Microencapsulation of β -Carotene by Self-Aggregated Caseinates

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β -Carotene was encapsulated by self-aggregated caseinates, and the encapsulation efficiencies and storage stabilities of the resultant spray-dried powder were investigated. The pH of the sodium caseinate solution, which determined the degree of aggregation, tremendously affected the encapsulation yield and storage stability of β -carotene in the spray-dried powder. We found that at pH 6.0, the resultant powder showed excellent encapsulation property compared to other pH conditions (pH=6.5, 5.5). Small angle X-ray scattering analysis revealed that the representative sizes of the aggregates in the sodium caseinate solution at pH 6.5, 6.0, and 5.5 were approximately 91, 111, and 166 nm, respectively. This analysis also clarified that our system represented fractal growth aggregation while the surface fractal dimension increased with decreasing pH. Apparently, the encapsulation property was affected by the physical properties of the aggregates, and there existed an optimal aggregation degree, which maximized the performance of an encapsulated system.

Key words: microencapsulation; casein; aggregation; nanoparticle; spray-drying

1. Introduction

Casein offers several advantages when used as a shell matrix for encapsulation, such as it is inexpensive, non-toxic, highly emulsifying, highly stable, biodegradable, and nonodorous. It is commonly obtained from defatted milk through acid treatment in the form of precipitates. This precipitate is then solubilized using an alkali to obtain caseinates such as sodium caseinate (CAS) and calcium caseinate. These compounds have applications in the pharmaceutical and food industries wherein suitable processing technologies endow them with functional properties for use in nano- and microencapsulated systems [1,2]. CAS forms self-aggregated structures (nanoparticles) under acidic pH condition. Lipophilic molecules could be associated with the hydrophobic chains of CAS. As a result, casein-based nanoparticles could be potential delivery vehicles for nutraceuticals and pharmaceuticals, which have low water solubility [3–9]. Semo et al. reported that the fat-soluble vitamin D₂ was successively incorporated with reassembled casein micelles prepared from CASs. They suggested that vitamin D₂ could be associated with the hydrophobic domains of the caseins, and certain portions of the caseins would be incorporated into the reassembled

micelle while others would not [3]. Perhaps, simple aggregates could stabilize a considerable amount of lipophilic substances. As shown by several researchers, CAS solutions mixed with ethanol that dissolved lipophilic substances such as β -carotene [9], bixin [6], and curcumin [7]. Subsequently, their pH values were properly adjusted and spray-dried. This simple microencapsulation process resulted in the encapsulations with high efficiencies, and retaining of the antioxidative properties of the core materials. Although the affinities between the casein micelles and/or aggregates and core materials have been well studied [3,10], investigations that correlate the structural properties and encapsulation properties are scarce. We will discuss in this paper the relationships between the encapsulation and structural properties of the self-aggregated casein systems. Small angle X-ray scattering (SAXS) analysis is a powerful tool to give structural information of nanomaterials, it could also be applied to protein-based micellar systems and aggregates [11,12]. Our approach was first to set-up self-aggregated caseinate systems that stabilized lipophilic component, and then, to compare the SAXS profiles of the aggregates and the encapsulation properties of the resultant dried powders.

In this study, β -carotene was first associated with the caseinates in the CAS solution, and the pH of the solution was adjusted to obtain the casein self-aggregates. These suspensions were then spray-dried to obtain powder

products. The encapsulation efficiencies of β -carotene in the resultant powders were investigated. The stabilities of the encapsulated β -carotene during storage were assessed in terms of the remaining amount of β -carotene and color change of the powders. The structural characteristics of the casein aggregates and micelles were also studied using small angle X-ray scattering (SAXS).

2. Materials and Methods

2.1 Materials

Sodium caseinate from bovine milk and β -carotene (type I, synthetic, >93%) were purchased from Sigma-Aldrich (St Louis, MO 63178, USA). Acetic acid (99.9%), trisodium citrate, dipotassium hydrogen phosphate, calcium chloride, lithium chloride, sodium chloride, hexane, and acetone were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other chemicals used in this work were of analytical grade and used as received.

