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Effect of Microfluidization on in Vitro Micellization and Intestinal Cell Uptake of Lutein from *Chlorella vulgaris*

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ABSTRACT: *Chlorella* is a nutrient-rich microalga that contains protein, lipid, minerals, vitamins, and high levels of lutein. This study evaluated the bioavailability of lutein from *Chlorella vulgaris* using a coupled in vitro digestion and human intestinal Caco-2 cell model. Lutein bioaccessibility was low, and approximately 75% of total *C. vulgaris* lutein was not micellized during the digestion process but remained in the insoluble digestate. Microfluidization improved lutein micellization efficiency during *C. vulgaris* digestion. *C. vulgaris* was microfluidized at a pressure exceeding 10000 psi, and the cell surface disruption was visualized by scanning electron microscopy. The mean *C. vulgaris* particle size was reduced from 3.56 to 0.35 μm with the microfluidization treatment. *C. vulgaris* microfluidization at 20000 psi was three times more efficient for aqueous lutein micelles production as compared with untreated *C. vulgaris*, and the final lutein content accumulated by intestinal Caco-2 cells was also higher with microfluidization. *C. vulgaris* lutein stability was not affected by microfluidization. These results indicate that microfluidization may be useful for improving lutein bioaccessibility from *C. vulgaris* during food processing.

KEYWORDS: *Chlorella vulgaris*, lutein, microfluidization, bioaccessibility, in vitro digestion, micellization, Caco-2 cells

INTRODUCTION

Chlorella is a green microalga that has been used as a traditional food since ancient times and is recognized as a potential source of various nutrients, such as protein, functional lipids, minerals, and carotenoids.[1,2] Rapid developments in overproduction and metabolic engineering technology have facilitated large scale cultivation of *Chlorella* for industrial use.[3] *Chlorella* is widely produced and marketed as a food supplement in Japan, Korea, China, the United States, and Europe.[4]

Lutein is abundant in dark, leafy green vegetables such as spinach and kale and is used as an important natural food colorant and additive. Lutein is an effective bioactive compound, which inhibits cataracts and atherosclerotic development.[5] *Chlorella* accumulates high levels of lutein and produces a free nonesterified lutein with higher bioavailability as compared with esterified lutein, which provides an interesting alternative to usual plant sources.[6] Thus, considerable research activity is focused on the development of *Chlorella* culture technology and lutein extraction technology.[7−9]

The bioactivity of carotenoids requires the intake of carotenoid-rich foods, before carotenoids and their metabolites are absorbed by intestinal cells. Carotenoids are released from the food matrix and transferred to lipid droplets and then incorporated into mixed bile salt micelles during digestion.[10] Carotenoid bioavailability is affected by numerous factors, including physicochemical properties, food sources, the matrix, processing, and interactions with other dietary compounds, such as lipids, fiber, and other carotenoids.[11] Simulated in vitro digestion models coupled with differentiated human intestinal Caco-2 cells have been used to mimic the human gastrointestinal environment and investigate the complex influences of different factors on carotenoid absorption.[12−14]

Microfluidization is a wet milling technique that creates fine emulsions from large particles by high pressure homogenization. A sample dispersion of large particles is passed through specially designed interaction chambers at high pressure. The specialized geometry of the chambers and the high pressure cause the liquid stream to reach extremely high velocities. The streams collide with each other and the walls of the chamber, thereby resulting in particle size reduction.[15] Microfluidization is an emerging technology in food processing, which is widely used in the preparation of nanoemulsions and liposomes and the production of submicrometer pharmaceutical suspensions.[16]

Few publications report the bioavailability of lutein in *Chlorella*, whereas numerous reports detail the extraction of lutein from *Chlorella*. The aim of this study was to evaluate lutein bioavailability after *Chlorella vulgaris* ingestion by using a coupled in vitro digestion and human Caco-2 cell model. Microfluidization was used to assess the effect of *C. vulgaris* pulverization on lutein bioaccessibility and resolve the limits on digestion imposed by the thick cell wall of *Chlorella*.

