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Effect of ultrasonic treatment on the morphology of casein particles

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ABSTRACT

In this study, the effect of ultrasonic treatment duration on the morphology of self-assembled casein particles was investigated by atomic force microscopy (AFM), low vacuum scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In the case of AFM images, the particle analysis which was carried out by the SPIP program showed that the self-assembled casein particles after being ultrasonically treated for 2 min got smaller in size compared to the casein particles that have not been exposed to any ultrasonic treatment. Surprisingly, however, increasing the ultrasonic time exposure of the particles resulted in an opposite effect where larger particles or aggregates seemed to be present. We show that by comparing the results obtained by AFM, SEM and TEM, the information extracted from the AFM images and analyzed by SPIP program give more detailed insights into particle sizes and morphology at the molecular level compared to SEM and TEM images, respectively.

1. Introduction

Casein is one of the phosphor-proteins and the main protein in bovine milk which forms an integral part of the daily diet. The supramolecular structure of casein makes it easily to be bound by calcium phosphate into roughly spherical aggregations and have wide applications in producing or developing new dairy products [1]. Casein supramolecules are mainly composed of four different kinds of phosphoproteins named α_{1s}, α_{2s}, β-casein and κ-casein. The difference between the four kinds of phosphoproteins is due to containing 8, 9–11, 5 and 1 phosphate by phosphorylated serine residues respectively[2].α_{1s}, α_{2s}, and β-casein are partly precipitated by calcium and exist in the inner section of casein supramolecule; while κ-casein belongs to calcium in-sensitive and mostly makes up the surface.

α_{1s} and α_{2s} casein can be found in most milk and seem to show the same behavior[3]. For instance, α_{1s} casein has a 22 negative charge at neutral. Its hydrophobic ends point out of the polymeric chain and hydrophilic end points in [4]. The hydrophobic/hydrophilic distribution forms the intermolecular linkages which results in the polymeric chain [5,6]. β-Casein is divided into a hydrophilic part [7] in the 50 first residues present the net charge of 12, and a hydrophobic part [8]. The two parts make it possible to be served as a natural diblock copolymer [9] and adsorbed at air–water interfaces [10]. The hydrophilic part is the reason to form the aggregates, for instance, the micelles in solution. Compared to the other three proteins, κ-casein mostly consists of the outer part of casein supramolecules which can dissolve in calcium solution interacts with other three proteins and stabilizes the colloid [11]. NMR studies have shown that the C-terminal is mobile at 20 °C [12],extends into the aqueous solution, presents negative charge and is hydrophilic, and keeps from hydrophobic contact and stabilizes the molecular structure.

Recently, there is an increasing demand for understanding the exact structure and the mechanism of casein supramolecular formations for synthesizing new products with essential elements. Even though the protein has been studied for many years, it is still a major barrier of understanding and controlling the stability of aggregation properties. The size distribution of casein particles was significantly impacted by the casein supramolecules formations. Many studies have focused on the parameters in the formation processes of casein supramolecules such as pH, temperature, ionic strength and solvent. However, the effect of ultrasonic treatment time has not been investigated as our knowledge so far. It is expected that ultrasonication will produce free radicals [13]that might have an impact on the morphology and particle size of casein molecules in solution and upon their adsorption on the surface. Atomic force microscopy (AFM) is capable of obtaining nanoscale resolution of super molecular structures [14–16]. This method also allows for the analysis of the self-assembly particles. However, the research of outer parameters that based on particle analysis by means of (AFM) is still scarce.

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In this study, the effect of ultrasonic treatment time on the morphology and particles' size of self-assembled casein particles was investigated by AFM, SEM and TEM.

2. Materials and methods

2.1. Casein particles formation

The casein peptide from bovine milk was obtained from the sample provider by hydrolysis with trypsin enzyme and acetonitrile. A casein solution of 2% was prepared by adding purified water into the samples followed by centrifugingwith 1×10^4 r/m for 20 minutesand then the solution was cooled in an ice-bath at 4 °C. An ultrasonic generator (Wuxi Finebio biological engineering Ltd.Co.) with 20 kHz, 600 W and each pulsed cycle 2s/2s was used to obtain the homogenous solution. The type of ultrasonic generator is horn type sonicator. Then the casein solution was freeze dried and dehydrated with a CHRIST ALPHA2-4 vacuum freeze dryer. NaOH(0.5 M) and HCl(1 M) were used to regulatethe pH value.

