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Preparation of Chitosan Sub-Micron Beads as Bacteriostatic Materials by Phase Separation with Polyvalent anion

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Chitosan sub-micron particles were prepared by ion exchange phase separation method as follows: 1) chitosan was dissolved in lactic acid aqueous solution: 2) the obtained chitosan solution was dropped in polyvalent anion salt aqueous solution, i.e. dianion salt, trianion salt solution: 3) desalinating and deacidifying from aqueous dispersion of chitosan submicron particles was carried out by dialyzing tube method. The aqueous dispersion of chitosan particles, which was prepared using aqueous solution of polyvalent anion salt appeared cloudy. This phenomenon was attributed to cross-linking by ion interaction between sulfate anion and amino groups in glucosamine unit. The chitosan aggregates were confirmed to promote with increase of amount of Na_2SO_4 by dynamic light scattering method. This indicates that agglutination sites among chitosan particles increased as the number of the sulfate anion coupled with amino group increased. As results, the chitosan particles of sub-micron size were prepared by adding 1.0 equivalent of Na_2SO_4 against an amount of amino groups to dispersion medium. In addition, we investigated the antibacterial activity test for *Escherichia coli* of obtained chitosan particles. The chitosan sub-micron particles showed significantly antibacterial activity at concentration of 5.0 mg/ml, in spite of incubation in neutrality condition (pH 7.1-7.6).

Key words: Chitosan, particles, Phase separation, Polyvalent anion, Antibacterial activity

1. INTRODUCTION

Chitosan is a cationic biopolymer obtained from N-deacetylation of chitin, a β-(1,4)-linked N-acetyl-D-glycan[1]. The non-toxic, biodegradable and biocompatible properties of chitosan provide potential for many types of applications. Chitosan and derivatives have become useful polysaccharides in biomedical area. Especially, these microparticles were utilized as chromatographic packings[2,3], enzyme-immobilized support[4,5], affinity adsorbents for proteins[6], endotoxin adsorbents[7] and drug carrier[8,9].

The methods for producing porous and spherical particles from chitosan derivative aqueous or organic solution, such as the "suspension evaporation method[3]", "suspension crosslinking technique[10]" using chitosan acid aqueous solution have been reported. These methods require the use of organic solvents that are unfavorable to environment.

While the sphering method by "spray drying[11,12]" using chitosan acid aqueous solution has a wide range of application in the chemically industry, these methods have difficulty of controlling for particle size and sizing below tens of micron and require a heating process for drying as an unfavorable factor.

Recently, "rapid expansion of supercritical fluid technology[13]" has also been described for preparing method of sub-micron particles. Since rapid expansion of supercritical fluid technology" utilized supercritical CO_2, the process have environmentally safe. However, it has been known that the control of particle size, shape and composition are difficult.

In this paper, we describe that the chitosan particles with series of sub-micron size or micron size could be prepared by "ionic exchange technique" using from inorganic polyvalent anion salt alone, without organic substance such as organic emulsifier and oil solvent. In addition, we also reported that the obtained particles showed antibacterial activity for *Escherichia coli*.

2. EXPERIMENTAL

2.1 Preparation of Chitosan Particles

Chitosan particles were prepared by phase separation method using Na_2SO_4 salt. The types of 70-100 kDa (CS85) or 440-530 kDa (CS485) of M_w of chitosan (deacetylation degree: 85 mol%) were used, respectively. 20 ml of 1.5 wt% chitosan-lactic acid solution (1.3 wt%) were dropped into a given concentration of Na_2SO_4 (1.0, 2.0, 5.0 and 10.0 equivalent for amino groups in glucosamine unit) aqueous solution at 30 °C. In addition, its mixture was cooled off at 5 °C. The dispersion was desalinated and deacidified by dialyzing in a dialysis tube (MWCO, 12000-14000 (Spectra/por 5)) against distilled water for 4 days. The obtained particles were freeze-dried.

2.2 Measurement

The identification of obtained particles was carried out by Fourier transformed (FT-IR) spectroscopy (FT/IR-700, JASCO Co. Ltd., Japan). The turbidity of dispersion was evaluated by measurement of optical transmittance at 500 nm using UV/VIS spectrophotometer (V560, JASCO Co. Ltd., Japan). 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, Hyrox Co. Ltd., Japan).