MATERIALS AND METHODS

Samples and Chemicals. *C. vulgaris* culture broth was obtained from Daesang Corp. (Kunsan, Korea). All-*trans*-lutein was used as a standard and was obtained from Sigma-Aldrich (St. Louis, MO). Sodium cacodylate, glutaraldehyde, osmic acid, sodium bicarbonate, pepsin, pancreatin, pancreatic lipase, and bile extract were also purchased from

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Sigma-Aldrich. Fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), nonessential amino acids, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Invitrogen, Carlsbad, CA). High-performance liquid chromatography (HPLC)-grade *tert*-butyl methyl ether (TBME), ethanol, methanol, *tert*-butanol, and water were purchased from Fisher Scientific (Springfield, NJ). Unless indicated, all other chemicals were supplied by Sigma-Aldrich.

Microfluidization Procedures. *C. vulgaris* culture broth was pulverized using a Microfluidizer M-110EH (Microfluidics Co., Newton, MA). The *Chlorella* was microfluidized with a single treatment of 10000 psi or 20000 psi, using a combination of a 200 μm auxiliary chamber H30Z and a 100 μm interaction chamber H10Z. Individual batches were processed in the microfluidizer for 1 min at a speed of 400 mL min^{-1} and collected in glass beakers. Treated solutions were cooled under running water, where the temperature was maintained at 40 °C using a metal coil that dissipated the heat from the microfluidization process. A portion of the treated *C. vulgaris* was retained for cell size analysis and scanning electron microscopy. The remaining microfluidized *C. vulgaris* (MC) solution was freeze-dried using a vacuum freeze-drier (Ilshin Laboratory, Korea) and refrigerated prior to simulated digestion.

Scanning Electron Microscopy. Two hundred fifty microliters of *C. vulgaris* solution was fixed using 250 μL of 0.2 M sodium cacodylate, 250 μL of 4% (v/v) glutaraldehyde, and 250 μL of 0.2 M phosphate buffer (pH 7.4) and fixed once more using 250 μL of 0.2 M sodium cacodylate, 4% osmic acid, and 250 μL of 0.2 M phosphate buffer (pH 7.4). The fixed sample was dehydrated sequentially with 50, 70, 80, 90, and 100% (v/v) ethanol. Finally, 100% ethanol was replaced with *tert*-butanol. The sample was then freeze-dried and coated with osmium tetroxide. Samples were observed using a field emission type scanning electron microscope (FE-SEM, Hitachi SU-70, Japan) at an accelerating voltage of 15 kV. Micrographs were taken at 2000× and 10000× magnification.

Measurement of Cell Size. *C. vulgaris* particle size was analyzed by laser diffraction using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom) to evaluate the impact of microfluidization on *Chlorella* cell wall breakdown. A minimum of five parallel measurements were performed at 25 °C. The average size was then calculated.

In Vitro Digestion. The in vitro digestion procedure was performed according to the method of Garrett et al.[17] with minor modifications as described by O'Sullivan et al.[12] Briefly, *C. vulgaris* samples were weighed (200 mg) and mixed in 5 mL of HBSS. Simulated gastric digestion was performed by acidifying the mixed samples to pH 2 and digesting with porcine pepsin (40 mg mL^{-1} 0.1 N HCl) followed by incubation at 37 °C in a shaking water bath (Lab companion, Jeio Tech, Korea) for 1 h. The pH was increased to 5.3 after gastric digestion, which was followed by the addition of glycodeoxycholate (0.8 mmol L^{-1}), taurodeoxycholate (0.45 mmol L^{-1}), taurocholate (0.75 mmol L^{-1}), and porcine pancreatin (0.08 g mL^{-1}). The pH of each sample was increased up to 7.4. The final volume of the digestate was approximately 20 mL. Samples were incubated for 2.5 h at 37 °C in a shaking water bath, which completed the intestinal phase of the in vitro digestion.

