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Abstract. Nanofibrils were formed from soy protein isolate (SPI), which was isolated from black soybean, and commercial SPI by heating 2% w/w SPI suspensions at pH 2 and 80°C for 16 h. Nanofibrils from both types of SPI have branches and curvy structures with diameters between 10 and 20 nm. These fibrils, together with high methoxyl pectin (HMP), were used to make up to seven layered microcapsules at pH 3.5. Initially, negatively charged HMP (0.1 %w/w) was adsorbed onto oil droplets stabilized by unheated SPI (0.1 %w/w) which was positively charged. Onto this base, following layers were added by alternately adsorbing positively charged SPI nanofibrils and negatively charged HMP until the desired numbers of layers were achieved. Both types of SPI resulted in similar morphology of microcapsules. The microcapsules had a diameter between 12 and 18 μm with shell thickness of around1.7 μm and a final charge of +3.5 mV. This research showed that SPI nanofibrils can be used effectively as building blocks for constructing multilayer microcapsules that have much thicker shells for the same number of layers as examples from literature.

1. Introduction

Whey protein isolate (WPI) originates from milk and consists of mixed proteins, i.e., β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulin [1]. WPI is pretty much the golden standard ingredient in food, and well-known for its functionalities such as foaming, emulsifying, thickening/gelling and texturizing properties [2]. Since proteins of animal origin are much less sustainable than their plant counterparts, many researchers are looking for plant-based proteins that

have similar, or is possibly even better properties than whey protein isolate. A good candidate is soy protein isolate (SPI) that also consists of mixed proteins, i.e., soy glycinin and β-conglycinin [3].

Both WPI and SPI can form amyloid fibrils at acidic conditions under heating. For WPI it was reported that these fibrils, could be used very efficiently as building blocks for microcapsules prepared by the layer-by-layer adsorption method [4] and [5]. SPI forms branched and curvy structures of fibrils instead of the long and straight WPI fibrils. The WPI fibrils strengthened the capsules to a large extent due to their persistence length, and it is expected that the branched SPI fibrils could even be better building blocks due to their persistence length that is now related to surface coverage. The potential of SPI nanofibrils as building blocks of multilayer microcapsules using layer-by-layer adsorption method has been indicated by Warji et al. [5], who isolated SPI from yellow soybean. The protein content of both yellow and black soybeans, particularly from superior cultivars, is about 40% or higher than this, but it is certainly much higher than the protein content of imported soybeans. The current study specifically elucidates the details on preparation and physical properties of the microcapsules reinforced by SPI nanofibrils, in which the SPI is isolated from local black soybean. Nanofibrils prepared from commercial SPI were also used in this study as a reference.

2. Materials and methods

2.1. *Materials and chemicals*

Local black soybean was obtained from farmers in Yogyakarta. Commercial soy protein isolate (Newpro 90 #20140319NP) was kindly provided by PT. Pintu Mas Mulia Kimia, Indonesia. High methoxyl pectin (HMP) with a degree of methoxylation about 70-75% was obtained from Sigma Aldrich, USA (CAS. No. 9000-69-5). Other chemicals used in synthesis were n-hexane (AR, Cat. No. A-1045, Smart-Lab, Indonesia), 2-Mercaptoethanol for synthesis (Cat. No. 805740, Merck, Germany), Tris(hydroxymethyl) aminomethane (Cat. No. 108382, Merck, Germany), n-hexadecane (Cat.No. 820633, Merck, Germany) and HCl 37% (Cat.No. 1000317, Merck, Germany). Double distilled water was used to disperse the proteins.