2.2 Particle preparation

25 g of CAS was dissolved in 400 mL distilled water by stirring overnight. The CAS solution was mixed with 62.5 mg of β -carotene (dissolved in acetone *ca.* 4 mL). This made a CAS- β -carotene solution with the mass ratio of 1:400 (dry basis, β -carotene: CAS). The resultant mixture was stirred in a water bath at 37°C for 2 h. In order to prepare the self-aggregated caseinates, the pH values of the solutions were adjusted by adding an aqueous acetic acid solution and using a pH meter (SK-620PH, Sato Keiryoki MFG Co., Ltd, Tokyo, Japan). Finally, the solution was messed up to 500 mL.

The reassembled casein micelle was also prepared using a previously published method [3]. Briefly, 5.0% (w/v) of CAS solution was mixed with 4 mL of β -carotene solution. After stirring in a water bath at 37°C for 2 h, 10 mL of 1 mol/L trisodium citrate was added to the prepared solution. Then, 2.5 mL of 0.2 mol/L K_2HPO_4 and 3 mL of 0.2 mol/L $CaCl_2$ were consecutively added twelve times every 10 min. Finally, distilled water was added to bring the final volume to 500 mL, pH was adjusted to 6.7 with an acetic acid solution, and the final solution was gently stirred for 1 h.

The prepared solution was fed into an LB-8 spray-dryer with an atomizer (Ohkawara Kakohki Co., Ltd, Yokohama, Japan) at a flow rate of 10 mL/min. The rotational speed of the atomizer was 5000 rpm, and the temperatures of the air inlet and outlet were 150°C and *ca.*

110°C, respectively. The resulting dried powders were kept in tightly capped vials and stored in the dark under dry conditions in vacuum desiccators until analysis. The specimen IDs of the prepared spray-dried powders are listed in Table 1 with the specifications.

Table 1 Specification of spray-dried powders.

Specimen ID	pH	note
control	–	spray-dried without any pH adjustment
A65	6.5	
A60	6.0	
A55	5.5	
RCM	–	reassembled casein micelle

2.3 Encapsulation efficiency measurement

The encapsulated amounts of β -carotenes were measured by extracting them from the dried specimens. Briefly, 0.1 g of dried specimen was soaked in 10 mL of hexane, and stirred for 5 min before the first sampling. The sampled solution was filtered using a membrane filter (pore size 0.45 μ m) while the concentration was determined through the absorbance at 450 nm, measured using UV-visible spectrophotometer (U-5100 Ratio-Beam, Hitachi High-Technologies Corporation, Tokyo, Japan). This concentration was used to evaluate the surface loading amount of β -carotene in particles. The second sampling was done after 24 h. The resultant measurement values were used to calculate the total loading amounts. The encapsulation efficiencies were calculated using the following equations:

Encapsulation efficiency=

$$\left[\frac{\text{Extracted amount of carotene after 24 h}}{\text{Total carotene load in the original solution}} \right] \times 100 \quad (1)$$

Surface loading efficiency=

$$\left[\frac{\text{Extracted amount of carotene by the first sampling}}{\text{Total carotene load in the original solution}} \right] \times 100 \quad (2)$$

$$\text{Inner loading efficiency} = (\text{Encapsulation efficiency}) - (\text{Surface loading efficiency}) \quad (3)$$

The total mass of β -carotene loaded in the original solution was used to standardize the encapsulation efficiencies.

2.4 Measurement of β -carotene retention and color change of powders during storage

One-tenth g of the dried samples were placed in the glass vials and exposed to a controlled relative humidity environment in desiccators at ambient temperature. The relative humidities were set to 11% and 75% using the saturated solutions of lithium chloride and sodium chloride,

respectively. At the predetermined time intervals, the sample vials were removed from the desiccators. The color coordinates of the vials were analyzed and the remaining amounts of β -carotenes were measured using the protocol described in the previous section). The tristimulus color coordinates (L^* , a^* , and b^*) were measured using the NF 333 Handy spectrophotometer (Nippon Denshoku Industrires Co., Ltd., Tokyo, Japan).

2.5 Small angle X-ray scattering measurement

The SAXS measurements were performed at the beam line BL402B2 at Spring-8, Hyogo, Japan. The measurement was carried out at a length of 4139 mm in the camera, which covered a q -range of 0.05–2.0 nm⁻¹. q is the scattering vector; $q = (4\pi/\lambda)\sin(\theta/2)$, where, λ is the wavelength of the beam and θ is the scattering angle. The wavelength of the incident X-rays was set to 0.1 nm. The sample solution was poured into a quartz capillary tube with a diameter of 2 mm diameter and wall thickness of 0.01 mm. The capillary tube filled with the specimen was placed on the measurement stage, and an imaging plate of 30 cm² was used to record the 2-dimensional X-ray scattering patterns.

