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Alpha casein micelles show not only molecular chaperone-like aggregation inhibition properties but also protein refolding activity from the denatured state

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ABSTRACT

Casein micelles are a major component of milk proteins. It is well known that casein micelles show chaperone-like activity such as inhibition of protein aggregation and stabilization of proteins. In this study, it was revealed that casein micelles also possess a high refolding activity for denatured proteins. A buffer containing caseins exhibited higher refolding activity for denatured bovine carbonic anhydrase than buffers including other proteins. In particular, a buffer containing α-casein showed about a twofold higher refolding activity compared with absence of α-casein. Casein properties of surface hydrophobicity, a flexible structure and assembly formation are thought to contribute to this high refolding activity. Our results indicate that casein micelles stabilize milk proteins by both chaperone-like activity and refolding properties.

1. Introduction

Casein micelles comprising several subclasses of casein proteins (α-, β- and κ-) are well known as the major component of milk proteins [1]. Casein molecules are present as random coils and do not possess well-defined secondary or tertiary structures [2,3]. Casein micelles are polydisperse, roughly spherical aggregates, with diameters ranging between 150 and 300 nm [4]. The structure and stability of casein micelles are still a matter of debate and various models are discussed in the literature [5].

Recently, it was found that casein micelles have chaperone-like activity. Bhattacharyya and Das first reported that the α-casein micelle was working as an aggregation inhibitor similar to a molecular chaperone [6]. Inhibition of protein aggregation was identified in the presence of α-casein after applying a number of stresses. This aggregation inhibition effect of casein has been shown in many types of proteins including insulin [6,7], γ-crystallin [6], ovotransferrin [8], β-lactoglobulin [7,9], α-lactalbumin [7] and milk whey proteins [10]. Inhibition of aggregation by molecular chaperones, including a small heat shock protein, is achieved by complexation through hydrophobic interaction between denatured proteins and the molecular chaperones [11,12]. It was also reported that the hydrophobicity of the α-casein micelle is increased when complexes between casein micelles and denatured proteins are formed [7]. Therefore, the mechanism for inhibition of protein aggregation by casein micelles is thought to be same as that of molecular chaperones. Based on the chaperone-like activity of the casein micelles, casein is considered to play an important role in the stabilization of milk whey proteins under several types of stress.

In the present study, we first demonstrate that α-casein micelles show not only molecular chaperone-like properties but also protein refolding activity from the denatured state. It is revealed that a protein in fully denatured state is renatured to the native state effectively in the presence of casein micelles. Our results thus indicate that casein micelles stabilize milk whey proteins by both chaperone-like activity and refolding properties. Additionally, we show that casein micelles might be a valuable additive that assists refolding at an industrial level because separation of casein micelles from the refolding buffer is easily achieved after the refolding reaction.

2. Materials and methods

2.1. Reagents

All proteins, *p*-nitrophenylacetate and 8-anilino-1-naphtalenesulfonic acid were purchased from Sigma–Chemical Company (St. Louis, MO). The other chemicals were obtained from Wako Pure Chemical Company (Osaka, Japan).

Abbreviations: BSA, bovine serum albumin; CAB, bovine carbonic anhydrase; LYZ, lysozyme; CMC, critical micellar concentration; oval, ovalbumin; ANS, 8-anilino-1-naphtalene-sulfonic acid.

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2.2. Preparation of denatured CAB

Carbonic anhydrase (CAB) was dissolved at 10 mg/ml in 50 mM Tris–HCl buffer solution (pH 7.5) containing 8 M urea. The denaturation solution was incubated for 3 h at room temperature to completely denature the CAB.

2.3. Refolding of denatured CAB mediated by casein

Alpha casein was dissolved in 50 mM Tris–HCl buffer solution (pH 7.5). The solution containing denatured CAB was added to the α-casein solution and the final CAB concentration diluted 50-fold. This solution was incubated for 24 h at room temperature. After incubation, the CAB activity was measured by the following method.

2.4. Measurement of CAB activity and calculation of refolding ratio

The esterase activity of CAB was measured by the increase of absorbance at 348 nm due to the hydrolysis of *p*-nitrophenylacetate with a UV–Vis spectrophotometer (Jasco V-570, Tokyo, Japan). The CAB regeneration yield was determined by comparing the catalytic activities between native and regenerated CABs under the same conditions.

2.5. Analysis of protein hydrophobicity by ANS

Fluorescence studies were carried out with a fluorescence spectrometer (LS55C, Perkin–Elmer). A refolding buffer containing each protein was equilibrated with 200 μM 8-anilino-1-naphtalenesulfonic acid (ANS) at room temperature for 15 min. The excitation monochromator was set at 350 nm and the emission scanned from 360 to 600 nm in the correct spectrum mode to monitor the emission maxima of ANS.

