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Efficient Preparation of Liposomes Encapsulating Food Materials Using Lecithins by a Mechanochemical Method

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Abstract: In order to evaluate to the feasibility of using lecithins for nanocapsules including functional food materials, liposomes were prepared from different commercially available lecithins (SLP-WHITE, SLP-PC70 and PL30S) by the Bangham method, and their physicochemical properties were examined by using a confocal laser scanning microscopy (CLSM) and the measurements of trapping efficiency. There was little difference in the trapping efficiency among the three types of liposomes. In all cases, the trapping efficiency clearly increased with an increase of the lecithin concentration up to 10 wt % , and the maximum efficiency reached at approximately 15%. CLSM observation showed the particle size of liposomes prepared from SLP-WHITE is significantly smaller than that prepared from other lecithins. In addition, liposomal solution prepared from SLP-WHITE remained well dispersed for at least 30 days, while two other liposomal solutions showed a phase separation due to aggregation and/or fusion of liposomes. These results indicated that SLP-WHITE is the most appropriate for the preparation of stable liposomes with well dispersed among the lecithins tested. SLP-WHITE liposomes were then prepared by the mechanochemical method using a homogenizer and microfluidizer, aiming at improving the preparation efficiency and liposome stability. The particle size of the prepared SLP-WHITE liposomes decreased with increasing inlet pressure and the number of processed cycles, and reached between 73 and 123 nm based on the measurement using dynamic light scattering. Moreover, freeze-fracture transmission electron microscopy revealed that the prepared liposomes are small unilamellar vesicles (SUV) with a diameter of approximately 100 nm. The extract of *Curcuma longa* Linn. (Ukon), which contains curcumins as a functional food material, was then subjected to the mechanochemical method with SLP-WHITE to give liposomes including the functional materials. Interestingly, the trapping efficiency of the liposomes for curcumins was found to reach over 85%. From these results, the present mechanochemical method is very likely to allow us to efficiently prepare stable and functional liposomes from the low-cost lecithin. The method may thus have a potential for manufacturing practical nanocapsules, which serves as a novel carrier of functional food materials.

Key words: liposome, nanocapsule, lecithin, functional food, mechanochemical method, microfluidizer, food delivery

1 INTRODUCTION

Liposomes are colloidal structures formed by the self-assembly of phospholipids in aqueous solutions[1]. They have been attracting increasing attention as a drug carrier for drug delivery systems (DDS) because they are able to retain water-soluble substances in the inner aqueous phase and oil-soluble substances in the bilayer wall[2]. In DDS, liposomes are highly advantageous for the reduction of

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drug toxicity and/or targeting of drugs to the diseased part[3-6]. They also have proved to be useful carriers for gene delivery to cells in culture, and for gene therapy in preclinical and clinical trials, as cationic liposomes can easily form stable complexes with DNA[7-9].

There have been several reports on the use of liposomes for the oral administration of drugs including insulin[10] and cyclosporin[11] as an alternative to the delivery of drugs by injection into the blood stream. Drugs enveloped with liposomes bearing a lipid bilayer can be protected from digestion in the stomach, and show great absorption in the gastrointestinal tract ; this leads to the high bioavailability of the drugs. Liposomes have thus a great potential not only in advanced medical and pharmaceutical fields but also in other fields such as the food industry.

Recently, various kinds of functional foods have been manufactured and marketed. Some of the active ingredients in these foods, however, are decomposed by stomach digestion and are not efficiently absorbed into the body. In addition, polymeric active ingredients such as β-glucan and related compounds tend to aggregate to large-sized molecules in aqueous solutions and result in the low intestinal absorption[12, 13]. Lipidic active ingredients such as crucumin in *Curcuma longa* Linn. (Ukon) also show low bioavailability due to their low water-insolubility and dispersibility[14]. In order to improve the absorption of such active ingredients into the body, it is essential to suppress the decomposition in the stomach and to accelerate the intestinal absorption by breaking the aggregation or by enhancing the water-solubility. Um *et al*. reported that unilamellar vesicles with a mean diameter of 1000 nm or less are appropriate for efficient intestinal absorption[15]. We thus focused our attention on the feasibility of using liposomes for an efficient carrier of functional food materials.

