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Characterization and Bioavailability of Liposomes Containing a Ukon Extract

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In order to use liposomes as an efficient carrier of functional food materials, liposomes encapsulating a ukon extract (LUE) were prepared by the mechanochemical method under different conditions, and were physico-chemically and biochemically characterized.

After a homogenization treatment, the size of LUE decreased with decreasing concentration of the extract from 10 to 2.5 wt %, but did not decrease below 570 nm. LUE were thus subjected to microfluidization. The LUE solutions obtained from less than 5 wt % of the extract remained well dispersed for at least 14 d, whereas those from 10 wt % showed phase separation. With 5 wt % of the extract, the size of LUE obtained at an inlet pressure of 100 MPa was smaller than that obtained at 20 MPa, and reached below 180 nm. Under optimal conditions, resulting LUE was confirmed to be small unilamellar vesicles (SUV) with a diameter of approximately 100 nm by freeze-fracture electron microscopy (FFEM).

When used for treating simulated gastric and intestinal fluids, LUE obtained by microfluidization showed a 2-fold higher residual rate of curcumin than the uncapsuled extract itself. The bioactivity of LUE was further examined for its suppressive effect on carbon tetrachloride (CCl_4)-induced liver injury by using mice. Orally administrated LUE at a dose of 10 mg/kg as the extract had a much higher suppressive effect on the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, compared to the uncapsuled extract at a dose of 33 mg/kg.

Key words: liposome; mechanochemical method; curcumin; gastrointestinal fluid; liver injury

The rhizome of *Curcuma longa* L. (ukon) is widely used for different therapeutic purposes in India and other Asian countries. A variety of food supplements using ukon have recently been manufactured and marketed. Curcumins, which are polyphenols and one of the most important lipidic ingredients in ukon, have been reported to be effective as a traditional remedy possessing anti-HIV,[1)] anti-tumor,[2,3)] anti-oxidative,[4,5)] and anti-inflammatory activities.[6–8)] However, such lipidic bioactive compounds have suffered from low bioavailability[9)] due to their low watersolubility[10)] and low stability against gastrointestinal fluids and/or alkaline pH conditions.[11)] These limitations should be overcome in order to enhance the bioavailability and food functions of such compounds by using new food processing and delivery methods.

One of the most appropriate breakthroughs towards solving the problems is the use of liposomes which are microcapsules prepared from phospholipids. There have been several reports on the employment of liposomes to protect pharmaceuticals such as insulin,[12)] peptides,[13)] calcitonin[14)] and cyclosporin[15)] from enzymatic degradation, and to efficiently deliver them into the blood stream. We thus focused our attention on the feasibility of using liposomes as an efficient carrier of the bioactive ingredients in ukon.

We have previously reported a new mechanochemical method[16)] for the large-scale preparation of liposomes using a homogenizer and microfluidizer. This mechanochemical method allowed us to prepare liposomes containing a ukon extract by using commercial soybean lecithins. However, details of the preparation conditions

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Abbreviations: LUE, liposomes containing a ukon extract; CCl_4, carbon tetrachloride; DLS, dynamic light scattering; FFEM, freeze-fracture electron microscopy; SUV, small unilamellar vesicle; AST, aspartate aminotransferase; ALT, alanine aminotransferase

have not yet been investigated, and available information on the physico-chemical and biochemical properties of the liposomes is still limited. It is thus of great interest to determine the conditions leading to stable and functional liposomes, and to characterize the upgraded properties of the liposomes compared to an uncapsuled ukon extract.

We prepared in this study liposomes from the extract and lecithins under different conditions by using both homogenization and microfluidization, and investigated their physico-chemical characteristics. We also treated the liposomes with simulated gastric and intestinal fluids, and evaluated the protective effect of the encapsulated extract in terms of the residual proportion of curcumin. The upgraded food functionality of the liposomes was also examined against carbon tetrachloride (CCl_4)-induced liver injury in mice. This is the first report on the physico-chemical and biochemical characterization of liposomes containing a ukon extract.

Materials and Methods

Materials. Curcumin (> 98% purity) was purchased from Wako Pure Chemical Co. (Osaka, Japan). The soybean lecithin, SLP-WHITE (> 96% total phospholipid content) was purchased from Tsuji Oil Mill Co., Ltd. (Mie, Japan), and exclusively used as the lecithin. Pepsin and pancreatin were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Sigma Ltd. (Tokyo, Japan), respectively. Carbon tetrachloride was purchased from Kanto Chemical Co. (Tokyo, Japan). All other chemicals and reagents were from Wako Pure Chemical Co. (Osaka, Japan).

