Nanosuspended branched chain amino acids: the influence of stabilizers on their solubility and colloidal stability

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Abstract This study examined the influence of stabilizers with different hydrophilic-lipophilic balances on the solubility of branched chain amino acids (BCAA) and colloidal stability of nanosuspended BCAAs. Initial BCAA solubility increased by homogenization as evidenced by the BCAA solubility in Tween 80-based nanosuspensions, which remained at almost 97% of their initial solubility after 20 days of storage. However, the contents of solubilized BCAAs in Span 80-based nanosuspensions decreased to approximately 85% of their initial solubility after 20 days of storage. In fact, the BCAA:Tween 80 ratio had no effect on the colloidal stability but the same variable changed according to the BCAA:Span 80 ratio. Based on this study, it can be concluded that stabilizers with a hydrophilic trait (Tween 80) could be more effective in improving BCAA solubility and the colloidal stability of nanosuspended BCAAs compared to those with a lipophilic trait (Span 80).

Keywords Branched chain amino acid · Colloidal stability · Nanosuspension · Solubility · Stabilizer

Introduction

Owing to the many biological functions of branched chain amino acids (BCAA) that include metabolism modulation in muscle cells [1–3], being precursors of other amino acids and proteins [4, 5], as well as being anti-oxidants [6], BCAAs such as L-leucine, L-isoleucine, and L-valine are utilized in the food and pharmaceutical industries as functional ingredients. When the BCAAs are supplied through food pharmaceutical products in a similar ratio to that they are naturally present in an animal cell (2:1:1 = L-leucine:L-isoleucine:L-valine), the maximization of biological benefits could be expected [7].

However, similar to most of natural bioactive compounds, a main obstacle of utilizing BCAAs in food and pharmaceutical products is their low water solubility. This is a possible reason for that most of commercially available BCAA-related food and pharmaceutical products exist in the powder form. Therefore, the solubilization of BCAAs is a key hurdle to overcome in food and pharmaceutical industries in developing food and pharmaceutical products with high bioavailability and absorption [8–10]. Increasing interest has been given various solubilization techniques including emulsions, suspensions, capsules, and micelles for the increase in the solubility of poorly water-soluble functional materials and for the increase in their bioavailability. In addition, recently, these solubilization strategies are combined with several nanonization techniques, developed into nanoemulsion, nanosuspension, and nanocapsulation [11–13]. Among these solubilization techniques, nanosuspension systems created through high-

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pressure homogenization have received increasing interest as no harsh solvents are used [12]. High-pressure homogenizer generates the intense disruptive forces by passing colloidal solutions containing poorly water soluble bioactive compounds through very small orifice of the homogenizer at a high pressure. High-pressure homogenization is a widely accepted technique for creating nanosuspension in the food and pharmaceutical industries. When poorly water-soluble particles are dispersed into an aqueous solution as nanosuspension, the area-to-volume ratios of the poorly water-soluble materials increase. As a result of this phenomenon, the dispersed particles (regardless of their sizes) are vulnerable to flocculation and/or coalescence due to Ostwald ripening [14]. Since stabilizers generally have the ability to prevent flocculation and/or coalescence of nanoparticles and nanodroplets through electrostatic repulsion and a steric stabilization effect, the incorporation of stabilizers into the nanosuspensions is required to maintain their initial sizes and to give them long-term colloidal stability [15].

Therefore, in this work, the effects of stabilizers with the different hydrophilic-lipophilic balances on the solubility of BCAAs and the colloidal stability of BCAAs in nanosuspensions were determined with the aim of providing helpful information on the selection of the appropriate stabilizer to develop BCAA-related products having liquid or colloidal forms with the high BCAA solubility and the colloidal stability of BCAA-nanosuspended solutions.

Materials and methods

Materials

Branched chain amino acids (BCAA, L-leucine (purity 99.7%), L-isoleucine (purity 99.4%), and L-valine (purity 99.2%)) were a gift from Daesang Inc. (Seoul, Korea). Tween 80 and Span 80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). According to the supplier's information on Tween 80 and Span 80, the values for their molecular weights and hydrophilic-lipophilic balances are 1310.0 and 428.6 g/mol, and 15.0 and 4.3, respectively. All reagents were used as food grade.

