medical Industries, Los Angeles, CA; molecular weight cutoff 12 000–14 000), and 10 mL of 6.25×10^{-3} M β-CD or 4.67×10^{-3} M modified β-CD (hydroxyethyl-β-cyclodextrin; Mβ-CD) in 50 mM phosphate buffer, pH 8.0, was placed outside the tube. Prior to use, the dialysis tubing was treated in accord with the recommendations of Cooper (1977). The starting molar gradient of β-CD (Mβ-CD) to β-casein was 150:1, and that for Mβ-CD to β-casein was 112:1. $CaCl_2$ was not present in these samples.

To determine the concentration of β-CD or Mβ-CD in the inner and outer solutions, the phenol–sulfuric acid assay was used (Dubois et al., 1956). Two drops of the 80% phenol solution was added to 1 mL of the sample and thoroughly mixed, and 2.5 mL of concentrated sulfuric acid was added. The tubes were allowed to stand 10 min, shaken, and cooled to 25–30 °C, and absorbance at 490 nm was measured. Blanks consisted of phosphate buffer solution. The concentrations of β-CD or Mβ-CD were determined from standard curves prepared at room temperature.

Statistical Analysis. Data were analyzed by one- or two-way ANOVA (analysis of variance), and either Fisher's LSD (least significant difference) T test or Tukey's test was used to determine significance of differences between pairs. Significance of differences is reported at the 95% level of confidence.

RESULTS AND DISCUSSION

Effect of β-Cyclodextrin and Incubation Time on Aggregation of β-Casein at 31 °C. The effect of incubation times, ranging from 2 to 20 h at 31 ± 1 °C, and a molar ratio of 8:1 β-CD to β-casein on the ability of β-CD to inhibit aggregation of β-casein is shown in Figure 1. The behavior of control samples containing all constituents except β-CD is also shown in Figure 1. All samples contained 0.002% NaN_3 to prevent growth of microorganisms.

The most important point from Figure 1 is that β-CD, regardless of incubation time, exerted a significant ($P < 0.05$) inhibitory effect on aggregation of β-casein (31 °C data) as compared to that exhibited by the appropriate control sample (no β-CD). Also, solubility of β-casein (percent of the total β-casein present in the supernatant fluid) decreased significantly ($P < 0.05$) during 20 h at 31 °C in both the treated and control samples. These decreases in absorbance with time undoubtedly indicate

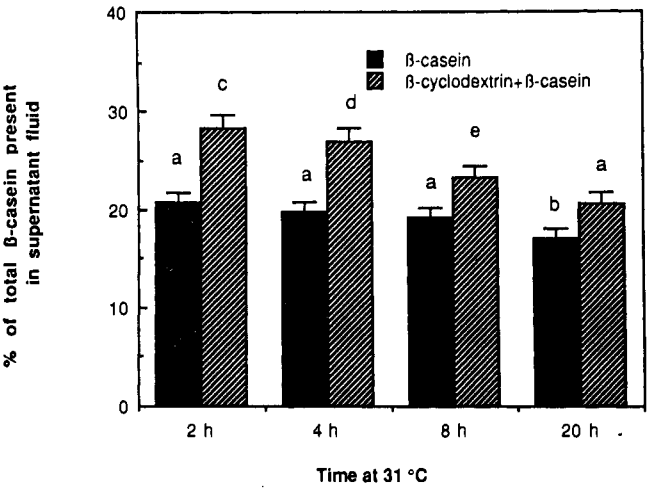

Figure 1. Effect of incubation time at 31 °C on the ability of β-cyclodextrin to inhibit aggregation of β-casein at 31 °C. Error bars indicate standard deviations. Data in columns headed by a common letter do not differ significantly ($P < 0.05$).

Table II. Effect of the Molar Ratio of β-Cyclodextrin to β-Casein on the Ability of β-Cyclodextrins To Inhibit Aggregation of β-Casein during a 2-h Holding Period at 31 °C

concn of β-cyclodextrin, M	molar ratio, β-cyclodextrin: β-casein[a]	% of total β-casein present in the supernatant fluid
0	0:1 A[b]	10
1.25×10^{-4}	0.3:1 B	22
2.50×10^{-4}	0.6:1 B	23
4.17×10^{-4}	1:1 B	25
8.34×10^{-4}	2:1 B	25
2.09×10^{-3}	5:1 B	25
3.34×10^{-3}	8:1 B	25
4.17×10^{-3}	10:1 B	25

[a] β-Casein concentration 4.17×10^{-4} M in all samples. [b] Values with suffixes not containing a common letter differ significantly ($P < 0.05$).

that the aggregation process of β-casein was still continuing after 20 h at 31 °C. On the basis of these results, a 2-h incubation time at 31 °C was generally used in the remaining studies.

