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Encapsulation efficiency of vitamin D_3 in α-lactalbumin during storage

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Abstract

Vitamin D deficiency causes rickets in children and will precipitate and exacerbate osteopenia, osteoporosis, and fractures in adults. It has been associated with increased risk of common cancers, autoimmune diseases, hypertension, and infectious diseases. A circulating level of 25-hydroxyvitamin D of >75 nmol/L, or 30 ng/mL, is required. Fortification program of vitamin D in daily food products is one of an effective way to increase vitamin D in food. Since vitamin D is a sensitive vitamin, encapsulation of α-lactalbumin is needed to protect it from the environments, such as light, heat, and oxidation. In this study, vitamin D_3 was encapsulated with α-lactalbumin. The objective was to obtain the stability and the structure of vitamin D_3 encapsulated with α-lactalbumin. Encapsulation efficiency (EE) measured by UV-VIS spectrophotometer with wavelength of 264 nm. Structure of vitamin D_3 encapsulated with alpha-lactalbumin obtained by scanning electron microscope (SEM) with four different magnification. In this study, vitamin D_3 attached inside α-lactalbumin as seen by SEM. The morphology showed the hydrophobic binds between vitamin D_3 and α-lactalbumin. Vitamin D_3 was successfully encapsulated with α-lactalbumin with an EE 94.89%. The highest EE was from the vitamin D_3/α-lactalbumin ratio 5:1. The coating of α-lactalbumin increased the solubility of vitamin D_3 in the system. System stored in a cool temperature (5°C). The encapsulation efficiency of the system is 16.44% on the seventh day. It means that it decreases a lot from 100% to 16.44% in seventh day and proof that attachment between the system is weak. Finally, it can conclude that the encapsulation efficiency of α-lactalbumin is high, but the shelf-life is short and attachment between the system is week.

1. Introduction

Vitamin D is a fat soluble vitamin that plays a significant role in the regulation of calcium and maintenance of phosphorus levels in the blood. These factors are vital for maintaining healthy bones. People need vitamin D to allow the intestines to stimulate and absorb calcium and reclaim calcium that the kidneys would otherwise excrete. Vitamin D deficiency in children can cause rickets, which leads to a severely bowlegged appearance due to the softening of the bones. Similarly, in adults, vitamin D deficiency manifests as osteomalacia or softening of the bones. Osteomalacia results in poor bone density and muscular weakness. A vitamin D deficiency can also present as osteoporosis, for which over 53 million people in the United States either seek treatment or face an increased risk. Vitamin D can be obtained through food and supplementation. Vitamin D deficiency can be prevented by sufficient vitamin D intake (Lhamo *et al.,* 2017). Study in 280 samples found that 174 subjects (62.1%) insufficient vitamin D and 66 subjects (23.6%) deficiency vitamin D (Amer *et al.,* 2013). Study in Indonesia showed that 45.1% of children 1-18 years old deficiency vitamin D (Schaafsma *et al.*, 2013). Another study showed that osteopenia prevalent that caused by vitamin D deficiency in 25 years old adult is 37.1%. Vitamin D_3 has a big potential to increase 25-hydroxyvitamin D (25(OH)D) serum in the liver. Study in pelvic fracture patients that caused by vitamin D deficiency showed that 25 µg vitamin D_3 consumption is more effective than vitamin D_2 consumption with the same dose in increasing the 25 (OH)D serum level (Mahony *et al.*, 2011).

Vitamin D_3 is sensitive towards light, heat, oxidation, and is insoluble in water, vitamin D also not stable in

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acidic environment (Carolyn *et al*., 1995). Those things can affect the bioavailability of vitamin D in the body (Hasanvand *et al.,* 2015). Vitamin D_3 has the ability to isomerize in some condition. Isotachysterol is an isomer that forms an acidic condition (Mahmoodani *et al*., 2018). Supplementation of 2.000 IU vitamin D_3 for 20 weeks can increased level serum 25(OH)D significantly (Kerley et al. 2017) but it can't be used for long term consumption. Vitamin D fortification can be used to overcome vitamin D efficiency. But, since vitamin D_3 is sensitive to light, acid, and oxidation, encapsulation can be an alternative to increase the stability and bioavailability of vitamin D_3 for protection and delivery function.

