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Caseins: Utilizing Molecular Chaperone Properties to Control Protein Aggregation in Foods

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Denaturation and aggregation of proteins are reactions that are relevant to functional applications of proteins in foods. Depending on concentration, ionic strength, and pH, aggregation can result in turbidity, precipitation, or gelation. Aggregation may be desirable, as in the case of gelation, or undesirable, as in the case when it causes phase separation in beverages. One approach to improve the stability of globular proteins against heat stresses is through the addition of other compounds that alter aggregation. Numerous studies have shown the ability of molecular chaperones to assist proper folding/unfolding and assembly/disassembly of proteins, especially during stressed conditions. Recently, several papers have reported the molecular chaperone-like properties of caseins, especially using α_s- and β-caseins. Caseins appear to function like small heat shock proteins (sHSP). We have compared the results among investigations from the perspective of food processing conditions and related them to the mechanism for sHSP. Caseins possess three of the four common features among sHSP; lacking a similar sequence domain. Their function may be explained in part by having structures fitting the intrinsically unfolded class of proteins. With a few exceptions, most investigations were done at solution conditions that poorly represent foods; lacking investigations at pH < 4.5 and concentrations above 20 mg/mL. While it is clear that caseins can alter aggregation at neutral pH, their effectiveness at low pH, high protein concentration, and high thermal treatment ($T \geq 100$ °C) remains to be fully established.

KEYWORDS: β-Casein; α_s-casein; molecular chaperone; intrinsically unstructured protein

INTRODUCTION

Proteins are structurally sensitive macromolecules that constantly face stresses from their environments. One challenge in developing protein-based beverages is that thermal processing (i.e., heat stress) can cause undesirable levels of aggregation leading to excessive turbidity and possibly precipitation or gelation. In biological systems, proteins that inhibit undesired aggregation are called molecular chaperones. Caseins, the major protein found in milk, have been reported to have molecular chaperone properties (*1*).

Three decades ago the term molecular chaperone was first used by Laskey et al. (*2*) to describe the ability of thermostable nucleoplasmin to prevent the aggregation of folded histone proteins with DNA during the assembly of nucleosomes. However, it was only in 1987 that the initial concept of molecular chaperone was established by R. John Ellis (*3*), extending the possibility of many proteins to be identified as molecular chaperones. The term molecular chaperone has been continually reviewed for its precise meaning and usefulness (*4*−*8*). Molecular chaperones are defined as "a large and diverse group of proteins that share the functional property of assisting the non-covalent folding/unfolding and the assembly/disassembly of other macromolecular structures, but are not permanent components of these structures when they are performing their normal biological functions" (*7*, *8*). On the basis of this definition, only two criteria need to be fulfilled by a macromolecule to be assigned as a molecular chaperone (*6*, *9*). The first is that it must assist the noncovalent assembly or disassembly of other macromolecular structures regardless of the mechanism(s) involved. Second, it must not be a permanent component of these structures during their normal biological functions. While the latter is essential to biological function, it may not be required in food applications.

The chaperone concept has had a major impact. On the basis of searches using Entrez PubMed, total publications with the term molecular chaperone(s) in titles or abstracts alone have grown to more than 4200 in 2008 (**Table 1**). The first claim that caseins have molecular chaperone properties was by Bhattacharyya and Das in 1999 (*1*). The study was related to the observation that α_s-caseins (a combination of α_{s1}- and α_{s2}-caseins) have micellar structure that is similar to the oligomeric structures of many molecular chaperones. They were shown to decrease the aggregation (turbidity) of proteins caused by heating (40−70 °C) or chemical modification (disulfide cleavage and photoaggregation). Since then, our search has found 18 publications (including one review paper) that describe various molecular chaperone properties of caseins (as in July 2009) (**Table 2**). Most of the studies were published in the last five years (2005−2009). Among the studies,

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as shown in **Table 2**, 13 involved α_s-casein (*1*, *10−21*), while 14 investigated β-casein (*10−13*, *15−19*, *21−25*). Although caseins in other forms have also been studied, they are much fewer and include κ-casein (*11*, *26*), micellar casein (*13*), whole casein (*25*), and sodium caseinate (*26*).

Since α_s- and β-caseins are the two most studied caseins for their molecular chaperone properties, further discussions will focus on these two proteins. The majority of substrate proteins investigated thus far are globular proteins (including enzymes), although a few nonglobular proteins from milk have been studied, namely, κ- (*12*, *21*) and α_{s2}-caseins (*16*). While most investigations focus on suppression of amorphous or particulate type aggregation, studies using κ- and α_{s2}-caseins investigated the prevention of amyloid fibrils. Chaperone effects on inhibiting amyloid fibril formation have been reviewed recently (*19*) and are beyond the scope of this article. The review by Thorn et al. (*19*) is primarily focused on biological functions and chaperone effects in relation to protein misfolding diseases. The review also briefly concluded that such chaperone activity is important to proteins during food processing. Here, we will discuss in detail the potential importance of α_s- and β-casein chaperone functions in food systems, taking account of different conditions commonly used in food processing. We will only cover amorphous type aggregation (common in food processing) and focus on the chaperone effects of α_s- and β-caseins at different protein concentrations, types of stresses, as well as solution conditions such as pH and ionic strength. These are all key factors to determining the relevance to food systems.