Effect of Molar Ratio of β-Cyclodextrin to β-Casein on Aggregation of β-Casein at 31 °C. In this experiment, the β-CD to β-casein molar ratio was varied from 0:1 to 10:1. The sonification treatment at 3 °C was omitted for this study. From the 31 °C results in Table II, it is evident that the percent β-casein in the supernatant fluid was 10% in the control sample (no β-CD) and increased significantly ($P < 0.05$) to 22% in the sample containing a β-CD to β-casein ratio of 0.3:1. Further increases in the ratio of β-CD to β-casein caused no significant ($P < 0.05$) increase in the percent β-casein in the supernatant fluid. This result is interesting since it indicates that the full effectiveness of β-CD is achieved, on average, when less than one molecule of β-CD interacts with one molecule of β-casein.

It is noteworthy that the control sample at 31 °C (no β-CD) had a β-casein concentration that was about half that obtained in other experiments (see Figures 1–5). This low value probably occurred because samples in this experiment were not sonified.

β-Cyclodextrin–β-Casein Interaction As Influenced by Pretreatment at pH 8–11. The possibility that interaction between β-CD and β-casein could be improved by manipulation of pH was explored by testing pH values of 8, 9, 10, and 11 (Figure 2). These were "intermediate"

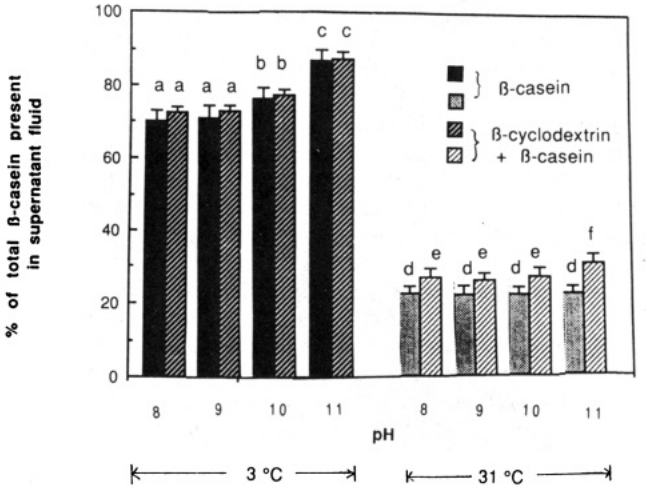

Figure 2. Effect of pH on the ability of β-cyclodextrin to inhibit aggregation of β-casein at 31 °C. Values of pH shown are intermediate pH values. Error bars indicate standard deviations. Data in columns headed by a common letter do not differ significantly ($P < 0.05$).

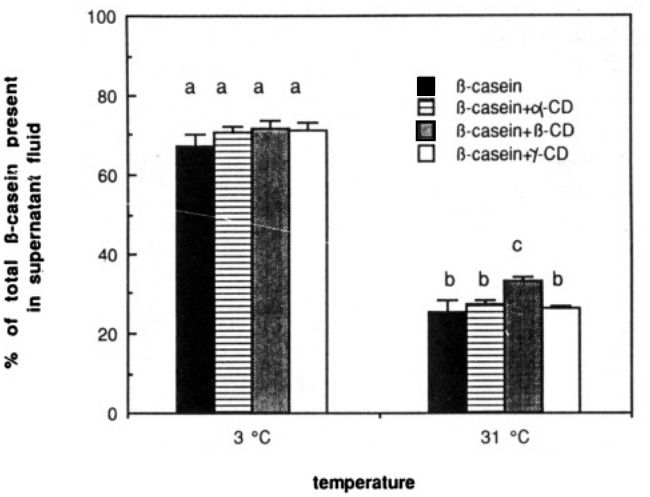

Figure 3. Comparative abilities of α-, β-, and γ-cyclodextrins to inhibit aggregation of β-casein at 31 °C. Error bars indicate standard deviations. CD is cyclodextrin. Data in columns headed by a common letter do not differ significantly ($P < 0.05$).

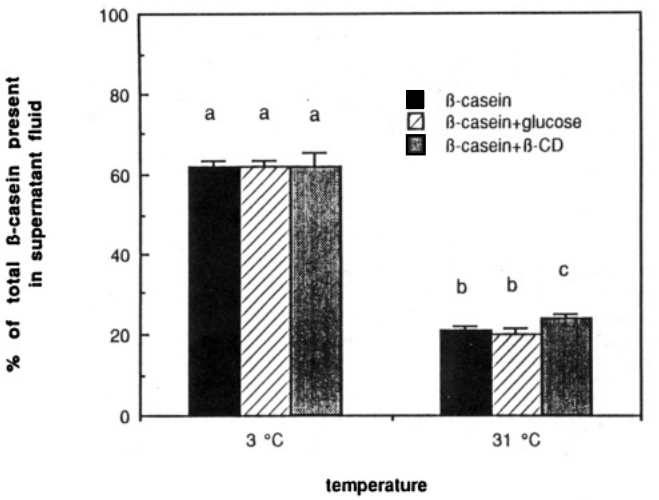

Figure 4. Comparative ability of glucose and β-cyclodextrin to inhibit aggregation of β-casein at 31 °C. Error bars indicate standard deviations. CD is cyclodextrin. Data in columns headed by common letters do not differ significantly ($P < 0.05$).