Few applications of liposomes, however, have been attained in food manufacturing processes. This is probably due to 1) the high cost of pure lecithins[16, 17], 2) use of organic solvents or detergents, 3) complicated fabrication equipment for solvent evaporation and/or sonication and 4) low encapsulating efficiency of conventional techniques. Recently, supercritical reverse phase evaporation method was reported on the efficient preparation of liposomes without any organic solvents other than ethanol[18]. Nevertheless, the possibility of using the conventional liposome technique in food processes is far from straightfoward due to the high cost and manufacturing efficiency.

On the other hand, microfluidization[19, 20] allows the ink industry and pharmaceutical industry to obtain different formulations, from emulsions and suspensions to nanoparticles, and is thus often employed for the production of liposomes[21, 22]. This mechanochemical method is readily available with a commercial ultra high-pressure homogenizer and needs no organic solvents. However, there have been limited studies on the large-scale preparation of liposomes from edible lipids of low purity by using the method[23-25] from the viewpoint of developing the formulations into an efficient carrier of functional food materials.

In this study, we undertook the preparation of liposomes from different edible lecithins by the conventional Bangham method, and examined their physicochemical properties using trapping efficiency measurements and optical microscopy. We then prepared liposomes from the selected lecithin by the mechanochemical method with a microfluidizer, and investigated the particle size by dynamic light scattering measurements and electron microscopy. We also tried to prepare liposomes from the lecithin and extract of Ukon. This is the first report on the large-scale preparation of liposome-encapsulated crucumins as a functional food material.

2 EXPERIMENTAL

2.1 Materials

Two kinds of soybean lecithins, SLP-WHITE and SLP-PC70, were supplied by Tsuji Oil Mill Co., Ltd (JAPAN), and an egg yolk lecithin, PL-30S, was supplied by QP Co., Ltd (JAPAN). These lecithins were used for liposome preparation as received. The average compositions of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA) as given by the supplier are summarized in Table 1. Other components include carbohydrates and other phospholipids, although the detailed compositions remain undisclosed by the suppliers.

3, 3'-Bis [*N*, *N*-bis (carboxymethyl)-aminomethyl] fluorescein (Calcein), a water-soluble marker purchased from DOJINDO Lab. was used for the determination of the trapping efficiency of liposomes. Crucumin I [1, 7-Bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione] of biochemical grade (purity, $>$ 98%) was purchased from Wako Pure Chemical (Osaka, Japan) and was used without further purification.

2.2 Liposome preparation

2.2.1 Preparation of liposomes by the Bangham method

The liposomes were prepared from three kinds of lecithins (SLP-WHITE, SLP-PC70, and PL-30S) as follows. Each lecithin (40 to 200 mg) was dissolved in chloroform (2 mL) in a test tube. The solvent was then removed under the stream of nitrogen gas into the test tube, and the residual solvent was further dried overnight under vacuum. A Tris-HCl buffer (10 mM, pH 7.3, 2 mL) was added to this lipid film and warmed (55 - 60℃) above its phase transition temperature (T_c) for 10 min. The test tube was then shaken on a vortex mixer for 5 min. The total lecithin concentrations were 2 to 10 wt %.

The extract of Ukon was prepared as followed. The dried

Table 1 The Compositions of Edible Lecithins used in This Study.

Type of lecithin	(wt %)					
	PC	PE	PI	PA	Others[a]	Total PL[b]
SLP-WHITE (soybean, unhydrogenated)	20	26	16	10	16	96
SLP-PC70 (soybean, unhydrogenated)	70	— [c]	—	2	8	80
PL-30S (egg yolk, unhydrogenated)[d]	30	5	—	—	—	35

[a]Not precisely determined. [b]Total phospholipid content.
[c]Trace amount. [d]This product consists of 62% of triacylglyecrols, 35% of phospholipids, and 3% of cholesterols.