Preparation of nanosuspensions

The stabilizer solutions were prepared by dissolving Tween 80 or Span 80 into 10 mM phosphate buffer (pH 6). The mixture of BCAAs (L-leucine:L-isoleucine:L-valine = 2:1:1) was directly added into the stabilizer solution to reach a final concentration of 5% (w/v). All BCAA suspensions were stirred for 2 h at 25 °C. All sample solutions were stirred for 2 h at 25 °C. The sample solution

was first preheated at 70 °C and subsequently, homogenized over 5 cycles at 100 MPa using a high pressure homogenizer (MN400BF, Micronox, Seongnam, Korea) at 70 °C. The freshly prepared nanosuspension was divided into the storage containers or measuring glass vials for a Turbiscan LAB optical analyzer (Formulation, L'Union, France). Nanosuspended BCAAs were stored under inert conditions at 25 °C for up to 20 days.

Measurement of BCAAs' solubility in nanosuspensions

Following the method of Starcher [16], the content of solubilized BCAAs was determined by measuring the UV absorbance of a ninhydrin reaction's product. A 4 N sodium acetate buffer was prepared by dissolving 6.542 g of sodium acetate in 2 mL of glacial acetic acid and distilled water up to 10 mL total volume. A stannous chloride solution was prepared by dissolving 1 g of SnCl_2 in 10 mL of ethylene glycol. The ninhydrin reagent was prepared by dissolving 0.16 g of ninhydrin in the mixed solution of 6 mL ethylene glycol and 2 mL sodium acetate buffer, followed by the addition of 200 μL of stannous chloride solution under stirring. To measure the BCAA solubility in nanosuspension, sample was collected from the upper layer of storage container and filtered through syringe filter having pore sizes of 0.02 μm to remove the insoluble BCAA crystals. Equal volumes (100 μL) of the ninhydrin reagent and sample solution were added to a glass vial, and the ninhydrin reaction was accelerated by heating at 100 °C for 10 min. The reaction was stopped by adding 1 mL of 50% ethanol followed by cooling at 0 °C for 2 min. The ninhydrin reaction product was measured by using a spectrophotometer (UV1650PC, Shimadzu, Kyoto, Japan) at 575 nm.

Measurement of BCAA nanosuspensions' colloidal stability

To determine the colloidal stability of BCAA nanosuspension, a Turbiscan Lab optical analyzer (Formulation) was used. The nanosuspension was first transferred into a measuring glass vial of a flat-bottomed cylindrical shape (70 mm in height, 2 mm in bottom thickness, and 25 mm in internal diameter) to a height of 50 mm (48 mm of sample height). The vial was later placed in the instrument, which utilizes a near-infrared electroluminescent diode as the light source ($\lambda = 880 \text{ nm}$). Optical sensors periodically recorded the intensities of transmitted light at 40 μm intervals at 25 °C, while moving along the sample height ($\approx 55 \text{ mm}$). The percentage of transmitted light is measured as a function of sample height and storage time. Each sample vial was divided into three equally sized layers (bottom layer:

0–16 mm, middle layer: 16–32 mm, and top layer: 32–48 mm of sample height). The obtained variation of transmission was used to determine the colloidal stability of the BCAA nanosuspensions. The sum of the percentage of transmitted light at each layer was used to determine the colloidal stability of BCAA nanosuspensions.

Statistical analysis

All of the experiments were performed in triplicate, and the data were expressed as the mean $\pm$ SD. Analysis of variance (ANOVA) was performed, and the mean separations were performed using Duncan's multiple-range test ($p < 0.05$). All of the statistical analyses described above were conducted using SAS (version 9.3., SAS Institute Inc., Cary, NC, USA).