Table 1. Papers Published with the Term Molecular Chaperone(s) in the Title and/or Abstract from 1987−2008[a]

year	no. of papers	year	no. of papers
1987	1	1998	230
1988	1	1999	256
1989	4	2000	233
1990	17	2001	283
1991	28	2002	270
1992	45	2003	305
1993	82	2004	356
1994	137	2005	322
1995	173	2006	357
1996	206	2007	342
1997	210	2008	368

[a] The PubMed search engine (http://www.ncbi.nlm.nih.gov/sites/entrez/) was used to determine the number of papers published each year that contain the term molecular chaperone or molecular chaperones in the title or abstract. Chaperone or chaperones alone was not used for the search to avoid inclusion of medical papers that refer to human rather than molecular activities. Therefore, the actual numbers regarding molecular chaperone(s) are higher when including those with chaperone or chaperones.

EXPERIMENTAL PROCEDURES

Sources of α_{s1}- and β-Caseins. Although milk can be obtained from all types of mammals, so far only caseins from bovine (*Bos taurus*) and camel (*Camelus dromedarius*) have been investigated (**Table 2**). For the most part, these proteins have not been purified directly from milk but directly purchased from commercial sources. Most of the α_s- and β-caseins used were from Sigma-Aldrich and used with or without further purification. In addition, some studies have used caseins purified in their laboratories (*13*, *16*, *20*, *23−25*) or supplied by other sources (*18*). Despite the different sources of α_s- and β-caseins, they showed the ability to act as molecular chaperones; although chaperone activity varied.

Specific Proteins Investigated. Globular proteins are folded into a native state that is only 8−42 kJ mol^{-1} more stable than their unfolded states (*27*). The native state is held by a number of weak noncovalent forces such as hydrogen bonds and hydrophobic interactions, as well as covalent disulfide bonds in some proteins (*28*). Globular proteins used as chaperone substrates are whey proteins (β-lactoglobulin, α-lactalbumin, and bovine serum albumin) (*1*, *11*, *13*, *18*, *21*, *26*), ovotransferrin, and ovalbumin from egg (*10*, *21*, *25*), β- and γ-crystallins from the eye lens (*1*), insulin (*1*, *20*, *22*), tubulin (*14*), and beef sarcoplasmic protein (*17*). Whey and egg proteins are common food ingredients that are used in processed foods; therefore, understanding how caseins can improve the stability of these proteins under stressed conditions will provide information on how they can be used in various applications.

Alcohol dehydrogenase is the most frequently used enzyme to monitor the chaperone activity of α_s- and β-caseins (*1*, *21−24*). Other enzymes studied are carbonic anhydrase (*1*, *25*), lysozyme (*22*), α-chymotrypsin (*15*), μ-calpain, and MMP13 enzyme (*17*).

Table 2. Type of Caseins, Substrate Proteins, and Stresses Used in Papers Published with Studies Related to Caseins as Molecular Chaperone from 1999−2008[a]

			caseins				milk	substrates[d]				stresses
	authors	year	α_s[b]	β	κ	others[c]	sources	wheys	eggs	enzymes	others	H/C/L[e]
1.	Bhattacharyya and Das	1999	yes				Bovine	yes		yes	yes	H, C, L
2.	Matsudomi et al.	2004	yes	yes			Bovine		yes			H
3.	Zhang et al.	2005		yes			Bovine			yes	yes	H, C
4.	Morgan et al.	2005	yes	yes	yes		Bovine	yes			yes	H, C
5.	Thorn et al.	2005	yes	yes			Bovine				yes	C
6.	O'Kennedy and Mounsey	2006	yes	yes		yes	Bovine	yes				H
7.	Mitra et al.	2007	yes				Bovine				yes	C
8.	Rezaei-Ghaleh et al.	2008	yes	yes						yes	yes	H
9.	Barzegar et al.	2008		yes			Bovine, Camel			yes		H
10.	Hassanisadi et al.	2008		yes			Bovine, Camel			yes		H
11.	Thorn et al.	2008	yes	yes			Bovine				yes	C
12.	Khodarahmi et al.	2008		yes		yes	Bovine		yes	yes		H, C
13.	Pulford et al.	2008	yes	yes			Bovine			yes	yes	H
14.	Yong and Foegeding	2008	yes	yes			Bovine	yes				H
15.	Thorn et al. (review)	2009										
16.	Guyomarc'h et al.	2009			yes	yes	Bovine	yes				H
17.	Badraghi et al.	2009	yes				Camel				yes	C
18.	Koudelka et al.	2009	yes	yes			Bovine	yes	yes	yes	yes	H, C

[a] Search engines PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez/), Scopus (http://www.scopus.com/home.url), and Web of Knowledge (http://isiwebofknowledge.com/) were used to determine the published papers that correlated caseins to their molecular chaperone properties. The search was accurate as of July 2009. [b] α_s-caseins used were a combination of α_{s1}- and α_{s2}-caseins except for study numbers 6 (α_{s1}/β-casein), 11, and 17 (α_{s1}-casein). [c] Non-purified casein was used in study numbers 6 (micellar casein), 12 (whole casein), and 16 (sodium caseinate). [d] Type of substrates: whey proteins (whey protein isolate, β-lactoglobulin, α-lactalbumin, and bovine serum albumin), eggs (ovotransferrin and ovalbumin), enzymes (alcohol dehydrogenase, carbonic anhydrase, catalase, lysozyme, α-chymotripsin, μ-calpain, and MMP13 enzyme), and others (insulin, Crystallin, κ-casein, α_{s2}-casein, tubulin, and sarcoplasmic protein). [e] Type of stresses: heat (H), chemical (C), and light (L).

