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N-Carboxyacyl-*N*-acylchitosan Gels as Novel Media for Gel Chromatography

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SUMMARY

Three partially substituted N-*carboxyacyl and six* N-*carboxyacyl*-N-*acyl derivatives of chitosan were prepared and their practical use as media for gel chromatography was examined.* N-*(3'-Carboxy-2'-propenoyl)*-N-*stearoyl-chitosan gel was a relatively good medium for gel chromatography (solvent, water), and had a wide fractionation range (MW* $= 2 \times 10^4$–6×10^5*). Its chromatographic properties were compared with those of* N-*methylene-chitosan gel (solvent, 0·5 M NaCl).*

INTRODUCTION

Chitin (a (1→4)-linked 2-acetamido-2-deoxy-β-D-glucan) and chitosan (its *N*-deacetylated derivative) are naturally abundant polysaccharides. They are structurally related to cellulose, in which hydroxyl groups at C-2 are replaced with acetamido and amino groups in the molecules. Cellulose is commercially used as a medium in ion-exchange and affinity chromatography. The chemical modification of chitin and chitosan is important for developing new properties which may be of value in the scientific and technological fields.

Several reports have dealt with the use of chitin and chitosan as media for anion-exchange (Noguchi *et al.*, 1965), thin layer (Takeda, 1978), chelation and ligand-exchange (Muzzarelli, 1978) and affinity (Bloch & Burger, 1974; Baba *et al.*, 1980) chromatographies. Recently we isolated a novel polysaccharide gel produced by the chemical *N*-substitution of chitosan with a series of carboxylic anhydrides and

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aldehydes in aqueous acetic acid–methanol (Hirano *et al.*, 1980), and we reported that *N*-methylenechitosan (MC) gel was usable as a medium for gel chromatography (Hirano *et al.*, 1979).

We are now able to report on the preparation of novel *N*-carboxyacyl-*N*-acyl derivatives of chitosan and their practical properties in comparison with those of *N*-methylenechitosan (MC) gel as media for gel chromatography.

MATERIALS AND METHODS

Chitosan was kindly supplied by Kyōwa Yushi Co. Ltd, Chiba, Japan and was retreated with 40% NaOH at 100°C for 5 h. The product ($[\alpha]_D^{14} = -6°$, concentration = 0·5, 2% acetic acid) effectively contained no acetyl groups when examined by ^{1}H-NMR (D_2O: DCO_2D, 8 : 2, v/v) and by infrared spectra (KBr), and its elemental analyses were in agreement with theoretical values within 0·3% error.

The other general methods have been cited previously (Hirano *et al.*, 1979).

N-Carboxyacyl and *N*-carboxyacyl-*N*-acyl derivatives of chitosan

An intermolecular carboxylic anhydride (0·4 mol of GlcN) was added with stirring, to a chitosan (3·22 g) solution in 2% aqueous acetic acid–methanol (400 ml) and the mixture was allowed to stand at room temperature for 18 h. A portion of the reaction products was used to analyse the degree of substitution (DS) by *N*-acyl groups. Acetic or stearic anhydride (1·6 mol of GlcN) was added to the remainder, and the mixture allowed to stand at room temperature for 18 h. When stearic anhydride was used, the mixture was heated in a boiling water bath for 10 min. The gel was ground and excess acetic and dicarboxylic acids were removed by washing with distilled water and ethanol. Excess stearic acid was removed by successive washing with chloroform–methanol (2 : 1, v/v), ethanol and ether. The products were air-dried. Excessively fine or granular portions were removed. The xerogel (50–500 mesh) thus prepared was used for the present study.

Potentiometric titration

The xerogel was treated with 0·1 M HCl solution and washed with distilled water to afford a regenerated gel (—COOH and —NH_3Cl form). The gel was suspended in 0·5 M NaCl solution (150 ml), and the suspension titrated with 0·5 M NaOH solution with stirring. Changes in pH were followed with a pH meter.

Ion-exchange capacity

The gel (—COOH and —NH_3Cl form) prepared above was stirred in 0·1 M NaOH solution (200 ml) for 18 h. The amount of NaOH used was determined by titration with 0·1 M HCl solution, and the ion-exchange capacity of the xerogels was calculated on the basis of available —COOH and —NH_2 groups.