Aliquots of the digested samples (5 mL) were frozen at −80 °C after overlaying the headspace with nitrogen gas. The remaining digestate was ultracentrifuged at 194270*g* using a Beckman Ultracentrifuge (Optima L-100 XP Ultracentrifuge, Beckman Coulter, Fullerton, CA) for 95 min to isolate the aqueous micelles. The micellar fraction was prepared by filtration (0.2 μm; Millipore, Bedford, MA) of the aqueous fraction to eliminate microcrystalline aggregates of lutein. The resulting micelles were stored at −80 °C prior to the cellular uptake experiment.

Synthetic lutein micelles were prepared as the positive control, according to the method of Lakshminarayana et al.[18] Micelles were suspended in phosphate-buffered solution (PBS) (4 mL) containing monooleoyl glycerol (2.5 mM), oleic acid (7.5 mM), sodium taurocholate (12 mM), lutein (200 μM), and lyso-phosphatidylcholine (3 mM). Appropriate amounts of these chemicals were dissolved in methanol or dichloromethane and mixed to achieve the final concentration. The solvent was evaporated to dryness using nitrogen, and the mixture was suspended in PBS (pH, 7.0) with vigorous mixing using a vortex mixer to obtain an optically clear solution. The resulting solution was used as the lutein micelle standard. The lutein concentration in the micelles was ascertained by HPLC analysis

Uptake of Carotenoids by Caco-2 Cells. Caco-2 cells (HTB-37, American Type Culture Collection, Rockville, MD) were maintained for cellular uptake experiments, according to the method of Hubatsch et al.[19] Cultures of Caco-2 were used between passages 25 and 34. Experiments were conducted with monolayers that were 21−25 days postconfluent. Monolayers were washed with DMEM before adding 2 mL of the test medium containing 1.5 mL of DMEM and 0.5 mL of micellar fraction. Cultures were incubated at 37 °C in a humidified 95% air and 5% CO_2 atmosphere for 4 h. The medium was eliminated by aspiration, and monolayers were washed once with PBS containing 5 mmol L^{-1} sodium taurocholate at 0 °C to remove any micelles and carotenoids adhering to the surface of the monolayer. Cells were collected by scraping into 1.4 mL of ice-cold PBS and pelleted by centrifugation. The supernatant was aspirated before the cell pellets were overlaid with nitrogen gas and stored at −80 °C prior to carotenoid analysis. The total protein content was measured with a Bio-Rad assay system (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard.

Extraction and HPLC Analysis of Carotenoids. Carotenoids were extracted from raw and MC and from aliquots of digestate, micellar fractions, and Caco-2 cells, using the method of Chitchumroonchokchai et al.[20] Thawed samples (1−3 mL) of homogenized food, digestate, and aqueous micellar fraction were extracted by adding 3.0 mL of petroleum ether:acetone (2:1) containing 4.5 mM butyl hydroxytoluene (BHT), mixing on a vortex mixer for 1 min, and centrifuging at 5000 rpm for 5 min to accelerate phase separation. The extraction procedure was repeated three times, and petroleum ether fractions were combined and dried at room temperature with nitrogen purging, followed by resolubilization in a methanol/TBME (90:10) solution that was used as mobile phase.

All extracts were analyzed using an analytic UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA) equipped with an UltiMate 3000 autosampler column compartment, HPG3200 binary pump UltiMate 3000 UV detector, and Chromeleon software. A YMC C30 carotenoid column (3 μm particle size, 150 mm × 4.6 mm, Waters, Milford, MA) was used to analyze carotenoids. The optimum mobile phase system comprised methanol/TBME (10:90) (solvent A) and water/methanol (5:95) (solvent B) with the following conditions: a linear gradient from 10 to 35% A for 20 min; a linear increase to 95% A for 5 min; isocratic at 95% A for 5 min; and a linear decrease to 10% A in 1 min. The column was equilibrated for 10 min at the starting conditions before each injection. The flow rate was 1.0 mL min^{-1} at 30 °C with an injection volume of 20 μL. Detection was conducted at 445 nm for lutein and β-carotene.

