Carbohydrate Polymers **20** (1993) 139–143

Site specific binding of calcium ions to anionic chitin derivatives

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(Received 23 January 1991; revised version received 21 June 1991; accepted 14 July 1991)

6-*O*-carboxymethylated chitin (6-*O*-CM-chitin) and *N*-succinylated chitosan (Suc-chitosan) are shown to have a high affinity for calcium ions on a 10^5 mol^{-1} dm^3 order of adsorption constants. It is suggested that 6-*O*-CM-chitin especially forms a tight complex with calcium ions, which is restricted even in acidic media. The Scatchard plot of 6-*O*-CM-chitin shows linear behavior, suggesting that each adsorption site is independent for calcium adsorption, while that of 3,6-*O*-CM-chitin indicates negative cooperativity suggesting a slight interaction among adsorption sites. Thus 6-*O*-CM-chitin may form more stable complexes with calcium ions than 3,6-*O*-CM-chitin. These results revealed that a secondary hydroxyl group of an *N*-acetylglucosamine (GlcNAc) residue at the C-3 position contributed significantly to calcium chelation for 6-*O*-CM-chitin.

INTRODUCTION

It is suggested that chitin, a mucopolysaccharide supporting the cuticle of crustaceans, does not adsorb metal ions owing to its inert surface (Tokura *et al.*, 1983*a*). Chitosan, a deacetylated cationic product of chitin, does adsorb various kinds of metal ions in addition to having remarkable affinities for heavy metal ions (Muzzarelli, 1973). Chitin derivatives which have anionic groups were reported to act as ion exchangers and adsorbents for organic compounds (Nishi *et al.*, 1987; Nishimura *et al.*, 1987; Muzzarelli *et al.*, 1989). An *N*-carboxymethyl chitosan in particular shows much higher chelating ability for heavy metal ions than chitosan, but it does not bind calcium ions at all (Delben & Muzzarelli, 1989). It was suggested that 6-*O*-CM-chitin prepared from Queen Crab shells retained the highest affinity for calcium ions among divalent metal ions, and formed a specific chelating complex with calcium ions by geometrical support with acetamide and hydroxyl groups of GlcNAc residue in addition to carboxyl groups (Uraki & Tokura, 1988). However, the adsorption constant and the mechanism of dissociation of calcium ion complexes with 6-*O*-CM-chitin have not been elucidated. Moreover, the relationship between the adsorption properties of chitin derivatives and the substitution site on GlcNAc residues is not yet clear.

In the present study, various anionic chitin derivatives substituted by carboxymethyl groups at the C-6 and C-3 hydroxyl sites of the GlcNAc residue were prepared to investigate the chelation mechanism of calcium ions. A succinylated chitosan (Suc-chitosan) which was substituted by a carboxyethyl group at the C-2 amino group was also prepared, to elucidate the geometrical relationship among functional groups.

Crab shell is believed to consist of α-chitin and calcium carbonate together with proteins (Carlstrom, 1957; Minke & Blackwell, 1978). If 6-*O*-CM-chitin adsorbs calcium ions by a similar mechanism to crab shell, the carboxyl group of 6-*O*-CM-chitin might be considered to be a side chain of the supporting protein. The adsorption abilities of these chitin derivatives for metal ions may relate closely to the natural occurrence of chitin. Two types of crystalline structures of chitins were utilized to investigate the influence of the flexibility of chitin molecules, accessibility to chemical modification and metal adsorption. α-Chitin is less flexible than β-chitin which is prepared from squid bones (Gardner & Blackwell, 1975), since the extent of hydrogen bonding of β-chitin is less than that of α-chitin. In this paper, the relationship between metal adsorption properties of chitin derivatives and chitin crystalline structures is discussed, and a model for calcium adsorption of 6-*O*-CM-chitin is proposed.

MATERIALS AND METHODS

Materials

α-Chitin was prepared from Queen Crab shell according to the method of Hackman (1954) and powdered to 45–60 mesh before use. β-Chitin was kindly supplied by Nissui Co. Ltd, Tokyo, Japan.