3. Results and Discussion

3.1 Encapsulation efficiencies

The encapsulation efficiencies of β -carotenes in the spray-dried powder are summarized in Fig. 1. The total encapsulation efficiencies have been presented as the sum of the surface and inner loading efficiencies. Spray-drying yielded aggregated casein powder, which encapsulated a certain amount of β -carotenes. Our data showed that the encapsulation efficiency was approximately 53%. The yield could be improved by adjusting the pH of the original solution. The solution with pH 6.5 could load 84% of β -carotene after spray-drying; however, approximately 6% of β -carotene were located on the surface (β -carotene was initially released from the powder when extracted). Specimens A60 and A55, which were prepared from the solution using highly acidic conditions, could encapsulated approximately 91% and 86% of β -carotenes, respectively. The surface load for A60 remained at 7%, while that for A55 was 9%. Assuming that good encapsulation involved maximizing inner loading efficiency and minimizing surface loading, the processing conditions for A60 were the best among the available conditions. The spray-drying of a reassembled casein micelle solution yielded powder (RCM) containing a

small amount of surface loading and 59% inner loading. This, however, suggested that the encapsulation tendency of the aggregated system was different from that of the micelle system.

3.2 Retention and color change of β -carotene during storage

The prepared specimens were stored under constant relative humidity conditions as the remaining amounts of β -carotene were measured, as depicted in Fig. 2, where the β -carotene retention was calculated by dividing the remaining amount of β -carotene, C [kg- β -carotene/kg-powder], by the initially encapsulated amount, C_0 . As expected, increasing relative humidity (RH) decreased the amount of β -carotene. The degradation trend suggested first order kinetics. The specimen A65 lost approximately 50% of its β -carotene over nearly one week under 75%RH, whereas A60 could hold 50% over two weeks. Both A65 and A60 could hold approximately 43% for 60 days at 11%RH. A55 could not hold its β -carotene at higher levels. The retention was down to less than 20% in 30 days at 75%RH and 25% at 11%RH. RCM showed considerable stability at 11%RH; however, the retention after storing for 60 days was approximately 46%. However, RCM did not show high stability at 75%RH. The remaining β -carotene decreased to less than 30% over 30 days and 20% over 60 days. This trend was not seen during the storage of the aggregation-based specimens, A65, A60 and A55.

Fig. 1 Encapsulation efficiencies; inner loading efficiency (white), surface loading efficiency (grey), the values on the top represent the sum of the inner and surface loading efficiencies. Specimen IDs are listed in Table 1.

Fig. 2 β -Carotene retention in powder during storage. Specimen IDs are listed in Table 1.

The color changes were plotted as a function of the storage time (Fig. 3), where $a^*-a_0^*$ was the absolute value of the color change from the starting point ($a^*=a_0^*$). The color coordinate a^* was only discussed in this paper because its changes were obvious. The change in color reflected one kind of degradation of β -carotene; nevertheless their time dependent trends did not perfectly correspond to the retention trends shown in Fig. 2. The storage condition 75%RH led to a larger change in the color compared to the 11%RH for all the specimens. This sug-

gested that the stabilization mechanism was influenced by the moisture content. However, considering that the chemical components of the present specimens were identical (except reassembled micelle containing calcium salts), it would be reasonable to suggest that the location of the encapsulated β -carotenes in the particles largely differed depending on the physical structure of the particles. The color changes followed zero order kinetics, as the changes were linear. A marked difference, which appeared among the specimens, was a retardation in the

Fig. 3 Color change of powder during storage. Specimen IDs are listed in Table 1.

color change. This clearly appeared in A65, A60, and RCM. The obvious color changes were not confirmed in A65 and A60 for approximately 30 days at 11%RH, and approximately two weeks in RCM. This retardation was not observed at 75%RH. A55 changed its color both in the cases of 75%RH and 11%RH.