Protein Concentrations. The details of the studies listed in **Table 2** are shown in **Table 3**. The concentrations of caseins used were from 0.01 mg/mL (*1*) to 28.6 mg/mL (*11*). The substrate protein concentrations were 0.05 mg/mL (*15*) to 60 mg/mL (*18*), giving a total protein concentration from 0.1 mg/mL (*15*) to 80 mg/mL (*18*). Total protein concentration is important because as the concentration increases, the complexity of molecular crowding effects become more of a factor (*9*). Our study provided an example of where casein chaperone ability was observed at protein concentrations as high as 80 mg/mL. A chaperone effect (i.e., reduction in turbidity) was observed in solutions containing 20 mg/mL α_s- or β-casein and 60 mg/mL β-lactoglobulin, after heating at 70 °C for 20 min (*18*). This is significant as it demonstrated that casein could be used as a chaperone at protein concentrations desirable/necessary for the food industry.

Type of Stresses. Three types of stresses generally applied are thermal, chemical, and light. Among these, high temperature was one of the first used and continues to be the main stress applied to evaluate chaperone activity (*29*). This is directly relevant to foods where thermal processing for safety and thermal stability (i.e., no undesirable physical changes) is required for product quality. The majority of the studies in **Table 3** used a heat treatment (40−90 °C) for 5 to 480 min to cause protein denaturation and aggregation. Chaperone activity of caseins changes with temperature. At 70 °C, both α_s- and β-caseins have chaperone activity, while only β-casein maintained the chaperone behavior at 75 to 90 °C (*18*). Similar results were observed with sarcoplasmic proteins, in that β-casein was a stronger chaperone than α_s-casein and maintained activity at > 65 °C (*17*). Our recent investigations with β-casein have shown that chaperone activity is maintained up to 145 °C (unpublished data), making this viable for ultra high temperature processing. In a study using bovine and camel β-caseins, bovine β-casein displayed greater chaperone ability in delaying the onset of aggregation temperature and had a greater effect on reducing aggregate formation (*23*). In these cases, the higher effective hydrophobic surfaces of bovine β-casein as compared to α_s-casein (*30*) and camel β-casein (*23*) might have played an important role.

Besides heat, reducing agents (dithiothreitol, DTT (*31*)) and denaturants (guanidinium chloride, GuHCl (*32*)) were also used to chemically destabilize substrate proteins for studying casein chaperone activity. Disulfide bonds in insulin (*1*, *11*, *20*, *22*), α-lactalbumin (including apo- and holo-forms) (*11*, *21*), and lysozyme (*22*) were reduced using DTT, or 6 M GuHCl was used to denature carbonic anhydrase (*25*) at 18−37 °C. In the case of 6 M GuHCl, aggregation was observed when the denaturant was diluted 50-fold. Like heat treated substrate proteins, addition of α_s- or β-caseins decreased the turbidity of these solutions significantly in a concentration dependent mode. However, the prevention of chemically induced aggregation by α_s- and β-casein also seemed to differ from that observed with thermal stress. While α_s-casein increased its chaperone activity on DTT-reduced substrates when the temperature dropped from 37 to 25 °C or lower (*1*, *11*), β-casein showed similar chaperone ability at 25 and 37 °C. The behavior of α_s-casein was correlated to cold shock proteins (*1*), whereby decreasing temperature results in increasing of certain activities (*33*). It is also noteworthy to mention that for DTT-induced aggregates, when α_s- or β-casein was added after about half of the turbid aggregates were formed, not only was further insoluble aggregate formation prevented but also some of the formed aggregates were resolubilized (*1*, *22*).

Ultraviolet light-stressed aggregation is another option for studying molecular chaperone ability (*34*); however, only one study has used this approach with caseins (*1*). It showed that the addition of α_s-casein to UV-light-stressed γ-Crystallin completely prevented the formation of turbid aggregates.

pH and Ionic Strength. Structural and functional properties of proteins are greatly influenced by electrostatic interactions; therefore, the effects of pH and ionic strength have been extensively studied (*35*−*44*). A pH range of 6.0 to 7.5 is commonly used for heat-stress studies (*1*, *10*, *11*, *15*, *17*, *18*, *21*−*25*) with the exception of one where a lower pH range (5.55 to 6.40) was investigated (*13*). DTT-induced aggregation studies were conducted in a pH range of 7.0−7.4 (*1*, *11*, *12*, *20*−*22*), and GuHCl-induced aggregation was at pH 7.75 (*25*). The ionic contribution would depend on the buffering system and minerals inherent in the protein preparations. For most proteins investigated, the pH range of 6.6−7.5 was too high (i.e, above the isoelectric point) to show much aggregation at low ionic strength; therefore, ions in the range of 0.1−0.2 M NaCl (*11*, *21*, *26*) or 0.5 mM $MgCl_2$ (*14*) were added in addition to a buffer (10−50 mM phosphate buffers were the most common) to increase aggregation. The overall conclusion was that α_s- and β-caseins exhibit their chaperone ability to completely or partially reduce the turbidity of studied samples regardless of the pH values tested (5.55−7.75). The effect on aggregation at lower pH values remains to be established.

In the preceding discussion, the ionic conditions were fixed while evaluating chaperone ability; which raises the question, what happens to the chaperone ability of caseins when salts are added to increase ionic strength? A few of the studies listed in **Table 3** addressed this question. Ionic strengths were created either using increasing NaCl concentrations (*10*, *25*) or different types of salt using NaCl, $NaNO_3$, and KCl (*20*). There were contrasting results in the chaperoning ability of α_s- and β-caseins with increasing ionic strength. Ionic strength weakened the chaperone activity of α_s-casein toward ovotransferrin, which suggests that electrostatic interactions are involved in chaperone activity (*10*). Also, camel α_{s1}-casein loses its chaperone activity in solutions containing 0.2 M NaCl, $NaNO_3$, or KCl (*20*). The loss of chaperone ability of α_{s1}-casein could be related to conformational changes in α_{s1}-casein, the aggregating protein, or both. Alternatively, it could be due to a more general electrostatic attraction mechanism. The latter is consistent with synthetic nanoparticle chaperones based on electrostatic interactions (*45*). In contrast, adding up to 0.1 M NaCl only slightly decreased the chaperone ability of β-casein toward ovalbumin (*25*). On the basis of these results, it appears that the importance of ionic interactions will vary among the casein molecules and possibly with the substrate protein. This is of particular relevance to thermal stability in protein-fortified beverages where minerals are often added for nutritional purposes.