2.6. Measurement of critical micellar concentration

The surface tensions of α-casein solutions in the refolding buffer were determined using a drop volume tensiometer (Lauda TVT-2S). The tensiometer measures the volume of a drop detaching from a capillary of known diameter. Measurements were carried out at room temperature.

3. Results and discussion

Several refolding buffer samples were prepared containing a protein, such as α-, β- or κ-caseins, lysozyme (LYZ), bovine serum albumin (BSA) or ovalbumin (oval), and the effect of these refolding buffers on denatured CAB refolding was examined. A fully denatured CAB solution with 8 M urea was diluted fifty times using each refolding buffer and the mixed solution was then incubated for 24 h at room temperature. As a control, refolding buffer containing no proteins was added to the denatured solution and incubated under the same conditions. Fig. 1A shows the refolding yield obtained from measurement of CAB activity after incubation. The results indicate that a buffer containing caseins exhibited higher refolding activity than the other buffers. In particular, the buffer containing α-casein showed approximately a twofold higher refolding activity than that of the control. Refolding buffers containing protein other than caseins showed low refolding activity.

We also assessed the production of CAB aggregates in the refolding buffer solution. The turbidity change of the solution caused by the accumulation of aggregated CAB was measured using UV spectroscopy. Fig. 1B shows the time-course of the optical density at 500 nm with various refolding buffers. In the control,

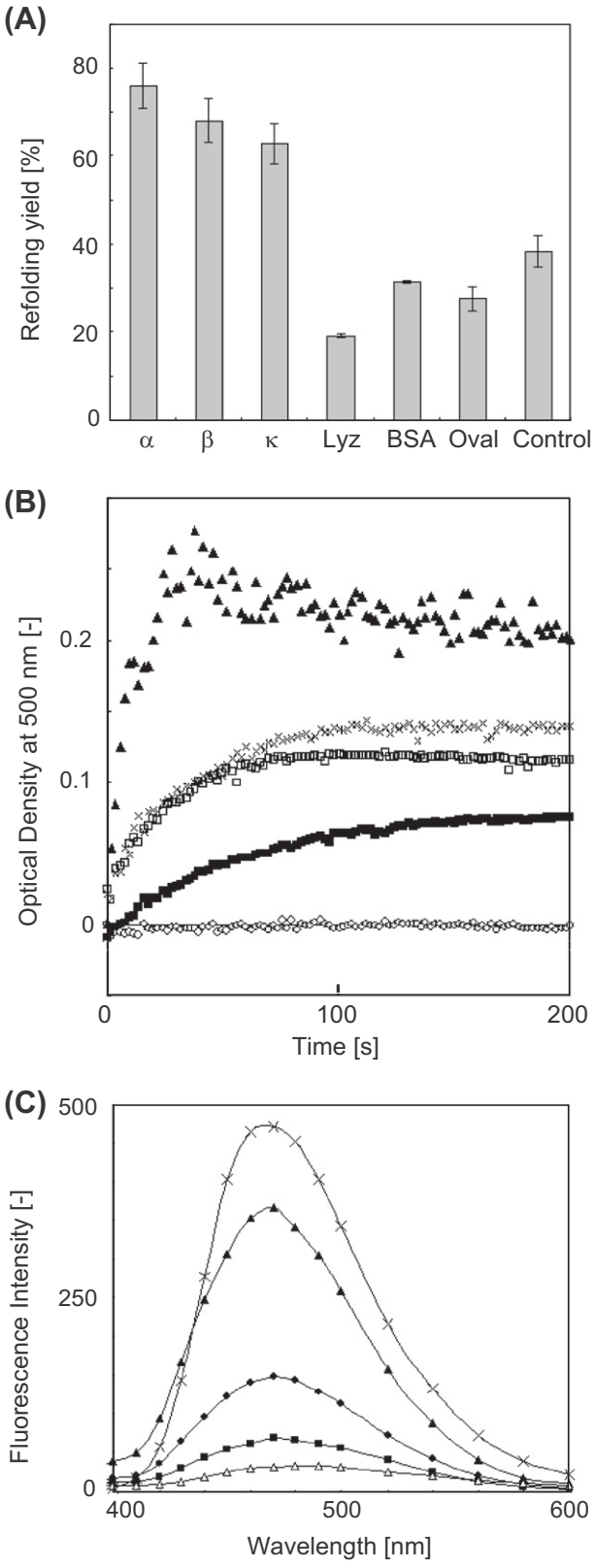

Fig. 1. Influence of dissolved mature protein on refolding of denatured CAB. (A) Refolding yield of CAB in the presence of mature protein. (B) Measurement of protein aggregates production by optical density (triangle: LYZ, cross: oval, open square: control, filled square: BSA, and circle: α-casein). (C) Surface hydrophobicity analysis of coexisting protein with ANS probe (cross: BSA, triangle: κ-casein, circle: β-casein, square: α-casein, and triangle: oval).