Gel chromatography

The xerogel was equilibrated in distilled water or 0·5 M NaCl solution at room temperature and loaded into a glass column. Gel chromatographic properties were analysed with several standard compounds: blue dextran 2000, dextran (MW = 176 000, Pharmacia Fine Chemicals), dextran T70 (MW = 75 800, Pharmacia Fine Chemicals), dextran (MW = 61 500), dextran T40 (MW = 40 100, Pharmacia Fine Chemicals), dextran (MW = 37 000), amylose (MW = 21 000), dextran T10 (MW = 10 500, Pharmacia Fine Chemicals), amylose (MW = 2900), maltose (MW = 342) and several monosaccharides. The screening tests were carried out for aldohexoses by the anthrone method (Trevelyan & Harrison, 1952) and for proteins by observing absorption at 280 nm.

The total gel bed volume (V_t) and water regain (W_r) values were determined by conventional methods.

RESULTS

Three novel, partially substituted *N*-carboxyacyl derivatives (DS = 0·30–0·39) were prepared from chitosan by *N*-acylation with 0·4 mol of

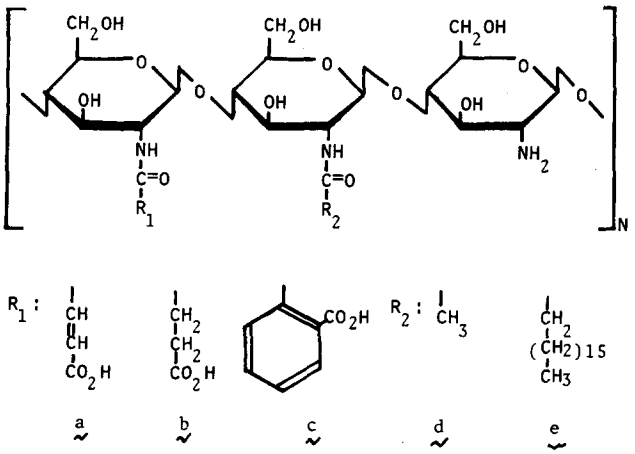

Fig. 1. A proposed structure of *N*-carboxyacyl-*N*-acyl derivatives of chitosan.

Compound	R_1	R_2
1	**a**	**d**
2	**a**	**e**
3	**c**	**d**
4	**c**	**e**
5	**b**	**d**
6	**b**	**e**

The distribution of each group is unknown.

GlcN, respectively, malonic, phthalic and succinic anhydrides in 2% aqueous acetic acid–methanol (Hirano & Moriyasu, 1981). They were further *N*-acylated with acetic or stearic anhydride (1·6 mol of GlcN) to produce six novel *N*-carboxyacyl-*N*-acyl derivatives (Fig. 1): *N*-3′-carboxy-2′-propenoyl-*N*-acetyl(**1**), *N*-3′-carboxy-2′-propenoyl-*N*-stearoyl(**2**), *N*-2′-carboxybenzoyl-*N*-acetyl (**3**), *N*-2′-carboxybenzoyl-*N*-stearoyl (**4**), *N*-3′-carboxypropionyl-*N*-acetyl (**5**) and *N*-3′-carboxypropionyl-*N*-stearoyl (**6**) derivatives. *N*-Carboxyacyl (DS > 0·4) derivatives and even their *N*-peracetyl derivatives were soluble in 0·1 M NaOH solution. In order to decrease the solubility, the DS values for *N*-carboxylacyl

TABLE 1

Analytical Data for Some *N*-Carboxyacyl- and *N*-Carboxyacyl-*N*-acyl Derivatives of Chitosan