The encapsulated β -carotene in A55 was more easily degraded (oxidized) compared to those in A65 and A60. Considerable differences among A65, A60, and A55 arose owing to the differences in the degrees of aggregation in the original solution, based on the pH values. These

aggregates further aggregated during spray-drying and formed particles. A small surface area could reduce the reactive degradation by the external gases. However, the size of the primary particles (aggregates in solution) increased in the following order: A55 < A60 < A65 (discussed in the following section). As a result, the trends in degradation could not be simply explained by the differences in the particle sizes. Although the amount of the surface β -carotene of A55 was larger compared to those of A65 and A60, these differences were not large enough to cause any variations in the retentions and color

changes. We thus postulated that these differences emerged from the structural differences in the aggregates. This was verified using SAXS analysis, as explained in the next section.

3.3 Small angle X-ray scattering (SAXS) analysis

The SAXS profiles of the precursor solutions of A65, A60, A55, and RCM have been compared in Fig. 4. The discrepancies in these profiles indicated that the structures of these specimens were significantly different on the measured scale, *i.e.*, from 3 to 125 nm. A scattering profile gives the pair distance distribution function (PDDF) by Fourier Transform [13]. The radius of gyration, R_g , of the scattering medium (*i.e.* aggregated CAS) could be estimated from the PDDF. The values of R_g for the present specimens are listed in Table 2. It was observed that the R_g of the aggregates in the solution with pH 6.5, 6.0, and 5.5 were *ca.* 91, 111, and 166 nm, respectively, while the R_g of the reassembled casein micelle was approximately 76 nm. The scattering profile also reflected the fractal structure of the scattering medium. The slope obtained from the logarithmic plot of the scattering intensity, $I(q)$, yielded the fractal dimension, $D=n$ (*i.e.*, $I(q) \sim q^{-n}$). If the value of the slope lay over the range of 2–4, one could consider it as the surface fractal, D_s , given by $D_s=6-n$ [14]. The present scattering profiles commonly exhibited a peak at the lower end of the q range, so that the power exponent values (*i.e.*, fractal dimension) could be estimated using the

higher end of the q range rather than the peaks. The power exponents, $-n$, estimated from the values lying the range covering the peak, was approximately 4, thereby suggesting that the corresponding scale indicated a smooth surface. The power exponents estimated using the higher end values of the q range were approximately 2, thereby suggesting mass fractal nature over such a scale. In this study, a power exponent value was therefore estimated from a q range lying in between the ranges, *i.e.*, approximately $q=0.4$ – 0.9 . The surface fractal dimensions, D_s , was calculated using $D_s=6-n$, and are listed in Table 2. Interestingly, the D_s values of the aggregates in the solution at pH 6.5, 6.0, and 5.5 were approximately 2.4, 2.8, and 2.9, respectively. These values corresponded to the increase of the particle sizes when the pH of the CAS solution was down from 6.5 to 5.5. So, this aggregate growth was a fractal growth, that is, a random fractal structure formed in the aggregated particle. We opine that this fractal growth nature influenced the encapsulation efficiencies. As discussed in the earlier sections, A60 was found to have the highest encapsulation efficiency and stability during storage. The encapsulation tests and stability analysis suggested a difference in the structures among the different aggregated specimens. The SAXS data clarified that the present aggregations represented a fractal growth system, wherein the randomness of the surface increased with increasing particle size. It was noteworthy that A60 was the best among the tested specimens, which suggested that there existed an optimal aggregation degree, which maximized the performance of an encapsulated system. Consequently, it is important to control the aggreration mechanism during the syntheses of aggregation-based nanoencapsulation systems.

Fig. 4 Small angle X-ray scattering profiles. Specimen IDs are listed in Table 1.

Table 2 Estimated representative aggregate sizes and surface fractal dimensions.

Specimen ID	R_g [nm]	D_s
control	70.7	2.4
A65	91.1	2.4
A60	111	2.8
A55	166	2.9
RCM	76.2	2.4

4. Conclusions

In this study, β -carotene was encapsulated by self-aggregated caseinates, and the encapsulation efficiencies and storage stabilities of the resultant spray-dried powders were investigated. SAXS analysis was performed to correlate the structural insights to the encapsulation

properties.