2.2. *Protein isolation*

Protein from soybean was isolated using adapted methods from [3] and [6]. In brief, soybean meal (SBM), which was prepared from black soybean, was defatted five times with hexane at room temperature (SBM/hexane ratio of 1:5). The mixture was filtered using filter papers (KRTS60, Indonesian) and then, the defatted SBM was left to dry at room temperature. The protein was isolated by suspending defatted SBM in 30 mM Tris-HCl buffer pH 8 that contained 10 mM 2-mercaptoethanol (145 g of defatted SBM in 1.5 L of buffer). The suspension was stirred at room temperature for 1.5 h followed by centrifugation at 19,000 × *g*, 20 °C for 30 min (Beckman, Model J2-21 Ultracentrifuge, USA) and filtration (Whatman, Schleicher & Schuell, type 595, Cat. No.1001125). The supernatant was set to pH 4.8 using 6 M HCl solution to induce protein precipitation while stirring for 1 h. The resulting suspension was centrifuged at 19,000 × *g*, 20 °C for 30 min and the precipitates ware harvested. The precipitates were washed twice using double distilled water and centrifuged after each washing step. The precipitate was then re-suspended in double distilled water and set to pH 8 using 2 M Tris solution. The suspension was stirred overnight; its pH was re-adjusted again to pH 8 and then, freeze-dried at 200 millitorrs and -25 °C for at least 24 h (depends on the sample size) to obtain SPI powder.

2.3. *Preparation of protein nanofibrils*
Nanofibrils were prepared following the method of [5]. From SPI, originating from black soybean and the commercial one, suspensions were prepared by dispersing 2 %w/w of the proteins in double distilled water. The protein suspensions were stirred overnight to complete hydration and then, the pH was set to 2.0 using 6 M HCl solution. The protein suspensions at this pH were then heated in a water bath at 80 ºC (internal temperature) while stirring for 16 h.

2.4. *Microcapsules preparation using layer-by-layer (LbL) adsorption method*
Multilayer microcapsules made from SPI nanofibrils and HMP were prepared using LbL adsorption method following [5] that adopted the method of [7]. HMP solution and unheated SPI suspension were prepared by dispersing 0.1% w/w HMP and SPI in 25 mM sodium chloride solution at pH 3.5. The suspension/solution was centrifuged at 925 xg in a Kokusan centrifuge (H-103N Series, Japan) for 30 min to precipitate undissolved materials and then, filtered through 0.45 μm cellulose acetate syringe filter (25CS045AS, DISMIC-25CS, ADVATEC®, Japan). Oil-in-water emulsions were prepared as the template for the capsules. Hexadecane (1% w/w) was dispersed in 0.1 %w/w unheated SPI solution pH 3.5 using a rotor-stator dispersion tool (T25 Ultra-Turrax®, Ika, Germany) equipped with S25N – 25F dispersing element (IKA, Germany) at 9500 rpm for 1 min. The emulsion droplets had positive charges because pH of the protein was below its isoelectric point. The droplets were separated from the remaining protein solution using centrifugation at 70 x and then dispersed in HMP solution that was set at pH 3.5. A layer with negative charges was formed on top of positively charged emulsion droplets because HMP carries negative charges at this pH. These bi-layered droplets were harvested by centrifugation and then dispersed in positively-charged solution of protein fibrils. The protein nanofibrils deposited onto the droplets as the third layer. Subsequently, additional layers of HMP and protein nanofibrils were deposited onto the droplets by repeating the same procedures until seven layers were formed around the oil droplet. The final harvested droplets were freeze-dried at 200 millitorrs and -25 ºC for 24 hours to evacuate hexadecane; therefore, dried hollow microcapsules were obtained.

2.5. *Transmission Electron Microscopy (TEM)*
TEM method was adapted from [8] to observe the morphology of the protein nanofibrils. The fibrils were diluted to 0.05 wt % in HCl solution of pH 2.0. A drop of the diluted sample was deposited onto a 5- nm thick carbon support film on a copper grid (400 mesh). The excess was removed after 15 s using a piece of filter paper. Electron micrographs were made using TEM (G^2F20, FEI Technai™, USA) operated at 120 kV.

