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Chitosan Sub-Micron Particles Prepared Using Sulfate Ion Salt as Bacteriostatic Materials in Neutral pH Condition

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ABSTRACT

In this paper, the newly developed ion exchange phase separation method to create chitosan sub-micron particles is introduced: 1) *chitosan was dissolved in a lactic acid aqueous solution*; 2) *the obtained chitosan solution was added stepwise in a sodium sulfate aqueous solution and cooled down to* 5℃ *to become slightly turbid through agglutination*; 3) *desalinating and deacidifying of the mixture was carried out by a dialyzing tube method. IR spectroscopy and elemental analysis indicated that the agglutination of chitosan was induced by crosslinking effect with an electrostatic interaction between sulfate anions and amino groups in the glucosamine unit although large excess of* Na_2SO_4 *caused undesirable further agglutination of the resultant chitosan particles. As a result, the proper amount of* Na_2SO_4 *was approximately* 1.0 - 10.0 *equivalent for the amino group to create the chitosan particles with a sub-micron size. In addition, we investigated an antibacterial activity test for Escherichia coli of the obtained chitosan particles. The significant antibacterial activity was observed in incubation even at neutral pH condition while the chitosan microbeads* (*size*: *ca* 200 ㎛) *prepared by the conventional method and chitosan granules* (*size*: *ca* 600 ㎛) *as starting materials showed almost no antibacterial activity in the same condition.*

Keywords*: *Chitosan*, *Particle*, *Crosslinking*, *Microbeads*, *E. coli

1. Introduction

Chitosan is a cationic biopolymer obtained from *N*-deacetylation of chitin, *β*-(1,4)-*N*-acetyl-D-glycan [1]. The non-toxic, biocompatible and biodegradable properties of chitosan provide potential for many types of applications [2-4]. Chitosan and derivatives have become useful polysaccharides in the biomedical field. Especially, these microparticles have been utilized as chromatographic packings [5,6], enzyme-immobilized support [7,8], affinity adsorbents for proteins [9], endotoxin adsorbents [10] and drug carriers [11,12]. Generally, it was popular to use chitosan as antibacterial compounds in agriculture, as elicitors of plant defense responses [13], as additives in the food industry, as flocculating agents for wastewater [14] and as pharmaceutical agents in biomedicine [15,16]. In this content, environmentally antibacterial activity of chitosan has received considerable attention recently. However, these activities are limited to acidic conditions because of its poor solubility above pH 6.5, where chitosan starts to lose its cationic nature [17-20].

Chitosan is generally insoluble under a neutral pH conditions because of a strong hydrogen bonding and lower pKa (ca. 6) of a residual amino group. Thus, the molding, investigation and application of chitosan have been restricted. The methods for producing porous and spherical chitosan microbeads, such as the “suspension evaporation method” [6] and the “suspension crosslinking technique” [9,21] using chitosan acid aqueous solutions, have been reported. These methods require the use of organic solvents and emulsifier. Also a sphering method by spray drying [22,23] is known and widely applicable. However, these methods have some disadvantages: for example, the particle size control is difficult, especially in below tens of micron but also a heating process as a cost up factor is necessary. Recently, the “rapid expansion of supercritical fluid technology” [24] has been

developed as a method preparing sub-micron particles. Since this technique uses supercritical CO_2, the process is environmentally safe but it is also known that the control of particle size, shape and composition are difficult [25].

In this paper, we introduce a new method as the "ion exchange phase separation method" to create submicron particles from chitosan without using any organic emulsifier and solvent as well as with no heating process. This method can be characterized by solidification with Na_2SO_4 and following dialysis. It is also reported that the obtained chitosan particles shows excellent antibacterial activity even at neutral pH 7 towards *Escherichia coli* although chitosan exhibits higher antibacterial activity only in an acidic medium [26,27].