DISCUSSION

α_s-Casein versus β-Casein. There appears to be differences in the degree of activity of α_s- and β-caseins expressed under various conditions. Plausible reasons for these differences might be found in differences in primary structure. Complete primary sequences of bovine α_{s1}- (*46*), α_{s2}- (*47*), and β-caseins (*48*) as well as camel β-casein (*49*) were used to obtain amino acid composition (**Table 4**). Monomers of these caseins have similar molecular weight range of 23.6−25.0 kDa. However, they are quite different in their phosphoserine (*SerP*) and proline (*Pro*) residues. α_{s1}- and α_{s2} caseins have more phosphoseryl and much less prolyl residues than the bovine and camel β-caseins.

Commonly, these caseins have at least one *-SerP-SerP-SerP-Glu-* peptide in their primary structures. This peptide is negatively charged and is very different from the remaining parts of the protein chain that have many hydrophobic residues (*30*). Because of the distinctive charged and hydrophobic regions, monomers of caseins are amphiphilic in nature. The different sequential patterns of polar and nonpolar amino acids and varied content of phosphoseryl residues, causes the overall hydrophobicity of bovine caseins to decrease in $\beta > \alpha_{s1} > \alpha_{s2}$ order (*30*).

Another important structural feature is the high amount of proline, especially in β-casein. The proline residues introduce kinks or bends into proteins causing distortion of α-helices and β-sheet formation (*30*, *50*, *51*), giving caseins their open structure. Caseins do not have well-defined secondary and tertiary structures (*1*); therefore, they are considered neither globular nor fibrous in nature (*52*). They are categorized as intrinsically unstructured proteins, a group of proteins that can exist and function without a well-defined folded structure (*53*−*55*).

A couple of studies have modified the degree of phosphorylation of α_s- and β-caseins to understand the importance of phosphorylation on chaperone activity (*10*, *21*). The results produced two important conclusions. First, α_s- (*10*, *21*) and β-caseins' (*21*) chaperone ability was weakened by dephosphorylation. Second, the phosphopeptide (residues 1−25) from β-casein, which is highly hydrophilic and not amphiphilic, failed to show any