2.3 Assay of Antibacterial activity

A Cation Supplemented Mueller-Hinton Broth Agar, which contained Mueller-Hinton broth (Difco), 92 mg/l (50 mg, Ca^{2+}/l) of $CaCl_2 \cdot 2H_2O$, 105 mg/ml (25 mg, Mg^{2+}/l) of $MgCl_2 \cdot 6H_2O$ and 15 g/l of agar was used for assay of antibacterial activity. *Escherichia coli* NBRC 3972 (*E. coli*) was incubated at 37°C for 4-6h in the Cation Supplemented Mueller-Hinton Broth until logarithmic phase was reached. 0, 0.5, 1.0 and 5.0 mg/ml (F. C.) of chitosan granules (starting materials for particulate, size: 600 μm) or obtained chitosan particles were added into sterile glass-plates containing chitosan particles, respectively. 15 ml of Cation Supplemented Mueller-Hinton Broth Agar was added to each sterile glass-plate. *E. coli* culture was diluted by sterile distilled water. 1.0 x 10^2,10^4 and 10^6 cfu/plate of *E. coli* were respectively spread on above-mentioned plates using *E. coli* culture diluent. Their plates were incubated at 37 °C for 16 h. The antibacterial activity was defined in comparison of each chitosan plate with control plate (a lack of chitosan). For the each chitosan plates, no colonies determined as bactericidal or strong bacteriostatic. It was determined as bacteriostatic that the colonies on test plates grew smaller than those on control plate.

3. RESULTS AND DISCUSSION

3.1. Preparation of chitosan particles

Figure 1 show the FT-IR spectra of products prepared from CS85. As shown in Fig. 1-a, the products without dialyzing showed IR spectra with the significant

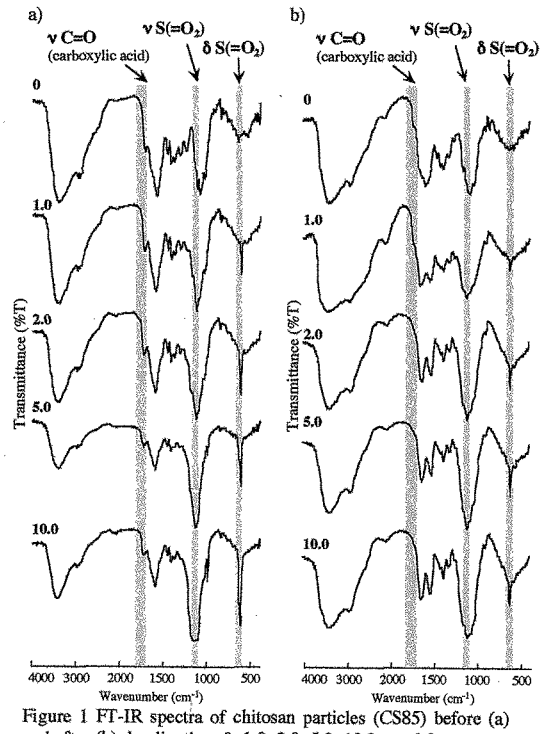

Figure 1 FT-IR spectra of chitosan particles (CS85) before (a) and after (b) desalinating. 0, 1.0, 2.0, 5.0, 10.0 stand for amount of Na_2SO_4 addition (eq.) for -NH_2 group.

adsorption at 1700 cm^{-1} corresponded to $\nu_{C=O}$ (carboxyl of Lactic acid). The adsorption of $\nu_{S(=O)_2}$ at 1110 cm^{-1} and $\delta_{S(=O)_2}$ at 620 cm^{-1} for these products increased with increasing of Na_2SO_4. The dispersion was dialyzed in a dialysis tube for 4 days. IR spectra showed significant change. As shown in Fig. 1(b), the adsorption at 1700 cm^{-1} disappeared. The IR spectra of products prepared with 1.0 equivalent or over of Na_2SO_4 for amino group changed little, the adsorption of $\nu_{S(=O)_2}$ at 1110 cm^{-1} and $\delta_{S(=O)_2}$ at 620 cm^{-1} showed no increase phenomenon of intensity. These results indicate that the removal of lactic acid and extra Na_2SO_4 could be completed, and anion-exchange from lactate ion to sulfate ion around ammonium ion groups in chitosan occurred. Additionally, IR spectra of CS485 products corresponded to those of CS85.