2. Materials and Methods

Chitosan sub-micron particles were prepared as follows: two kinds of chitosan materials (CS85 and CS485) were selected. Their chitosans are 85 mol% in the deacetylation degree, and were 70 - 100 kDa and 440 - 530 kDa in the molecular weight, respectively. Chitosan was dissolved in a 1.3 wt% lactic acid aqueous solution to be 1.5 wt% and then 20 ml of the solution was added stepwise into Na_2SO_4 aqueous solutions (1.0, 2.0, 5.0 and 10.0 equivalents for the amino group in a glucosamine unit) at 30˚C. The mixture was cooled down to 5˚C and kept for 10 min to become turbid. The mixture was desalinated and deacidified by dialyzing with a dialysis tube (MWCO, 12000 - 14000, Spectra/por 5) in distilled water for 4 days. The obtained chitosan particles are abbreviated as PCS85-sm and PCS485-sm.

The other type of chitosan spherical microbeads was prepared by slight modification of the suspension evaporation method reported previously [6]. 25 ml of a 1.5 wt% chitosan (CS85 or CS485) lactic acid solution was added to 250 ml of decahydronaphthalene containing 5 g of polyethylene glycol mono-4-nonylphenyl ether (polymerization degree, 10) and suspended by stirring at 80˚C for 24 h. By gradual removal of water, the chitosan-containing suspension particles were solidified to be spherical. The obtained microbeads were deacidified with 1 M NaOH, and successively washed with ethanol and ether. The obtained chitosan particles are abbreviated as PCS85-m and PCS485-m.

Surface area analysis of the particles was carried out by the Brunau-Emmet Teller (BET) method using Autosorb-1 (Aionics Co. Ltd., Japan). Fourier transformed infrared (FT-IR) spectroscopy was carried out with JASCO FT/IR-700. The particle size and distribution were measured by dynamic light scattering (DLS) method (Zetasizernano-ZS, Sysmex Corp., Japan). The particles were also observed using a field emission scanning electron microscope (FE-SEM) (S-4000, Hitachi, Co. Ltd., Japan) and stereomicroscope (KH-7700S, Hirox Co. Ltd., Japan).

For assay of antibacterial activity, Mueller-Hinton Broth (Becton Dickinson and Company, USA) adjusted by cation, which contained 92 mg of $CaCl_22H_2O$, 104.5 mg of $MgCl_26H_2O$ and 15 g of agar for 1l of broth, respectively, was used as culture media. *Escherichia coli* NBRC 3972 (*E. coli*) was incubated at 37˚C for 4 - 6 h in the Mueller-Hinton Broth until logarithmic phase was reached. The assay was carried out with 0, 0.5, 1.0 and 5.0 mg/ml of the chitosan sub-micron particles (PCS85-sm and PCS485-sm), the chitosan microbeads (PCS85-m and PCS485-m) and chitosan granules as starting materials (abbreviated as CS85-g and CS485-g). The chitosans were put into sterile glass-plates, 15 ml of Mueller-Hinton Broth was added to each sterile glass-plate containing *E. coli* culture and then were spread on the plates to be 1.0×10^2, 1.0×10^4 and 1.0×10^6 cfu/plate, and these were incubated at 37˚C for 18 h. Antibacterial activity was observed by comparison of with the control plate without chitosan.