Biomolecules like protein can be used to encapsulate vitamin and nutraceuticals. The previous study showed that β-lactoglobulin and casein used as nano vehicle to deliver vitamin D_3, docosahexaenoic acid (Chen *et al*., 2006; Abbasi *et al*., 2014), and genistein (Anjani, 2014). Whey protein is a globular protein. Milk protein contains 20% whey protein. α-lactalbumin is whey protein that able to bind hydrophobic ligands such as vitamin D_3 (Teng *et al.,* 2015), oleic acid (Gustafsson *et al*., 2005; Kehoe and Brodkorb, 2014), retinol (Livney, 2010), hydrophobic column chromatography phases, hydrophobic peptides, and melittin of bee venom (Barbana *et al*., 2006). It is a small globular protein that contains 123 essential cysteine amino acid with a molecular mass of 14.2 kDa, acidic (pI 4.8 present in the milk of all mammals where it is involved in the synthesis of lactose. The concentration of α-lactalbumin is 1-1.5 g/ L in bovine milk (about 3.4% of total milk protein). It is one of the dominant protein in human milk (Permyakov and Berliner, 2000; Jackson *et al*., 2004). It has an affinity for ligation to a group of hydrophobic ligands like some fatty acids, retinol and some peptides. The natural structure of bovine α-lactalbumin consists of a large helical domain and a small β-sheet domain, which is connected by a loop (Permyakov and Berliner, 2000). The helical domain contains three main α-helices (residue 5-11, 23-24 and 86-98) and two main smaller 310 helices (residue 18-20 and 115-118). The structure of β-sheet domain composes of three antiparallel β-strands (residue 41-44, 47-50 and 55-56), a 310 helix (residue 77-80) and some loops. The α-lactalbumin has a deep cleft between two domains, four disulfide bridges and one hydrophobic pocket. Native α-lactalbumin contains a calcium ion, bound to its high affinity binding site in the loop connecting two domains. Depletion of calcium ion changes the α-lactalbumin tertiary structure (Permyakov and Berliner, 2000). α-lactalbumin is the main precursor of glutathione which can be used for detoxification and antioxidant. It can be a good protein to deliver vitamin D_3 through hydrogen, van der waals, and hydrophobic binding (Delevari *et al.,* 2015).

Vitamin D is very sensitive to light, pressure and oxidation and to aqueous media (Fessi *et al*., 1989; Ibrahim *et al*., 1992; Chen *et al*., 2006; Abbasi *et al*., 2014). Previous studies have shown that the enrichment of liquid milk alone is not enough. Foods should be supplemented with vitamin D_3 (Holick, 1992; Vieth, 1999). Enrichment of other dairy products can compensate for the deficiency of vitamin D_3 in consumed foods (McKenna *et al*.,1995). Some proteins such as casein and β-lactoglobulin were proposed as nano vehicle of vitamin D due to their low cost and availability (Forrest *et al*., 2005). α-lactalbumin is a whey protein with the ability to interact with hydrophobic ligands. A study on vitamin D_3 enriched cheddar cheese has shown that their whey protein binding is loose. Compared with caseins, whey proteins can be better vehicles for vitamin D_3 (Banville *et al*., 2000). β-lactoglobulin is allergic so we can offer the less allergic one, which is α-lactalbumin (Selo *et al*., 1999; Hernandez-Ledesma *et al*., 2007). The stability of vitamin D_3 has previously been studied in natural foods during their storage and processing. This study analyzed the encapsulation efficiency of α-lactalbumin to encapsulate vitamin D_3 during storage and its morphology. Utilization of α-lactalbumin as an encapsulation material is expected to protect vitamin D_3 in gastrointestinal so that it can be absorbed optimally in the body and can be fortified in food products.