Ukon (1,000 g) was sliced into small pieces and then extracted with ten volumes of water at 90℃ for one hour. The aqueous solution was filtrated, and the resulting precipitate was again extracted with ten volumes of 50% (v/v) of aqueous ethanol solution. All the water and ethanol extracts were mixed and subjected to freeze-dry process, and the obtained powder (100 g) was used in the following experiment.

2.2.2 Preparation of liposomes by the mechanochemical method using homogenizer and microfluidizer

The liposome preparation by the mechanochemical method was performed according to following procedure. SLP-WHITE (500 g) was added into deionized water (9.5 L), and then dispersed by a homogenizer (TK HOMO MIXER MARK II, PRIMIX. Co. Ltd., Japan) at 35℃ with a revolving rate of 8,000 rpm for 30 min, unless otherwise indicated.

The obtained liposomal suspension (5 wt %) was then processed with a microfluidizer (M110-E/H, MIZUHO Industrial Co. Ltd., Japan) using the non-recirculation mode. The operation was carried out with an inlet pressure of 100 MPa for 5 min, unless otherwise indicated. The resulting homogeneous solution was then characterized by the following experiments.

2.2.3 Preparation of liposomes encapsulating functional food materials

The Ukon extract (100 g) and SLP-WHITE (100 g) were separately suspended into deionized water (900 mL), and mixed well with a stirrer. These solutions were mixed and subjected to the above homogenization at 4,000 rpm for 15 min. The resulting solution was then treated once with the microfluidizer at 100 MPa.

2.3 Measurement of the tapping efficiency of liposomes

The trapping efficiency for calcein as a water-soluble marker was determined by a fluorescence method using a spectrofluorometer (FP-6500, JASCO Co. Japan). The calcein (E_x = 490 nm, E_m = 520 nm) concentration (0.1 mM) was diluted 50 times by a Tris-HCl buffer, and the total fluorescence intensity was measured (I_{Total}). The calcein present in the outer aqueous phase was then quenched by complexation with Co^{2+}, and the fluorescence intensity was measured again (I_{In}). Finally the liposomes were destroyed by adding Triton X-100, and the fluorescence intensity was measured again (I_{TX}). The trapping efficiency (TE) was calculated according to Equation 1:

$$TE\,(\%) = \frac{I_{In} - I_{TX}\cdot r}{I_{Total} - I_{TX}\cdot r} \quad (1)$$

where r is the volume correction factor.

2.4 Observation of liposomes

2.4.1 Confocal laser scanning microscopy (*CLSM*)

CLSM were taken with an LSM 5 PASCAL (Zeiss, Germany), equipped with a 200 × objective lens with a numerical aperture of 0.5. Helium-neon laser excitation at 543 nm was used in combination with a 560 long-pass filter and an HFT 543 dichroic mirror. The assemblies were made visible by solubilizing hydrophobic Nile red in colloidal structures as a fluorescence probe (lecithin/Nile red = 700/1). The concentrations of liposomal solution were 5 wt %.

2.4.2 Freeze-fracture electron microscopy (*FFEM*)

FFEM was used to determine the structure of lecithin (SLP-WHITE) liposomes (2 wt %). This sample was frozen with liquid nitrogen at -189℃. The fracture process was performed with a JFD-9010 (JEOL, JAPAN) at -130℃ and the fractured surface was then replicated by evaporating platinum at an angle of 60°, followed by carbon at an angle of 90° to strengthen the replica. The replicate was placed on a 400 mesh copper grid after being washed with water, methanol, and chloroform. It was then examined and photographed using a JEM-1011 (JEOL, JAPAN) transmission electron microscope.