3. Results and Discussion

3.1. Preparation of Chitosan Sub-Micron Particles

Figure 1 shows the FT-IR spectra of the product prepared by the ion exchange phase separation method from CS85. As shown in **Figure 1(a)**, the product before dialyzing showed a distinct adsorption at 1700 cm^{-1} corresponding to $\delta_{C=O}$ of a carboxyl group of lactic acid. The adsorptions of $\delta_{S=O}$ at 1110 cm^{-1} and $\tau_{S=O}$ at 620 cm^{-1} increased with increasing amount of Na_2SO_4 added in the sphering process. This indicates that the product was the mixture of Na_2SO_4, chitosan and lactic acid. After dialyzation for 4 days, the resultant product showed that the adsorption at 1700 cm^{-1} for $\delta_{C=O}$ disappeared completely but also the adsorptions based on $\delta_{S=O}$ at 1110 cm^{-1} and $\tau_{S=O}$ at 620 cm^{-1} decreased remarkably. However, $\delta_{S=O}$ at 1110 cm^{-1} and $\tau_{S=O}$ at 620 cm^{-1} of the products remained regardless of sufficient dialyzing. Similar IR spectrum was observed for the product prepared from CS485. These results indicate that large excesses of lactic acid and Na_2SO_4 could be removed by dialysis, but that anion-exchange from a lactate ion to a sulfate ion towards an ammonium ion of chitosan occurred. The final products, PCS85-sm and PCS485-sm were insoluble in water.

3.2. Microscopic Observation of Chitosan Particles

In the ion exchange phase separation method, a chitosan

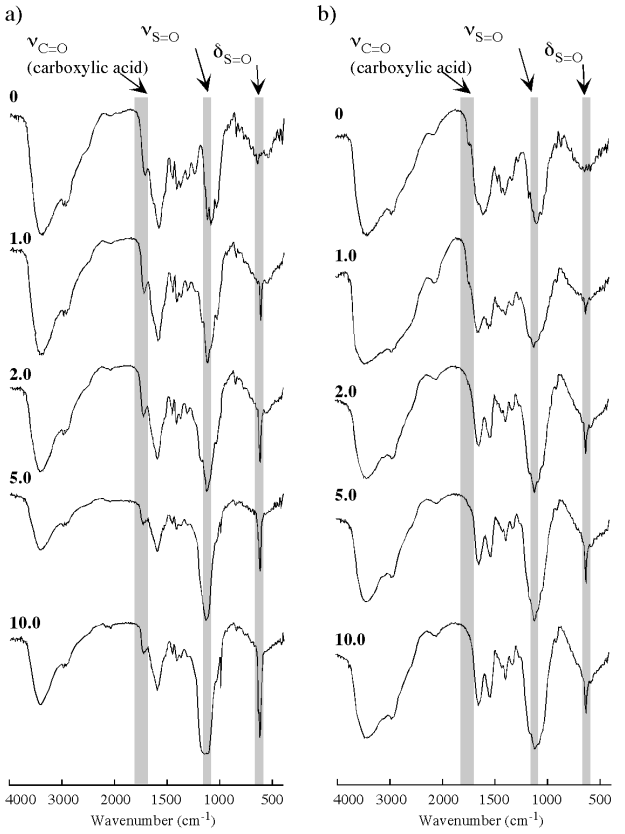

Figure 1. FT-IR spectra of PCS85-sm and PCS485-sm. a) before and b) after desalinating. Added amount of Na_2SO_4: 0, 1.0, 2.0, 5.0, 10.0 (eq.) for -NH_2 group.

lactic acid aqueous solution becomes turbid gradually by addition of Na_2SO_4 and cooling down to 5˚C. The turbidity remained after removing excesses of lactic acid and Na_2SO_4 by dialysis. On the other hand, similar turbidity increase was observed when a monoanion salt such as NaCl was used instead of Na_2SO_4 but the dialysis made it clear. This significant difference between Na_2SO_4 and NaCl can be attributed to a divalent property based on SO_4^{2-} because IR spectroscopy showed a distinct absorption based on $\delta_{S=O}$ even after successive dialysis as shown in **Figure 1**. Therefore, it is estimated that SO_4^{2-} works as a sort of a crosslinker.