Data Analysis. A minimum of three independent observations were made for each experimental group, and each experiment was repeated at least once to provide a minimum of $n = 6$ to determine any significant differences in treatments. All data are expressed as means ± standard errors of the mean (SEMs). Statistical comparisons between untreated and MC were performed using Student's t test. The Student−Newman−Keuls (SNK) test was performed to separate treatment means where significant differences ($p < 0.05$) occurred. Statistical software was GraphPad Prism 4.

RESULTS AND DISCUSSION

Effect of Microfluidization on the Size of *C. vulgaris* and Carotenoid Extraction. *C. vulgaris* was pulverized by microfluidization, which increased lutein bioaccessibility after disruption

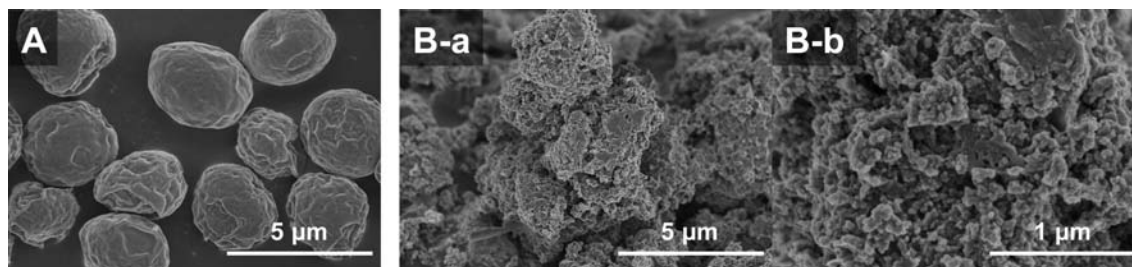

Figure 1. Scanning electron microscope images of the *C. vulgaris* culture (A) without microfluidization and (B) with microfluidization at 20000 psi. The bar at the right lower side of panels A and B-a represents 5 μm and that in panel B-b represents 1 μm.

Table 1. Particle Size and Lutein Content of UC and Microfluidized *C. vulgaris* (MC10000 and MC20000)[a]

	UC	MC10000	MC20000
mean particle size (μm)	3.56 ± 0.21 a	0.49 ± 0.18 b	0.35 ± 0.11 b
lutein content (mg g^{-1} chlorella)	1.42 ± 0.10 a	1.65 ± 0.15 a	1.61 ± 0.09 a

[a] Analyses of particle size and carotenoid content in *C. vulgaris* are described in the Materials and Methods. Data represent the mean ± SEM of six independent experiments. Different letters indicate significant differences ($P < 0.05$) between treatment means.

of the thick *Chlorella* cell wall. Untreated and microfluidized *Chlorella* were observed using a scanning electron microscope to evaluate the influence of microfluidization on *Chlorella* cell wall disruption (Figure 1). As shown in Figure 1, *Chlorella* treated without microfluidization exhibited the typical appearance of *C. vulgaris*, that is, 3−5 μm diameter and a globular cell shape (Figure 1A). This observation was consistent with previous studies.[6,21] Microfluidization of *C. vulgaris* at 20000 psi resulted in complete surface damage, as shown in the scanning electron microscope image (Figure 1B). Observation at 10000× magnification (Figure 1B-b) showed that the MC was smaller than 1 μm. The sizes of untreated *C. vulgaris* (UC) and MC treated at a pressure of 10000 and 20000 psi (MC10000 and MC20000) were 3.56 ± 0.21, 0.49 ± 0.18, and 0.35 ± 0.11 μm, respectively (Table 1). The nanosize produced with MC20000 was approximately 10 times smaller than UC, which indicates that *C. vulgaris* was effectively pulverized by microfluidization in a liquid form. It is known that food processing by microfluidization can generate nanoparticles or nanoemulsions from food materials, which results in better solubility, stability, and bioactivity.[22,23] This study showed that microfluidization was an appropriate tool for pulverizing *C. vulgaris* to a nanoscale size.