6-*O*-CM-chitins (Degree of Substitutions (D.S.) less than 1·0) were prepared by a method reported previously (Tokura *et al*., 1983*b*) from α- or β-chitin.

3,6-*O*-CM-chitin was prepared from 6-*O*-CM-chitin as reported previously (Nishimura *et al*., 1986). Briefly, 1 g of 6-*O*-CM-chitin (D.S. 0·74) was dissolved in 4 ml of 50% (w/w) NaOH aqueous solution and kept at −20°C overnight. It was thawed in 5 ml of *i*-propanol at room temperature. The alkaline-CM-chitin was neutralized by the stepwise addition of monochloroacetic acid (4 g) under vigorous stirring, to give crude 3,6-*O*-CM-chitin. The crude product was dissolved in 45 ml of distilled water and saturated $BaCl_2$ solution was added to precipitate the highly substituted 3,6-*O*-CM-chitin. After the precipitate was collected by centrifugation, it was dissolved in aqueous EDTA solution and dialyzed against deionized water repeatedly to remove the metal ions. The supernatant left after centrifugation was dialyzed against deionized water to collect the poorly substituted 3,6-*O*-CM-chitin and, following the EDTA treatment, was dialyzed against deionized water. Salt free solutions were concentrated, and the product was obtained by acetone precipitation or lyophilization.

Suc-chitosan: Succinic anhydride (15 times equivalent for amino groups) was added stepwisely to 20 g of chitosan suspension in 500 ml of 1·0 M Na_2CO_3–$NaHCO_3$ buffer (pH 8·5) at room temperature. The pH range of the reaction mixture was maintained between 7 and 9 during the reaction by the addition of aqueous NaOH solution. After the precipitate was removed by centrifugation, the crude product was dialyzed against deionized water thoroughly, followed by acetone precipitation or lyophilization.

Characterization and estimation of degree of substitution were carried out by IR, NMR spectra and potentiometric titration.

The degree of substitutions (D.S.) of 6-*O*-CM-chitin from α, β-chitin and 3,6-*O*-CM-chitin from α, β-chitin and Suc-chitosan were 0·58, 0·74, 1·4, 1·5 and 0·86, respectively.

Adsorption of metal ions to various chitin derivatives

Ten ml of 0·2% aqueous solution of chitin derivative was mixed with various concentrations of aqueous solutions of metal ions and shaken for 24 h (final concentrations of chitin derivatives = 0·1%). Following the equilibrium dialysis of the solution against deionized water at room temperature for 48 h, the metal ion concentrations of inner and outer solutions were measured by ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectroscopy, SPS1100, Seiko Electric Co. Ltd, Tokyo, Japan). The concentrations were estimated from the working curves by applying commercial standard solutions. Since the spray volume of polymer solution from the nebulizer was deviated from that of the standard solutions owing to the viscosity of mixed polymer–metal solution, the deviation was corrected by the calibration curves of intact mixed solution.

Amount of calcium ion released from 6-*O*-CM-chitin by pH shift

Batch method was applied to measure the amount of calcium ions adsorbed for CM-chitin. 0·3 g of poorly substituted 6-*O*-CM-chitin powder was immersed in 20 ml of 0·05 M $CaCl_2$ aqueous solution, agitated for 24 h at room temperature to form a complex and then washed thoroughly with water to remove free calcium ions. The amount of Ca^{2+} released from the complex was estimated from the supernatant concentration by mixing the CM-chitin-Ca complex with various concentrations of aqueous HCl solution and shaking for 24 h.

Selective adsorption of 6-*O*-CM-chitin in Ca–Mg mixture system

Adsorption capacities of Ca^{2+} or Mg^{2+} to water-insoluble 6-*O*-CM-chitin (poorly substituted) were measured by batch method in a buffer solution (0·05 M Tris-HCl, pH 7·4) containing Ca^{2+} and Mg^{2+} in several ionic strengths.

The total metal ion concentration in the supernatant was titrated by EDTA using BT (Eriochrome Black T) at pH 10 (Nishimura & Nakamura, 1967). After precipitation of $Mg(OH)_2$ at a pH higher than 12, the calcium ions were titrated using *N*, *N* (2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid) (Patton, 1956).