Stereomicroscopic images of PCS85-sm and PCS485-sm in aqueous dispersions are shown in **Figures 2** and **3**, respectively. The particle size was affected by the amount of Na_2SO_4 used in the sphering process especially before dialysis. It is clearly shown that the agglutination of chitosan particles was promoted with increase of the amount of Na_2SO_4 and extreme agglutination was observed in PCS485 as shown in **Figure 3(a)**. This is due to the fact that increase of amino groups as crosslinking sites in chitosan promotes agglutination among chitosan particles. As a result, the aggregate size reached over 50

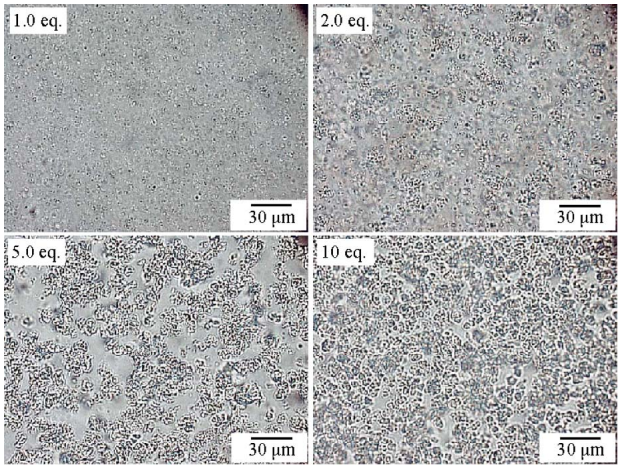

Figure 2. Stereomicroscope images of PCS85-sm in dispersion after desalinating. 0, 1.0, 2.0, 5.0, 10.0 stand for added amount of Na_2SO_4 (eq.) for -NH_2 group.

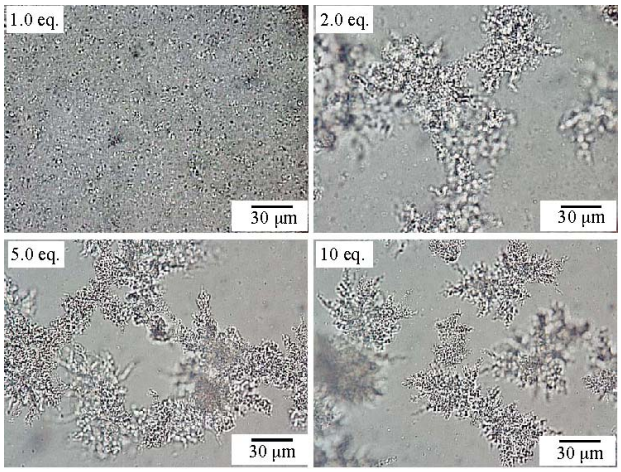

Figure 3. Stereomicroscope images of PCS485-sm in dispersion after desalinating. 0, 1.0, 2.0, 5.0, 10.0 stand for added amount of Na_2SO_4 (eq.) for -NH_2 group.

⌠m in PCS485-sm and to 5 - 10 ⌠m in PCS85-sm by agglutination. Therefore, the sub-micron particles without agglutination can be produced by a proper combination of the concentration of Na_2SO_4 and the polymerization degree of material chitosan.

On the other hand, the PCS485-m microbeads prepared by the suspension evaporation method showed a typical spherical shape and the particle size was about 200 - 300 ⌠m as shown in the SEM image of **Figure 4(a)**. **Figure 4(b)** showed the SEM image of material chitosan granules (CS485-g, ca 600 ⌠m).

3.3. Size Distribution of Chitosan Particles in Dispersions

As shown in **Figure 5**, the DLS diagrams showed a couple of peaks in both the PCS85-sm and PCS485-sm dis-

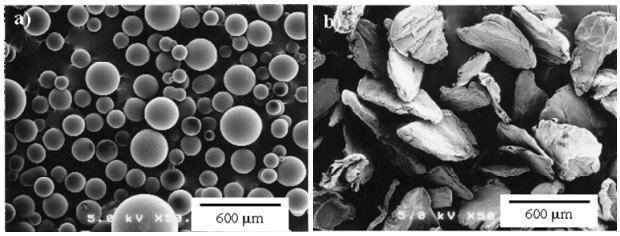

Figure 4. SEM images of PCS-m (a) and PCS-g (b).