Preparation of the curcuma longa Linn (ukon) extract. Dried ukon rhizomes were supplied by Kanehide Bio Co., Ltd. (Okinawa, Japan). The dried rhizomes (40 kg) were extracted with ten volumes of water at 90 °C for one hour. The aqueous solution was filtered, and the resulting residue was further extracted with ten volumes of a 50% (v/v) aqueous ethanol solution. All the water and ethanol extracts were combined, filtered and concentrated under reduced pressure. The concentrated ukon extract was diluted to an appropriate concentration and used in the subsequent experiments.

Preparation of liposomes containing the ukon extract (LUE). LUE was prepared by the mechanochemical method. Equal volumes of the lecithin solution (10 wt %) and ukon extract (5.0, 10.0 and 20.0 wt %) were well mixed by a homogenizer (TK Homo Mixer Mark II, Primix Co., Ltd., Japan) at 35 °C and 4,000 rpm for 15 min. The obtained liposomal suspension (6.0 liters) was then processed with a microfluidizer (M110-E/H, Mizuho Industrial Co., Ltd., Osaka, Japan) at an inlet pressure of 100 MPa in a single pass for 5 min, unless otherwise indicated. The total lecithin concentration was 5 wt %, and the total ukon extract concentrations were 2.5, 5.0 and 10.0 wt %, respectively.

Measurement of the particle size of LUE. The particle size of LUE was estimated by dynamic light scattering (DLS) at 24 °C with an FPAR-1000 instrument (Otsuka Electronics Co., Ltd., Osaka, Japan). The light source was a diode-pumped solid-state laser with a light source of 658 nm wavelength, and the scattering angle was 90°. The diffusivity of the liposomal suspension (D) was obtained from this measurement, and the average LUE diameter (d_{hy}) was calculated as

$$d_{hy} = kT/3\pi\eta D$$

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

Observation of LUE by freeze-fracture electron microscopy (FFEM). FFEM was used to determine the structure of LUE. The sample was frozen with liquid nitrogen at −189 °C. The fracture process was performed with a JFD-9010 (Jeol, Tokyo, Japan) at −130 °C, and the fractured surface was replicated by evaporating platinum at an angle of 60°, this being followed by carbon at an angle of 90° to strengthen the replica. The replica was placed on a 400-mesh copper grid, after being successively washed with water, methanol, and chloroform, before being examined and photographed with a JEM-1011 (Jeol, Tokyo, Japan) transmission electron microscope.

Effect of the ukon extract and LUE on simulated gastric and intestinal fluids. The ukon extract and LUE were treated with simulated gastric and intestinal fluids according to following procedures. With the simulated gastric fluid, 200 µl of the sample (5 wt %) was mixed with 800 µl of a pepsin solution (250 U) in a 0.1 M HCl/KCl buffer (pH 2.0), and the mixture was incubated at 37 °C for 2 h. With the simulated intestinal fluid, 200 µl of the sample (5 wt %) was mixed with 800 µl of a pancreatin solution (2 wt %) in a 0.1 M phosphate-buffered saline (PBS) buffer (pH 8.0), and the mixture was incubated at 37 °C for 3 h. These treated samples were each boiled for 10 min, and 4 ml of methanol was added. They were then vortexed for 3 min and centrifuged at 3,000 rpm for 15 min at 4 °C. The curcumin content in the clear supernatant as an index of the stability of the ukon extract and LUE was measured by high-performance liquid chromatography (HPLC), and the residual curcumin content (%) was calculated as

(Concentration of curcumin (enzymatic treatment)
/Concentration of curcumin (control)) × 100

The total digestive effects (%) of the simulated gastric and intestinal fluids on the ukon extract and LUE were calculated as

$$R_g \times R_I \times 0.01$$

where R_g is the residual curcumin content in the gastric

fluid, and R_I is the residual curcumin content in the intestinal fluid.

HPLC conditions. An HPLC system (LC-VP, Shimadzu Co., Kyoto, Japan) equipped with a UV detector was used to detect the curcumin at 420 nm. Separation was performed in an ODS column (5 μm Puresil, 4.6 × 150 mm), using a mobile phase consisting of water and acetonitrile (55:45, v/v) with 10 mM trifluoroacetic acid. The flow rate and oven temperature were 1 ml/min and 40 °C, respectively. The curcumin content was determined by the peak area ratio of a curcumin standard to the sample on the HPLC chromatogram.