Chitosan derivatives	*Yield*[a] *(%)*	*Formula*[b]	*Calculated (%)*			*Found (%)*		
			C	*H*	*N*	*C*	*H*	*N*
N-3′-Carboxy-2′-propenoyl	n.d.[c]	$[X(C_4H_3O_3)_{0\cdot39}(H)_{0\cdot61}1\cdot55H_2O]_n$	39·94	6·60	6·16	39·80	6·73	6·12
N-2′-Carboxybenzoyl	n.d.	$[X(C_8H_5O_3)_{0\cdot30}(H)_{0\cdot70}2\cdot18H_2O]_n$	41·20	6·82	5·72	41·32	6·57	5·69
N-2′-Carboxypropionyl	n.d.	$[X(C_4H_5O_3)_{0\cdot36}(H)_{0\cdot64}1\cdot63H_2O]_n$	39·44	6·98	6·18	39·16	6·90	6·08
1	82	$[X(C_4H_3O_3)_{0\cdot22}(C_2H_3O)_{0\cdot60}0\cdot62H_2O]_n$	44·33	6·31	6·40	44·41	6·30	6·19
2	n.d.	$[X(C_4H_3O_3)_{0.20}(C_{18}H_{35}O)_{0\cdot40}(H)_{0\cdot40}0\cdot38H_2O]_n$	57·11	8·82	4·76	57·29	8·63	4·76
3	95	$[X(C_8H_5O_3)_{0\cdot29}(C_2H_3O)_{0\cdot70}(H)_{0\cdot01}0\cdot43H_2O]_n$	48·39	6·02	5·81	48·49	5·90	5·69
4	63	$[X(C_8H_5O_3)_{0\cdot27}(C_{18}H_{25}O)_{0\cdot39}(H)_{0\cdot34}1\cdot03H_2O]_n$	56·34	8·53	4·33	56·25	8·42	4·39
5	97	$[X(C_4H_5O_3)_{0\cdot20}(C_2H_3O)_{0\cdot64}(H)_{0\cdot16}0\cdot44H_2O]_n$	44·93	6·51	6·49	44·90	6·39	6·49
6	60	$[X(C_4H_5O_3)_{0\cdot20}(C_{18}H_{35}O)_{0\cdot26}(H)_{0\cdot54}0\cdot36H_2O]_n$	53·66	8·38	5·45	53·50	8·33	5·48

[a] On the basis of the corresponding formula.

[b] X connotes a 2-amino-2-deoxy-D-glucose residue: $C_6H_{10}NO_4$.

[c] n.d. = not determined.

groups were limited to <0.4, and a hydrophobic *N*-stearoyl group was introduced. The xerogels (H^+ form) displayed infrared absorption (KBr) at (in cm^{-1}) 3500–3200 (OH, NH) 1710 (CO_2H), 1650 and 1550 (C=O and NH of *N*-acyl), 1150–1000 (C—O—C) and 890 (β-D). The infrared absorption at 1710 cm^{-1} was shifted to 1650 cm^{-1} (CO_2^-) in the Na^+ form. An additional absorption at 740 cm^{-1} (*o*-phenyl) appeared in the infrared spectra of **3** and **4**. Table 1 shows the analytical data of these xerogels.

As shown in Table 2, **5** and **6** have appropriate V_t and W_r values and are usable as gel media for chromatography, but **1–4** are not usable because of their small V_t and W_r values. The gel media of **5** and **6** were colorless, semitransparent and heat-irreversible.

TABLE 2
Some Properties of *N*-Carboxyacyl-*N*-acylchitosan Gels as Media for Gel Chromatography

Chitosan derivatives	*DS*[a]	*Total bed volume (ml g⁻¹ of the xerogel)*	*Water regain (ml g⁻¹ of the xerogel)*	*Particle size (μm)*	*Ion-exchange capacity*[b] *(meq g⁻¹ of the xerogel)*	
					Calc.[c]	*Found*
1	0·2:0·6	~1	~1	50–300	1·83	0·7
2	0·2:0·4	~1	~1	50–500	n.d.[d]	
3	0·3:0·7	~1	~1	50–500	1·24	0·7
4	0·3:0·4	5–10	~2	50–500	1·88	1·5
5	0·2:0·6	80–90	40–50	50–500	1·67	0·8
6	0·2:0·3	50–70	30–40	50–500	2·88	1·1
N-Methylene[e]	1·0	30–35	20–30	50–350	–	–

[a] Degree of substitution for *N*-carboxyacyl and *N*-acyl groups; the values are based on the elemental analyses, as shown in the formula (Table 1).
[b] See text for the experimental detail.
[c] Based on the elemental analyses for both carboxylic and amino groups present in the gel.
[d] Not determined.
[e] Hirano *et al.* (1979).