2. Materials and methods

2.1 Materials

Cholecalciferol vitamin D_3 (≥98%, C9756), Ethylenediaminetetraacetic acid (EDTA), tris (hydroxymethyl) and the Ca^{2+} - depleted α-lactalbumin from bovine milk (≥85% pure) were purchased from Sigma-Aldrich. Methanol and aquadest were purchased from Indrasari, Semarang, Indonesia.

2.2 Preparation of vitamin D_3 encapsulated with α-lactalbumin

About 450 µL α-lactalbumin (10 µM) was dissolved in tris buffer (20mM) containing EDTA (3.5 mM). Vitamin D_3 dissolved in ethanol. Then α-lactalbumin is titrated by vitamin D_3. At the end of the titration, the molar ratio of vitamin D_3/α-lactalbumin is 5 with ethanol below 3% (v/v). The mixture then vortexed for 10 s and incubated for 4 mins to form vitamin D_3- α-lactalbumin complex. It prepared on 4 different ratios of vitamin D_3 and α-lactalbumin, which are 2:1, 3:1, 5:1, 14:1.

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2.3 Encapsulation efficiency

To determine the amount of vitamin D_3 loaded in the system, UV-VIS spectrophotometer was used with the wavelength of 264 nm. Linearity was calculated for two different solutions (a) vitamin D_3 in methanol solution and (b) vitamin D_3 in 80% aqueous methanol solution in methanol (80:20 v/v). The calculation uses the following equation that obtained from the standard curve of vitamin D_3: $y = 0.1033x – 0.0381$

2.4 Stability of vitamin D_3 during storage

The vitamin D_3 encapsulated with α-lactalbumin were distributed in transparent test tube that covered with alumunium foil. The stability of vitamin D_3 encapsulated α-lactalbumin during storage was tested for seven days for stability. The first measurement was on the first day, then the measurements were examined every day until the seventh day. Residual vitamin D_3 was measured using UV-VIS Spectrophotometer, were analyzed triplicate.

2.5 Morphology analyzed

Morphology of vitamin D_3 encapsulated with α-lactalbumin analyzed used Scanning Electron Microscope (SEM) with 4 magnitudes (3,000x; 5,000x; 7,500x; and 10,000x).

3. Results and discussion

3.1 Encapsulation efficiency

Encapsulation efficiency (EE) measured by UV-VIS spectrophotometer with the wavelength of 264 nm. Four different ratios of vitamin D_3/α-lactalbumin were observed 2:1, 3:1, 5:1, and 14:1. The result of encapsulation efficiency (Table 1) showed that the highest EE is on vitamin D_3/α-lactalbumin ratio 5:1. Vitamin D_3 was successfully encapsulated with alpha-lactalbumin with an EE 94.89%. To achieve the optimal effects of the protected compound towards its target, high efficiency encapsulation is desired in the bioactive compound. α-lactalbumin can self-assemble into nanotube structures that could be used to modify food texture and to encapsulate micronutrients. The structure of the encapsulation shows that α-lactalbumin has one site for vitamin D_3. Based in the research held by Clara, α-lactalbumin nano tube were highly effective to encapsulate caffeine, as the encapsulation efficiency was near 100% and loading capacity near 10% (Fucinos, 2017). The values of %EE obtained with vitamin D_3 encapsulated with α-lactalbumin were higher than those achieved by caffeine encapsulation into other biopolymer nano particle, like the alginate nano particle with around 85% EE (Bagheri *et al*., 2013), or lactoferrin-glycomacropeptide nano hydrogel with around 90% EE (Bourbon *et al*., 2016). This fact is even more evident when the results obtained here are compared with those obtained when other bioactive compounds, different from caffeine, were encapsulated in whey protein nano structure. For example, the 70–80% EE reached by metoprolol succinate encapsulation into α-lactalbumin micro particle (Vijayaragavan *et al*., 2014), the 55% EE obtained by anthocyanin extract (Arroyo-Maya *et al*., 2012) or the encapsulation of date palm pit aqueous extract into WPI nano particle (Bagheri *et al*., 2013). Efficiency encapsulation may affect the release properties that depend on the structure of nano particle system (Tamjidi *et al.,* 2013). The proteins such as β-lactoglobulin and α-lactalbumin may encapsulate vitamin D by hydrophobic interaction, and they inhibit from degradation of vitamin D by air as the oxidizing agent. The encapsulation of bioactive substances with different kind of liposomes have been reported between 50-99%, depend on the concentration ratio, method of preparation, and lipid composition (Rovoli *et al*., 2014.)