2.5 Measurement of particle size of liposomes

The particle size of liposomes was estimated by dynamic light scattering at 24℃ using a FPAR-1000 (Otsuka Electronics Co., Ltd. JAPAN). The light source was a diode-pumped solid state laser with a wavelength of 658 nm as a light source. The scattering angle was 90°. The diffusivity of liposomal suspension (D) was obtained with above meas-

urement, and the average liposome diameter (d_{hy}) was calculated according to Equation 2[26]:

$$d_{hy} = kT/3\pi\eta D \quad (2)$$

where k is the Boltzman constant , T is the absolute temperature, π is the circular constant, and η is the viscosity.

2.6 Measurement of trapping efficiency of liposomes for crucumins

The trapping efficiency of SLP-WHITE liposomes for crucumins was estimated by high performance liquid chromatography (HPLC) using crucumin I as a standard. The aliquot (1 mL) of liposome solution, which was prepared from SLP-WHITE and the power of Ukon extract by the above mechanochemical method, were diluted 50 times with 10 mM Tris-HCl buffer (pH 7.3) and then subjected to ultra-filtration with a centrifuged tube (Vivaspin 20, molecular cut-off weigh: 5,000). The residual liposomes were added to 10 mL of methanol, and the solution was subjected to HPLC analysis after filtration.

Crucumin quantification was carried out by HPLC (LC-VP, Shimadzu, Japan) on an ODS column (Puresil 5 μm, 4.6 × 150 mm) with a UV detector using a solvent system consisting of water and acetonitrile (55 : 45, v/v) including 10 mM trifluoroacetic acid[27]. The aliquot (1 mL) of liposome solution without the ultra-filtration treatment was extracted with methanol (10 mL), and the whole extract was used as a control sample.

3 RESULTS AND DISCUSSION

3.1 The trapping efficiency of liposomes prepared from three kinds of lecithins

The aim of study is the feasibility of using edible lecithins for the preparation of liposomes servings as a functional food carrier. The three kinds of lecithins commercially available, SLP-WHITE, SLP-PC70 and PL-30S, were thus used in this study. The trapping efficiency is one of the most important characteristics of liposomes; this shows an ability to encapsulate target compounds in their cavity. Different liposomes were prepared from the three lecithins by the Bangham method, and were examined for the efficiency at a concentration range of 2 to 10 wt %, considering the lipid concentration leading to practical manufacturing processes.

Figure 1 shows the trapping efficiency of the individual liposomes prepared from the three lecithins. The plots in Fig. 1 represent the averages of the values measured at least three times independently. In all cases, the trapping efficiency increased with an increase in the lecithin concentration up to 10 wt %, and reached approximately 15%. There was little difference in the trapping efficiency among the three liposomes. With SLP-PC70, further increases in the lecithin concentration up to 20 wt % resulted in a great increase of the efficiency over 30%, as well as in a significant increase of the solution viscosity due to lipid gelation (data not shown). These results showed that the edible lecithins of low PC content are available for the preparation of liposomes.

3.2 The CLSM observation on the prepared lecithin liposomes

The prepared lecithin liposomes were then observed by CLSM, using Nile red as a hydrophobic fluorescence probe. Fig. 2 (a, b, and C) shows CLSM images of liposomal solutions prepared from 5 wt % of SLP-WHITE, SLP-PC70 and PL-30S, respectively. The black bar length was 50 μm. Figure 2 (a) exhibits liposomal particles with a diameter less than 10 μm. In contrast, Figs. 2 (b) and (c) show multilamellar liposomes with a diameter from 5 to 30 μm whose size is clearly larger than SLP-WHITE liposomes. SLP-WHITE has the highest content of phospholipids (96 wt %) among the three lecithins used in this study (Table 1). It seems thus reasonable that SLP-WHITE efficiently gives a greater number of liposomes compared to other lecithins.