Table 3. Papers Published with Caseins as Molecular Chaperone from 1999−2008

Paper (reference)	Casein/Source (in mg/mL, unless specified)	Substrate Protein (in mg/mL, unless specified)	Temperature (°C)/ Time (min, unless stated otherwise)	pH	Assay of aggregation of substrate protein	Buffers
Bhattacharyya and Das (*1*)	α_s-casein[a] (0.1)	alcohol dehydrogenase (0.4)	40/∼35	7.0	OD[b] at 400 nm	10 mM Pi buffer[c]
	α_s-casein[a] (0.03−0.06)	β_L-Crystallin (0.2)	60/∼33	7.0	OD at 400 nm	
	α_s-casein[a] (0.35)	carbonic anhydrase (0.1)	60/∼9	7.0	OD at 400 nm	
	α_s-casein[a] (0.4)	whey protein isolate (0.5)	70/∼58	6.6	OD at 400 nm	
	α_s-casein[a] (0.5)	bovine serum albumin (0.5)	70/30	6.6	OD at 400 nm	
	α_s-casein[a] (4−6)	α-lactalbumin (2) + β-lactoglobulin (2)	70/5	7.0	Gel filtration at 280 nm	
	α_s-casein[a] (0.01, 0.1)	insulin B-chain (0.25)	27/∼60, DTT[d]	7.0	OD at 400 nm	
	α_s-casein[a] (0.4)	γ-Crystallin (0.2)	UV-light[e]	7.0	scattering[f] at 295 nm	
	α_s-casein[a] (0.7)	insulin (0.35)	DTT	7.0	OD at 400 nm	
Matsudomi et al. (*10*)	α_s-casein[a] (0.1−1)	ovotransferrin (0.5)	80/20	7.0	OD at 500 nm	10 mM Pi buffer
					electrophoresis[g]	(0−100 mM NaCl)
	dephosphorylated α_s-casein[h] (0.1−1)				OD at 500 nm	10 mM Pi buffer
	β-casein[a] (0.1−1)					
	β-casein phosphopeptide[h] (0.1−1)					
Zhang et al. (*22*)	β-casein[a] (0.5−1)	alcohol dehydrgenase (1)	50/∼27	7.4	OD at 360 nm	50 mM Pi buffer
	β-casein[a] (0.2−0.5)	catalase (0.8)	60/∼27		OD at 360 nm	
	β-casein[a] (10)	catalase (10)	60/30		SEC[i] at 280 nm, SDS−PAGE	
	β-casein[a] (0.3−0.9)	insulin (0.5−0.6)	37/∼18, DTT		OD at 360 nm	
	β-casein[a] (0.2−0.4)	lysozyme (1)	37/20, DTT		OD at 360 nm	
	β-casein[a] (0.4), α_s-casein[a] (0.4)	lysozyme (0.94)	37/NA[j], DTT		OD at 360 nm	
		catalase (6.5)	55/NA		OD at 360 nm	
Morgan et al. (*11*)	α_s-casein[a] (0.5−5)	β-lactoglobulin (5)	70/480	7.1	OD at 360 nm	50 mM Pi buffer, 0.1−0.2 M NaCl, 2.5 mM EDTA[l]
	α_s-casein[a] (20)	β-lactoglobulin (10)	70/180	7.2	size exclusion HPLC[k]	50 mM imidazole buffer, 0.1 M NaCl, 5 mM $CaCl_2$
	α_s-casein[a] (20)	apo α-lactalbumin (10)	25/120, DTT	7.1	OD at 360 nm	
	α_s-casein[a] (0.0125−0.5)	insulin (0.25)	25, 37/120, DTT	7.2	real-time ^{1}H NMR[m]	
	β-casein[a] (0.0125−0.5)	apo α-lactalbumin (14.3)	37/∼25, DTT	7.1	OD at 360 nm	
	κ-casein[a] (0.0125−0.5)	apo α-lactalbumin (2)	25, 37/360, DTT	7.1	OD at 360 nm	
	α_s-casein[a] (28.6)	holo α-lactalbumin (2)	25, 37/360, DTT			
	α_s-casein[a] (0.5−8)					
	α_s-casein[a] (0.5−12)					
Thorn et al. (*12*)	α_s-casein[a] (1.88−11.25)	reduced κ-casein[m] (3)	37/40 h, DTT	7	thioflavin T (ThT) fluorescence at 490 nm	50 mM Pi buffer, 10 μM ThT, 0 or 20 mM DTT
		nonreduced κ-casein (3)				
	β-casein[a] (1.88−11.25)	reduced κ-casein[m] (3)				
		nonreduced κ-casein (3)				
	α_s-casein[a] (7.5)	reduced κ-casein[m] (3)	37/20 h, DTT		transmission electron microscope	50 mM Pi buffer, 10 μM ThT, 20 mM DTT
	β-casein[a] (7.5)					
O'Kennedy and Mounsey (*13*)	α_{s1}/β-casein[h] (0−0.5% w/w)	whey protein isolate	85/10	6	OD at 600 nm	deionized water, 1 N NaOH or 1N HCl
	α_{s1}/β-casein[h] (0.2% w/w)	(0.5% w/w)		5.55−6.4	OD at 600 nm	simulated milk ultrafiltrate
	micellar casein[h] (0.5% w/w)			6	OD at 600 nm,	
	micellar casein[h] (0.1−0.5% w/w)			5.5−6.0	particle size analysis	
					particle size analysis	
Mitra et al. (*14*)	α_s-casein[a] (0−15 μM)	tubulin (1.65)	37/30 (NT)	7.0	percent polymerization at 360 nm	50 mM PIPES[n] buffer, 1 mM EGTA[n], 0.5 mM $MgCl_2$, 1 mM GTP[n]
Rezaei-Ghaleh et al. (*15*)	α_s-casein[a] (0.5−2)	PMSF-inhibited[o]	65/30	7.0	OD at 350 nm	50 mM KPi buffer
	β-casein[a] (0.05−0.5)	α-chymotripsin (0.05)				
Barzegar et al. (*23*)	bovine β-casein[h] (0.014−0.14)	alcohol dehydrogenase (0.14)	48/60	7.5	OD at 360 nm,	50 mM Pi buffer
	camel β-casein[h] (0.014−0.14)				degree of aggregation, A/A_0	
	bovine β-casein[h] (0.03)		45−60/60		OD at 360 nm,	
	camel β-casein[h] (0.06)				aggregation rate constant, k_t	
	bovine β-casein[h] (0.014−0.14)		20−90/NA		OD at 360 nm,	