Figures 2 and 3 show the relationship between molecular weight (log) and elution volume (V_e) found on a column (1·7 × 138 cm) of **6** (Na^+ form) eluting with distilled water, and on a column (1·4 × 201 cm) of MC eluting with 0·5 M NaCl, respectively. The fractionation range (molecular weight) of **6** was 2×10^4–6×10^5 and that of MC was $2{\cdot}9 \times 10^3$–6×10^4, which is a more detailed result than in our previous report (Hirano *et al.*, 1979). The recovery yields of the neutral saccharides used as the molecular weight standards were in the range 80–100% on both the gel media. An affinity with monosaccharides was observed with **6** but not with MC (Figs 1 and 2). In addition, blue dextran 2000 was adsorbed on the H^+ form of **6** but not on the Na^+ form.

Figure 4 shows the potentiometric titration curve of **4** (—COOH and $-NH_3Cl$ form), which is typical for the cation-exchangers of carboxylic type (Peterson, 1970). The curve has an inflection point at pH 9·9 due to —COONa and $-NH_2$ groups completely titrated with 0·5 N NaOH (1·3 meq g^{-1} xerogel). This value corresponds to a DS for —COOH and $-NH_3Cl$ groups of 0·42 (calc. 1·88) and $pK_a = 7{\cdot}5$. Furthermore, the ion-exchange capacities of these xerogels were in the range 0·67–1·4 meq g^{-1} of the xerogel (Table 2).

DISCUSSION

Dextran gel (Sephadex) and agar are commonly used as polysaccharide media for gel chromatography (Fischer, 1969); starch gel has also been evaluated (Hanus & Kučera, 1974). It now appears that chitosan gels may also be used as a medium for gel chromatography. The present gels may be formed by the cross-linking of chitosan chains by micelles (Rees, 1972; Hirano *et al.*, 1978).

Partially *N*-carboxyacylated chitosans were *N*-acylated with stearic anhydride (DS = 0·26–0·40) or with acetic anhydride (DS = 0·6–0·70). The low DS values for *N*-stearoyl groups may be due to their steric hindrance. An increase in the hydrophobic property of the gels was successfully performed by *N*-stearoylation. All the xerogels except **3** are ampholites containing both carboxylic and amino groups in the molecules. *N*-2′-Carboxybenzoyl groups were stable in 0·5 M NaOH. *N*-3′-Carboxy-2′-propenoyl and *N*-3′-carboxypropionyl groups were slightly hydrolyzed in 0·5 M NaOH solution but stable in 0·1 M NaOH solution.