3.3 The dispersibility of lecithin liposomes

The dispersibility of liposomes is also an important character when liposomes are used in practice. The effect of lipid composition on the liposome dispersibility was thus examined by visual observation. In all cases, the prepared liposomal solution was homogeneous and turbid without any phase separation (data not shown). Figure 3 shows the time-dependence of the visual observation on the three liposomal solutions prepared from 5 wt % of lecithin. One

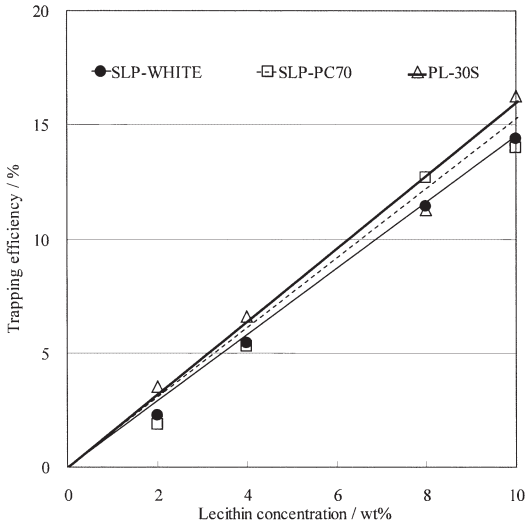

Fig. 1 The Relationship between the Trapping Efficiency and Lecithin Concentration.

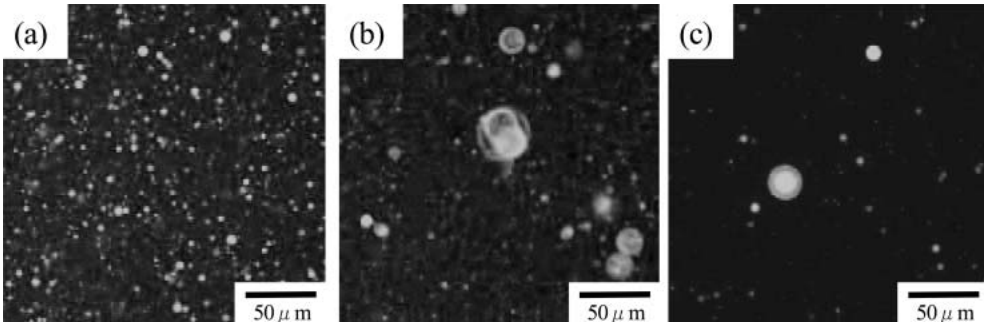

Fig. 2 Confocal Laser Scanning Micrographs of Liposomes Prepared from Different Lecithins by the Bangham Method.
(a) SLP-WHITE; (b) SLP-PC70; (c) PL-30S

day after preparation, the SLP-WHITE and SLP-PC70 liposomal solutions remained still homogeneous, while the PL-30S solution showed a slight phase separation (Fig. 3a). Interestingly, the SLP-WHITE solution remained well dispersed for at least 30 days, whereas the other two solutions developed phase separation and precipitation probably due to the aggregation and/or fusion of liposomes (Fig. 3b).

Generally, the dispersibility of liposomes depends on the physicochemical properties such as the particle size and value of surface charge. As indicated above, the SLP-WHITE liposomes showed smaller sizes compared to other lecithin liposomes. SLP-WHITE includes a greater amount of charged phospholipids such as PI and PA than that of other lecithins (Table 1). SLP-WHITE should thus give stable liposomes with well dispersed, and is likely to be the most appropriate for food encapsulation among the three edible lecithins tested. Moreover, the selected lecithin is highly advantageous from the practical point of view, as it costs six to ten times less than other edible lecithins in Japan.

3.4 Liposome preparation by the mechanochemical method

In order to improve the stability and preparation efficiency of liposomes, the SLP-WHITE liposomes were then prepared by the mechanochemical method using a homogenizer and microfluidizer. In this study, the liposome preparation was undertaken at a much larger-scale, aiming at constructing a practical process. SLP-WHITE (500 g) was added to twenty volumes of water, and vigorously mixed with the homogenizer at a moderate revolving rate of 8,000 rpm.