Experimental design for the protective effect on CCl_4-induced liver injury. Male ICR mice (4-week-old, Japan SLC Inc., Hamamatsu, Japan), weighing 25–35 g were used for the experiments. The mice were kept in an air-conditioned room at 23 ± 1 °C under a 12 h light/dark cycle. They were allowed free access to distilled water and commercial feed (Oriental yeast Co., Ltd., Tokyo, Japan). They were used for experiments after 7 d of acclimatization. The mice were randomly divided into six groups: i) The normal group (receiving water and not treated with CCl_4), ii) The control group (receiving water and treated with CCl_4), iii) The low-dose ukon extract group (L-ukon group, 17 mg of the ukon extract/kg), iv) The high-dose ukon extract group (H-ukon group, 33 mg of the ukon extract/kg), v) The low-dose LUE group (L-LUE group, 10 mg of the ukon extract/kg), vi) The high-dose LUE group (H-LUE group, 20 mg of the ukon extract/kg). Mice were orally administered with each sample for 3 d. One hour after the last administration, CCl_4 (5 ml/kg of body weight; diluted 1:200 in corn oil) was given intraperitoneally to all the mice, except those in the normal group, to induce liver injury. The normal group received the corn oil vehicle with H_2O.

After the CCl_4 treatment followed by an overnight fast, blood was taken by decapitating the mice in each group. The serum was immediately prepared by centrifugation and stored at −80 °C until being analyzed. This study was carried out in accordance with the Guidelines for Animal Experimentation of Kanehide Bio Co., Ltd.

Assessment of the liver functions. The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum were determined with an assay kit (TA-LN Kainols; Kainos Laboratories, Inc., Tokyo, Japan) according to the manufacturer's protocol. The enzyme activity is expressed as I.U. (μmol/min/l of serum at 25 °C).

Statistical analysis. Each result is expressed as the mean ± SE. Data were analyzed by one-way ANOVA, and differences among the means of groups were analyzed by the Bonferroni procedure as a post hoc test. Differences were considered significant at $P < 0.01$.

Results and Discussion

Effect of the ukon extract concentration on the particle size of LUE

We had previously developed a new mechanochemical method for the large-scale preparation of liposomes from commercially available soybean lecithins.[16] Liposomes encapsulating the ukon extract including curcumin were obtained by this method which applied homogenization and microfluidization. The preparation of liposomes containing the extract (LUE) was further investigated in this study in order to obtain the optimum conditions leading to a stable and functional liposomal carrier.

Table 1 shows the relationship between the mean particle size of LUE and the concentration of the ukon extract after treating by homogenization with and without microfluidization. The particle size of LUE decreased with decreasing concentration of the extract from 10.0 to 2.5 wt %, but did not reach less than 577 nm. In addition, phase separation and precipitation were observed in the LUE solutions within 1 d after preparation (data not shown). Accordingly, a stable LUE solution was difficult to prepare by only homogenization.

The foregoing LUE solutions were thus subjected to a treatment by microfluidization. As shown in Table 1, the particle size of LUE decreased with decreasing concentration of the extract, and reached less than 150 nm. Figure 1 shows the time-dependent visual observation of the three LUE solutions containing 2.5, 5.0 and 10.0 wt % of the extract. Immediately after preparation, all LUE solutions were well dispersed. Interestingly, the LUE solutions containing 2.5 and 5.0 wt % of the extract remained well dispersed for more than 14 d, whereas that containing 10.0 wt % exhibited significant phase separation and preparation.

In general, the dispersibility and stability of liposomes

Table 1. Relationship between the Mean Size of LUE and Concentration of the Ukon Extract after the Treatment by Homogenization with/without Microfluidization

Concentration of ukon extract (wt %)	Mean particle size (nm ± S.D.)	
	Homogenization	Homogenization and microfluidization
2.5	577.2 ± 17.8	142.4 ± 8.7
5.0	623.5 ± 19.3	185.1 ± 5.6
10.0	849.2 ± 24.7	309.9 ± 9.8

The respective LUE solutions were composed of 5 wt % lecithin and 2.5, 5.0, and 10.0 wt % of the ukon extract. Microfluidization was carried out with an inlet pressure of 100 MPa in 1 pass. The particle sizes of the LUE solutions were then determined by dynamic light scattering measurements. Each result is the mean of three experiments.