Table 1. Encapsulation Efficiency of three different ratio of α-lactalbumin and vitamin D_3

Vitamin D_3 : α-lactalbumin	Encapsulation Efficiency (%)
2:01	6.5
3:01	19.8
5:01	94.89
14:01	20.49

3.2 Stability of vitamin D_3 during storage

Stability if vitamin D_3 was observed during storage in cool temperature (4-5°C) for a week (Table 2). On the first day, EE was 94.89%, but on the second it was 47.01%. The EE reduced by half for two days. On the seventh day, the EE decrease to 16.44%. Abbasi et al determined the stability of vitamin D_3 encapsulated in NPC were 34% while, this value in two other particles reached to 21% and in the control samples reached to less than 10% after 7 days. The coating of α-lactalbumin increased the solubility of vitamin D_3 in the system better than NPC. Calcium ions can create ion binding between protein molecules. These reaction cause to formation of a compact structure with low porosity that slows down oxygen diffusion into particles and the rate of vitamin D degradation (Britten and Giroux, 2001). Teng *et al*. (2013) founded that carboxymethyl chitosan–soy protein complex nano particle or greater particles encapsulated higher concentration of vitamin D_3 than the ones prepared with the single ingredient or small nano particle. They concluded that these particles had higher capacity for vitamin D incorporation. Some vitamins are unstable when exposed to light, particularly UV light, air, oxidizing agents and heat treatment. Depending on the packaging type, the storage conditions (under light or dark conditions) and the initial quality of oil (e.g. peroxide and acid values), lipids and lipophilic vitamins can be lost within 2 weeks of storage (Laillou *et al*., 2013). Vitamin D stability in fortified foods depends on light and oxygen exposure (Jakobsen and Knuthsen, 2014). During the spray-drying or storage of fortified dairy products, the vitamin D3 added is stable (Kazmi *et al*., 2007; Wagner *et al*., 2008), whereas it degrades and isomerizes in an acidic environment or during sunlight drying of fish (Suzuki *et al*., 1988; Jin *et al*., 2004).

Table 2. Shelf –life of Vitamin D_3 encapsulated α-lactalbumin

Day	Encapsulation Efficiency (%)
1	94.89
2	47.01
3	44.47
4	31.16
5	30.6
6	24.68
7	16.44

3.3 Morphology analyzed

The morphological observations of α-lactalbumin alone and vitamin D_3 encapsulated with α-lactalbumin are shown in Figure 1 and Figure 2. It was evaluated by scanning electronic microscopy (SEM) at 3,000; 5,000; 7,500, and 10,000 magnifications. The SEM image of vitamin α-lactalbumin showed a rounded external surface containing characteristic of spheres (Figure 1). No visible holes and fractures were observed in the outer surfaces of the particles. Relative uniformity in particle size was observed. After encapsulation with α-lactalbumin, the shape of the particles became spherical and smooth. The morphology shows the hydrophobic binds between vitamin D_3 and alpha-lactalbumin (Figure 2). Moreover, the dispersion of vitamin D_3 into the α-lactalbumin affected size of the nano particles, and a significant change in the particle size was observed. The structure of the encapsulation shows that alpha-lactalbumin has one site for vitamin D_3. During the hydrophobic interactions of vitamin D_3 with alpha-lactalbumin, the conformation of protein changes and the hydrophobic surface of the alpha-lactalbumin increases. Previous studies reported particles exhibited a spherical shape and smooth shapes (Hsieh *et al*., 2006; Luo *et al*., 2012).