Figure 4 shows the relationship between the mean diameter of liposomes and the mixing time, The size of the liposomes decreased with an increase of the mixing time, and reached below 400 nm after 30 min. Further mixing resulted in a slight decrease of the liposome size. The following experiments were thus carried out with the homogenization for 30 min.

The obtained solutions, which include liposomes with a diameter of approximately 400 nm, were then subjected to the treatment with the microfluidizer. As shown in Fig. 5, the resulted liposome size decreased along with the number of process cycles (pass) as well as the inlet pressure. In all cases, the size of the liposomes was clearly decreased from the untreated size of 400 nm below 130 nm. More significantly, one cycle of the treatment was efficiently

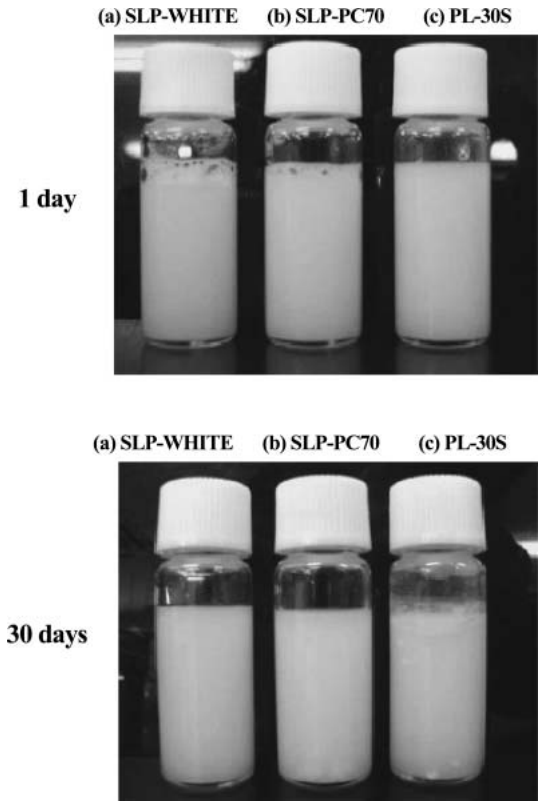

Fig. 3 The Visual Observation of Liposomal Solutions Prepared from Different Lecithins by the Bangham Method.

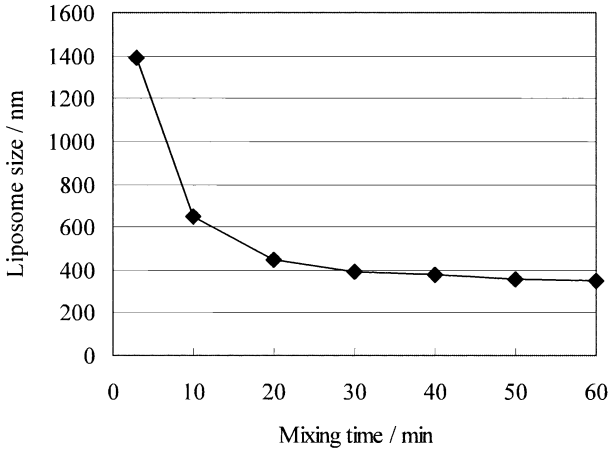

Fig. 4 The Effect of Homogenization Time on the Particle Size of SLP-WHITE Liposomes.

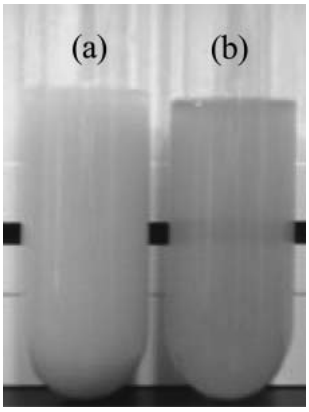

Fig. 6 The Visual Observation of Liposomal Solutions Prepared from SLP-WHITE by the Mechanochemical Method.
(a) with the homogenizer
(b) with the homogenizer and microfluidizer

attained within 5 min even for a large volume of the liposomal solution (10 L).