Results and discussion

Solubility of BCAAs in nanosuspensions

Low-energy consumption techniques (e.g., stirring and heating at 70 °C) were insufficient in dissolving the BCAAs completely (Table 1). Although heating at 70 °C improved the initial solubility of the BCAAs, thus agreeing with previous reports on the proportional relationship between temperature and the solubility of amino acids

[17, 18], heating alone was insufficient to dissolve the BCAAs completely. In contrast, the homogenization process following heating at 70 °C dissolved the BCAAs completely. The increased initial BCAA solubility could be due to powerful disruptive forces that arise from the homogenizer effect, which is achieved by pushing liquid solution through a very narrow orifice under a high pressure. Disruptive forces such as cavitation, collision, and shearing have a great impact on the BCAA particles, resulting in the disintegration of the coarse particles (coarsely suspended state) to nanoparticles (nanosuspended state) [11]. Although the low-energy consumption techniques could increase the initial BCAA solubility to some extent, the complete dissolution of BCAAs was not achieved by these approaches. Therefore, the complete dissolution of BCAAs could be achieved through high-energy consumption techniques (including high-pressure valve homogenization, microfluidization, and ultrasonication) that are able to generate intense disruptive forces [19].

In the case of the nanosuspension that was prepared via high-pressure homogenization without using a stabilizer, most of the BCAAs were initially dissolved but only 82% of the initially soluble BCAAs remained after 20 days of storage (Table 1). In comparison to samples prepared using stabilizers, it seemed that stabilizers had no effect on the initial solubility of the BCAAs and there was no distinct difference between nanosuspensions prepared with Tween 80 and Span 80. This suggests that the powerful disruptive

Table 1 Time dependence of the BCAA solubility in nanosuspensions at pH 6

Sample	BCAAs:surfactant (w:w)	Storage time (day)				
		0	1	5	10	20
C1	–	$^z3.87 \pm 0.05^c$	$^z3.77 \pm 0.04^g$	$^z3.75 \pm 0.03^f$	$^y3.63 \pm 0.09^d$	$^y3.61 \pm 0.09^e$
C2	–	$^z4.74 \pm 0.08^b$	$^y4.17 \pm 0.07^f$	$^x4.01 \pm 0.02^e$	$^x3.98 \pm 0.05^c$	$^w3.84 \pm 0.03^d$
C3	–	$^z4.94 \pm 0.04^a$	$^y4.40 \pm 0.01^c$	$^x4.30 \pm 0.02^d$	$^x4.27 \pm 0.02^b$	$^w4.04 \pm 0.01^c$
T1	100:1.00	$^z4.97 \pm 0.02^a$	$^y4.89 \pm 0.02^a$	$^y4.89 \pm 0.04^a$	$^yx4.83 \pm 0.05^a$	$^x4.80 \pm 0.04^a$
T2	100:0.67	$^z4.96 \pm 0.03^a$	$^y4.88 \pm 0.04^a$	$^yx4.85 \pm 0.03^{ab}$	$^yx4.82 \pm 0.04^a$	$^x4.79 \pm 0.06^a$
T3	100:0.50	$^z4.95 \pm 0.04^a$	$^y4.83 \pm 0.04^a$	$^y4.80 \pm 0.06^b$	$^y4.80 \pm 0.05^a$	$^y4.78 \pm 0.03^a$
T4	100:0.40	$^z4.96 \pm 0.02^a$	$^yx4.83 \pm 0.05^a$	$^y4.85 \pm 0.02^{ab}$	$^yx4.83 \pm 0.03^a$	$^x4.79 \pm 0.02^a$
S1	100:1.00	$^z4.95 \pm 0.03^a$	$^y4.68 \pm 0.02^b$	$^x4.39 \pm 0.03^c$	$^w4.23 \pm 0.03^b$	$^w4.24 \pm 0.05^b$
S2	100:0.67	$^z4.94 \pm 0.05^a$	$^y4.54 \pm 0.04^d$	$^x4.34 \pm 0.11^{cd}$	$^x4.26 \pm 0.07^b$	$^x4.22 \pm 0.04^b$
S3	100:0.50	$^z4.96 \pm 0.03^a$	$^y4.52 \pm 0.04^d$	$^x4.35 \pm 0.06^{cd}$	$^w4.25 \pm 0.03^b$	$^w4.24 \pm 0.01^b$
S4	100:0.40	$^z4.96 \pm 0.03^a$	$^y4.61 \pm 0.03^c$	$^x4.41 \pm 0.04^c$	$^w4.31 \pm 0.02^b$	$^w4.26 \pm 0.04^b$