Table 3. Continued

Paper (reference)	Casein/Source (in mg/mL, unless specified)	Substrate Protein (in mg/mL, unless specified)	Temperature (°C)/ Time (min, unless stated otherwise)	pH	Assay of aggregation of substrate protein	Buffers
	camel β-casein[h] (0.014−0.14)				thermal scanning derived parameters, T_{oa}, T_{ma}, Agg_{max}, k_t	
Hassanisadi et al. (*24*)	bovine β-casein[h] (0.15)	alcohol dehydrogenase (0.15)	50/45	7.5	OD at 360 nm	50 mM Pi buffer
	camel β-casein[h] (0.15)					
	bovine β-casein[h] (0.03)		40−60/∼46		MCR-ALS[p] calculated concentration profiles	
	camel β-casein[h] (0.03−0.06)					
Thorn et al. (*16*)	α_{s1}-casein[a] (1.4−8.5)	α_{s2}-casein (1.4−8.5)	37−50/160 h, DTT	7.0	ThT fluorescence at 490 nm,	50 mM Pi buffer
	β-casein[a] (1.4−8.6)				electron micrograph	
Khodarahmi et al. (*25*)	whole casein[a] (0.1−2.0)	carbonic anhydrase (0.2)	25/5, GuHCl[q]	7.75	OD at 400 nm, extent and initial rate of aggregation	20 mM Tris-sulfate
	whole casein[a] (0.2−2.0)	carbonic anhydrase (0.2)	25/2, 24 h, GuHCl	7.75	activity recovery	
	whole casein[a] (0.5)	carbonic anhydrase (0.2−0.6)	25/10, GuHCl	7.75	OD at 400 nm, apparent aggregation reaction order	
	β-casein[h] (0.5−2)	carbonic anhydrase (0.2)	25/∼6, GuHCl	7.75	OD at 400 nm, Extent of aggregation	
	whole casein[a] (0.015−1)	ovalbumin (1)	80/∼23	7.0	OD at 400 nm	50 mM NaPi buffer
	β-casein[h] (0.5−2)	ovalbumin (1)	80/∼23	7.0		
	whole casein[a] (1)	ovalbumin (1)	80/20	7.0	OD at 400 nm	50 mM NaPi buffer (0−100 mM NaCl)
	β-casein[h] (1)	ovalbumin (1)	80/20	7.0		
Pulford et al. (*17*)	β-casein[a] (4)	sarcoplasmic protein (2)	25−90/10	7.4	OD at 360 nm	25 mM Pi buffer
	α_s-casein[a] (4)				SDS−PAGE	
	β-casein[a] (NA)					
	α_s-casein[a] (NA)	μ-calpain and MMP13 enzymes in sarcoplasmic protein (NA)	25−58/10	∼7.0	enzyme activity (fluorescent measurements at 405 and 485 nm)	complex (refer to original paper)
Yong and Foegeding (*18*)	β-casein[a] (0.1−20)	β-lactoglobulin (60)	70−90/20	6.0	OD at 400 and 600 nm	deionized water, 1 N HCl
	β-casein[r] (20)					
	α_s-casein[a] (20)					
	β-casein[a] (20), α_s-casein[a] (20)		90/120		OD at 400 and 600 nm	
	β-casein[a] (20), α_s-casein[a] (20)		75/20 and 90/10		SEC-MALS[s]	
Thorn et al. (*19*)	review paper					
Guyomarc'h et al. (*26*)	κ-casein[h] (0−10)	whey protein isolate (0−25)	80/24 h	7.0	OD at 600 nm	distilled water with 0.1 M NaCl, 3 mM NaN_3
	sodium caseinate[r] (0−50)				static and dynamic light scattering	
					SEC-RP-HPLC[t]	
Badraghi et al. (*20*)	camel α_{s1}-casein[h] (0.02−0.15)	insulin (0.5)	37/50, DTT	7.0	OD at 360 nm	10 mM Pi buffer
Koudelka et al. (*21*)	α_s-casein[a]	α-lactalbumin (2)	37/360, DTT	7.1	scattering at 340 nm	2 mM EDTA and 0.1 NaCl
	β-casein[a]	ovotransferrin (0.5)	60/240	7.4	scattering at 340 nm	50 mM Pi buffer
	dephosphorylated α_s-casein[h]	alcohol dehydrogenase (1)	42/120	7.4	scattering at 340 nm	2 mM EDTA
	dephosphorylated β-casein[h] (molar ratios of casein: substrate are 0.5:1 to 3:1)	reduced and carboxymetylated κ-casein (1)	37/> 20 h	7.4	ThT fluorescence at 490 nm	50 mM Pi buffer

[a] Purchased from Sigma-Aldrich. [b] OD: optical density was measured at stated wavelength(s) using a spectrophotometer. [c] Pi buffer: phosphate buffer at stated concentration(s). [d] DTT: 20 mM 1,4-dithiothreitol (DTT) was used to chemically disrupt the disulfide bonds in the substrates. [e] UV-light: ultraviolet light was used to induce aggregation of γ-Crystallin. [f] Scattering: light scattering was measured at stated emission wavelength(s) using a spectrofluorometer. [g] Electropheresis: native polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (SDS) and two-dimensional gel electrophoresis (first dimension, native PAGE; second dimension, SDS−PAGE with 2-mercaptoethanol). [h] Purification and/or modification of the casein(s) was conducted in the stated lab; refer to the corresponding paper for details. [i] SEC: size exclusion chromatography. [j] NA: information was not available. [k] HPLC: high-performance liquid chromatography. [l] EDTA: ethylenediamine tetraacetic acid. [m] ^{1}H NMR: proton nuclear magnetic resonance. [n] PIPES: piperazine-*N,N′*-bis(2-ethane-sulphonic acid); EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′*,N′-tetraacetic acid; GTP, guanosine-5′-triphosphate. [o] PMSF-inhibited: phenyl methane sulphonyl fluoride inhibited. [p] MCR-ALS: multivariate curve resolution by means of alternating least squares. [q] GuHCl: 6 M guanidinium hydrochloride (GuHCl) was used to chemically denature the substrate. [r] Casein was provided by another party. [s] SEC-MALS: size exclusion chromatography and multiangle laser light scattering detector. [t] SEC-RP-HPLC: size exclusion chromatography and reverse phase HPLC.

Table 4. Chemical Composition of Bovine (*Bos taurus*) α_{s1}-, α_{s2}-, β-Caseins and Camel (*Camelus dromedarius*) β-Casein

	bovine α_{s1}-casein	bovine α_{s2}-casein	bovine β-casein	camel β-casein
accession number[a]	P02662	P02663	P02666	Q9TVD0
reference	*46*	*47*	*48*	*49*
content in α_s-caseins (%)	80[b]	20[b]		
total amino acid residues[c]	199	207	209	217
phosphoserine residues (reference)	8 (*46*)	11 (*47*)	5 (*48*)	4 (*49*)
proline residues[c]	17	10	35	37
cysteine residues[c]	0	2	0	0
hydrophobic residues[d] (% content)	58 (29.2)	54 (26.1)	66 (31.6)	69 (31.8)
molecular weight[c], M_w (kDa)	22.97	24.35	23.58	24.65
M_w including phosphate[e] (kDa)	23.61	25.15	23.98	24.97
hydrophobicity[e]	$\alpha_{s2} < \alpha_{s1} < \beta$[b]	most hydrophilic[b]	most hydrophobic[b]	
calcium sensitivity (mM)	<2	3−8	8−15	

[a] Accession number of caseins was attained from protein database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein). [b] Information was taken from ref *30*. [c] Information was obtained from analysis through DNASTAR Lasergene Protean version 7.2.1(1), 410. [d] Hydrophobic residues are Ala, Ile, Leu, Phe, Trp, and Val (based on categorization by DNASTAR Lasergene Protean version 7.2.1(1), 410). [e] Molecular weight including phosphate in serine residues = calculated molecular weight + (phosphoseryl residues) × (79.98).

chaperone activity (*10*). When phosphate groups were eliminated from the caseins, the proteins became less amphiphilic (*10*) and increased in ordered structure (*21*, *56*). These observations suggest that amphiphilicity is essential to maintain the chaperone ability of caseins.

