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Note

Some *N*-acyl derivatives of *O*-carboxymethylchitosan

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Chitinase (EC 3.2.1.14) and lysozyme (EC 3.2.1.17) hydrolyse chitin [(1→4)-2-acetamido-2-deoxy-β-D-glucan], partially *N*-deacetylated chitin[1–3], and *N*-acyl analogues[4] prepared from lower fatty acids. Because of the insolubility of these derivatives in water, both the structure and physical form have significant effects on the enzymic hydrolysis[5]. We now describe the preparation of novel water-soluble *N*-acyl derivatives of *O*-carboxymethylchitosan[6], and their hydrolyses by chitinase and lysozyme under homogeneous conditions.

O-Carboxymethylchitin[7] (d.s. 0.59) was *N*-deacetylated by heating in aqueous 10% sodium hydroxide containing sodium borohydride for 9 h at 80° to give *O*-carboxymethylchitosan (d.s. 0.59). These conditions are milder than those for the *N*-deacetylation of chitin (*e.g.*, aqueous 45% sodium hydroxyde, 5 h, 100°)[8–10]. The d.s. was calculated from the elemental analyses for C and N (see Experimental).

The *O*-carboxymethylchitosan was treated with a series of carboxylic anhydrides (5 equiv. per GlcN) in aqueous acetic acid–methanol[11] to give *N*-acyl derivatives in yields of 59–97%. The lower *N*-acyl (C_2–C_{10} and benzoyl) and hihger *N*-acyl (C_{12}–C_{16}) derivatives formed gels and precipitates, respectively, but the *N*-succinyl and *N*-phthaloloyl derivatives did not. The d.s. for *N*-acyl, calculated from the elemental analyses for C and N, varied from 0.25 to 0.75 (Table I), which probably reflects steric effects. The distribution of the *O*-carboxymethyl and *N*-acyl groups in the derivatives is not symmetrical[12], but is not known in detail. The lower *N*-acyl ($< C_6$ for fatty acyl), *N*-succinyl, and *N*-phthalolyl derivatives (Na salts) were soluble in water, aqueous 0.05% hydrochloric acid, and aqueous 0.05% sodium hydroxide (Table I). The lower *N*-acyl derivatives had isoelectric points in the pH range 3–5, at which a precipitate was produced.

Chitinase and lysozyme hydrolysed the water-soluble lower *N*-acyl derivatives (Table II). The results indicate that the susceptibility to enzymic degradation decreased as the length of the *N*-acyl group increased. However, in the absence of values for K_m and V_{max}, the data in Table II should be regarded as qualitative.

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TABLE I

Some *N*-acyl derivatives of *O*-carboxymethylchitosan

N-*Acyl group*	*Yield (%)*	$[\alpha]_D$ *(degrees) (c, aq. 0.05% NaOH)*	*D.s.*	*Formula*[a]	*Calc.*			*Found*		
					C	*H*	*N*	*C*	*H*	*N*
Acetyl	86	−12 (0.5)	0.75	$R(C_2H_3O)_{0.75}(H)_{0.66}\cdot 0.51H_2$	41.85	5.71	5.62	41.77	5.86	5.73
Propionyl	76	−9 (0.5)	0.54	$R(C_3H_5O)_{0.54}(H)_{0.87}\cdot 0.41H_2O$	42.96	5.97	5.69	42.98	6.18	5.89
Butyryl	74	−10 (0.5)	0.60	$R(C_4H_7O)_{0.60}(H)_{0.81}\cdot 0.49H_2O$	44.38	6.29	5.40	44.30	6.48	5.41
Pentanoyl	59	−18 (0.7)	0.50	$R(C_5H_9O)_{0.50}(H)_{0.91}\cdot 0.34H_2O$	45.32	6.39	5.46	45.31	6.56	5.54
Hexanoyl	94	−16 (0.7)	0.40	$R(C_6H_{11}O)_{0.40}(H)_{1.01}\cdot 0.41H_2O$	45.12	6.49	5.49	45.13	6.53	5.62
Octanoyl	83		0.51	$R(C_8H_{15}O)_{0.51}(H)_{0.90}\cdot 0.40H_2O$	48.31	7.03	5.00	48.30	7.00	5.10
Decanoyl	80		0.41	$R(C_{10}H_{19}O)_{0.41}(H)_{1.00}\cdot 0.20H_2O$	49.23	7.09	5.09	49.28	7.30	5.28
Lauroyl	67		0.35	$R(C_{12}H_{23}O)_{0.35}(H)_{1.06}\cdot 0.41H_2O$	48.89	7.25	5.01	48.94	7.43	5.18
Palmitoyl	60		0.25	$R(C_{16}H_{31}O)_{0.25}(G)_{1.16}\cdot 0.23H_2O$	49.35	7.24	5.15	49.34	7.51	5.42
Benzoyl	97		0.35	$R(C_7H_5O)_{0.35}(H)_{1.06}\cdot 0.58H_2O$	45.31	5.59	5.48	45.31	5.42	5.34
Succinyl	n.d.	−8 (0.6)		Not analysed						
Phthaloyl	n.d.	−2 (0.7)		Not analysed						

[a] A proposed repeating unit is shown. $R = C_6H_9NO_4(C_2H_2O_2Na)_{0.59}$.