pH values; i.e., the pH was first adjusted to 6 at 3 °C, then to the desired intermediate pH for 2 h at 3 °C, then back to pH 6.0 at 3 °C, followed by elevation of the temperature to 31 °C.

As expected, β-casein was significantly ($P < 0.05$) more stable at 3 °C than at 31 °C (Figure 2). Furthermore, at 3 °C, the presence or absence of β-CD had no significant effect ($P < 0.05$) on β-casein solubility, and changing the intermediate pH over the range 8–11 had no significant effect on β-casein except for pH 11.0, which significantly improved ($P < 0.05$) β-casein solubility.

At 31 °C, the presence of β-CD had a significant ($P < 0.05$) stabilizing effect regardless of the intermediate pH, but the stabilizing effect was significantly ($P < 0.05$) greater following the pH 11.0 treatment than it was following treatment at the other intermediate pH values.

The beneficial effect of using an intermediate pH of 11 probably occurred because β-casein assumed a greater net negative charge, unfolded, and interacted more effectively with β-CD. Inoue and Miyata (1981), studying phenylalanine–β-CD inclusion complexes, pH 2 and 11.3 at 34 ± 1 °C, also found that exposure to pH 11.3 ± 0.1 favored formation of a strong complex.

Comparative Abilities of α-, β-, and γ-Cyclodextrins To Inhibit Aggregation of β-Casein at 31 °C. A point of obvious interest is the comparative abilities of α-, β-, and γ-CDs to stabilize β-casein. These CDs have different cavity sizes, and this should have a bearing on their ability to form inclusion complexes with β-casein.

Each of the three types of CDs was mixed with β-casein at an 8:1 molar ratio and tested according to the standard procedure. At 3 °C, the three CDs did not differ significantly ($P < 0.05$) in their ability to solubilize β-casein (Figure 3). At 31 °C, β-CD exhibited a significantly greater solubilizing effect on β-casein than either α- or γ-CD, and the latter two solubilized β-casein to an extent that did not differ significantly ($P < 0.05$) from that of the control samples (no CD). Thus, the ability of β-CD to stabilize β-casein would appear to involve some specific attribute, such as cavity size and nature, that favors formation of an inclusion complex rather than attributes that are common to all CDs.

Comparative Abilities of Glucose and β-Cyclodextrin To Stabilize β-Casein at 31 °C. Hydrolysis of β-CD yields seven molecules in glucose. If, during the course of sample manipulation, some hydrolysis of β-CD occurs, this could be a factor in the β-casein solubilizing influence of β-CD. To determine the effect of possible hydrolysis, samples containing β-CD or an equivalent amount (seven times the molar concentrations of β-CD) of glucose were prepared and tested. These samples contained an 8:1 molar ratio of β-CD to β-casein or a 56:1 molar ratio of glucose to β-casein.

At 3 °C, no significant difference was observed among the samples (Figure 4). At 31 °C, samples containing β-CD exhibited significantly greater ($P < 0.05$) solubilities than those devoid of β-CD (with or without glucose). The latter two types of samples did not differ significantly in their ability to solubilize β-casein at 31 °C. Thus, if β-CD does hydrolyze partially during sample handling, this cannot account for the stabilizing effect of β-CD on β-casein.

An experiment was also conducted to determine whether the stabilizing effect of β-CD on β-casein might occur because of β-CD's ability to increase the viscosity of the test medium. The viscosities of 1.668×10^{-3} M β-CD in water and 1.168×10^{-2} M glucose (56 times the molar concentration of β-CD) in water were determined at 22 °C by using an Ostwald viscosimeter. The β-CD solution had a viscosity of 0.95 ± 0.01 cP, and the glucose solution had

Table III. Equilibrium Interaction Concentrations for β-Casein with β-Cyclodextrin and Modified β-Cyclodextrin

sample type	weight of cyclodextrin at equilibrium, mg/mL at 3 °C: inside dialysis tube	outside dialysis tube	inside − outside
β-cyclodextrin + β-casein	0.389	0.300	0.089
Mβ-cyclodextrin[a] + β-casein	0.387	0.303	0.084

[a] Modified β-cyclodextrin (hydroxyethyl-β-cyclodextrin).