CAB aggregates were produced within about 100 s after CAB refolding was initiated. The response of the ovalbumin buffer was similar to that of the control. However, BSA showed a more effective ability to inhibit protein aggregation than the control. Moreover, the production of CAB aggregates was not observed using the refolding buffer containing α-casein. The above results suggest that the high refolding activity of α-casein in Fig. 1A was caused by effective inhibition of protein aggregate production during the refolding reaction. In the case of LYZ, the production of CAB aggregates was more rapid and the amount of aggregates was much larger than that of the control. As the isoelectric point of LYZ is around 11.0, the surface electric charge of LYZ is cationic in the refolding buffer solution. The CAB refolding was inhibited by the electrostatic interaction between LYZ and the CAB, which possessed a negative electric charge in the buffer solution, so that the production of the LYZ–CAB amorphous complex was enhanced.

We also examined the surface hydrophobicity of the proteins identified as having good CAB aggregation inhibition effects using the ANS probe. Fig. 1C shows the intensity of the ANS probe in each protein solution. Results show that BSA possessed high hydrophobicity. In the case of caseins, κ-casein showed high hydrophobicity, whereas β-casein exhibited the lowest value. Hydrophobicity was almost undetectable in ovalbumin. Generally, re-aggregation of denatured protein during protein refolding is caused by hydrophobic interactions between folding intermediates [13]. Therefore, inhibition of hydrophobic interaction is important for the suppression of protein aggregation. As the surface hydrophobicity of ovalbumin is weak, the assistance to refolding was small. We predicted that BSA possessed high chaperone activity because BSA showed a high hydrophobicity. However, the effect of BSA on aggregation inhibition was smaller than that of caseins. The above results indicate that inhibition of aggregation is not just dependent on the surface hydrophobicity of the assistant protein.

Therefore, it can be assumed that another factor is associated with the high refolding activity of casein. We hypothesize that the formation of a molecular assembly by caseins influences protein refolding. The structure of casein micelles is regarded as possessing a variable and flexible form [5]. The inhibition of aggregation between denatured proteins appears to be due to the steric hindrance of micelles that interact with denatured proteins on their surfaces. The weak hydrophobic property of casein micelles facilitates dissociation of denatured protein from the micellar surface and then spontaneous protein refolding is achieved.

We examined the influence of casein micelle formation on protein refolding. A critical micellar concentration (CMC) of α-casein possessing high refolding activity was measured using surface tension analysis. Results show that the CMC value of α-casein was about 0.14 mg/ml (Fig. 2A). The effect of casein concentration within the refolding buffer on protein refolding was investigated. Fig. 2B shows the refolding yield of denatured CAB with casein at various concentrations. An elevation of refolding yield accompanied an increase in the casein concentration up to 3 mg/ml, above which the refolding yield was saturated. It was found that the refolding yield was the lowest at around the CMC value. This result indicates that casein micelle formation is critical for the refolding activity. Accordingly, increasing the concentration of casein micelles in the refolding buffer enhances the prevention of denatured protein interactions.

Several serines contained within the α-casein molecule are known to be phosphorylated [5]. It is now known that phosvitin, which has phosphorylated serine within the molecule, possesses a high aggregation inhibition property. The effect of phosphorylated amino acid within the α-casein molecule on protein refolding was examined. The refolding of denatured CAB was conducted by addition of α-casein with or without phosphorylation of serine. Fig. 3 presents the time-course of the CAB refolding yield, showing