2.6. *Scanning Electron Microscopy (SEM)*
SEM (EVO MA 10, Zeiss, Germany) was used to observe the morphology of the microcapsules using the adapted method of [4]. The freeze-dried microcapsules were placed onto brass holders with double- sided sticky carbon tape. The microcapsules were attached to the sticky layer with pressurized air. The microcapsules were sputter coated with gold and the analysis was performed at room temperature at a working distance of 15 mm using electron high tension (EHT) detection at 17 kV.

2.7. *Zeta potential of nanofibrils*
Charges of the proteins, HMP and nanofibrils were measured in terms of ζ-potential using ZetaSizer Nano ZS (Malvern Instruments, UK). Five to ten measurements were performed for each sample.

3. Results and discussion

3.1. *SPI and nanofibrils*

Protein from local black soybean had a protein content of 82.9% on wet basis or 87.0% on dry basis with yield of isolation around 54.7% (calculated based on the protein content in the beans). The protein content of SPI obtained in this research is close to the one indicated by Codex Standard [9], i.e., 90% on dry basis. No particular structures were observed (figure 1a and c) in unheated SPI suspensions, but after heating 2% w/w SPI suspensions at the conditions described previously, protein fibrils with branched and curvy structures with diameter between 10 and 20 nm were obtained (figure 1b and 1d). There was no obvious difference between fibrils made of SPI isolated from black soybean and of commercial SPI. During heating above denaturation temperature, polypeptides broke down and the low pH stimulated formation of new bonds, which is a process known as fibril configuration [3], [10], [11] and [12].

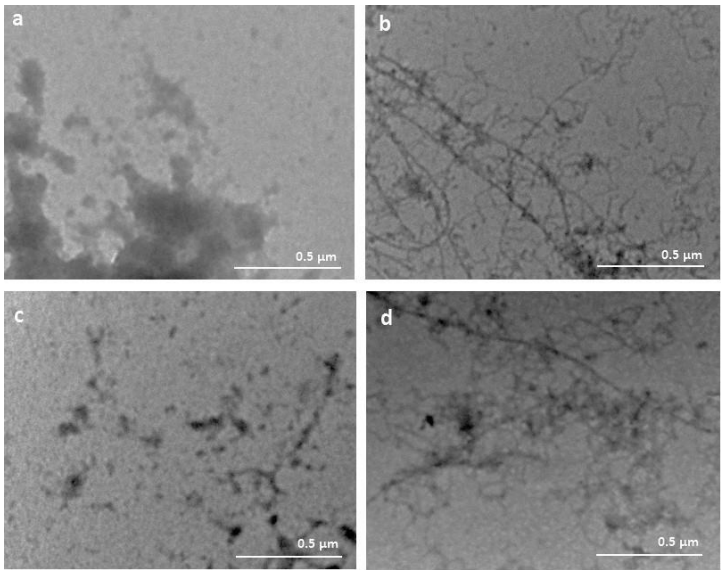

Figure 1. TEM images of (a) SPI of local black soybean, (b) SPI nanofibrils of local black soybean, (c) commercial SPI, and (d) nanofibrils of commercial SPI.

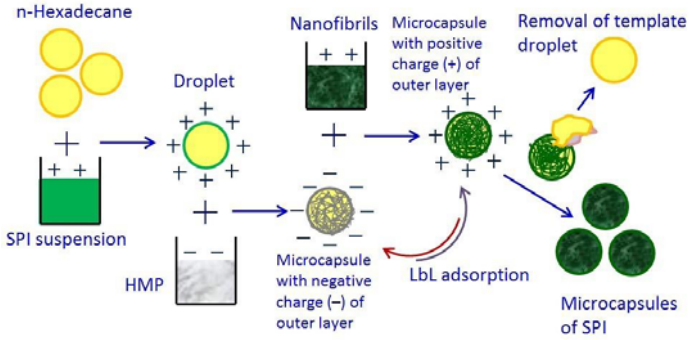

Figure 2. Schematic overview of layer-by-layer adsorption method.