With the inlet pressure of 100 MPa, the liposome sizes clearly became smaller than that with 20 MPa. The number of pass was also an important factor for controlling the liposome size as previous reported on lecithins[19]. Accordingly, the liposomes with a mean diameter between 73 and 123 nm were efficiently prepared from SPL-WHITE with the present mechanochemical method. Figures 6 (a) and (b) show the liposomal solutions obtained after the homogenization (8,000 rpm, 30 min) and after the microfluidization (1 pass, 100 MPa), respectively. From these results, the microfluidization process is very effective to control and to downsize the lecithin liposomes. To determine the direct structure of the obtained SLP-WHITE liposomes after the microfluidization, dynamic light scattering (DLS) and FFEM were undertaken. Figure 7 shows the size distribution of the SLP-WHITE liposomes; the mean hydrodynamic diameter was determined to be 100 nm (S. D. 49.2 nm). Figure 8 shows the typical FFEM image of the SLP-WHITE liposomes, confirming that they are SUVs. The particle size of the liposomes was about 100 nm, which corresponds well to the mean diameter obtained from the above DLS

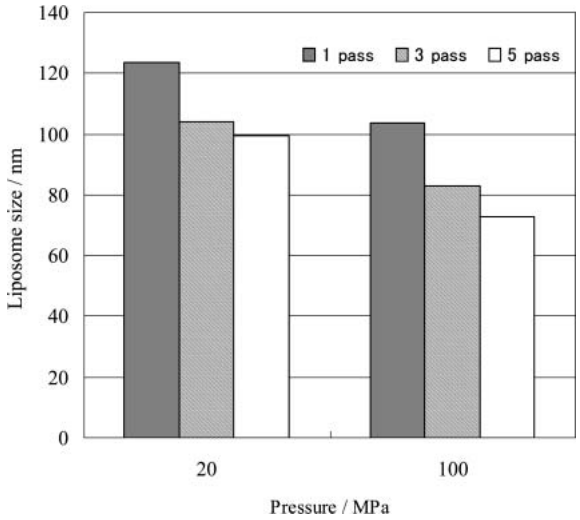

Fig. 5 The Effect of Inlet Pressure of Microfluidizer on the Particle Size of SLP-WHITE Liposomes.

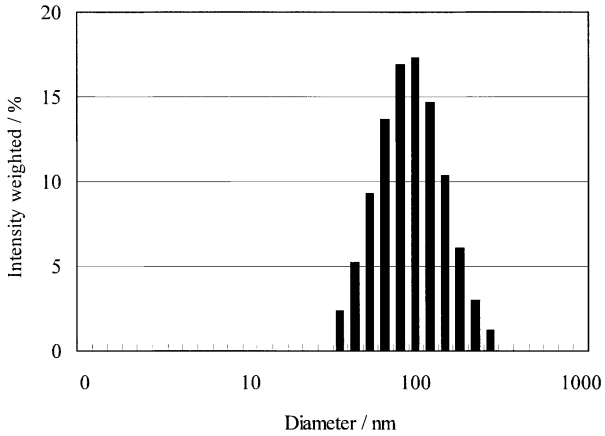

Fig. 7 Particle Size Distribution of SLP-WHITE Liposomes Prepared by the Mechanochemical Method.

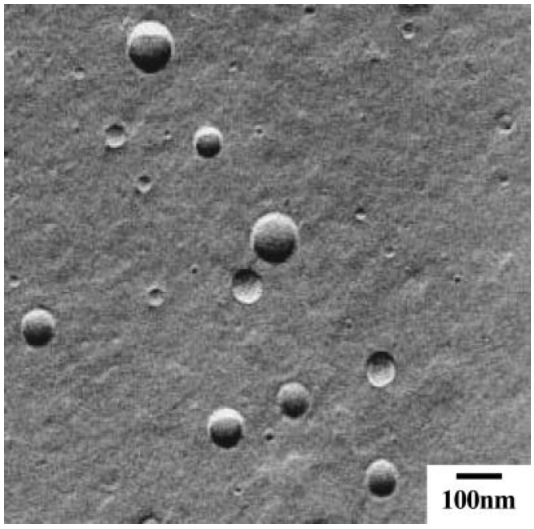

Fig. 8 Freeze Fracture Electron Micrograph of SLP-WHITE Liposomes Prepared by the Mechanochemical Method.

measurement.