Table 1 shows the carotenoid content for the UC, MC10000, and MC20000. Lutein was the major carotenoid in *C. vulgaris*. There was no significant difference between UC and MCs in terms of lutein. The lutein content per 1 g of *C. vulgaris* was approximately 1.5 mg. This indicates that the total *Chlorella* lutein content remained at the same level with all treatments, showing that no carotenoid loss was caused by the microfluidization treatment. The MC led to a slightly higher lutein concentration as compared with UC, which might be attributable to the improved extraction efficiency that resulted from the breakdown of the cell walls by microfluidization.

***Chlorella* Lutein Micellization during Simulated Digestion.** The utility of microfluidization in increasing lutein bioaccessibility

Table 2. Lutein Content of Digestate and Micelles in Digested Samples[a]

	lutein content (μg 20 mL^{-1})	
samples	digestate	micelles
UC	255.3 ± 11.4 a	77.0 ± 4.1 a
MC10000	258.6 ± 3.4 a	170.0 ± 6.6 b
MC20000	265.8 ± 29.8 a	218.2 ± 21.6 c
LT	243.3 ± 10.7 a	192.3 ± 23.2 bc

[a] Preparations of micellar fraction and lutein extract from each digestate and micelle are described in theMaterials and Methods. Data represent the mean value ± SEM (n = 3). Different letters indicate significant differences ($P < 0.05$) between treatment means.

from *C. vulgaris* was evaluated by testing the lutein content of the *Chlorella* digestate using the in vitro digestion model (Table 2). The initial amount of lutein was adjusted to 300 μg per total reaction solution (20 mL) for UC, MC10000, MC20000, and LT (synthetic lutein micelles used as a positive control), and the lutein of all *C. vulgaris* digestates was found to be similar. This shows that lutein stability was not affected by the microfluidization treatment. However, there was a significant difference in the amount of lutein in each micelle. The lutein content in MC20000 micelles was approximately three times higher than that with UC. Over 70% of the initial lutein in MC20000 was found in the micellar fraction, which is the bioavailable carotenoid fraction. The low lutein content of UC micelles indicates that *C. vulgaris* in its natural form limits the release of carotenoids during digestion. Carotenoid release from the food matrix is an important factor in the effective absorption of carotenoids.[10] Food processing such as heat, mechanical, and enzymatic treatments facilitates the disruption of the cell wall and organelles, which releases carotenoids from the food matrix and promotes their dispersal in the gastrointestinal tract.[24,25] The current study indicates that the application of microfluidization fully disrupted the *C. vulgaris* cell wall, which led to increased micellization of lutein. Thus, it is concluded that microfluidization effectively released lutein from *C. vulgaris* during digestion.

The lutein recovery and bioaccessibility during simulated in vitro digestion of UC, MC10000, MC20000, and LT were able to be calculated from Tables 1 and 2. The lutein recovery was defined as the amount in digestate divided by the amount in the initial raw material. The lutein bioaccessibility was defined as the amount in aqueous micelles divided by the amount in the raw material. There was no significant difference ($p > 0.05$) in the lutein recovery for each sample, with a recovery in the range of 75−85%. As previously mentioned, this might be attributable to