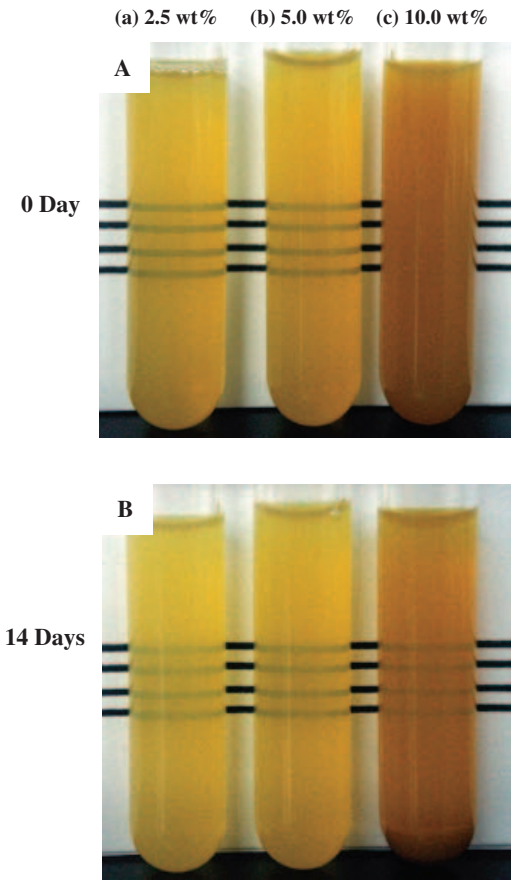

Fig. 1. Visual Observation of LUE Solutions Prepared from Different Concentrations of the Ukon Extract.

LUE solutions were subjected to a microfluidization treatment (100 MPa, 1 pass) after a homogenization treatment. A, Immediately after preparation; B, 14 d after preparation. The concentrations of the ukon extract in the three types of LUE solution were (a) 2.5 wt %, (b) 5.0 wt %, (c) 10.0 wt %, respectively.

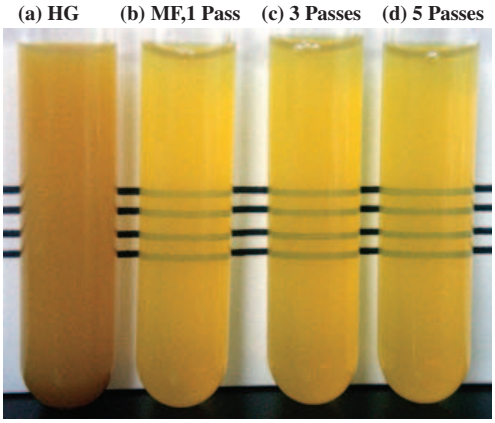

Fig. 3. Visual Observation of LUE Solutions Treated with and without Microfluidization.

HM, treatment by homogenization. MF, treatment by microfluidization after homogenization.

depend on the particle size, liposomes with a diameter of approximately 200 nm or less giving better dispersibility and practical use.[17] In addition, higher concentrations of the extract are more desirable for commercial products. Taken together, the optimum concentration of the extract was set at 5 wt %, the subsequent experiments were carried out at this concentration.

Effect of the inlet pressure and number of passes on the size of LUE

In order to improve the efficiency of the liposome preparation, the effects of the inlet pressure and number of processing cycles (passes) on the size of LUE were further investigated. The LUE solution prepared from the extract of 5 wt % with homogenization (600 nm, Table 1) was then treated by microfluidization under different conditions (Fig. 2). The particle size of LUE obtained with 100 MPa was clearly smaller than that

