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Characterization of casein-based nanoparticles formed upon freezing by in situ SAXS measurement

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ABSTRACT

The formation of casein-based nanoparticles from sodium caseinate and sodium caseinate–pectin solutions was investigated in a frozen system by protein self-aggregation and protein–polysaccharide complexation, respectively. Casein-based nanoparticles were prepared by controlling the pH levels of the solutions followed by freezing. The formation of precipitates was confirmed in the casein solutions at pH < 5.5. However, an obvious effect of the freezing on the formation of aggregates could not be confirmed, although the freezing did have an effect on accelerating the formation of precipitates. The mean particle sizes analyzed from the produced nanoparticles suggested that freezing did not have any significant effects on altering the particle sizes. Similar trends were observed in the casein–pectin solution in terms of phase separation and particle sizes. A difference was confirmed in the solution at pH 4.6; that is, a clear phase separation was observed due to freezing. Nevertheless, it was found, both in the casein and casein–pectin systems, that the degradation rates of the freeze–thawed nanoparticles were considerably slower than that of the original nanoparticles. This suggested that the casein-based nanoparticles formed through freezing had structural features different from the ones in the unfrozen solution. It could be concluded from the SAXS analysis that the formation of the protein-based particulate systems certainly occurred in the cryoconcentrated phase associated with freezing. The present technique is advantageous for the encapsulation of heat-sensitive and/or acid-sensitive ingredients in protein nanoparticles.

1. Introduction

Casein-based nanoparticles are being recognized as potential delivery vehicles for nutraceutical and pharmaceutical materials, and much study has been dedicated to applying them to food and drug systems [1–4]. Casein molecules have the ability to self-assemble into spherical micelles (ca. 50–500 nm in diameter) owing to their hydrophobic and hydrophilic amino acid residues. These hydrophobic residues can be incorporated into hydrophobic compounds (e.g., vitamins, polyphenols, lipophilic drugs), thus providing the potential for encapsulation into nano/microparticles [5–12]. When the caseinate system is associated with anionic polysaccharides, interpolymer complexations are induced by attractive electrostatic interactions. This complex system is regarded as an interesting encapsulant, of which the functional properties are largely different from those of the polymers before complexation [2]. Studies on caseinate-based polymer complexation, also known as complex coacervation, have successfully proved that the particulate system is potentially advantageous in the protection and controlled release of the ingredients [13–17]. Caseinate self-aggregation and caseinate-based coacervation are controlled by isoelectric conditions. At a pH below the isoelectric point, polymer phase separation (due to self-aggregation or complex coacervation) can be observed, and the degree of phase separation is dependent on the charge up to the polymer system [2,18,19]. The charge density and the distribution also play important roles to the complex formation [20,21].

The use of protein-based phase separation in the production of nanoparticles would ideally be a simple process but still, it is an important challenge for engineers to ensure a high reproducibility by minimizing lot-to-lot variations. Since the protein-based nanostructure formation is greatly influenced by the ionic charge balance (controlled by pH adjustment) and thermal conditions during processing, we were convinced that a process controlling both pH and temperature profiles was worth studying in terms of the production of nanoparticles. The aim of this study was to use freezing for controlling the kinetics of protein nanostructure formation. The growth of ice crystals in a solution induces a liquid–solid phase separation, in which the liquid phase is known as the cryoconcentrated (or freeze-concentrated) phase. During the freezing process, the freezing front rejects the solutes and suspended particles when the front velocity is sufficiently low, whereas it engulfs them when the velocity is high. Because of this rejection and engulfment, freezing generates an ice microstructure when the velocity of the

