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Chitosan beads with pendant α-cyclodextrin: preparation and inclusion property to nitrophenolates

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Abstract

Highly porous beads having an ability to form inclusion complexes with specific substrates have been synthesized and preliminary experiments for the application to adsorbent for affinity column chromatography and controlled release were carried out. Water-insoluble chitosan beads were synthesized by adding an aqueous acetic acid solution of chitosan into ethanolic aqueous sodium hydroxide and subsequent crosslinking with hexamethylene diisocyanate in *N, N*-dimethylformamide. The resulting beads were further treated with 2-*O*-formylmethyl-α-cyclodextrin in the presence of sodium cyanoborohydride in acetate buffer at pH 4.4, giving the cyclodextrin-linked chitosan beads. Their inclusion ability was examined by the use of *p*-nitrophenol and its analogue as model compounds.

Keywords: Cyclodextrin; Chitosan; *p*-nitrophenol; Inclusion complex; Controlled release

1. Introduction

Chitosan is a polysaccharide prepared by de-*N*-acetylation of natural mucopolysaccharide, chitin, which consisted of shells of crustaceans such as crabs and shrimps (Horton & Lineback, 1965). AS chitosan is a multifunctional polymer that has primary and secondary hydroxyl groups as well as a highly reactive amino group, it has been regarded as a useful starting material for various purposes such as ion exchanger (Muzzarelli & Rocchetti, 1974) and anti-fungal substances (Stossel & Leuba, 1984). Further, numerous investigations on chemical modification of chitosan have also being carried out to introduce novel functions into this biopolymer (Muzzarelli, Jeuniaux & Goody, 1986).

However, cyclodextrins (CDs), cyclic oligosaccharides consisting of at least six glucopyranose residues that are joined together by α(1 → 4) linkages, have received much attention due to their unique property to form host–guest complexes with various organic compounds (Szejtli, 1982). As a result of this quite unique property, CDs have been singled out for studies on supramolecular chemistry. Further, CDs and their derivatives have been widely used, in food, cosmetic and pharmaceutical industries, as host compounds in order to increase the solubility of drugs, to stabilize sensitive compounds, and to fix volatile aromas. In our recent studies on the chitosan-based supramolecular species, we have succeeded in the synthesis of new type of CD derivatives by coupling of chitosan and CDs through condensation (Furusaki, Ueno, Sakairi, Nishi & Tokura, 1996) and reductive amination (Tanida *et al*., 1998; Tojima *et al*., 1998). It was found that these CD polymers were soluble in water and that the inclusion ability of the original CDs was maintained after the coupling reaction. Now, our interest was focused on the preparation of insoluble CD-linked chitosan, because chitosan beads have been widely used in various fields such as metal ion adsorptive material (Malot, Tobin & Guibal, 1998; Kawamura, Yoshida, Asai & Tanibe, 1998) and immobilization of enzymes (Iwasaki, Inoue & Matsubara, 1998). In this article, we describe the first preparation of CD-linked chitosan beads and results of preliminary experiments for molecular recognition.

2. Experimental

2.1. Materials and general methods

All reagents were purchased from Wako-pure Chemicals Co. Ltd. (Osaka, Japan) and used as received. *N, N*-dimethylformamide (DMF) was distilled under reduced