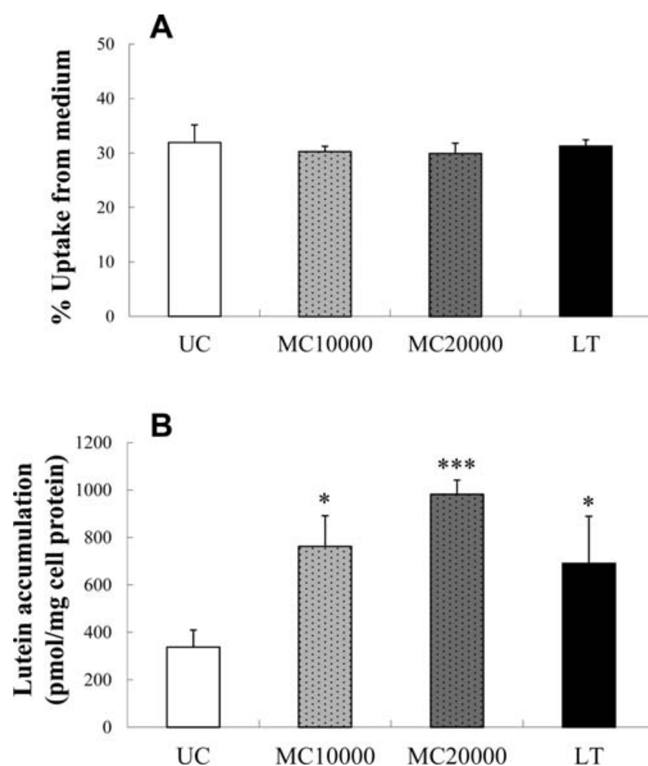

Figure 2. Effect of *C. vulgaris* microfluidization on (A) the efficiency of lutein uptake and (B) accumulation by Caco-2 cells. ***$P < 0.005$ and *$P < 0.05$, as compared to UC using Student's t test with $n = 3$.

the low effect of the microfluidization technology on lutein stability during *C. vulgaris* processing. By contrast, lutein bioaccessibility, which equated with micellization, was notably different between UCs and MCs. The bioaccessibility in MC10000 and MC20000 was 57 and 73%, respectively, while that in UC was only approximately 26%. The UC supernatant was almost colorless after digested samples were ultracentrifuged to separate the aqueous micellar fraction and the insoluble residue, whereas the micellar fractions of MC10000, MC20000, and LT had a clear yellow color, which indicates that few aqueous lutein micelles resulted from the digestion of UC. Thus, most of the lutein in the UC digestate was insoluble and was not absorbed from intestinal cells but remained in the indigestible residues. The inefficiencies of the UC treatment on lutein digestion were overcome by microfluidization. MC20000 was particularly effective in increasing lutein bioaccessibility compared with MC10000.

Uptake of Micellized *Chlorella* Luteins by Caco-2 Cells. Figure 2 shows the amount of lutein captured by Caco-2 cells from each micelle after 4 h of incubation with micelles from UC, MC10000, MC20000, and LT. The final lutein concentrations in the media per well were approximately 1.8, 4.2, 5.5, and 4.7 μM for UC, MC10000, MC20000, and LT, respectively. Figure 2A shows that the percentage lutein uptake by cells during the 4 h incubation was about 30% for all samples, and there were no significant differences ($p > 0.05$). This similarity suggests that the lutein types and conformations in micelles produced by simulated digestion were homogeneous. However, Figure 2B indicates that the absolute quantity of lutein accumulated in cells was significantly ($p < 0.005$) greater in MC20000 as compared with UC. This might be attributable to differences in the bioavailability of the lutein content among micellar fractions. There are similarities between the result of lutein bioaccessibility during simulated digestion and Figure 2B, which indicates that the amount of lutein captured by cells reflects the concentration of lutein in micelles, which was generated during simulated digestion. This result agrees with a previous study, which established that the amount of carotenoids absorbed by intestinal cells is proportional to the pigment content of the micellar fraction, thereby emphasizing the importance of efficient micellization in the maximization of the cellular concentration of carotenoids.[26]

In conclusion, the bioavailability of lutein from *C. vulgaris* was assessed using a coupled in vitro digestion and human intestinal Caco-2 cell model. There is an abundance of lutein in *C. vulgaris*, but without processing, there are restrictions on lutein bioaccessibility. Microfluidization improved the micellization of lutein from *C. vulgaris* during simulated digestion and accumulation by Caco-2 cells. Thus, microfluidization is an effective means of food processing that increases lutein bioaccessibility after the ingestion of *C. vulgaris*.

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ABBREVIATIONS USED

FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered solution; BHT, butyl hydroxytoluene; TBME, *tert*-butyl methyl ether; UC, untreated *C. vulgaris*; MC10000, microfluidized *C. vulgaris* at a pressure of 10000 psi; MC20000, microfluidized *C. vulgaris* at a pressure of 20000 psi; MC, microfluidized *C. vulgaris*; LT, micellized lutein; SEM, standard error of the mean

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