3.5 Preparation of liposomes encapsulating functional food materials by the mechanochemical method

In order to evaluate the feasibility of preparing liposomes encapsulating functional food materials, we further undertook the preparation from SLP-WHITE and the Ukon extract (powder) containing crucumins as an active ingredient. The lecithin and the extract were separately suspended into water, and the solutions were mixed and homogenized. The mixed solution was then treated once with the microfluidizer at 100 MPa.

The obtained solution was turbid but highly homogeneous, and remained unseparated for at least 30 days (data not shown). Based on DLS measurement, the solution was a well dispersed liposomal solution, and the mean diameter of the liposomes was approximately 114 nm, which is very similar to that of the liposomes prepared only from SLP-WHITE. This means that the Ukon extract gives little effect on the liposome formation from the lecithin used, and that the ingredients are likely to be efficiently encapsulated in the liposomes. Crucumins are one of the most important active ingredients in Ukon (Turmeric), and are widely known to exhibit anti-oxidant and anti-inflammatory properties[28]. To estimate the trapping efficiency for the ingredients, crucumins were thus extracted from the liposomes and were quantified by HPLC using crucumin I as a standard. On HPLC analysis, the trapping efficiency of the lecithin liposomes for crucumin I was found to be 85.2%, indicating that the mechanochemical method makes the convenient encapsulation of food materials possible. Crucumins are poorly soluble in water and are thus likely to be incorporated into the phospholipid bilayer. Began *et al*. reported that crucumins show high binding affinity toward PC[29]. The active ingredients of Ukon seem thus significantly preferable for the liposome encapsulation; this would lead to the bioavailability of the functional food materials by enhancing the intestinal absorption.

There have been a few reports on the application of liposomes to food processes and/or food delivery systems[30]. In these studies, PC of high purity and single food materials such as vitamins and flavors were extensively used for the liposome preparation. In addition, the characterization of the prepared liposomes has not yet been fully undertaken. In the present study, we succeeded in preparing thermodynamically stable liposomes from a commercially available lecithin, which is inexpensive and contains different phospholipids in addition to PC. We also demonstrated that the liposomes encapsulating active food ingredients are readily prepared from the lecithin. To our knowledge, this is the first paper on the preparation of liposome-encapsulated crucumins.

4 CONCLUSION

In the present work, we undertook the feasibility of using lecithins commercially available for the preparation of liposomes by the mechanochemical method, aiming at applying the formulation to a practical carrier for functional food materials. In this study, we used three types of lecithins, two of soybean lecithins, SLP-WHITE and SLP-PC70, and an egg yolk lecithin, PL-30S. SLP-WHITE was the most appropriate for the preparation of thermodynamically stable liposomes among the three lecithins tested. On the microfluidization of liposomal solutions, the control of the inlet pressure and the number of pass would make the size control possible. With the combination of the homogenization and microfluidization, SUV with a diameter of 100 nm were efficiently prepared from SLP-WHITE. Finally, the liposome-encapsulated crucumins were easily obtained from the lecithin and the Ukon extract including crucumins by the mechanochemical method; the encapsulation efficiency for the active ingredients reached over 80%. The detailed physicochemical and biological properties of the newly obtained liposomes are now in progress, and the results will be appeared.

The present mechanochemical method is very likely to allow us to efficiently prepare stable and functional liposomes from commercial lecithins of low cost. The method may thus have a potential for manufacturing practical nanocapsules, which serves as a novel carrier of functional food materials.

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