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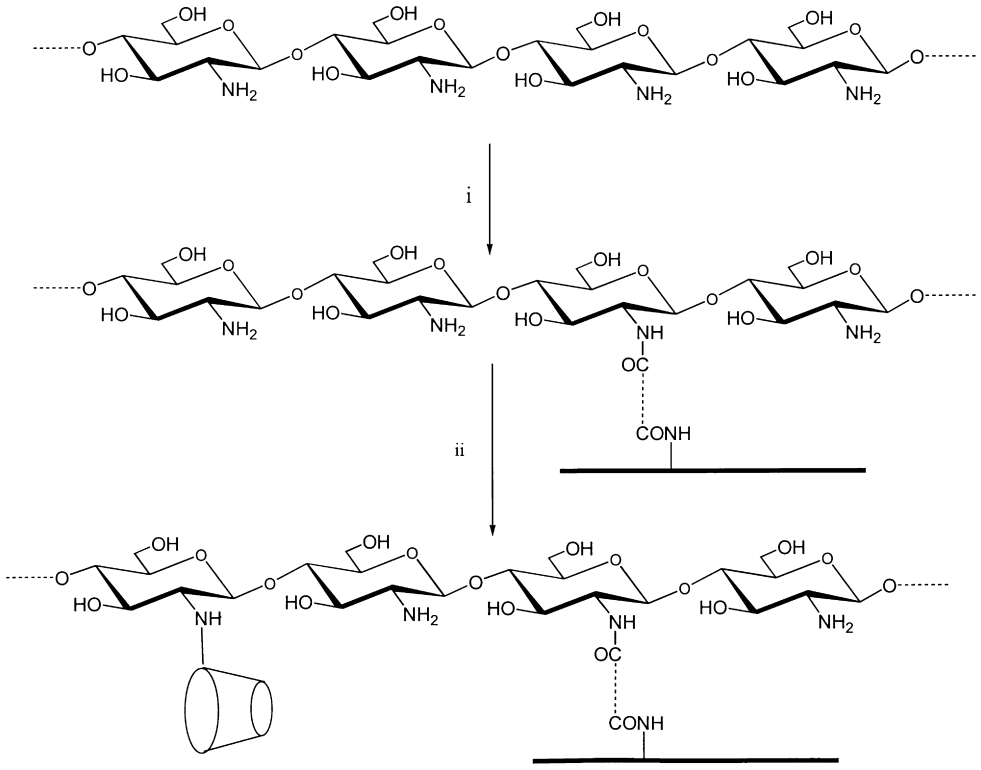

Fig. 1. Preparation of CD-linked chitosan beads. *Reagents and conditions:* (i) hexamethylene diisocyanate, DMF, r.t., overnight; (ii) formylmethyl-α-CD, $NaBH_3CN$, 0.2 M acetate buffer at pH 4.4, r.t., 4 d.

pressure from calcium hydride before use. Chitosan was prepared from Flonac C (Kyowa Technos Co. Ltd., Chiba, Japan), partially de-*N*-acetylated chitin with an average molecular weight 40 000. Flonac C was treated with 50% (w/v) aqueous NaOH that containing a small amount of sodium cyanoborohydride for 2 h at 120°C and washed with water; this procedure was repeated three times, washed with methanol, and air dried. α-CD was allylated with 2-bromopropene-lithium hydride-lithium iodide in dimethylsulfoxide (DMSO) (Hanessian, Benalil & Laferriere, 1995). Mono allyl-β-cyclodextrin **1** was isolated in 32% yield by column chromatography on Diaion HP-20 using aqueous methanol as an eluant and was subject to ozonolysis of the allyl group in 50% aqueous methanol to give 2-*O*-(formylmethyl)-α-cyclodextrin **2**. UV–visible spectrum was recorded with a Hitachi U-2000 A spectrometer. Surface structure of the α-CD-linked chitosan bead was observed with a scanning electron microscope (SEM) Hitachi S-2400.

2.2. Preparation of α-CD-linked chitosan beads

Chitosan (30 g) was dissolved in 3% aqueous acetic acid (515 ml) and diluted with water to 720 ml. The solution was filtered and added dropwise through a hypodermic needle (inside diameter; 0.7 mm), into a mixture of sodium hydroxide (45 g), ethanol (240 ml), and water (900 ml). The white beads were shaken in ethanol (2 l) overnight and the solvent was replaced by DMF (1 l) by decantation. Hexamethylene diisocyanate (2.5 ml) was added to the mixture, which was shaken at room temperature overnight. Elemental analysis of the chitosan beads was C, 44.35; H, 6.93, N, 8.68%. DMF was removed by decantation and the beads were suspended in 0.2 M acetate buffer at pH 4.4 (1 l) which contained sodium cyanoborohydride (1.42 g). 2-*O*-Formylmethyl-α-cyclodextrin **2** prepared from **1** (20 g) was added to the mixture and was shaken at room temperature for 4 d. The α-CD-linked chitosan beads thus obtained were washed several times with water. Elemental analysis of the α-CD-linked chitosan beads was C, 44.60; H, 6.57; N, 4.27%.