Comparison of Caseins with Small Heat Shock Proteins. The chaperone behavior of caseins has been said to be similar to that of small heat shock proteins (sHSP) (*11*, *12*). sHSP are a group of molecular chaperones that prevent their substrate aggregation but require the action of ATP-dependent chaperones for releasing their bound substrates (*57*−*59*). Bovine α-Crystallin was the first sHSP reported to have molecular chaperone activity (*60*), and there are 10 types of α-Crystallin-related sHSPs in humans alone (*61*). The α-Crystallin-related sHSPs share the following common features: monomeric molecular weight of 12−43 kDa; tendency to form oligomeric complexes; molecular chaperone activity shown under stressed conditions (i.e., those favoring unfolding); and a moderately conserved region known as the α-Crystallin domain (*62*, *63*). How many of these features are actually shared by α_s- and β-caseins will be discussed in the following section.

The molecular weight of α_{s1}-, α_{s2}-, and β-caseins are 23.6, 25.2, 24.0−25.0 kDa, respectively (**Table 4**). The molecular weight range of caseins falls within the sHSP molecular weight range. Monomers of caseins are amphiphilic in nature; therefore, caseins are prone to exist as colloidal casein−calcium complexes in milk which are termed as casein micelles (*64*) with a size distribution ranging from 40−280 nm (*65*). It is now generally agreed that casein micelles are roughly spherical with a hairy outer layer (*66*−*70*). However, there is still discussion as to whether submicelles exist inside casein micelles (*71*−*74*). Casein micelles are dynamic rather than fixed structures. Changes in temperature, pH, and ionic strength lead to changes in size distribution and dissociation of the micelles (*71*). Each type of casein can undergo association reactions with themselves to form homo-oligomeric micelles (*67*), as have been shown by α_{s1}- (*75*) and β-caseins (*76*, *77*). Therefore, micelle formation of caseins fits well with the oligomers requirement of sHSPs. The next feature, performing chaperone activity by suppressing protein aggregation under stressed conditions, has been explained in detail in previous sections.

This leaves the last feature: the α-Crystallin domain of sHSP. In order to answer this, pair wise alignment comparisons for the sequence homology of each casein with αA- and αB-crystallins were run. The result (**Table 5**) showed that all of the caseins' primary sequences have very low identical amino acids with the α-crystallins, ranging from only 10.3−16.0%. Therefore, an α-Crystallin domain is unlikely to occur in these caseins.

We are struck with the dilemma of having similarity in key structural properties (molecular weight and forming oligomers) and chaperone activity (altering aggregation of unfolded proteins) but lacking the key sequence associated with α-Crystallin-related sHSPs (*62*, *63*). Therefore, the chaperone ability of caseins may be due to some overlapping elements of structure that are not required for specific biological chaperone activity. As mentioned earlier, caseins are categorized as intrinsically unstructured proteins. The chaperone function of caseins is not an isolated case for intrinsically unstructured proteins. Clusterin (*78*) and synucleins (*79*, *80*) are intrinsically unstructured proteins that have chaperone-like activity, hinting that there might be some as yet to be defined structural elements in intrinsically unstructured proteins that provide chaperone properties.

Models. *Proposed Model for sHSP Chaperone Functions.* α_s- and β-caseins interact with different globular and nonglobular proteins and thereby alter amorphous and amyloid-fibril types of aggregations. The observed chaperone activity of caseins on a wide array of substrates (**Table 3**) showed that these intrinsically unstructured protein chaperones displayed no substrate specificity. Even though only 3 of the 4 common features of sHSP matched with α_s- and β-caseins, it is still feasible for us to propose a casein chaperone-like function model from the sHSP chaperone models.

Although there are different schematic models suggested for the chaperone functions of sHSPs (*58*, *59*, *62*, *63*, *81*−*85*), the models generally agreed that the chaperone action of sHSPs is not dependent on ATP-driven processes for preventing the aggregation of their substrate proteins. Under stress such as heat shock, two different modes are proposed to be responsible for the chaperone mechanisms of sHSPs: an inactive, low affinity sHSP oligomer either switches to an active, high affinity sHSP oligomer or the oligomer dissociates into smaller oligomers or dimers to bind with the partially unfolded, aggregation-prone intermediates (*58*, *59*, *82*, *83*). The transition between the different oligomeric states is thought to be regulated by phosphorylation (*86*). Unfolded substrate proteins are bound to the sHSP oligomer or dimer, preventing further aggregation. Upon returning to favorable conditions, their bound substrate proteins are released spontaneously from the protein−sHSP complexes or through the assistance of ATP-dependent chaperones such as HSP70 before refolding into their native states (*58*, *82*−*84*). In a multi-chaperone network, sHSP are thought to eventually hand over their bound substrates to other ATP-requiring chaperones for further processing (*63*, *85*).