3.2. Stereomicroscope image of chitosan particles in dispersion

As the mixture of chitosan lactic acid solution and Na_2SO_4 aqueous solution was cooled off at 5 °C, a

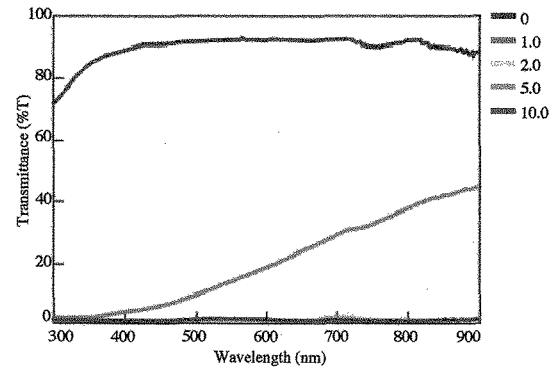

Figure 2 Optical transmittance for chitosan particles (CS85) aqueous dispersion after desalinating. 0, 1.0, 2.0, 5.0, 10.0 stand for amount of Na_2SO_4 addition (eq.) for -NH_2 group.

mixture solution became clouded gradually. The clouded mixture solution was dialyzed by dialysis tube in order to remove salt and acid. This solution remained clouded in spite of dialyzing. This indicates that these phenomena were attributed to cross-linking by ion interaction between amino group and sulfate ion. As a reference, in the case of use of monoanion salt such as NaCl instead of Na_2SO_4, although its mixture solution cooled off at 5 °C became clouded, the solution became transparent with dialyzing. Figure 2 showed in optical

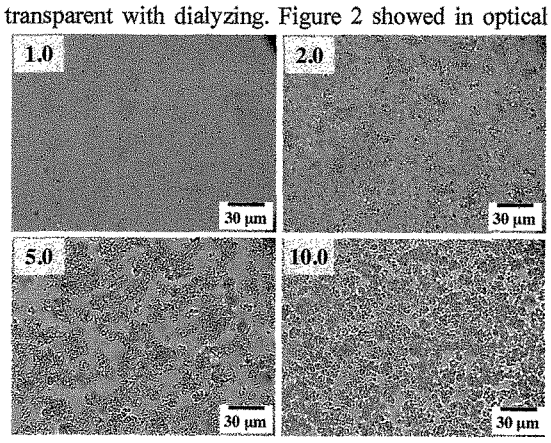

Figure 3 Stereomicroscope images of chitosan particles (CS85) in dispersion after desalinating. 0, 1.0, 2.0, 5.0, 10.0 stand for amount of Na_2SO_4 addition (eq.) for -NH_2 group.

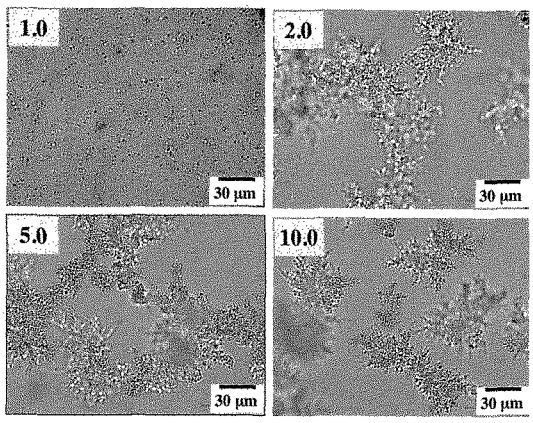

Figure 4 Stereomicroscope images of chitosan particles (CS485) in dispersion after desalinating. 0, 1.0, 2.0, 5.0, 10.0 stand for amount of Na_2SO_4 addition (eq.) for -NH_2 group.

transmittance at 500 nm for dispersion of CS85. The more increase of concentration of Na_2SO_4 in dispersion media produced higher turbidity for dispersion. Additionally, optical transmittance of CS485 products was similar to that of CS85, regardless of M_w. Stereomicroscope images of dispersion prepared from CS85 and CS485 were shown in Fig. 3 and 4, respectively. The particles were clearly observed in aqueous dispersion. The particle size of chitosan was affected by amount of Na_2SO_4. The aggregates were promoted with increase of amount of Na_2SO_4 in CS85 dispersion. In addition, the drastic agglutination was observed in CS485 dispersion prepared with 1.0 equivalent or over of Na_2SO_4 aqueous solution as shown in Fig. 4. These were attributed to fact that increase of the cross-linking moiety in molecular chain produced agglutination among chitosan particles. As results, the size of aggregate reached to 50 μm over in CS485 dispersion, and did to 5-10 μm in CS85 dispersion. The CS485 particle size is larger than CS85 particls size. These results indicate that the molecular domain of CS485 in aqueous solution was larger than that of CS85.

3.3 Size of chitosan particles by DLS measurement.

Figure 5 showed the size distribution of CS85 and CS485 measured using DLS. The particle sizes (Z-average) in CS85 and CS485 dispersion before dialyzing were 1060 nm and 1460 nm, respectively.