2.3. Column chromatographic separation

A suspension of the α-CD-linked chitosan beads (2.4 g) in water was poured into a glass column containing a small plug of cotton at the lower end, and allowed it to settle. A solution of *p*-nitrophenol (PNP) (1 mg) and 3-methyl-4-nitrophenol (MPNP) (1 mg) in 0.1 M phosphate buffer (pH 11; 10 ml) was applied on the column. The column was eluted with 0.1 M phosphate buffer (pH 11; 300 ml) and then 9:1, v/v methanol-buffer (100 ml). The flow rate was 1 ml/min and fractions were collected for each 10 ml. Chromatograms were obtained by the measurement of absorbance of the fractions at 401 nm.

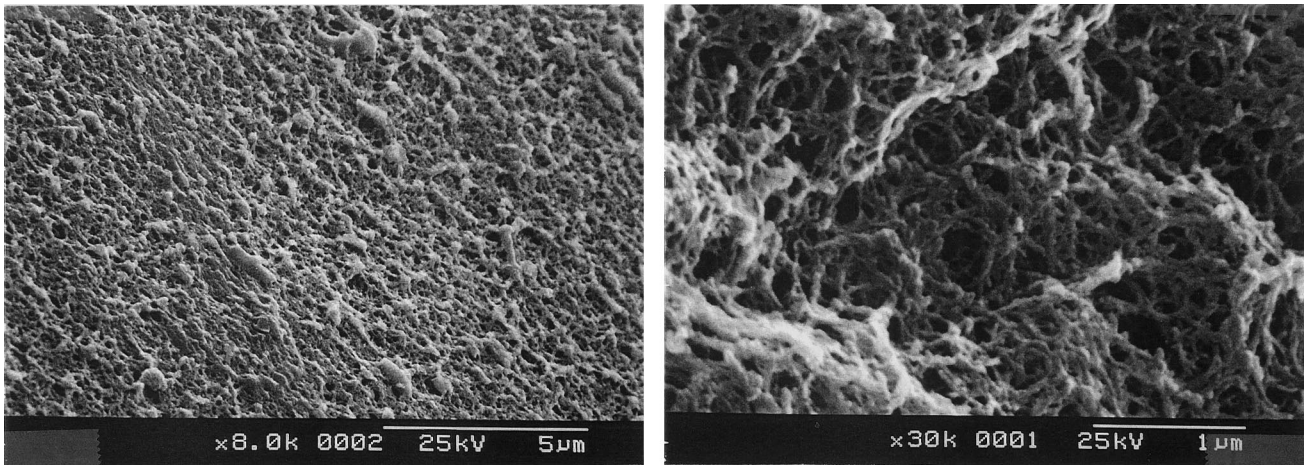

Fig. 2. SEM photograph of an α-CD-linked chitosan bead: (a) surface of the bead; and (b) cross-section.

2.4. Controlled release of p-nitrophenolate

α-CD-linked chitosan beads (4×10^{-2} g) were allowed to stand for 1 d in 1×10^{-2} M solution of PNP in 0.1 M phosphate buffer (pH 11; 3 ml) at room temperature. The beads were filtered and wiped with filter paper. The resulting beads were washed with aqueous methanol and the washings were diluted with the phosphate buffer. Total amount of PNP entrapped in the beads was estimated by measurement of absorbance of the solution at 401 nm.