Proposed Model of β-Casein Chaperone Function for Heat-Stressed Amorphous Aggregates. An overall model of the

Table 5. Pairwise Alignment Percent Identity of Bovine Proteins Calculated Using the ClustalW Method of DNAStar Lasergen MegAlign version 7.2.1 (1) with Gonnet 250 Protein Weight Matrix[a]

protein 1	protein 2	identical amino acids (%)
bovine β-casein	bovine α_{s1}-casein	15.9
bovine β-casein	bovine α_{s2}-casein	12.6
bovine α_{s1}-casein	bovine α_{s2}-casein	13.3
bovine β-casein	camel β-casein	67.8
bovine αA-Crystallin[b]	bovine αB-Crystallin[b]	58.9
bovine αA-Crystallin	bovine β-casein	13.3
bovine αA-Crystallin	bovine α_{s1}-casein	13.9
bovine αA-Crystallin	bovine α_{s2}-casein	14.5
bovine αB-Crystallin	bovine β-casein	16.0
bovine αB-Crystallin	bovine α_{s1}-casein	15.1
bovine αB-Crystallin	bovine α_{s2}-casein	10.3

[a] The accession number of each casein is shown in **Table 3**. [b] Bovine αA- and αB-crystallin accession numbers are P02470 and P02510, respectively (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein).

interactions of casein oligomers/micelles to prevent substrate protein aggregation that is similar to sHSP has been proposed (*19*). Casein micelles or oligomers of α_s- and β-casein will interact with intermediate unfolded structures before these intermediates enter the off-folding pathway to form insoluble amorphous aggregates or highly ordered amyloid fibrils which are irreversible. The model, however, did not take into consideration the conformational changes likely in caseins themselves and how this may relate to chaperone activity.

We therefore propose a model based specifically on β-casein chaperone activity against a globular protein under heat stressed conditions that results in altering amorphous aggregation (**Figure 1**). This has direct application to the food industry such as increasing the thermal stability of a protein-fortified beverage. Bear in mind that micelle conformational changes of β-casein are affected by concentration (*76*), temperature (*77*, *87*), pH (*77*, *88*), and type of solvent (*87*). Altered environmental conditions will either change the micelle structure or dissociate the micelle, resulting in changes in their effective surface hydrophobic and electrostatic interactions. Changes with temperature are of particular importance to applications involving thermal processing. β-Casein exists as a monomer at low temperature (< 10−15 °C) and starts to form micelles at 15−30 °C (*89*, *90*). As heating continues from 10 to 50 °C, there is a continuous increase in sedimentation coefficients of β-casein micelles, suggesting a continuous temperature-dependent alteration of the quaternary structure (*91*). Therefore, the precise quaternary structure of casein in solution will depend on concentration and temperature, and this in turn will alter the potential to act as a chaperone. In addition, the overall amphiphilic nature of β-casein monomers will depend on the degree of phosphorylation, which will alter chaperone activity (*21*, *92*).

We suggest the following reactions when β-casein and a substrate globular protein are heated together. When heated alone, globular proteins will unfold and aggregate when electrostatic barriers are overcome, producing insoluble amorphous aggregates. When β-casein is present during heating, the conformation of β-casein in micelles is such that β-casein-substrate protein mixed aggregates are favored. The normal aggregation pathway of partially unfolded intermediates is either terminated or the amount going into that pathway is decreased. This would suggest a combination of mixed β-casein-substrate protein aggregates and normal substrate protein aggregates. Although β-casein is consistently shown to reduce the aggregation of heat-stressed globular proteins, it fails to reverse the insoluble aggregates that are formed by heating (*23*, *24*). Therefore, we

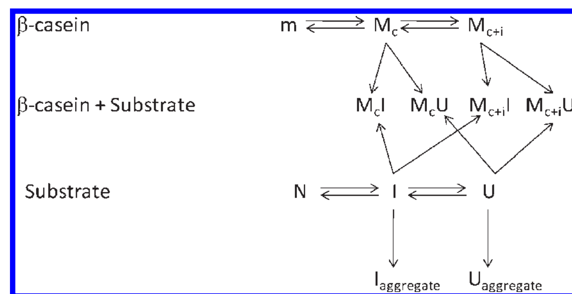

Figure 1. Model for the chaperone function of β-casein under heat-stress. β-Casein is a monomer (m) at low temperature that forms micelles (M) as the temperature increases. Micelles start at a critical number of molecules (M_c), then grow with temperature increase (M_{c+i}). A substrate protein is depicted as starting from a native state (N), then progressing to an unfolded (U) state with an intermediate (I) state of unfolding. Aggregation is a competition between substrate molecules aggregating with themselves or with β-casein.

do not suggest a reversal of aggregation and possible refolding. However, in the chemically stressed situation, α_s- and β-caseins are able to resolubilize some of the aggregates (*1*, *22*), suggesting that a different model may be necessary for this type of stress.

Conclusions. α_s- and β-Caseins consistently show a chaperone function in various conditions. However, these two caseins have some distinctive differences in their chaperone strength, probably due to the different structures that influence their mechanisms. The effectiveness of caseins as chaperones for common food ingredients such as whey and egg proteins is encouraging, as this property will be useful to expand the applications of these heat sensitive food proteins. For example, successful suppression of aggregation at a high whey protein/casein ratio demonstrates the possibility of using low amounts of caseins to improve the heat stability of high concentrations of whey protein isolate. However, food processing conditions involve a range of pH values, additional ingredients, and thermal processing conditions that have not been explored. Much work is still needed to convert these primarily model system observations to practical applications for the food industry.

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