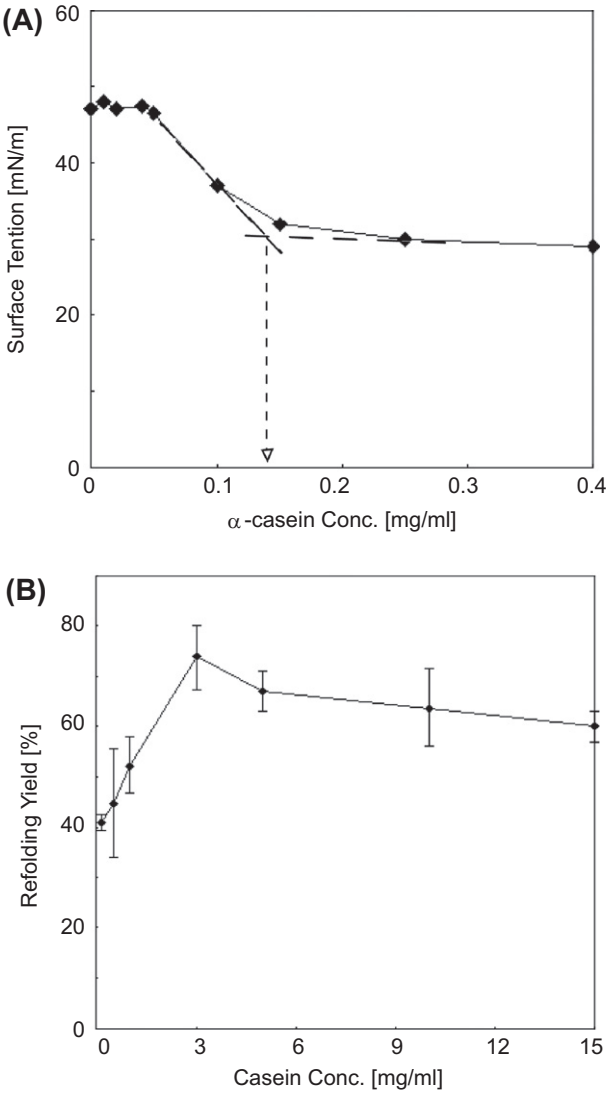

Fig. 2. (A) Surface tension of α-casein as a function of concentration at room temperature. (B) Effect of α-casein concentration on CAB refolding yield.

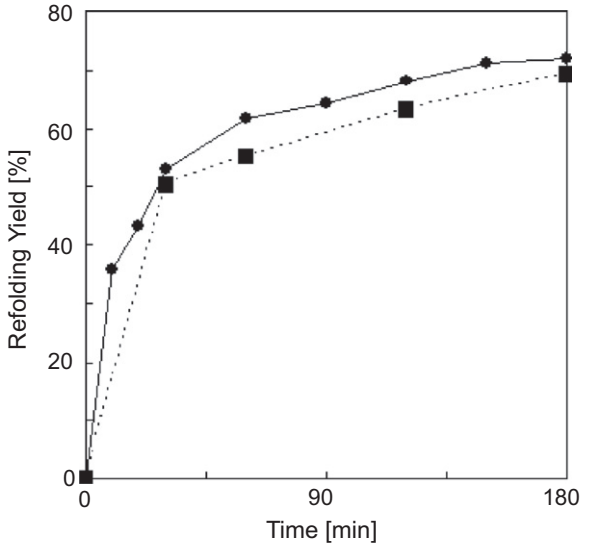

Fig. 3. Time-course of refolding yield in the presence of α-casein containing serine with (circle) or without (square) phosphorylation.

Table 1
Removal yield of α-casein micelles by calcium chloride addition.

Final $CaCl_2$ conc. (mM)	Removal yield of casein micelles (%)
2.5	0.52
12.5	95.3
25	95.5
50	95.6
100	95.9

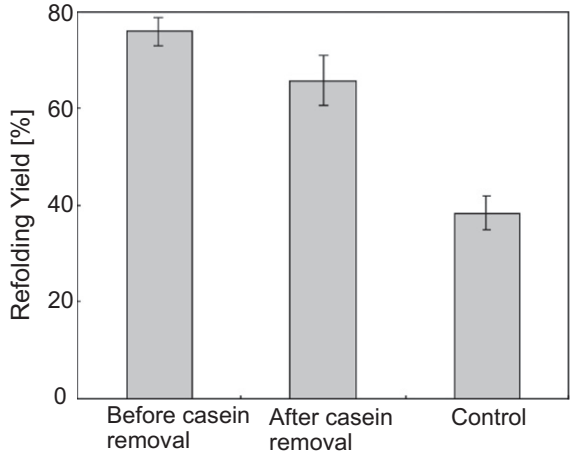

Fig. 4. Influence of α-casein micelle removal by calcium chloride addition on CAB activity.

that a comparable profile was obtained at each condition. These data indicate that the refolding activity of the casein micelle is not due to the effect of phosphorylated serine. Therefore, it is suggested that the refolding observed with casein micelles was caused by the formation of the micellar structure and not by the effect of specific amino acids.

