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Preparation of highly porous β-chitin structure through nonsolvent–solvent exchange-induced phase separation and supercritical CO_2 drying

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ABSTRACT

A highly open, porous, three-dimensional β-chitin network structure with meso- and macropores was successfully fabricated by exploiting nonsolvent–solvent exchange-induced phase separation and supercritical CO_2 drying processes. It was revealed that a formation of the highly porous structure, consisting of interconnected micro-scale mesoporous chitin particles and nano-scale fibrils, was favored by repeating the freeze–thaw cycle of the chitin–formic acid solution. The sodium hydroxide (NaOH) solution was used to remove the residual formic acid in the chitin gel network during the neutralization step. Nitrogen adsorption was used to characterize the properties of the mesopores, which were found to be in the range of 5–25 nm. The crystalline structure was also investigated by X-ray diffraction, which revealed a conversion of β-chitin to α-chitin during the fabrication and drying processes.

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1. Introduction

The production of highly porous materials has drawn much attention because of their potential applications not only in many areas of biotechnology, such as tissue engineering and drug delivery systems, but also in cleaning technologies for dye and heavy metal ion removal, for example. Depending on the application, different criteria for porosity, surface area, pore size, and pore structure are required. In addition, many applications prefer the use of nontoxic, biocompatible and biodegradable materials. Chitin is one of the materials that can satisfy these requirements. It is an aminopolysaccharide derived from various fungi, the shells of insects and crustacean shell waste. It is composed of $\beta(1\rightarrow4)$ linked 2-acetamido-2-deoxy-β-D-glucose units (or *N*-acetylglucosamine) forming a long linear polymer chain. Because of the presence of the acetamide group, which is similar to the amide linkage in proteins found in living tissues, chitin is biocompatible and safe for biological applications [1]. According to previous studies, chitin has exhibited the ability to enhance and accelerate wound healing [2]. It has also been shown to promote cell attachment and the spread of normal human keratinocytes and fibroblasts [3]. Moreover, lysozyme is capable of degrading chitin, generating harmless byproducts when present in the human body [4]. However, the utilization of chitin is fairly restricted because of the nature of its strong inter- and intramolecular hydrogen bonds, which make processing difficult. Several processing techniques such as leaching of water-soluble particles [5], leaching of water-insoluble particles [6], and lyophilization [7,8] have been proposed for the preparation of porous chitin matrices. Nevertheless, some limitations still remain for the use of porous chitin because of the time-consuming nature of the processing techniques as well as their inability to provide porous chitin with high interconnectivity. Moreover, residual organic solvents trapped inside the polymeric network could still be found after processing using the above techniques.

To prepare porous chitin with the desired properties, a supercritical fluid drying method has previously been employed as an alternative environmentally friendly technique. It was found that the use of supercritical carbon dioxide (sc-CO_2) was limited by the presence of water in the chitin framework (hydrogel). Some studies have shown that substituting water for the organic solvent before the supercritical drying process increases the efficiency of the process [9–11]. In this study, β-chitin derived from squid pens was used throughout the experiment. This is due to the fact that β-chitin has less intramolecular hydrogen bonding, which may result in intracrystalline swelling and solvent absorption, compared with α-chitin derived from arthropods and fungi [12]. A highly open porous three-dimensional chitin network with meso- and macropores was prepared from β-chitin by gelation induced by nonsolvent–solvent exchange and drying with sc-CO_2. It was hypothesized that nonsolvent–solvent exchange induced phase separation and sc-CO_2 drying could increase the interconnectivity and porosity of the porous chitin network. Morphological observations and nitrogen adsorption measurement were performed to evaluate the cell morphology and surface area of the so-obtained

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porous chitin. Changes in the crystalline structure of β-chitin resulting from the fabrication method and sc-CO_2 drying process were also detected by X-ray diffraction.

2. Materials and methods

2.1. Materials

Formic acid (98.0%) and all other chemicals were purchased from Wako Pure Chemicals Industries, Osaka, Japan. All chemicals were of analytical reagent grade and were used without further purification. Water used during the experiments was taken from a deionizer (Autostill WG240, Yamato Scientific Co., Ltd, Tokyo, Japan). β-Chitin was derived from squid pens, which were purified according to the procedure outlined below.

2.2. Experimental

2.2.1. Purification of squid pens

Decalcification was conducted by immersing squid pen flakes in 1 M hydrochloric acid (HCl) for 2 days. The squid pen flakes were separated and treated by a deproteinization process where the flakes were immersed in a 4% (w/v) sodium hydroxide (NaOH) solution for 24 h. The mixture was then heated on a hot plate at 80 °C for 4 h in order to obtain pure β-chitin flakes. Finally, the chitin flakes were washed with water and dried in an oven at 60 °C for 24 h. The degree of deacetylation (DD) of the obtained chitin was determined by FT-IR spectroscopy (VERTEX 70, Bruker Optics, Germany) following the method of Baxter et al. [13] and it was found to be around 10.58% (equal to degree of acetylation, DA, 89.42%).