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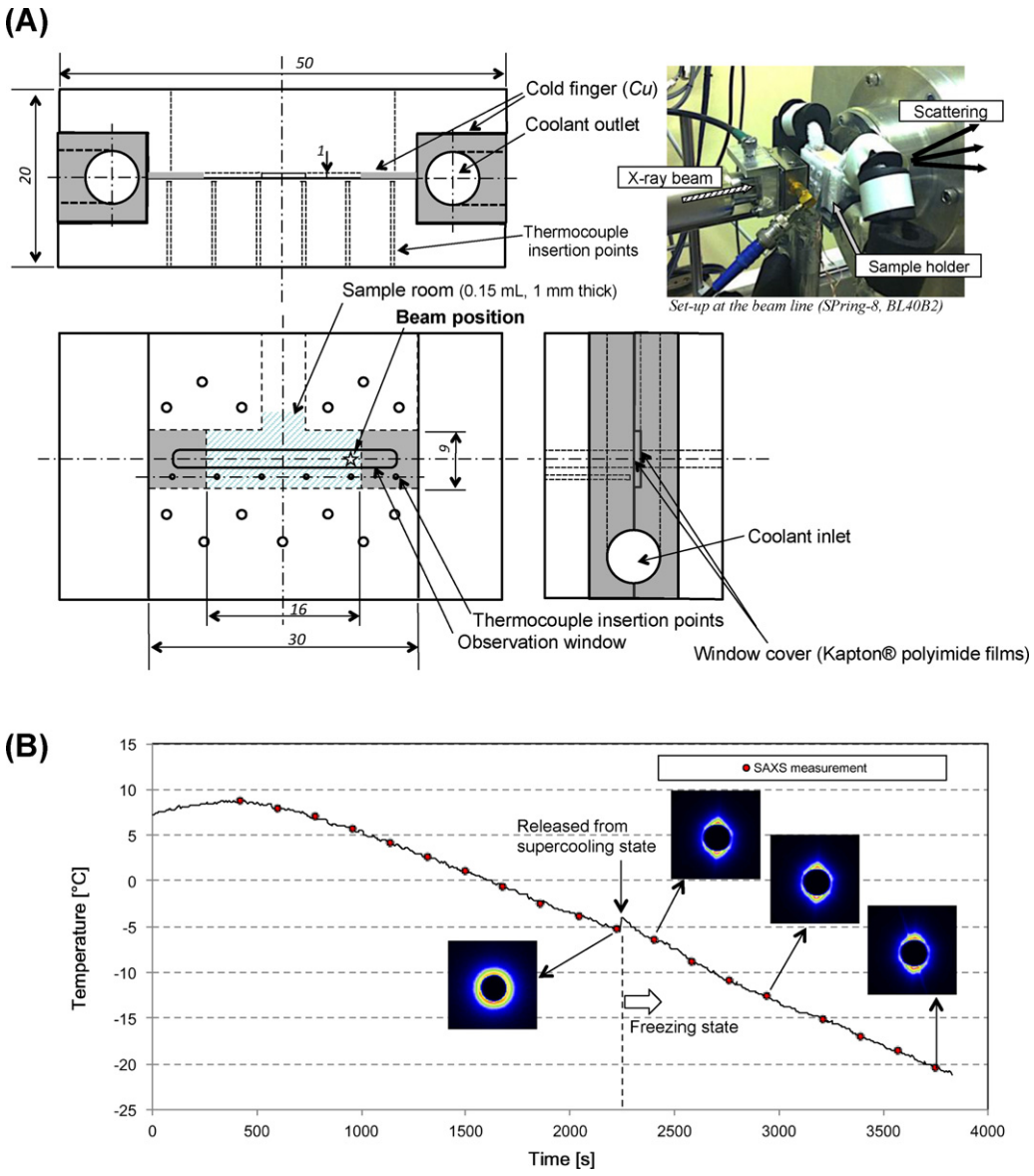

Fig. 1. Set-up for the SAXS measurement: (A) sample holder and beam position, and (B) example of the temperature and SAXS measurements.

freezing front is maintained at a certain value. The concentration of solutes in the cryoconcentrate is controlled by the phase equilibrium. When the temperature of the freezing solution was fixed, the concentration of the cryoconcentrate coincidently followed the phase equilibrium. In a eutectic system, the solute concentration of the cryoconcentrated phase can vary between the original and the eutectic phases. Freezing enables a highly concentrated microspace to be established without the loading of alternative additives. In industrial pharmaceutical freeze-drying, polymer phase separation is known to be a problem that leads to the denaturation of labile proteins due to the protein aggregation or the thermodynamic incompatibility of proteins and polysaccharides in the cryoconcentrated phase [22]. However, this may be useful for nanoparticle production by using polymer phase separation in the cryoconcentrated phase [23].

In this study, attempts were made to produce casein-based nanoparticles by freezing. A sodium caseinate solution, whose pH was adjusted with acetic acid, was prepared to observe the formation of self-aggregated nanoparticles before and after freezing. Similarly, a caseinate–pectin system was employed to observe the formation of nanoparticles due to protein–polysaccharide complexation. The rates of enzymatic digestion of the nanoparticles were compared to understand the influence of freezing on the properties of the particles. Finally, in situ small angle X-ray scattering (SAXS) measurements were applied to the frozen caseinate solutions to investigate the formation of the nanostructures in the systems during freezing.

2. Materials and methods

2.1. Materials

Sodium caseinate was purchased from Wako Pure Chemical Ind. Ltd., Japan, and used as provided. The total nitrogen of the reagent was 14% and it contained approximately 2% moisture. High methoxyl pectin was obtained from CP Kelco (GENU pectin, Type YM-100-H "food grade," Tokyo, Japan). This powder contained approximately 9% moisture and the degree of esterification was approximately 68%. Protease from *Bacillus licheniformis* (7–15 units) in aqueous propylene glycol solution (86 mg protein/58.14 ml) (Sigma Co. Ltd., Germany) were used for the