RESULTS AND DISCUSSION

Adsorption of metal ions to water-soluble chitin derivatives

Ten mM of metal ions were applied to estimate the adsorption capacities and constants of chitin derivatives from Scatchard plots. Since the primary relations of Scatchard plots (Fig. 1) for 6-*O*-CM-chitins prepared from α-, β-chitin and Suc-chitosan were shown on the adsorption of calcium ions, this adsorption seems to proceed independently of the adjacent adsorption sites.

As adsorption constants for the adsorption of calcium ions with 6-*O*-CM-chitins and Suc-chitosan

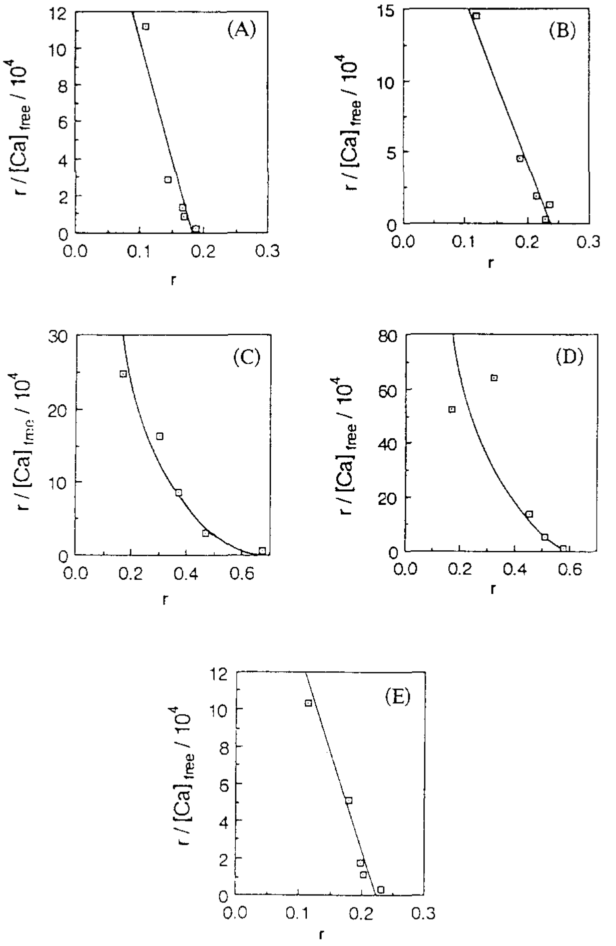


Fig. 1. Scatchard plots of Ca^{2+} for anionic chitin derivatives. (A), for 6-*O*-CM-chitin from *α*-chitin (D.S. 0·58); (B), 6-*O*-CM-chitin from *β*-chitin (D.S. 0·74); (C), 3,6-*O*-CM-chitin from *α*-chitin (D.S. 1·4); (D), 3,6-*O*-CM-chitin from *β*-chitin (D.S. 1·5); (E), Suc-chitosan (D.S. 0·86).

Table 1. Adsorption properties of Ca^{2+} to anionic chitin derivatives

Chitin derivative	*K*a[a] (/mol dm^{-3})	n[b]
6-*O*-CM-chitin from *α*-chitin	$5{\cdot}9 \times 10^5$	0·2
6-*O*-CM-chitin from *β*-chitin	$4{\cdot}1 \times 10^5$	0·3
Suc-chitosan	$9{\cdot}2 \times 10^5$	0·2

[a] *K*a, adsorption constant.
[b] n, number of adsorption sites in a glucosamine derivative residue.

were more than 10^5 mol^{-1} dm^3 (as shown in Table 1), the acetamide group at the C-2 position in GlcNAc residues seemed to have a high affinity for calcium ions. However, the plots for other metal ions (Fig. 2) and those of 3,6-*O*-CM-chitin for all ions were shown to deviate from straight lines, suggesting negative cooperativity on the adsorption of metal ions. This may suggest that the approach of metal ions to the adsorption site is influenced by the adjacent carboxyl adsorption site. As the disappearance of specificity of 3,6-*O*-CM-chitin for calcium ions seems to result from substitution of the secondary hydroxyl group at the C-3 position of the GlcNAc residue, the C-3 hydroxyl group may also contribute significantly to the tight complex formation with calcium ions.