On the other hand, the particles in dispersions which prepared with 2.0, 5.0 and 10 equivalent of Na_2SO_4 aqueous solution before dialyzing, was so large that those could not be measured using DLS. In contrast, after these dispersions were dialyzed, all the particle size of CS85 dispersion could be measured using DLS because the obtained particles became smaller, as shown in Fig. 6. However, in the case of CS485, the dispersion prepared with 1.0 and 2.0 equivalent of

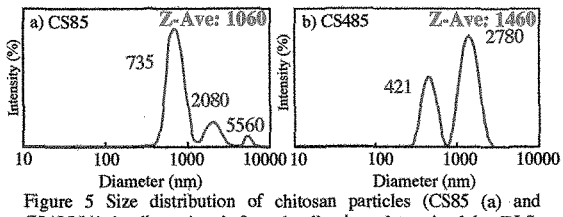

Figure 5 Size distribution of chitosan particles (CS85 (a) and CS485(b)) in dispersion before desalinating, determined by DLS. Amount of Na_2SO_4 addition for -NH_2 group: 1.0 eq..

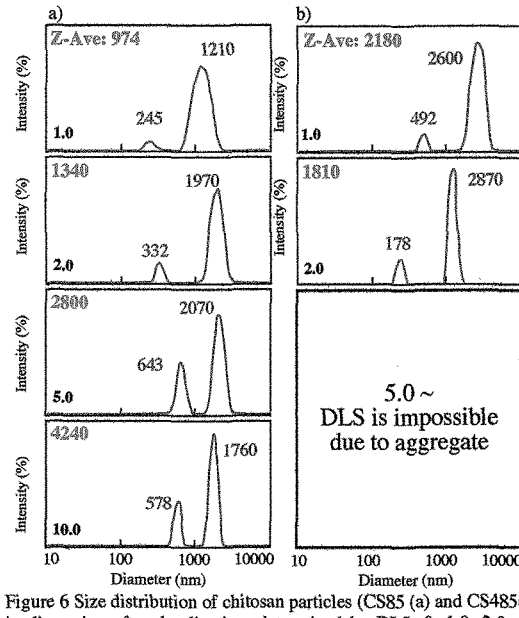

Figure 6 Size distribution of chitosan particles (CS85 (a) and CS485(b)) in dispersion after desalinating, determined by DLS. 0, 1.0, 2.0, 5.0, 10.0 stand for amount of Na_2SO_4 addition (eq.) for -NH_2 group.

Na_2SO_4 aqueous solution could be only measured using DLS. The measurements of dispersion prepared with 5.0 and 10.0 equivalent of Na_2SO_4 aqueous solution were impossible, because of occurrence of intensive aggregation, in accordance with stereomicroscope images shown in Fig. 4. In addition, the CS85 dispersion desalinated by dialyzing was treated in a centrifuge at 6200 rpm for 10 min, in order to separate the sub-micron and micron-particles in the dispersion. The size of particles in supernatant and precipitate obtained by centrifugation were also measured using DLS. In the case of particles obtained from dispersion prepared with 1.0 equivalent of Na_2SO_4, the Z-average size of particles in the supernatant was calculated at 631 nm, however, in the precipitate, that in the precipitate was 1470 nm. With increase of adding amount of Na_2SO_4, 2.0, 5.0, 10 equivalent in dispersion medium in preparing, the sub-micron particles of a few hundred-micrometer size were decreased, while the micron particles of micrometer size were increased. As a result, in the case of particles prepared with 10 equivalent of Na_2SO_4 dispersion medium, the Z-average size of particles in the precipitate was reached to the size of 5060 nm.

3.4 SEM image of particles freeze-dried

The scanning electron micrographs of freeze-dried CS485 products are shown in Fig. 7. In spite of concentration of Na_2SO_4 in dispersion, all the freeze-dried CS485 products became a porous aerogel. This indicates that the cross-linking moiety in molecular chain produce agglutination among particles

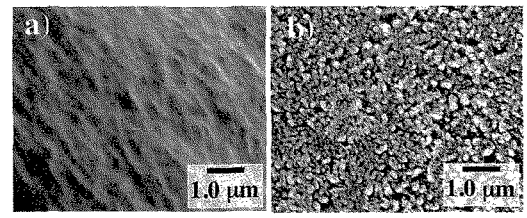

Figure 7 SEM images of chitosan aerogel (CS485) after desalinating and freeze-drying. Na_2SO_4 addition: a) 1.0, b) 2.0 eq. for -NH_2 group.