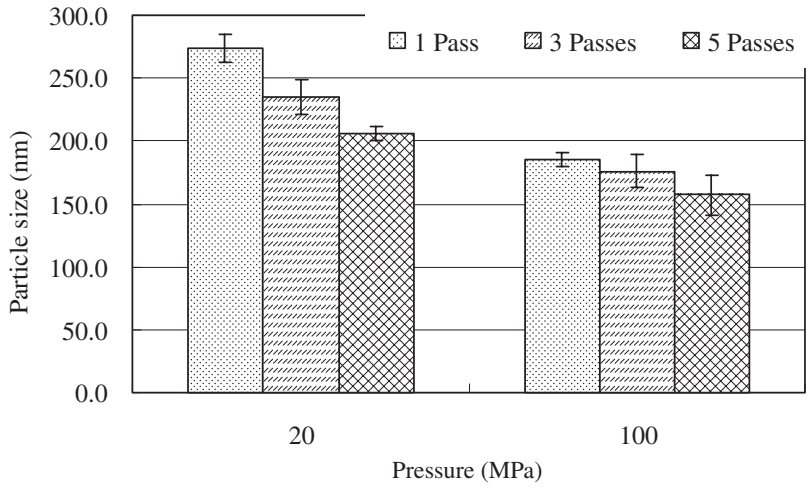

Fig. 2. Effect of Inlet Pressure and Number of Passes on the Particle Size of LUE.

LUE solutions composed of 5 wt % lecithin and 5 wt % of the ukon extract were prepared by a microfluidizer after homogenization. The operation was carried out with an inlet pressure of 20 MPa and 100 MPa for 1, 3 and 5 passes, respectively. The particle sizes of the resulting LUE solutions were then determined by dynamic light scattering measurements. The results are the mean of three experiments.

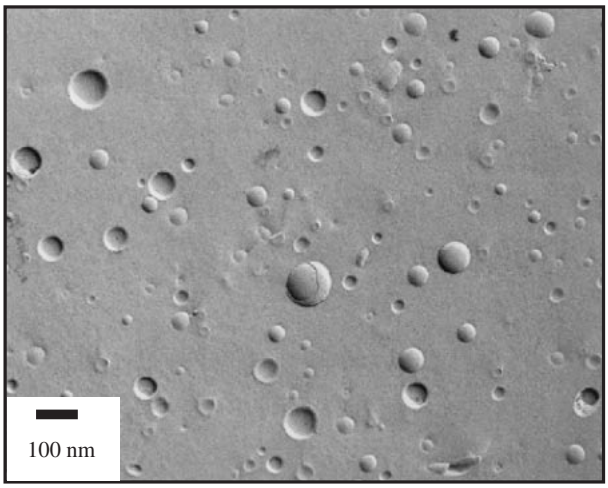

Fig. 4. Freeze-Fracture Electron Micrograph of LUE.
The LUE solution was composed of 5 wt % lecithin and 5 wt % of the ukon extract and treated by microfluidization (100 MPa, 1 pass) after homogenization. The scale is 100 nm.

with 20 MPa. The number of passes had a significant effect on the size of LUE at 20 MPa, whereas they had little effect at 100 MPa. This means that the size distribution of LUE could be more efficiently controlled at the higher inlet pressure. Figure 3 shows LUE solutions treated with and without microfluidization at 100 MPa. There seems almost no difference in the dispersibility among the three solutions obtained with different numbers of passes, supporting the foregoing speculation. Interestingly, the FFEM image shows that LUE obtained with one pass of 100 MPa was mainly composed of unillamellar vesicles (SUV) with a diameter of approximately 100 nm (Fig. 4). This result clearly reveals the effectiveness of the present microfluidization process.

Accordingly, LUE with high stability could be efficiently obtained from solutions of the ukon extract (5 wt %) and lecithin (10 wt %) with the combination of homogenization (4,000 rpm for 15 min) and microfluidization (100 MPa in 1 pass for 5 min). We then evaluated the biochemical properties of resulting LUE in the subsequent experiments.

Protection of curcumin in LUE against simulated gastric and intestinal fluids

The macronutrient composition of the ukon extract was 1.2% of protein, 3.7% of fat (including 1.67% of curcumin) and 1.6% of carbohydrate. In order to evaluate the protective effects on the bioactive compounds in the ukon extract by encapsulation against digestive fluids, we compared the differences between the curcumin contents of the ukon extract and LUE after their exposure to simulated gastric and intestinal fluids. Figure 5 shows the residual curcumin contents of the ukon extract and LUE after each treatment. In the case of the simulated gastric fluid assay, the residual curcumin contents of the ukon extract and LUE were approximately 84% and 90%, respectively. LUE showed a slightly higher residual curcumin content than ukon extract after simulated gastric digestion, but this result did not sufficiently explain the high protective effect on curcumin by encapsulating the extract. On the other hand, the residual curcumin contents of the ukon extract and LUE after simulated intestinal fluid was approximately 55% and 100%, respectively. The ukon extract without encapsulation clearly showed the decomposition of curcumin. Ansari *et al.* have reported that curcumin was unstable in the basic pH range and underwent alkaline hydrolysis in an alkaline pH solution.[18] It was therefore suggested that the bilayer wall of LUE was not decomposed by the simulated intestinal fluid at high pH