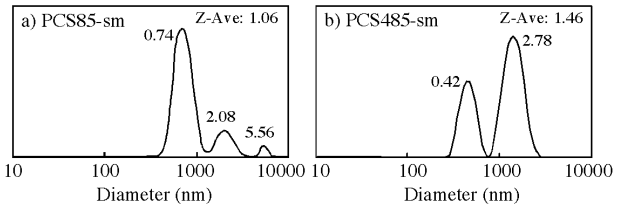

Figure 5. Size distribution of PCS85-sm (a) and PCS485-sm (b) before desalinating, determined by DLS. Added amount of Na_2SO_4 for -NH_2 group: 1.0 eq.

persions prepared with 1.0 eq. of Na_2SO_4 before dialyzing. Their Z-averages were 1.06 ⌠m and 1.46 ⌠m, respectively. This indicates that both the dispersions include sub-micron size particles but also agglutination of the sub-micron particles occurs somewhat. The agglutination was promoted with increase of Na_2SO_4 used in the preparation procedure as mentioned in the microscopic observation. As supporting this, the Z-average of the samples prepared with 2.0 or higher eq. of Na_2SO_4 could not be obtained because the detection limit of DLS was within 10 ⌠m and thus these samples might produce extremely large aggregates. On the other hand, to remove excess of lactic acid as well as Na_2SO_4 by dialysis suppressed this agglutination distinctly. For example, the Z-averages of the PCS85-sm dispersions after dialysis were estimated to be 0.97, 1.34, 2.80 and 4.24 ⌠m in the preparation with 1.0, 2.0, 5.0 and 10 eq. of Na_2SO_4, respectively.

However, the peaks indicated in **Figure 6(a)** showed smaller particle sizes than those Z-averages. This indicates that agglutination was not completely suppressed by dialysis.

3.4. Antibacterial Activity for Chitosan Particles

We investigated the antibacterial activity of the obtained chitosan particles for *E. coli.* PCS85-sm prepared with 1.0 eq. of Na_2SO_4 was selected for this purpose because the particle agglutination was most suppressed comparing with the others. Also PCS85-m and CS85-g were used for comparison.

The first application was carried out at pH 5.0 because it is known that usual chitosan exhibits antibacterial activity only in an acidic medium such as pH 5.4 - 6.5 [26,

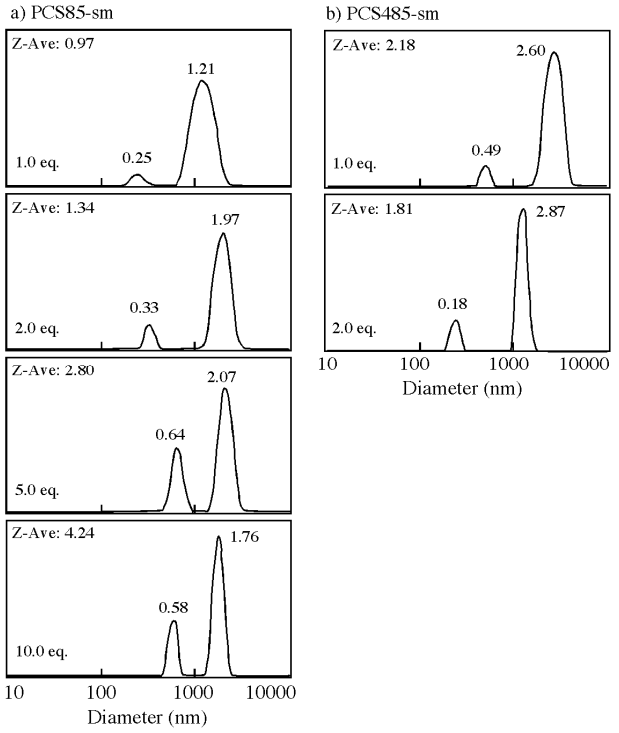

Figure 6. Size distribution of PCS85-sm (a) and PCS485-sm (b) after desalinating, determined by DLS. 0, 1.0, 2.0, 5.0, 10.0 stand for added amount of Na_2SO_4 (eq.) for -NH_2 group.