T# or S# sample; homogenization after heating at 70 °C under stirring was applied for BCAA solubilization with Tween 80 (T samples) or Span 80 (S samples)

C1 sample just stirring was applied for BCAA solubilization, C2 sample just heating at 70 °C under stirring was applied for BCAA solubilization, C3 sample homogenization after heating at 70 °C under stirring was applied for BCAA solubilization

The values with different superscripts (a, b, and c) in a same column are significantly different ($p < 0.05$)

The values with different superscripts (w, x, y, and z) in a same row are significantly different ($p < 0.05$)

forces derived from high-pressure homogenization mainly increased the BCAA solubility of freshly prepared nanosuspensions. However, there was a significant difference in the stabilizer's ability to retain the initial BACC solubility between Tween 80- and Span 80-based nanosuspensions. Over 97% of the initial solubility of BCAAs remained in the Tween 80-based nanosuspensions but when Span 80 was used, it declined by almost 15% after 20 days of storage. Although both Tween 80 and Span 80 are similar to non-ionic surfactants, they have very different molecular weights and hydrophilic-lipophilic balances (HLB) (1310 vs. 429 g/mol, and 15.0 vs. 4.3 for Tween 80 and Span 80, respectively). In particular, Tween 80 is hydrophilic in nature whereas Span 80 has the opposite characteristics. Although the effect of the stabilizer's molecular weight on BCAA solubility is unclear, it seems that hydrophilic stabilizers are more effective in retaining the initial solubility of the nanosuspended BCAAs compared to the hydrophobic ones. In other words, Tween 80 could increase the saturation solubility of BCAAs in water more efficiently. As shown in Table 1, sample C1, C2, and C3 exhibited very similar solubility values at 3.6, 3.8, and 4.0%, respectively, which indicates that the saturation solubility (*i.e.*, maximum solubility in water) of the samples is approximately 4.0% after 20 days of storage. When comparing the sample C1 with the sample C3, T, or S (Table 1), it is evident that both homogenization and the use of stabilizer increased the saturation solubility of BCAAs [20]; however, stabilizers clearly have a more pronounced effect [21] as evidenced by the improved solubility of stabilizer-based BCAA nanosuspensions. Additionally, the BCAA:stabilizer ratio affected neither the initial BCAA solubility nor the nanosuspensions' ability to retain the initial BCAA solubility.

Stability of BCAAs in nanosuspensions

Based on the contents of solubilized BCAAs after 20 days of storage, it was assumed that all freshly prepared BCAA nanosuspensions were supersaturated. The supersaturated BCAAs results in the crystal formation [22], thus influencing the colloidal stability [23]. The hydrophilic and hydrophobic interactions between the stabilizers and the BCAA particles could impede the BCAA crystal formation [24], thus allowing the stabilizers to finally increase the colloidal stability of the BCAA suspensions. The effects of stabilizers and BCAA:stabilizer ratio on the colloidal stability of the BCAA nanosuspensions are depicted in Figs. 1 and 2.

In the case of Tween 80-based nanosuspensions (Fig. 1), the transmittance of the top and middle layers rarely changed but that of the bottom layer decreased over