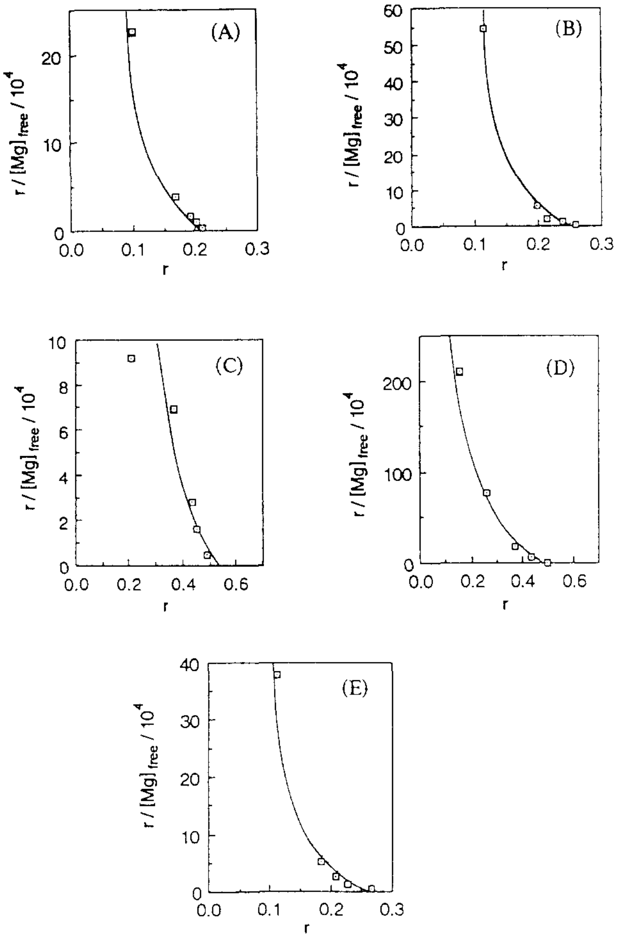
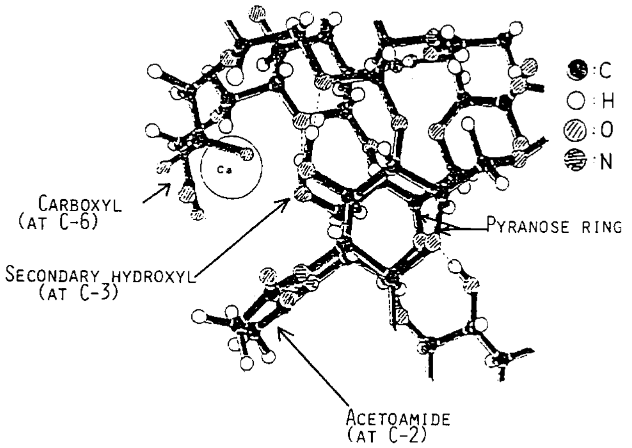


Fig. 2. Scatchard plots of Mg^{2+} for anionic chitin derivatives. (A), for 6-*O*-CM-chitin from *α*-chitin (D.S. 0·58); (B), 6-*O*-CM-chitin from *β*-chitin (D.S. 0·74); (C), 3,6-*O*-CM-chitin from *α*-chitin (D.S. 1·4); (D), 3,6-*O*-CM-chitin from *β*-chitin (D.S. 1·5); (E), Suc-chitosan (D.S. 0·86).

As the amount of calcium ion adsorbed to 6-*O*-CM-chitin was not enough to neutralize carboxymethyl groups, it was assumed that calcium chelation was achieved by a part of the carboxyl groups and other functional groups in a local domain. These domains may be distributed along the polymer chain without interaction between adsorption sites. These phenomena confirm a previous report, in which a specific adsorption site of 6-*O*-CM-chitin for calcium ions was suggested to be formed by the geometrical arrangement of a few functional groups such as amide and hydroxyl groups in addition to carboxyl groups (Uraki & Tokura, 1988).