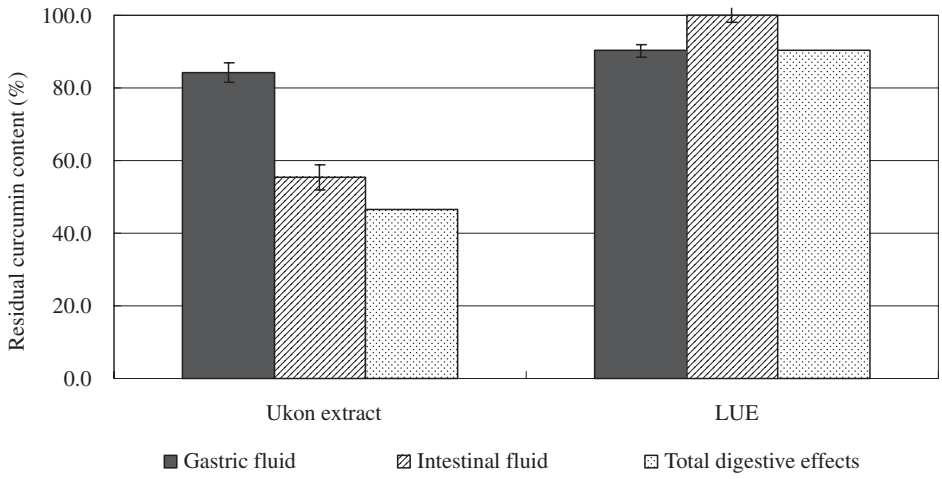

Fig. 5. Protective Effect of Curcumin on the Lipid Bilayer against Gastric and Intestinal Fluids.
The bar chart shows the residual curcumin contents (%) of LUE and the ukon extract after treatment by the simulated gastric fluid and simulated intestinal fluid, and the total effect (simulated gastric and intestinal fluids). The total effect (%) was calculated as $R_g \times R_I \times 0.01$, where R_g is the residual curcumin content in the gastric fluid, and R_I is the residual curcumin content in the intestinal fluid. Data are presented as the mean ± SD of three experiments.

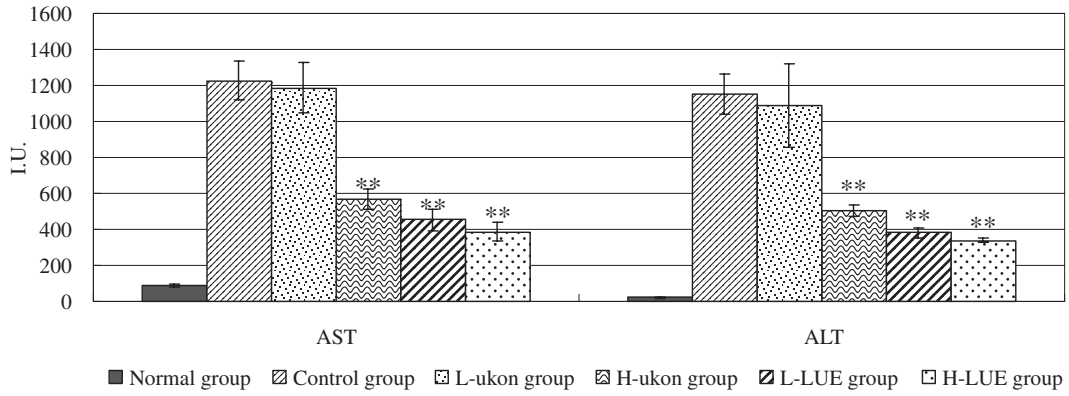

Fig. 6. Effect of LUE and the Ukon Extract on the Levels of Serum AST and ALT in the CCl_4–Induced Liver Injured Mice.