0.1 M Phosphate buffer (pH 11; 3 ml) was added to the resulting beads and the mixture was allowed to stand at room temperature. Absorbance of the supernatant was measured at 401 nm every 15 min. The amount of PNP released into the supernatant was estimated form the absorbance and was summarized in Fig. 4.

The PNP entrapped α-CD-linked chitosan beads were allowed to stand for 30 min in 0.1 M phosphate buffer (pH 11; 3 ml). The beads were separated by filtration, and absorbance of the filtrate was measured at the wavelength of 401 nm. The separated beads were treated with the fresh phosphate buffer in the same way, and a series of treatments described earlier was repeated 30 times. The change of absorbance of the filtrates was summarized in Fig. 5.

3. Results and discussion

3.1. Preparation of α-CD-linked chitosan beads

As the introduction of CD residue into chitosan has been successfully attained in a homogeneous system through reductive amination strategy (Tanida *et al*., 1998; Tojima *et al*., 1998), we examined a similar procedure for the preparation of insoluble derivatives as shown in Fig. 1. The starting beads were synthesized by dropping an acetic acid solution of chitosan into aqueous sodium hydroxide through a hypodermic needle followed by cross-linking with 10% molecular equivalents of hexamethylene diisocyanate in DMF. Introduction of α-CD to the beads was carried out in a similar way to that described for the homogeneous system. Thus, the chitosan beads were shaken with **3α** in acetate buffer at pH 4 to form the Schiff's base, which was directly reduced by sodium cyanoborohydride to give α-CD-linked chitosan beads. On the basis of elemental analyses of the starting chitosan beads and CD-linked product, D.S. was calculated to be 16%. Although introduction CD residue in the heterogeneous system was lower than that in homogeneous system, desired α-CD-linked chitosan beads were also prepared in a one-pot manner.

The α-CD-linked chitosan beads thus obtained were first examined by SEM. A SEM image of the surface of the beads shown in Fig. 2 showed that the beads had highly porous structure. The cross-section of the beads also showed that the beads had micro pores with an average pore size of 0.5–1.0 μm. The diameter of the beads was 2.5–3.0 mm and its dried and hydrated weights were 0.28 g/ml and 1.66 g/ml, respectively. The water content of the beads was 83%.

3.2. Selective adsorption

Next our interest focused on the inclusion ability of the α-CD-linked chitosan beads, as our previous experiments using water-soluble CD-linked chitosan revealed that they possessed strong inclusion ability similarly to original CDs (Tanida *et al*., 1998; Tojima *et al*., 1998). As a first step, the inclusion property of the beads was examined by the use of PNP and MPNP as the model guest molecules. Although these two guest molecules have closely resembling structures, the methyl group of the latter strongly inhibits the formation of an inclusion complex due to steric hindrance of the methyl group. The dissociation constants of inclusion complexes of PNP and MPNP with original α-CD are