20 days of storage, regardless of the BCAA:Tween 80 ratio. However, in the case of the Span 80-based nanosuspensions, the transmittance of freshly prepared nanosuspension was lower than that of Tween 80-based nanosuspensions, independent of the BCAA:Span 80 ratio. Additionally, the transmittance of the bottom layer (and sometimes, the middle layer) decreased over the storage period but no clear patterns of transmittance reductions were observed. Contrary to the Tween 80-based nanosuspensions, the changes in the transmittance of the Span 80-based nanosuspensions were strongly affected by the BCAA:Span 80 ratio. The low transmittance of freshly prepared Span 80-based nanosuspensions was due to the low water solubility of Span 80. When Span 80 is dissolved in water, its solution is not completely clear (*i.e.*, being somewhat translucent) owing to the low water solubility of Span 80. Presently, all nanosuspensions did not exhibit any correlation between the colloidal stability and the BCAA:stabilizer ratio, regardless of the stabilizer used. The transmittance of the BCAA nanosuspensions decreased owing to the crystal formation that can be explained by Ostwald ripening. The rate of Ostwald ripening in nanosuspensions with relatively high ratio of stabilizer to BCAAs could become slow by the interfacial film able to reduce the rate of attachment and detachment of BCAA molecules at the nanoparticle surface [14]. Also, because the poorly water-soluble materials solubilized into micellar structures did not attribute to the Ostwald ripening [25], it could be expected that the slow rate of Ostwald ripening was observed in BCAA nanosuspensions with the high ratio of stabilizer to BCAAs. However, the results obtained in this work indicates that the BCAA:stabilizer ratio was not a major concern in determining the colloidal stability of BCAA nanosuspensions. As Ostwald ripening rate is directly proportional to the concentration of the dispersed particles in the system [23, 26], the lower saturation solubility of BCAAs in Span 80-based nanosuspensions compared to that of Tween 80 may be responsible for the dramatic reduction in transmittance of their bottom layer. With respect to the Tween 80-based nanosuspensions, the reduction in transmittance observed for their bottom layer and the minimal changes in transmittance of their top and middle layers suggest that most BCAA crystals were sufficiently large to induce precipitation. However, in the Span 80-based nanosuspensions, it seemed like the BCAA crystals existed in various polymorphic forms. Some of the BCAA crystals were large enough to precipitate or they had a highly dense crystalline structure to facilitate precipitation, both of which resulted in the transmittance reduction of the bottom layer. Concurrently, since some of the BCAA crystals were not large enough to precipitate or they were in an amorphous state, they could easily spread throughout the entire solution, thus resulting

Fig. 1 Time dependence of the BCAA nanosuspensions' colloidal stability at various BCAA:Tween 80 ratios. (A) 100:1.00, (B) 100:0.67, (C) 100:0.50, (D) 100:0.40

in a decrease in transmittance for all the layers. Our findings disagree with a previous study that reported no correlation between the colloidal stability and the HLB of the non-ionic stabilizer [24]. Considering the data obtained, the colloidal stability of BCAA nanosuspensions appear to correlate with the saturation solubility of BCAAs in nanosuspensions, as well as the degree of supersaturation involving freshly prepared nanosuspensions. Since the contents of solubilized BCAAs in fresh nanosuspensions were approximately 5%, all nanosuspensions were supersaturated, regardless of the stabilizer used. The chosen stabilizer, which minimally increases the saturation solubility of BCAAs (*i.e.*, Span 80 in this work), showed a lower colloidal stability compared to other stabilizers that significantly increase BCAA saturation solubility. The huge gap between saturation and supersaturation solubility could lead to fast crystal formation (both the nucleation and growth steps, or just one of them), thus influencing crystal

morphology. Therefore, it could be concluded that the colloidal stability could be influenced by the stabilizer's ability to increase the saturation solubility of BCAAs.

The solubility of BCAAs and the colloidal stability of nanosuspended BCAAs were investigated under various BCAA:stabilizer ratios. High pressure homogenization alone increased the initial solubility of BCAAs. Increased initial solubility of BCAAs after high pressure homogenization was attributed powerful disruptive forces that arise from the homogenizer effect. However, although homogenization positively affected the saturation concentration of BCAAs, its effect was limited. In fact, the introduction of stabilizers increased the saturation concentration of BCAAs, resulting in that the initial BCAA solubility in nanosuspensions was retained to a high level. Interestingly, the BCAA:stabilizer ratio did not have effect on the initial solubility and saturation concentration of BCAAs. The Tween 80-based nanosuspensions showed the relatively

Fig. 2 Time dependence of the BCAA nanosuspensions' colloidal stability at various BCAA:Span 80 ratios. (A) 100:1.00, (B) 100:0.67, (C) 100:0.50, (D) 100:0.40

high colloidal stability but the Span 80-based ones did not. The colloidal stability of the Span 80-related nanosuspensions varied with BCAA:Span 80 ratio but correlation between the BCAA:Span 80 ratio and colloidal stability was not clear. Conclusively, the development of liquid-and/or colloid-type BCAA-related food products is possible through the combination of high pressure homogenization and stabilizer. However, to gain a detailed information about BCAA nanosuspensions, in the further study, the physical stability of BCAA nanosuspensions should be investigated under various stress conditions, such as storage temperature, mechanical agitation, etc.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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