Orally administered with the ukon extract (L-Ukon group, 17 mg of the ukon extract/kg body weight; H-Ukon group, 33 mg of the ukon extract/kg body weight), and with LUE (L-LUE group, 10 mg of the ukon extract/kg body weight; H-LUE group, 20 mg of the ukon extract/kg body weight). Each sample was orally administered once a day for 3 d. One hour after the final oral administration, the mice were treated with CCl_4 (0.5 ml/100 g body weight, i.p.). After overnight fasting, the mice were killed to obtain their serum. The obtained serum AST and ALT levels were assessed as markers of liver injury. A difference is considered statistically significant between the control group and a treated group (L-Ukon, H-Ukon, L-LUE or H-LUE) when $P < 0.01$ (**). Each value is the mean ± SE. (n = 7).

in the body, and protected not only curcumin but also other bioactive compounds in LUE. Since the total residual curcumin content of LUE after exposure to the simulated gastric and intestinal fluids was over 90%, approximately 43% more of the curcumin was protected by encapsulation when compared to the ukon extract (Fig. 5). These results lead to the high bioavailability of ukon extract using the encapsulation technique, and might show the potential for stable delivery of various food materials to the intestinal tract by encapsulation.

Effects of the ukon extract and LUE on liver functions

The preventive action against rat liver damage induced by CCl_4 has been widely used as an assay of the liver protective activities of food materials and components.[19–21] In this study, the effects of the ukon extract and LUE on the CCl_4-induced elevation of serum enzymes (AST and ALT) in the mouse were examined. The serum activities of AST and ALT are the most commonly used using biochemical markers of liver injury.[22,23]

Several studies have demonstrated the beneficial effects of lecithin on liver damage.[24] It could thus be possible that liposome lecithin itself may exert a protective effect on CCl_4-induced liver damage. However, our preliminary experiment revealed that liposome lecithin alone had no protective effect against the liver injury induced by CCl_4 (data not shown). For this reason, a control for the liposome treatment was not included in the experiment groups.

Figure 6 shows the effects of the ukon extract and LUE on serum AST and ALT in the CCl_4-exposed mice. The levels of serum AST and ALT in the normal group of mice were 88 ± 4 and 21 ± 1 I.U., respectively, which were raised to the respective levels of 1227 ± 108 and 1154 ± 111 I.U. by the dose of CCl_4 (control group). There was little protective effect against the increase of AST and ALT in the CCl_4-exposed mice at a ukon extract dose of 17 mg/kg (L-ukon group), whereas treatment with the ukon extract at a dose of 33 mg/kg (H-ukon group) showed a significant decrease in both AST and ALT in the CCl_4-exposed mice. These results suggest that the ukon extract possessed potent antihepatotoxic activity, but that its activity depended on a high dose of the ukon extract. As encapsulation was expected to increase the availability of the ukon extract, we therefore administered the LUE solution at a 40% lower dosage to the L- and H-ukon groups (10 and 20 mg/kg, respectively). A significant decrease of AST and ALT in the CCl_4-exposed mice was observed by the treatment of LUE at doses of both 10 and 20 mg/kg (L- and H-LUE groups). The administration of L-LUE (10 mg/kg) to the CCl_4-exposed mice compared to the effect of the ukon extract showed the much higher protective effect against increases of AST and ALT. These results confirm that the antihepatotoxic activity of the ukon extract was enhanced by encapsulation. Two possible explanations need to be considered for these results. Firstly, the total residual curcumin content of LUE in the simulated gastric and intestinal fluids was over 90% (Fig. 5). The bilayer wall of LUE might not be decomposed by digestive fluids in CCl_4-exposed mice and mostly protected by the antihepatotoxic compounds including curcumin in LUE. It is therefore suggested that these compounds almost all reached the intestinal tract and were then absorbed, and even the treatment with a low dose of LUE (10 mg/kg) to the CCl_4-exposed mice exhibited antihepatotoxic activity (Fig. 6). Secondly, Reyes *et al.* have reported that curcumin protected against acute liver damage in the rat.[25] Curcumin is poorly soluble in water and is thus likely to be incorporated into the phospholipid bilayer. Began *et al.* have reported that curcumin showed high binding affinity towards lecithin.[26] Accordingly, it seem that the

encapsulation of lipophilic compounds such as curcumin in ukon, led to a great improvement in the antihepatotoxic activity. However, to demonstrate the high functionality of LUE in detail, the increased curcumin absorption due to encapsulation needs to be tested. Experiments on this are in progress, using rats as the animal model, and the results will be reported later.

We developed an efficient preparation method for liposomes containing a ukon extract which is likely to enhance its food functionality. This LUE would also open new avenues for the development of liposomes in food delivery systems.

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