3.2. *Multilayer microcapsules*

In figure 2, microcapsules assembly using the LbL adsorption method is illustrated. The images of microcapsules of local black soybean shown in figure 3a were similar to those made of commercial SPI (figure 3b), although the first ones seem to be smoother. The microcapsules were spherical, compact and hollow, and due to the harsh conditions during freeze-drying they opened up which allowed us to measure shell thickness as discussed later. It is expected that the cavities of these microcapsules are suitable to deliver e.g. functional food materials, or drugs, to a specific location along gastrointestinal tract.

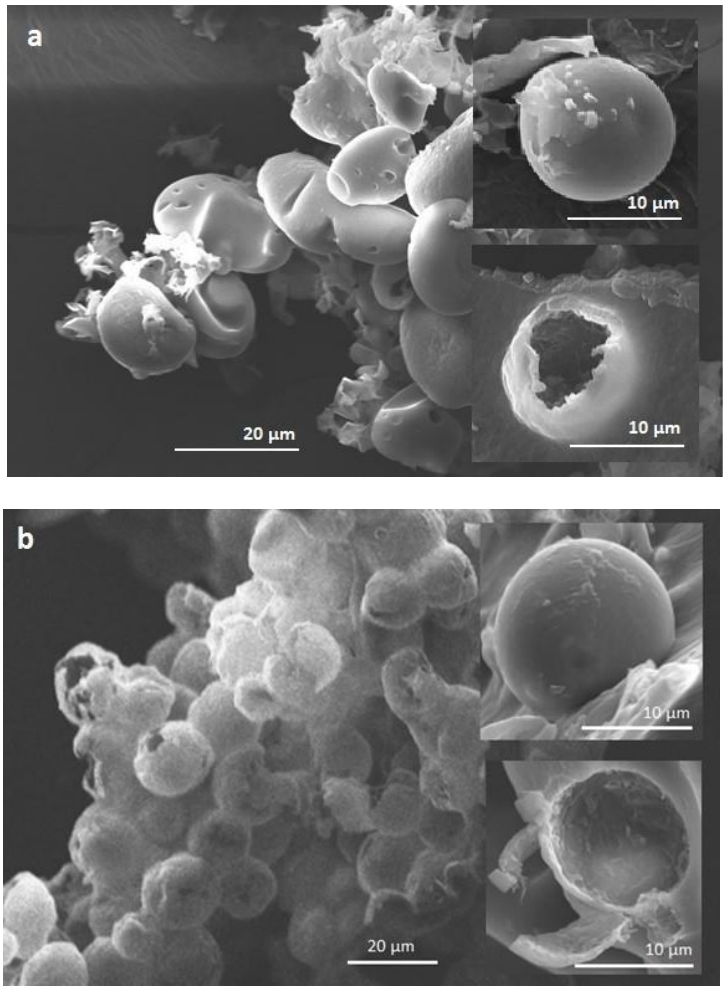

Figure 3. SEM images of microcapsules constructed by (a) SPI nanofibrils of local black soybean and (b) nanofibrils of commercial SPI.

The charge of the microcapsules after addition of each layer was as indicated in figure 4. The first layer, which was unheated SPI solution, was positively charged. The second layer, which was HMP, was negatively charged. Following odd-numbered layers corresponded to the layers constructed by SPI nanofibrils which were positively charged; even numbered layers corresponded to HMP. The final

charges of the microcapsules were about +3.5 mV, both for the ones made of SPI from local black soybean and commercial SPI.