2.2.2. Preparation of β-chitin gels

A specified amount of β-chitin flakes was suspended in formic acid (98%). A vial containing a suspension with 1 wt% chitin was then frozen at −20 °C and allowed to defrost at room temperature, so-called freeze–thaw process [14]. To improve the solubility of chitin in formic acid, this freeze–thaw process was repeated several times before further fabrications [15]. In this study, the defrost temperature during thawing were controlled at 30 °C by using a shaking incubator (AS ONE; model: SWB-17/25) maintained under continuous speed constant at 100 rpm for 30 min. By using the same chitin suspension, the freeze–thaw process was repeated four times as a cycle represented by FT-1, FT-2, FT-3 and FT-4, respectively. Each cycle provided each set of a chitin–formic acid solution. The obtained solution was then poured into a polypropylene (PP) plastic mold (21 mm diameter, 44.5 mm height). Distilled water, which is a poor solvent for chitin, was poured directly into the chitin solution to induce gelation. The volume ratio of chitin solution to distilled water was set to 1–2, which was found to be an appropriate ratio for the nonsolvent–solvent (water–formic acid) exchange to induce a phase separation and gel formation. The remaining water was rinsed out, and the obtained β-chitin gel was then immersed in a bath of 2-propanol for a few days. This immersion process was repeated several times to completely exchange any water in the gel to 2-propanol.

2.2.3. sc-CO_2 gel drying

After exchanging water in the gel for 2-propanol, the chitin gel obtained from each freeze–thaw cycle was loaded into a cup-shaped piece of aluminum foil and put in a high-pressure vessel. The vessel was pressurized with CO_2 to 10 MPa while keeping the temperature at 60 °C, conditions under which supercritical CO_2 formed. The sc-CO_2 drying process was conducted for 6 h. During the sc-CO_2 drying, the pressure was controlled by intermittent purging of a needle valve while CO_2 was continuously fed into the pressure vessel. After drying, the pressure was released gradually to prevent the β-chitin gel from foaming.

2.3. Characterizations

2.3.1. Morphology observations

The cell morphology of samples dried with sc-CO_2 was examined by scanning electron microscopy (Tiny-SEM 1540 and SEM JEOL, JSM-6340FS). The samples were coated with gold–palladium *in vacuo* prior to the SEM analysis. Transmission electron microscopy (TEM) analysis was also conducted (TEM JEOL, JEM-1010). The sc-CO_2 dried samples were dispersed in ethanol and poured onto a Cu grid before vacuum drying for TEM observation.

2.3.2. Nitrogen adsorption measurement

Nitrogen adsorption measurements were conducted using a BELSORP-mini II-KS (BEL Japan, Inc.). The prepared β-chitin gel was placed in a vacuum chamber for 24 h and was degassed at 90 °C for 3 h before the measurement. The associated analysis software was used to calculate the Brunauer–Emmet–Teller (BET) surface area.

2.3.3. GPC analysis

The weight average molecular weight of the chitin gel was determined by gel permeation chromatography (GPC) (Shimadzu, model DGU-20A3). A mixture of *N*,*N*-dimethylacetamide (DMAc), dehydrated (GPC grade, Wako Pure Chemicals Industries, Osaka, Japan) and lithium chloride (LiCl), 6% (w/v), was used as an eluent. By employing the method of Tsioptsias et al. [16], the sample was firstly dissolved in the DMAc–LiCl mixture. The obtained chitin solution, 20 μL, was then injected into a series of column (Shodex GPC; K-806L and K-800D, Showa Denko, Tokyo, Japan), which was heated up to 40 °C. The retention time and molecular weight were calibrated using Pullulan standards (Shodex standard; P-82, Showa Denko, Tokyo, Japan).

2.3.4. X-ray diffraction (XRD)

XRD patterns of the samples were collected on a Rigaku X-ray generator (RINT 2000) operated at 40 kV and 20 mA with a 2θ range of 5–40° using Ni-filtered Cu Kα radiation.

3. Results and discussion

3.1. β-Chitin gel structure

3.1.1. Effect of the freeze–thaw process

β-Chitin was dissolved in formic acid by undergoing the as-mentioned freeze–thaw process. The chitin gel was successfully fabricated from the so-obtained chitin–formic acid solution from each freeze–thaw cycle by a nonsolvent–solvent exchange-induced phase separation strategy where the water for formic acid exchange induced the gelation. Fig. 1a shows a picture of the resulting chitin gel from FT-4. After exchanging water with 2-propanol, sc-CO_2 drying step was performed. Fig. 1b shows a picture of the gel after undergoing the sc-CO_2 drying step. The sample exhibited slight shrinkage, which may have been caused by a rearrangement of the molecular hydrogen-bonding network of the chitin polymer chains during the sc-CO_2 drying process. Fig. 2 shows the morphology of the chitin gel obtained from each freeze–thaw cycle after sc-CO_2 drying. As shown in Fig. 2a, a highly porous chitin fibril network could be provided from the first cycle, FT-1. By following the next cycle, FT-2, the porous structure exhibits more well-dispersed in the chitin fibril network (Fig. 2b) as compare to that of FT-1. However, a dramatical change in the gel structure was observed during FT-3 and FT-4. It was found that the resulting fibril network structure was gradually transformed to micro-scale porous chitin particles (Fig. 2c and d) as increasing the freeze–thaw cycle. The

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