The study of refolding assistant additives for industrial use has recently attracted considerable attention [14–16]. The above results show that casein micelles are effective for protein refolding. After the refolding reaction, the purification of regenerated protein is achieved by removing refolding assistant reagents. The α-casein micelle is known to precipitate by addition of a bivalent ion such as calcium [17]. In an attempt to purify regenerated CAB from the refolding buffer, calcium was added and precipitated micelles were simply separated by filtration. The influence of calcium concentration on casein micelle removal is shown in Table 1. The results show that casein micelle removal is achieved by addition of calcium chloride at a concentration greater than 12.5 mM. Fig. 4 shows the CAB activity before and after calcium chloride addition. The CAB activity did not change after micelle removal and the removal of casein micelles did not interfere with regenerated CAB activity. The above results support the notion that a high refolding activity and the easy removal of casein are beneficial for industrial scale protein refolding as assistant reagents.

4. Conclusions

In this study, it was shown that casein micelles possess a high refolding activity. Properties of surface hydrophobicity, a flexible structure and assembly formation are thought to contribute to this high refolding activity. There are several reports concerning the aggregation inhibition properties of casein. Our results show that casein micelles possess not only an aggregation inhibition property but also protein refolding activity. Based on the results, it is concluded that casein plays the role of a molecular chaperone with refolding activity to stabilize milk whey proteins.

References

[1] D.G. Dalgleish, D.S. Horne, A.J.R. Law, Size-related differences in bovine casein micelles, Biochim. Biophys. Acta 991 (1989) 383–387.
[2] H. Susi, S.N. Timasheff, L. Stevens, Infrared spectra and protein conformations in aqueous solutions. I. The amide I band in H_2O and D_2O solutions, J. Biol. Chem. 242 (1967) 5460–5466.
[3] L.K. Creamer, T. Richardson, D.A. Parry, Secondary structure of bovine alpha s1- and beta-casein in solution, Arch. Biochem. Biophys. 211 (1981) 689–696.
[4] P. Muller-Buschbaum, R. Gebhardt, S.V. Roth, E. Metwalli, W. Doster, Effect of calcium concentration on the structure of casein micelles in thin films, Biophys. J. 93 (2007) 960–968.
[5] D.J. McMahon, B.S. Oommen, Supramolecular structure of the casein micelle, J. Dairy Sci. 91 (2008) 1709–1721.
[6] J. Bhattacharyya, K.P. Das, Molecular chaperone-like properties of an unfolded protein, alpha(s)-casein, J. Biol. Chem. 274 (1999) 15505–15509.
[7] P.E. Morgan, T.M. Treweek, R.A. Lindner, W.E. Price, J.A. Carver, Casein proteins as molecular chaperones, J. Agric. Food Chem. 53 (2005) 2670–2683.
[8] N. Matsudomi, Y. Kanda, Y. Yoshika, H. Moriwaki, Ability of alphas-casein to suppress the heat aggregation of ovotransferrin, J. Agric. Food Chem. 52 (2004) 4882–4886.
[9] Y.H. Yong, E.A. Foegeding, Effects of caseins on thermal stability of bovine beta-lactoglobulin, J. Agric. Food Chem. 56 (2008) 10352–10358.
[10] B.T. O'Kennedy, J.S. Mounsey, Control of heat-induced aggregation of whey proteins using casein, J. Agric. Food Chem. 54 (2006) 5637–5642.
[11] A.L. Horwich, E.U. Weber-Ban, D. Finley, Chaperone rings in protein folding and degradation, Proc. Natl. Acad. Sci. USA 96 (1999) 11033–11040.
[12] T.H. MacRae, Structure and function of small heat shock/alpha-crystallin proteins: established concepts and emerging ideas, Cell. Mol. Life Sci. 57 (2000) 899–913.
[13] M.K. Eiberle, A. Jungbauer, Technical refolding of proteins: do we have freedom to operate?, Biotechnol J. 5 (2010) 547–559.
[14] M. Sakono, Y.M. Kawashima, H. Ichinose, T. Maruyama, N. Kamiya, M. Goto, Direct refolding of inclusion bodies using reversed micelles, Biotechnol. Prog. 20 (2004) 1783–1787.
[15] K. Tsumoto, M. Umetsu, I. Kumagai, D. Ejima, J.S. Philo, T. Arakawa, Role of arginine in protein refolding, solubilization, and purification, Biotechnol. Prog. 20 (2004) 1301–1308.
[16] J. Okada, T. Maruyama, K. Motomura, K. Kuroki, K. Maenaka, M. Sakono, M. Goto, Enzyme-mediated protein refolding, Chem. Commun. (Camb.) (2009) 7197–7199.
[17] E.W. Bingham, H.M. Farrell Jr., R.J. Carroll, Properties of dephosphorylated s1-casein. Precipitation by calcium ions and micelle formation, Biochemistry 11 (1972) 2450–2454.