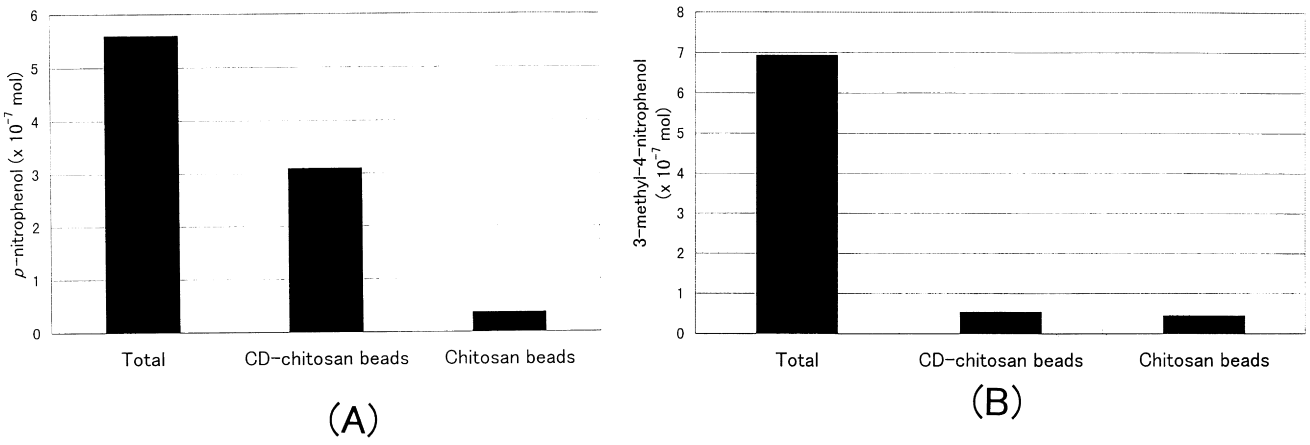

Fig. 3. Selectivity on the adsorption of: (a) MPNP; and (b) nitrophenol on chitosan beads and α-CD-linked chitosan beads.

reported to be 4.2×10^{-4} and 4.0×10^{-2} M, respectively (Bergeron, Channing, Gibeily & Pillor, 1977). The beads were dipped into 2×10^{-4} M solution of PNP and MPNP in phosphate buffer (pH 11) and the entrapped dyes were washed out with methanol. After evaporation of the washing, the residue was dissolved in the phosphate buffer and amount of the dyes were estimated by measuring absorption at 401 nm. As we expected, potent inclusion ability was observed on α-CD-linked chitosan beads toward PNP while MPNP was not adsorbed on the beads (Fig. 3). However, chitosan beads showed poor ability to form inclusion complex with both PNP and MPNP.

On the basis of the aforementioned experiments, we examined applicability of α-CD-linked chitosan beads to column chromatography. Recently the utilization of CD and their derivatives in high-performance liquid chromatography (HPLC) has achieved spectacular success. For example, CD-modified silica gels have been demonstrated to be exceptionally useful in resolving various kind of geometric and enantiomeric isomers (Armstrong, DeMond, Alak, Hinze, Riehl & Bui, 1985). In addition, CD-linked polymers have been used successfully as a stationary phase in affinity chromatography for separating such biopolymers as amylase (Weselake & Hill, 1982) and fibroblast growth factor (Sheng, Forkman, Weisz, Joullie & Ewing, 1990). These precedents prompted us to use our highly porous α-CD-linked chitosan beads for affinity chromatography. A mixture of PNP and MPNP was subjected to column chromatography on α-CD-linked chitosan beads; the chromatogram recorded by absorbance at 401 nm is shown in Fig. 4. Eluting from the column with phosphate buffer at pH 11.0, MPNP showed less affinity to α-CD and a band of PNP stayed on the top of the column. PNP was eluted after the mobile phase was changed to a less hydrophobic solution, such as aqueous methanol. These results suggest that the α-CD-linked chitosan beads may be useful as selective adsorption systems for organic compounds through differential molecular complexation.

3.3. Controlled release

As PNP binds strongly to α-CD-linked chitosan beads, our interest turned toward examining the controlled release of organic molecules that are entrapped within α-CD-linked chitosan beads. After dipping the beads in a PNP solution, we allowed them to stand in a phosphate buffer at pH 9.0 and PNP released into the supernatant was monitored by UV–visible spectroscopy. These results shown in Fig. 5 suggested that PNP entrapped with α-CD-linked chitosan beads was released slowly into the buffer and that equilibrium was reached after 15 h.