27]. **Figure 7(a)** shows the growth of *E. coli* in the presence of CS85-g. The sample preparation is as follows: CS85-g was dispersed to be 0.5 mg/ml, 1.0 mg/ml and 5.0 mg/ml in an incubation medium at pH 5.0 adjusted with a 0.2 M HCl aqueous solution. After the given amount of agar was added to each dispersion, *E. coli* culture was spread on the above-mentioned media. As expected, CS85-g showed significantly antibacterial activity with the increase of its concentration. On the other hand, when an incubation medium was adjusted at around pH 7.0 with a 0.2 M NaOH aqueous solution and then *E. coli* culture were spread and incubated with the same procedure, distinct growth of *E. coli* was observed even in the presence of 5.0 mg/ml of CS85-g as shown in **Figure 7(b)**. This indicates that CS85-g showed almost no effect in inhibition of growth of *E. coli* under neutral condition. Similar no inhibition at pH 7.0 in *E. coli* growth was observed in PCS85-m prepared by the suspension evaporation method as shown in **Figure 7(c)**.

Complete inhibition of *E. coli* growth at pH 7.0 was observed only in the presence of PCS85-sm prepared by the ion exchange phase separation method. **Figure 7(d)** shows the typical example. No *E. coli* colony was found for 48 h incubation even in the low concentration of 5.0 mg/ml. The mechanism of growth inhibition for *E. coli* is not yet specified but it is considered presumably that the

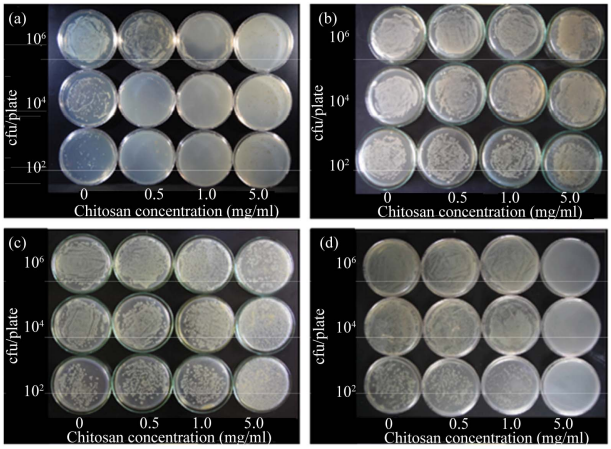

Figure 7. Growth of E. coli in culture containing PCS-g in pH5.0 (a) PCS-g in neutral condition (b) PCS85-m in neutral condition (c) and PCS-sm in neutral condition (d).

size effect may be included but also the specific surface area of the particles can play an important role. For example, the specific surface areas were determined by BET method to be 127 m^2/g, 15.19 m^2/g, and 2.82 m^2/g in PCS85-sm, PCS85-m and CS85-g, respectively. It is reasonable to consider that increase of the surface area promotes the adsorption of *E. coli* onto the chitosan particles and thus effective antibacterial activity can be derived from amino groups of chitosan.

4. Conclusions

The chitosan submicron particles have been prepared by the newly developed ion exchange phase separation method which is characterized by the facts that chitosan is dissolved in a lactic acid aqueous solution and solidification can be realized by addition of Na_2SO_4 and cooling down to 5 ˚C. By removal of excess of lactic acid and Na_2SO_4, particle agglutination can be sufficiently suppressed. Probably a stable dispersion state in water can be attributed to crosslinking effect between amino groups of chitosan and sulfate anion from Na_2SO_4.

In addition, antibacterial activity for *E. coli* has been investigated. It was confirmed that only the chitosan submicron particles prepared by the present method showed distinct inhibition of *E. coli* growth at neutral pH although the material chitosan granules showed effective antibacterial activity only in acidic condition as it has been known [26,27].

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