2.2. AFMand EM characterization

AFM images (Multimode microscope V, Bruker, Santa Barbara, CA)were obtained using Olympus cantilevers with resonant frequency about 300 kHz and typical spring of 26.1(N/m).SEM was used to obtain the overview information of casein particles morphology on a small silicon wafer in low vacuum with electrons of lower energy (5–6 keV).The solution and volume used were the same both in AFM and SEM observations.

2.3. Casein supramolecules formation

The effect of ultrasonic treatment time on casein morphologywas evaluated. The four protein stock solutions were diluted by adding14 μL stock solution to 14 mL purified water to obtain the protein concentration of 1 μg/mL, and then four samples were treated by pulsed ultrasound for 0, 2, 5 and 10 min, respectively. All samples were imaged by AFM.

2.4. Particle analysis

In this work, SPIP image analysis software was used, where the threshold method which is the simplest and fastest method in SPIP program for AFM particle detection was used. The detection in this method must be carried out on a relatively flat substrate surface. Considering the AFM as a topographic landscape, this threshold is a zero-point for measurements. The threshold level is significant above which any features are regarded as particles and below which are regarded as pores (Fig. 1a). Three points should be taken seriously into account: (1) the substrate of the sample should be horizontal rather than slant to avoid appearing high or low enough to be considered as particles or pores. (2) Avoid considering extreme noise signal as particles, and in this regard, filters are used to remove noise. (3) Manually exclude local artifacts or other high/low features. The height of the particles rests with the threshold level.

The SPIP program was also used to detect particle-shapes. It has different methods and in this study a method called contour smoothed was used. In the AFM image, a particle was considered as a group of pixels having a certain height. On the pixel edge various places were contour points. This method creates a smoother shape but also makes the perimeter shorter (Fig. 2). Particle size was measured by particles diameter and height. Data analysis was conducted that the area was based on the shapes' periphery and the height was one of the highest points inside each shape contour.

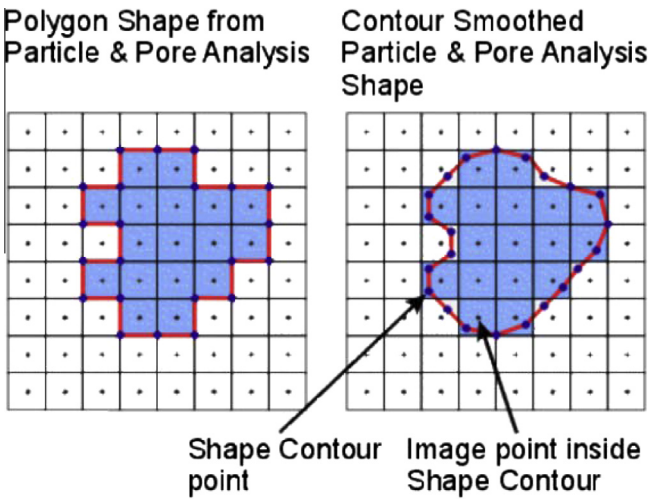

Fig. 2. The difference between the two SPIP detection methods,polygon shape form and contour smoothed. The small boxes indicate pixels and the black point in the middle of each box indicates the image point. The light blue boxes are the particle area according to the AFM image. The blue points in each image represent the contour points. The red line encircles the particle detected.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

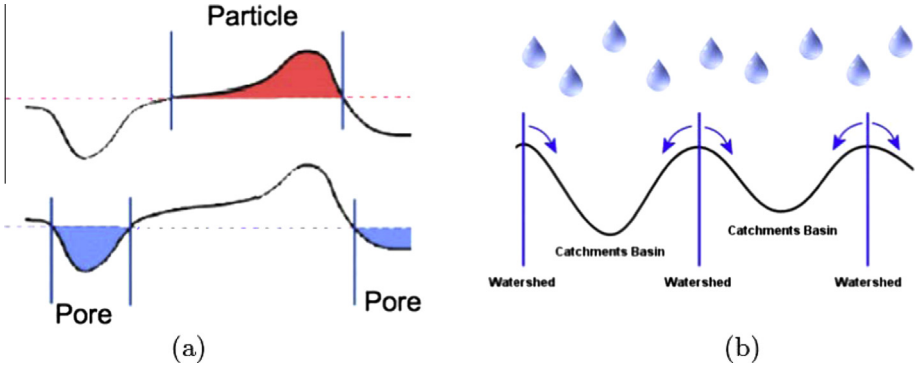

Fig. 1. (a) The principle of threshold segmentation method where the threshold level determines what will be detected as particles and pores; (b)The basic idea of the watershed method in a rain metaphor where water runs towards local minimum in the direction opposite to the gradient.The local minima will then work as shape boundaries in the SPIP.