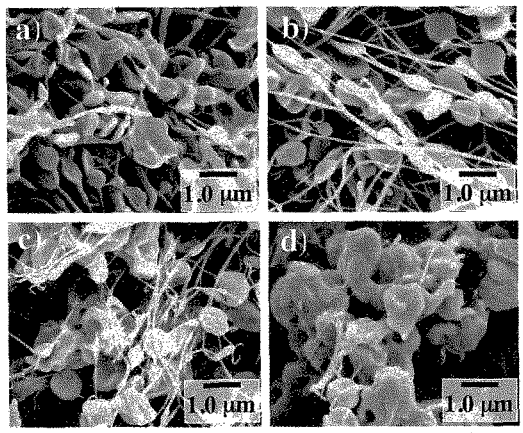

Figure 8 SEM images of chitosan particles (CS85) after desalinating and freeze-drying. Na_2SO_4 addition: a) 1.0, b) 2.0, c) 5.0, d) 10.0 eq. for $-NH_2$ group.

with freeze-drying gradually. In addition, the surface of CS485 was found to become more porous structure with increase of Na_2SO_4 adding into dispersion media. On the other hand, many particles were observed in CS85 products, as shown in Fig. 8. This attributed to the fact that the molecular domain of CS85 in aqueous solution was smaller than that of CS485. Therefore, shrinking speed of CS85 was fast than that of CS485, because the molecular motion of CS85 is fast than that of CS485 because with freeze-drying. It was observed that the aggregates of particles were promoted with increase of amount of Na_2SO_4 adding into dispersion media, in accordance with results of stereomicroscope images.

3.5 Antibacterial activity for chitosan particles

We investigated the antibacterial activity test for *E. coli* of obtained chitosan particles. Figure 9 showed the growth of *E. coli*, which coexisted with CS85 particles prepared with 1.0 equivalent of Na_2SO_4 aqueous solution or chitosan granule (size: ca 600 μm) as starting materials for microparticulation of chitosan. Antibacterial activity for chitosan at concentrations ranging from 0.5 to 5.0 mg/ml for culture media was examined in neutrality pH condition. Their pH in dispersion containing 0, 0.5, 1.0 and 5.0 mg/ml of CS85 particles are 7.56, 7.48, 7.38 and 7.14, respectively. The pH in dispersion containing chitosan granule also are same. The chitosan granule as starting materials showed no effective in growing smaller slightly with the increase of concentration of chitosan granule. In contrast, the obtained chitosan particles showed significantly antibacterial activity at concentration of 5.0 mg/ml. Generally, it is well known that chitosan microparticle shows antibacterial activity only in an acidic medium. The antibacterial activity of chitosan is usually influenced by solubility of chitosan in solution under or above pH 6.5[14]. In contrast, the prepared chitosan particles showed significantly antibacterial activity at concentration of 5.0 mg/ml, in spite of incubation of neutrality pH condition. These results were attributed to be microparticulated. Therefore, the antibacterial activity was considered to depend on the large surface area of chitosan particles.

In conclusion, the chitosan particles were prepared as follows. Chitosan lactic acid solution was dropped in inorganic salt aqueous solution. The salt and lactic acid were removed from aqueous dispersion of

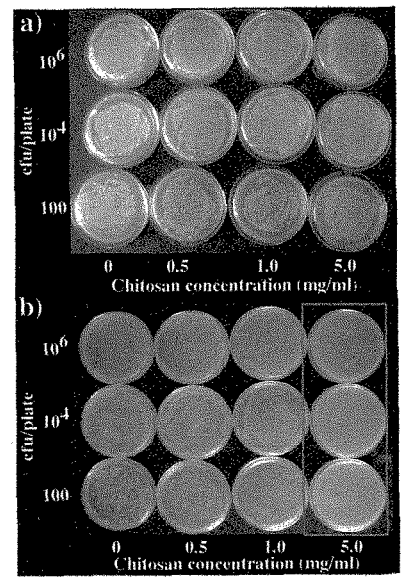

Figure 9 Growth of *E. coli* in culture containing chitosan granule (a) and chitosan particles (CS85) (b).

chitosan particles by dialyzing. As a result, the chitosan particles could be prepared using polyvalent anion, i.e Na_2SO_4. The formation mechanism of particle is attributed to crosslinking structure occurred by ion interaction between sulfate anion and amino groups.

In addition, we confirmed the antibacterial activity for *Escherichia coli* of obtained chitosan particles. The above-mentioned chitosan sub-micron particles showed significantly antibacterial activity at concentration of 5.0 mg/ml for culture media, in spite of incubation in neutrality condition.

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