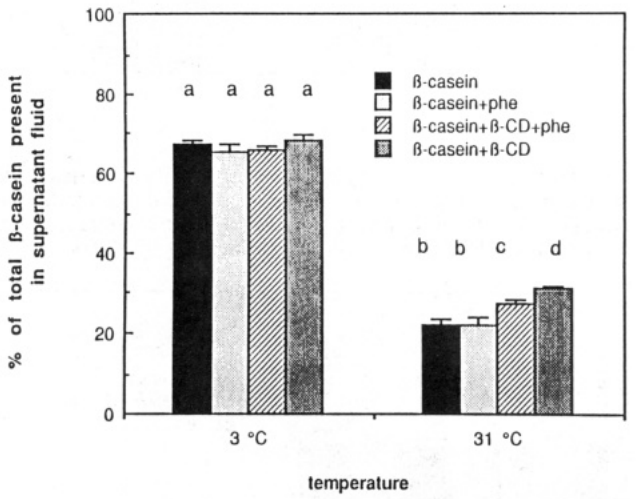

Figure 5. Effect of incubation of β-cyclodextrin with phenylalanine on the ability of β-cyclodextrin to inhibit aggregation of β-casein at 31 °C. Error bars indicate standard deviations. β-CD and Phe are β-cyclodextrin and phenylalanine, respectively. Data in columns headed by a common letter do not differ significantly ($P < 0.05$).

a viscosity of 0.98 ± 0.01 cP. Water has a viscosity of 0.9548 cP at 22 °C (Dean, 1979). Since the viscosity of the β-CD solution was slightly less than that of the glucose solution and essentially the same as that of pure water and since β-casein was solubilized more effectively by β-CD than by glucose, the solubilizing ability of β-CD cannot be attributed to its effect on viscosity.

Equilibrium Interaction Concentrations between β-Cyclodextrins and β-Casein. Equilibrium dialysis was used to determine the equilibrium interaction concentration (EIC) for β-casein with β-CD or modified β-CD (Mβ-CD). Following attainment of equilibrium, the inside concentration of CD exceeded the outside concentration by 0.089 mg/mL for β-CD and by 0.084 mg/mL for Mβ-CD (Table III). Therefore, the molar EIC was 1.88:1 for β-CD and β-casein and 1.34:1 for Mβ-CD and β-casein. This result is in fairly good agreement with the results in Table II.

Effect of Phenylalanine on the Ability of β-Cyclodextrin To Stabilize β-Casein. To help determine the mechanism by which β-CD partially solubilizes β-casein, an experiment involving phenylalanine (Phe) was devised. Phe will form an inclusion complex with β-CD (Inoue and Miyata, 1981; Kuan et al., 1985), and if formation of this complex causes β-CD to lose its ability to partially solubilize β-casein, this would be presumptive evidence that β-CD stabilizies β-casein by forming an inclusion complex with hydrophobic side groups of β-casein.

Four samples were evaluated for this experiment, and these are indicated in the inset of Figure 5. Phe, when used, was present at a concentration of 4.17×10^{-4} M. β-Casein was present at a concentration of 4.17×10^{-4} M, and β-CD, when added, was present at a concentration of 8.34×10^{-4} M. $CaCl_2$ and DMAA buffer, pH 6, were present at standard concentrations, and the standard 6–9–6 pH procedure was used.

The four samples did not differ significantly ($P < 0.05$) with respect to β-casein solubility at 3 °C (Figure 5). At 31 °C, the sample contaioning β-CD and β-casein (no Phe) exhibited significantly greater ($P < 0.05$) β-casein solubility than did the other samples. Thus, reacting Phe with β-CD before addition of β-casein did reduce significantly, but not totally negate, β-CD's ability to solubilize β-casein. If Phe had totally inhibited the solubilizing influence of β-CD, then samples containing β-casein plus β-CD plus Phe and samples containing β-casein plus Phe would not have differed significantly in β-casein solubility. A logical explanation for the 31 °C results in Figure 5 is that Phe and β-CD form an inclusion complex and that the addition of β-casein causes some, but not all, of the β-CD–Phe complexes to dissociate in favor of new β-CD–β-casein inclusion complexes. Alternative explanations are not obvious.

Throughout all of these experiments, β-CD exhibited a small, but statistically significant, ability to solubilize β-casein at 31 °C. Since β-casein possesses many hydrophobic groups, including three C-terminal amino acids (valine end two isoleucines) that are probably exposed, it seems likely that β-CD forms an inclusion complex with one or more of these groups. This result, which has not previously been reported, is interesting and potentially useful for the reasons previously stated. Although the stabilizing effect of the cyclodextrin–β-casein interaction is relatively small, it seems logical that this effect might be greater with peptides, since steric interference should be less.

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