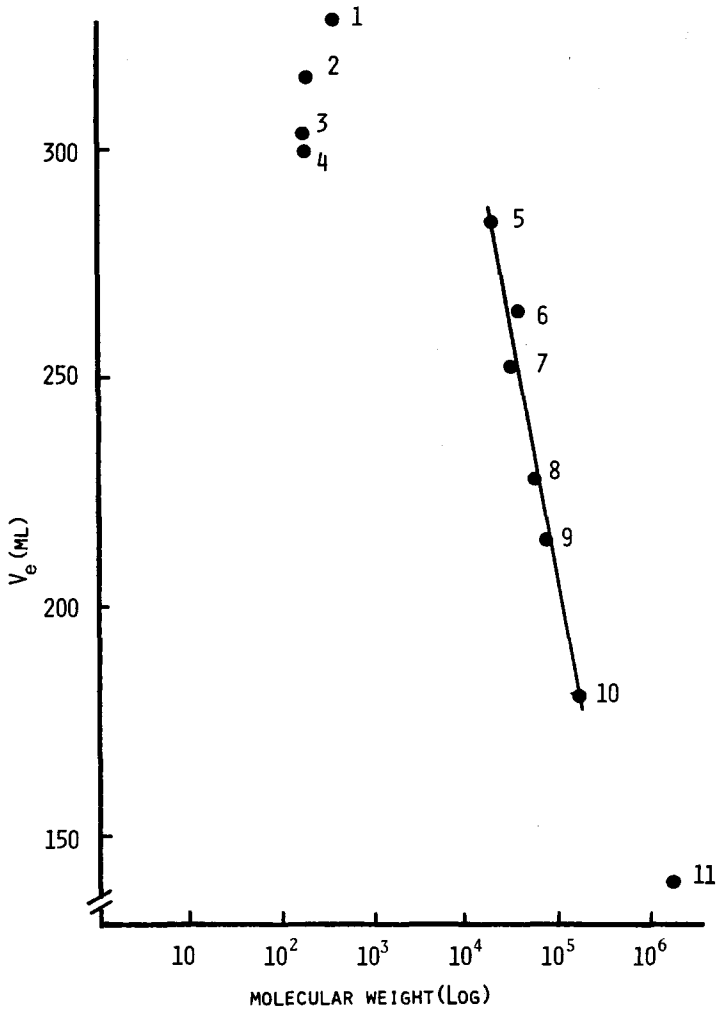

Fig. 2. Relationship between elution volume (V_e) and molecular weight (log) on a column (1·7 x 136 cm) of *N*-3′-carboxypropionyl-*N*-stearoylchitosan gel (**6**). The column was eluted with distilled water at room temperature (15–20°C). 1, Maltose (MW = 342); 2, D-mannose (MW = 180); 3, D-glucose (MW = 180); 4, D-galactose (MW = 180); 5, amylose (MW = 21 000); 6, dextran (MW = 37 000); 7, dextran (MW = 40 100); 8, dextran (MW = 61 500); 9, dextran (MW = 75 800); 10, dextran (MW = 176 000); 11, blue dextran (MW = 2000).

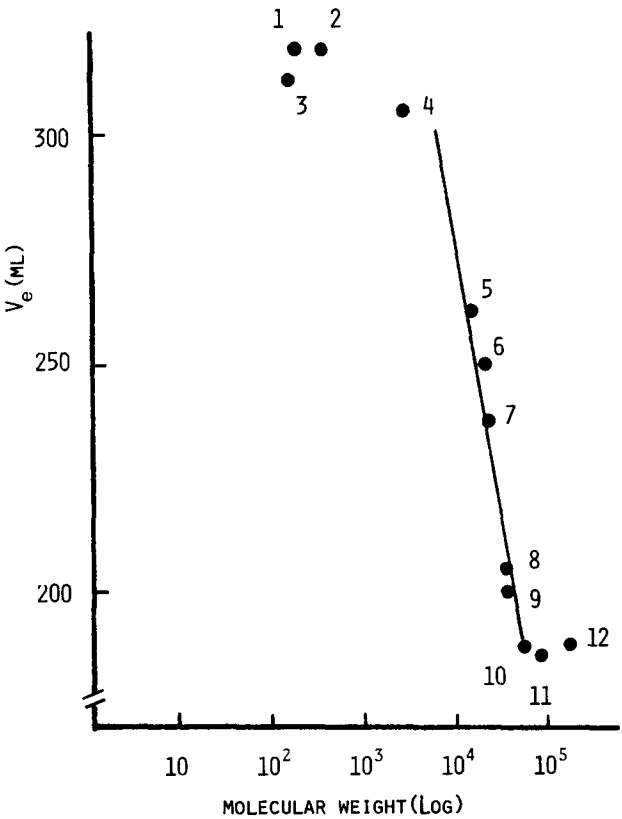

Fig. 3. Relationship between elution volume (V_e) and molecular weight (log) on a column (1·4 x 201 cm) of *N*-methylenechitosan gel. The column was eluted with 0·5 M NaCl solution at room temperature. 1, D-Glucose (MW = 180); 2, maltose (MW = 342); 3, D-xylose (MW = 150); 4, amylose (MW = 2900); 5, dextran (MW = 10 500); 6, papain (MW = 21 000); 7, amylose (MW = 21 000); 8, dextran (MW = 37 000); 9, dextran (MW = 40 100); 10, dextran (MW = 61 500); 11, β-glucosidase (MW = 85 000); 12, dextran (MW = 176 000).

This is revealed by a slight decrease in their DS values after treatment with 0·5 M NaOH solution.