Next, we allowed the PNP entrapped α-CD-linked chitosan beads to stand in a phosphate buffer at pH 9.0; the buffer solution was replaced every 30 min. The time course of the change in absorbance of the supernatant was recorded at 401 nm (Fig. 6). In contrast to these data, chitosan beads, which have little ability to form inclusion complexes, released almost all of the PNP within several hours. Our spectroscopic data indicated that PNP may become

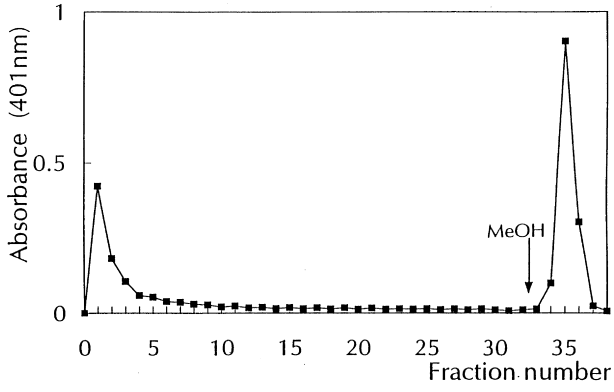

Fig. 4. Chromatogram of nitrophenols on a column of α-CD-linked chitosan beads: PNP and MPNP were subjected to the column and the column was eluted with 0.1 M phosphate buffer at pH 11.0 and then 9:1 v/v methanol-buffer (flow rate: 1 ml/min, fractions: 10 ml, detection: 401 nm).

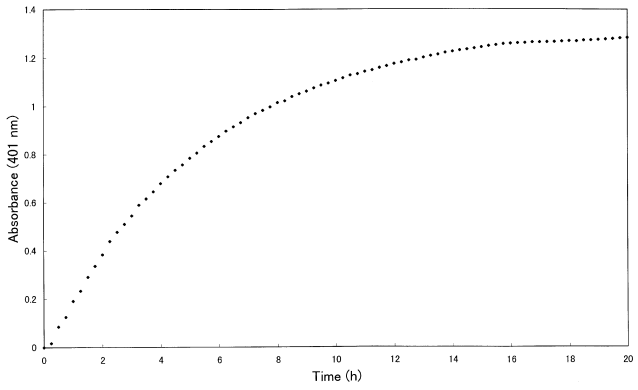

Fig. 5. Release of PNP from α-CD-linked chitosan beads using 0.1 M phosphate buffer at pH 11.0 and absorbance of the supernatant was recorded at 401 nm.

entrapped within α-CD-linked chitosan beads and be released slowly into the buffer and that more than 40% of the PNP may remain in the beads after 30 changes of buffer. These experiments suggest that CD-linked chitosan may serve as an adsorbent for controlled release of drugs and aromatics.

4. Conclusion

We described the successful synthesis of highly porous α-linked chitosan through the formation of the Schiff's base with the corresponding 2-formylmethyl derivatives and subsequent reduction with $NaBH_3CN$. The ability of these α-CD-linked chitosan beads to form inclusion complexes with PNP was validated and some preliminary experiments were performed in which the beads were used as a reversed-phase adsorption system, as an adsorbent in controlled release applications.

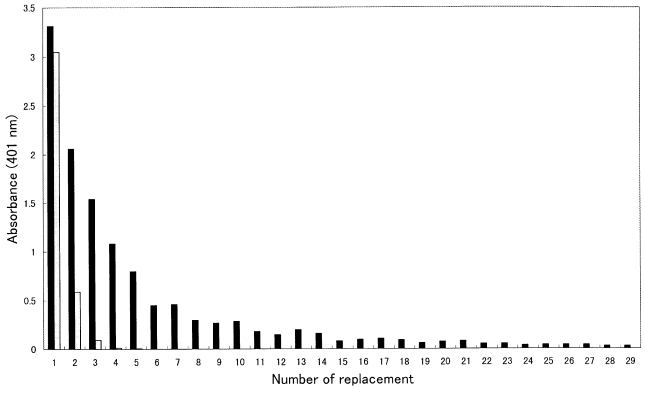

Fig. 6. Release of PNP from α-CD-linked chitosan beads (■) and form chitosan beads (□) using 0.1 M phosphate buffer at pH 11.0. The buffer solution was changed every 30 min and absorbance was recorded at 401 nm.