We found that the pH of the sodium caseinate solution, which determined the degree of the aggregation, immensely affected the encapsulation efficiencies of β -carotene in the spray-dried powder. Among the examined pH conditions (pH=6.5, 6.0, and 5.5), pH 6.0 yielded excellent encapsulation efficiency and storage stability. In the present study, these aggregation-based systems showed superior encapsulation properties compared to the micelle-based systems. All the encapsulated β -carotenes underwent increased degradation with increasing humidity, thereby suggesting that the stabilization mechanism was influenced by the moisture content. However, considering that the encapsulants were made from the same chemical components (except reassembled micelle, which contained calcium salts), it would be reasonable to suggest that the encapsulated β -carotenes were localized in the particles and their stability was affected by the physical structures of the particles. SAXS analysis estimated the representative sizes of the aggregates (radius of gyration) in the solution, and clarified that the present aggregation was a fractal growth system. The size of the aggregates increased with increasing pH in the following order: 5.5 < 6.0 < 6.5. Also, the surface fractal dimension increased in the same order. Thus, these differences were linked to the encapsulation characteristics, and suggested that there would be an optimal aggregation degree, which maximized the performance of the encapsulated systems.

5. Acknowledgement

This study was partially supported by The Japanese-Finland Cooperation Research Project on “Nano-emulsions and encapsulation for delivering functionality in foods” .

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自己凝集カゼイネートへの相互作用を利用した β カロテン噴霧乾燥マイクロカプセル

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カゼインは乳タンパク質のひとつであり、安価であること、安全性、乳化特性、生分解性、臭気特性などに優れることから様々な応用が期待されている。脱脂乳より酸の添加により分離される固形分中の主成分がカゼインであり、これをナトリウム塩により可溶化させたものがカゼインナトリウムである。カゼインナトリウムは、pH に依存してナノサイズの自己凝集体を形成する。疎水性相互作用を利用して脂溶性物質をカゼイネートの疎水鎖に吸着させた後、pH 調整によって凝集構造を形成させることで極めて簡単にナノカプセルを作製することができる。近年これを、栄養物質や薬効成分を送達するためのキャリアとして利用する技術が研究されている。本研究では、カゼインナトリウム溶液中に β カロテンを混合・安定化させた後、pH 調整によって自己凝集体を形成させた。この分散液を噴霧乾燥させることで乾燥粉末を作製し、得られた粉末のマイクロカプセルとしての特性を β カロテン包含率、貯蔵安定性、色変化の観点から評価した。本実験において混合させた β カロテンの量はカゼイネート 400 g に対して 1 g と比較的低く設定し、最終的に得られる噴霧乾燥粉末中への包含率も比較的高い値が得られるような系を採択した。また、pH 調整に伴って形成する凝集体構造に関する情報を小角 X 線散乱分析により得た。

初期溶液の pH を 6.5, 6.0, 5.5 とそれぞれ変化させた場合、pH を 6.0 に設定した溶液から得られた噴霧乾燥粉末において最も β カロテンの包含率が高いことが見出された。表面包含率、内部包含率と分けて分析したところ、この設定は表面包含率を低く、内部包含率を高く

する条件であることがわかった。一定湿度下における β カロテンの貯蔵安定性を評価したところ、やはり同条件にて作製された試料が優れた特性を示した。pH を 5.5 と設定することでより凝集度が高まるものの、包含率、貯蔵安定性などの観点では優れてはいなかった。貯蔵過程における色変化を測定したところ、pH を 5.5 と設定した場合には貯蔵期間に比例して退色が進行したのに対し、pH を 6.5, 6.0 に設定した場合においては室温にて約 30 日間、低湿度下では大きな退色は起こらなかった。本研究におけるマイクロカプセルは、賦形剤などを添加しないカゼイネートのみで安定化された系である。原料溶液中の凝集に加え、乾燥過程における凝集も起こると考えられるが、上記実験結果は β カロテンの包含のメカニズムに原液中の凝集構造が強く関わっていることを示唆している。

原液中の凝集体構造に関わる知見を得るために小角 X 線散乱にて凝集体のナノ構造を評価した。散乱プロファイルをフーリエ変換して得られる二体間距離分布関数より凝集体の慣性半径を算出し、また散乱強度の傾きより表面フラクタル次元を算出した。その結果、pH 変化に依存した凝集体サイズと表面フラクタル構造の変化が確認できた。pH を 6.5 から 5.5 へと低下させることで凝集体サイズは 91 nm から 166 nm へと増加するが、同時にフラクタル次元も 2.4 から 2.9 へと増加した。すなわち pH 変化に伴う凝集によって、よりフラクタル性の高い表面構造が形成していることが示唆された。恐らくこの物理的構造の違いが、凝集構造内に包含される β カロテンの安定性と関連していると考えられた。

(受付 2014 年 3 月 4 日, 受理 2014 年 5 月 14 日)

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