The observed DS values based on the potentiometric titration curve (Fig. 2) and the observed ion-exchange capacity (Table 2) are lower than the values calculated from their formulae. This may be due to the

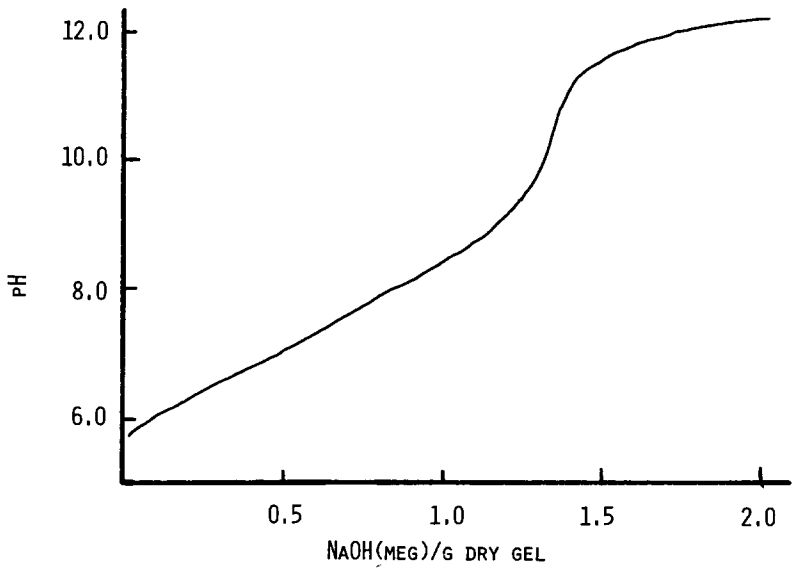

Fig. 4. A potentiometric titration curve of *N*-2′-carboxybenzoyl-*N*-stearoylchitosan (**4**) with 0·5 M NaOH in 0·5 M NaCl (150 ml).

presence of —COOH and $-NH_2$ groups buried or hidden in the xerogels, and these groups do not participate in the ion-exchange function.

The most striking characteristic of the present gels is their relatively wide fractionation range: **6** covers the fractionation ranges of Bio-Gel P-30 to P-300 and CM covers those of Bio-Gel P-10 to P-30. Distilled water, 0·1 M HCl and 0·1 M NaOH solutions can be used as eluting solvents for **6**, but aqueous acidic solutions cannot be used for CM because of the destruction of the Schiff base. CM has no free amino groups and may therefore be used for neutral, acidic and basic compounds as demonstrated in the present study, but **6** has an amphoteric property and is only of use for neutral but not acidic or basic compounds.

REFERENCES

Baba, R., Yamaguchi, R., Arai, Y. & Itoh, T. (1980). *Carbohydr. Res.* **86**, 161.
Bloch, R. & Burger, M. M. (1974). *Bichem. Biophys. Res. Commun.* **58**, 13.

Fischer, L. (1969). *An Introduction to Gel Chromatography*, North-Holland Publishing Company, Amsterdam.

Hanus, J. & Kučera, J. (1974). *J. Chromatogr.* **97**, 270.

Hirano, S., Yamaguchi, R., Matsuda, N. & Tanaka, H. (1978). *Int. J. Biochem.* **9**, 501.

Hirano, S., Matsuda, N., Miura, O. & Tanaka, T. (1979). *Carbohydr. Res.* **71**, 344.

Hirano, S., Kondo, Y., Miura, O., Yagi, Y., Tobetto, K., Hasegawa, M. & Nishiguchi, Y. (1980). *Proc. 3rd Recycling World Congr.* Basle, p. 3.6.1.

Hirano, S. & Moriyasu, T. (1981). *Carbohydr. Res.* **92**, 323.

Muzzarelli, R. A. A. (1978). *Proc. 1st Int. Conf. Chitin Chitosan*, MIT Sea Grant Program, p. 335.

Noguchi, J., Tokura, S., Inomoto, M. & Asano, C. (1965). *Kogyo Kagaku Zasshi*, **68**, 904.

Peterson, E. A. (1970). *Cellulosic Ion Exchangers*, North-Holland Publishing Company, Amsterdam.

Rees, D. A. (1972). *Chem. & Ind. (London)*, 630.

Takeda, M. (1978). *Proc. 1st Int. Conf. Chitin Chitosan*, MIT Sea Grant Program, p. 355.

Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.