This novel polymeric host compound with its carbohydrate skeleton shows promise of being useful in the production of biologically and/or environmentally friendly materials in a wide variety of fields. Further studies along this line are now in progress.

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References

Armstrong, D. W., DeMond, W., Alak, A., Hinze, W. L., Riehl, T. E., & Bui, K. H. (1985). Liquid chromatographic separation of diastereomers and structural isomers on cyclodextrin-bonded phases. *Anal. Chem., 57*, 234–237.

Bergeron, R. J., Channing, M. A., Gibeily, G. J., & Pillor, D. M. (1977). Disposition requirements for binding in aqueous solution of substrates in the cyclohexaamylose cavity. *J. Am. Chem. Soc., 99*, 5146–5151.

Furusaki, E., Ueno, Y., Sakairi, N., Nishi, N., & Tokura, S. (1996). Facile preparation and inclusion ability of a chitosan derivative bearing carboxymethyl-β-cyclodextrin. *Carbohydro. Polym., 29*, 29–34.

Hanessian, S., Benalil, A., & Laferriere, C. (1995). The synthesis of functionalized cyclodextrins as scaffolds and templates for molecular diversity, catalysis, and inclusion phenomena. *J. Org. Chem., 60*, 4786–4797.

Horton, D., & Lineback, D. R. (1965). *N*-deacetylation, chitosan from chitin. In R. L. Whistler (Ed.), (pp. 403). *Methods in carbohydrate chemistry*, 5. New York: Academic Press.

Iwasaki, K. -I., Inoue, M., & Matsubara, Y. (1998). Continuous hydrolysis of pectate by immobilized endo-polygalacturonase in a continuously stirred tank reactor. *Biosci. Biotech. Biochem., 62*, 262–267.

Kawamura, Y., Yoshida, H., Asai, S., & Tanibe, H. (1998). Elution of Hg (II) adsorbed on highly porous polyaminated chitosan. *J. Chem. Engng. Jpn., 31*, 115–118.

Malot, C., Tobin, M., & Guibal, E. (1998). Metal-anion sorption by chitosan beads: equilibrium and kinetic. *Ind. Engng. Chem. Res., 37*, 1454–1463.

Muzzarelli, R. A. A., & Rocchetti, R. (1974). The determination of vanadium is sea water by hot graphite atomic absorption spectrometry on chitosan after separation from salt. *Anal. Chim. Acta. , 70*, 283–289.

Muzzarelli, R. A. A., Jeuniaux, C., & Goody, G. W. (1986). Chitin in nature and technology. New York: Plenum Press.

Sheng, Y., Forkman, J., Weisz, P. B., Joullie, M. M., & Ewing, W. R. (1990). Affinity of fibroblast growth factors for β-cyclodextrin tetradecasulfate. *Anal. Biochem., 185*, 108–111.

Stossel, P., & Leuba, J. L. (1984). Effect of chitosan, chitin and some aminosugars on growth of various soil-borne phytopathogenic fungi. *Phytopath. Z., 111*, 82–90.

Szejtli, J. (1982). Cyclodextrin and their inclusion complexes. Budapest: Academi Kiado.

Tanida, S., Tojima, T., Han, S. -M., Nishi, N., Tokura, S., Sakairi, N.,

Seino, H., & Hamada, K. (1998). Novel synthesis of a water-soluble cyclodextrin-polymer having a chitosan skeleton. *Polymer, 39*, 5261–5263.

Tojima, T., Katsura, H., Han, S. -M., Tanida, F., Nishi, N., Tokura, S., & Sakairi, N. (1998). Preparation of an α-cyclodextrin-linked chitosan derivative via reductive amination strategy. *J. Polym. Sci. Part A. Polym. Chem., 36*, 1965–1968.

Weselake, R. J., & Hill, R. D. (1982). Cycloheptaamylose as an affinity ligand of cereal alpha amylase: characteristics and a possible mechanism of the interaction. *Carbohydr. Res., 108*, 153–161.