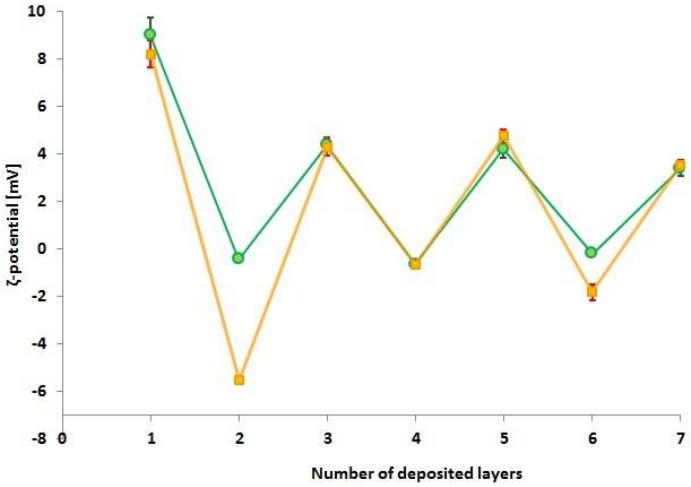

Figure 4. Zeta potential of multilayer microcapsules constructed by) SPI nanofibrils of local black soybean and) nanofibrils of commercial SPI.

The microcapsules had average diameters of 14.33 μm and 16.74 μm for the ones made with SPI of local black soybean and commercial SPI, respectively (figure 5). These diameters were similar to the ones reported by [4] and [5] for seven layers microcapsules reinforced by WPI nanofibrils and SPI nanofibrils (from yellow soybean), respectively. The microcapsules made from nanofibrils of SPI of local black soybean and commercial SPI also had a similar thickness, i.e., 1.13 and 1.42 μm, respectively, but these values were much higher than the thickness of seven-layer microcapsules reinforced by WPI nanofibrils, which was about 250 nm, as reported by [4]. This particular difference is suggested as the result of different structures of the protein fibrils and thickness of the fibrils; with soy protein, thicker layers can be built using fewer layers, which makes the production process simpler.

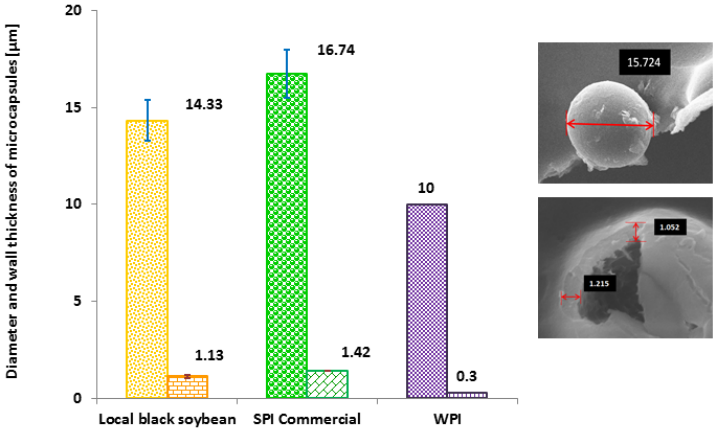

Figure 5. ( ) denotes diameter of microcapsules constructed with SPI nanofibrils of local black soybean, ( ) same but for commercial SPI, ( ) thickness of microcapsules constructed with SPI nanofibrils of local black soybean and ( ) same for nanofibrils of commercial SPI. ( ) denotes diameter and ( ) thickness of WPI microcapsules [7].

4. Conclusions

SPI, which was isolated from local black soybean, and commercial SPI can be converted into fibrillar structures that were curvy and branched by heating the suspension at pH 2 and 80 °C for 16 h. These structures successfully constructed multilayer microcapsules together with HMP using LbL adsorption method, making use of self-assembly of two polymers having opposite charges. Hollow microcapsules with similar diameter, thickness, and final charges were obtained using both types of SPI; compared to microcapsules made of WPI, the soy microcapsules reached higher thickness at a lower number of added layers, therewith making preparation simpler.

Acknowledgments

The research was financially supported by International Research Collaboration and Scientific Publication with contract number 011/SP2H/LT/DRPM/IV/2017, date April, 20th 2017. The authors also thank PT DKSH Indonesia for supporting ZetaSizer NanoZS (Malvern Instruments, UK).

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