Adsorption properties of calcium ions to chitin derivatives appears to be independent of the crystalline



Scheme 1. Speculative model of Ca^{2+}-6-*O*-CM-chitin complex.

structure of chitin, because of similar profiles shown by derivatives of α- and β-chitins. The adsorption sites for calcium ions may be restricted among a few residues, independent of the flexibility of the chitin molecule. A speculative model of Ca^{2+}-6-*O*-CM-chitin complex is proposed on the basis of these results in scheme 1.

Alternatively, negative cooperativity may be explained by rapid reduction of the surface charges of CM-chitin molecules, owing to neutralization with the adsorption of metal ions. Since the higher substitution of the carboxymethyl group caused the larger adsorption capacity, only carboxyl groups may contribute to the non-specific adsorption for metal ions. Therefore adsorption may be merely controlled by electrostatic interaction.

Specificity of calcium ion to 6-*O*-CM-chitin

Metal ions adsorbed to water insoluble 6-*O*-CM-chitin were only thoroughly released by pH shifts, except calcium ions which required chelating agents such as EDTA or EGTA for the complete decalcination (see Fig. 3). This suggests that 6-*O*-CM-chitin adsorbs calcium ions with a tight complex formation.

Adsorptions of calcium and magnesium ions to 6-*O*-CM-chitin were measured by the batch method in Ca^{2+}-Mg^{2+} mixed solutions as shown in Table 2. The distribution coefficient (relative capacity for calcium ions against that for magnesium ions) gives high values (greater than 1·0) under various ionic strengths; the highest coefficient was 4·3 at an ionic strength of 0·5. It was suggested again that 6-*O*-CM-chitin had a selective adsorption ability for calcium ions. A similar specific ion exchange phenomenon was also reported for calcium ions (Tokura, 1983*a*).

These phenomena might be explained as follows: Under the high magnesium ion concentration, the 6-*O*-CM-chitin-Mg^{2+} complex is unstable, probably due to the repulsion between the magnesium ions as shown by the negative cooperativity. Alternatively, calcium ions form a stable complex at specific adsorption sites on the 6-*O*-CM-chitin molecule.

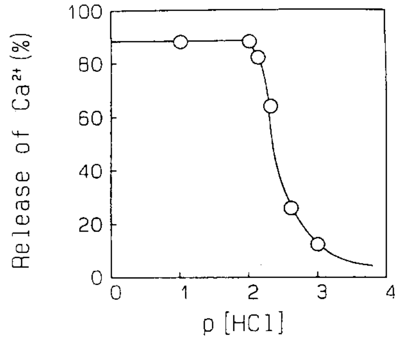


Fig. 3. Release of Ca^{2+} from 6-*O*-CM-chitin.

Table 2. Selective adsorption of Ca^{2+} to 6-*O*-CM-chitin at various ionic strengths

Ion	Ionic strength		
	0·35 (meq/g)	0·50 (meq/g)	0·65 (meq/g)
Total	0·56	0·48	0·44
Ca^{2+}	0·31	0·39	0·28
Mg^{2+}	0·25	0·09	0·16
D.C.[a]	1·2	4·3	1·8

[a] D.C., Distribution coefficient (adsorption capacity of Ca^{2+}/adsorption capacity of Mg^{2+}).

Relationship of metal ion complex with crystalline structure

6-*O*-CM-chitin from β-chitin and 3,6-*O*-CM-chitin from all sources were found to form precipitates following gel formation, even at low concentrations of transition metal ions. These phenomena suggest that transition metal ions bind with carboxyl groups by time dependent mechanisms at slow rates, owing to the gradual destruction of the rigid conformation of α-chitin and the flexibility of β-chitin, so that loose gel was formed by a small number of cross-linkages during the first stage of metal ion adsorption. The matrix may become tighter due to the increment of the number of cross-linkages by metal ions. Therefore flexibility of crystalline structure seems to regulate the profiles of metal ion complexes.

ACKNOWLEDGEMENT

This work was supported by a grant from the Ministry of Education of Japan (02555183).

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