4. Conclusion

In this study, the highest encapsulation efficiency (94.89%) was from the vitamin D_3/α-lactalbumin ratio 5:1. It showed that vitamin D_3 encapsulated with α-lactalbumin has high EE. To see the shelf life, system stored in cool temperature (5°C) for a week. During the second day, the EE decreased around 50%. On the last day, EE decreases to 16.44%. Morphology determined by SEM showed that vitamin D_3 stick inside the α-lactalbumin. During the hydrophobic interactions of vitamin D_3 with α-lactalbumin, the conformation of protein changes and the hydrophobic surface of the α-lactalbumin increases. The coating of α-lactalbumin increased the solubility of vitamin D_3 in the system, but it has a weak binding because the EE was drastically decrease during a week storage. Finally, we can conclude that the encapsulation efficiency of α-lactalbumin is high, but the shelf-life is short. The morphology shows the hydrophobic binds between vitamin D_3 and α-lactalbumin.

Conflict of interest

The authors declare no conflict of interest.

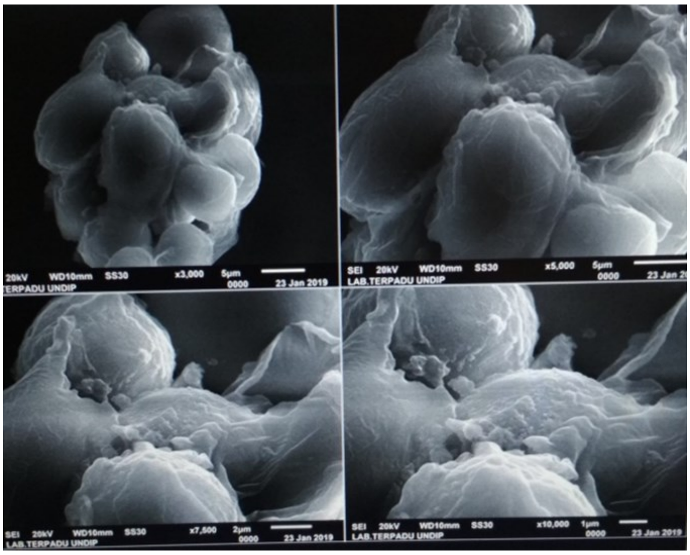

Figure 1. Morphology of α-lactalbumin using SEM

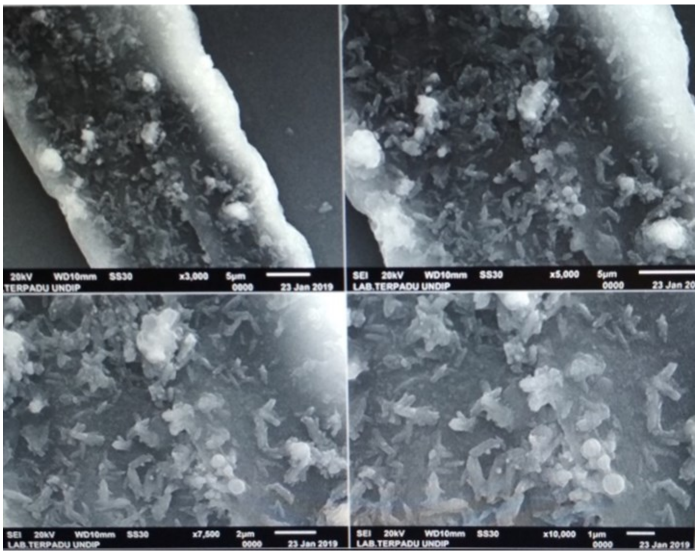

Figure 2. Morphology of Vitamin D3 encapsulated with α-lactalbumin using SEM

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