TABLE II

Increase in the reducing-sugar value per min in the hydrolyses of *N*-acyl derivatives of *O*-carboxymethylchitosan by lysozyme and chitinase

N-*Acyl group*	*Reducing-sugar value (μmol/min)*	
	Lysozyme	*Chitinase*
Acetyl	0.015	0.054
Propionyl	0.010	0.032
Butyryl	0.009	0.040
Pentanoyl	0.004	0.003
Hexanoyl	0.002	0.000

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded with a Hitachi R-24 spectrometer and the other analytical methods were as reported[13].

Materials. — *O*-Carboxymethylchitin (d.s. 0.59) was prepared from crab-shell chitosan[7]. Lysozyme from hen egg white (Sigma, Grade III) and chitinase from *Streptomyces griseus* (Sigma) were commercial products.

O-*Carboxymethylchitosan.* — A solution of *O*-carboxymethylchitin (Na salt, 2.6 g, d.s. 0.59), $[\alpha]_D^{26}$ − 13° (*c* 0.7, aq. 0.05% NaOH) in aq. 10% NaOH (130 mL) containing sodium borohydride (0.2 g) was heated at 80° for 9 h. The cooled solution was neutralised with acetic acid, dialysed against running water for 3 days and against distilled water for 1 day, then concentrated *in vacuo* to ~ 150 mL. Ethanol (3 vol.) was added with stirring, and the precipitate was collected by centrifuging at 4200*g* for 15 min, washed with EtOH, and dried to give the title product as the sodium salt (1.9 g, 87%), $[\alpha]_D^{22}$ − 4° (*c* 1, aq. 0.05% NaOH). The product was soluble in water, aq. NaOH and aq. acetic acid, had ν_{max}^{KBr} 1600 cm^{-1} (NH_2), but no absorption for NHAc at 1650 and 1500 cm^{-1}. ^{13}C-N.m.r. data (D_2O): δ 181 (C = O), 105 (C-1), 80.9 (C-4), 77.6 (C-5), 76.9 (C-3), 72.9 (C-6), 59.3 (C-2). The ^{1}H-n.m.r. spectrum (D_2O) revealed no signal for NAc.

Anal. Calc. for $[C_6H_{10}NO_4(C_2H_2O_2Na)_{0.59}(H)_{0.41}\cdot 0.13H_2O]_n$: C, 40.93; H, 5.67; N, 6.64. Found: C, 41.09; H, 5.97; N, 6.57.

N-*Acylation of* O-*carboxymethylchitosan.* — A solution of *O*-carboxymethylchitosan (Na salt, 0.25 g) in aq. 2% acetic acid (25 mL) was diluted with MeOH (30 mL), and the carboxylic anhydride (5 mol. equiv. per GlcN) was added with stirring at room temperature. The reaction mixture was stored overnight at room temperature to give a gel or precipitate. For the reaction with lauric and palmitic anhydrides, each mixture was heated for a few minutes at 80° to give a clear solution temporarily, and then to give a precipitate. Each product was mechanically homogenised in EtOH (200 mL). The precipitate was collected by filtration, and washed with EtOH several times to remove free fatty acids. Each precipitate was dissolved in aq. 0.05% NaOH, a small amount of

an insoluble material was removed by centrifugation at 4500*g* for 15 min, and then EtOH (3 vol.) was added to the supernatant solution. The precipitate was collected by filtration, washed several times with EtOH and ether, and dried to give the *N*-acyl derivative (see Table I).

Enzymic hydrolyses. — To a solution of the substrate (0.1 mmol for a proposed repeating unit based on the elemental analysis: 25.0 mg for *N*-acetyl and *N*-propionyl, 26.1 mg for *N*-butyryl and *N*-pentanoyl, 25.9 mg for *N*-hexanoyl) in 0.05M citric acid–0.1M Na_2HPO_4 buffer solution (1.5 mL, pH 6.8) was added a solution (0.20 mL) of lysozyme or chitinase (2.5 mg/mL). Each mixture was stirred for 30 min at 40°; the reaction was stopped by adding M NaOH (0.15 mL), and the increased reducing-sugar value was determined as μmol of 2-acetamido-2-deoxy-D-glucose by a modification[14] of the method of Schales and Schales. The results are shown in Table II.

REFERENCES

1 A. Ohtakara, M. Izume, and M. Mitsutomi, *Agric. Biol. Chem.*, 52 (1988) 3181–3182.
2 S. Hirano, H. Tsuchida, and N. Nagao, *Biomaterials*, 10 (1989) 574–576.
3 M. Mitsutomi, A. Ohtakara, T. Fukamizo, and S. Goto, *Agric. Biol. Chem.*, 54 (1990) 871–877.
4 S. Hirano and Y. Yagi, *Carbohydr. Res.*, 83 (1980) 103–108.
5 S. Hirano and N. Nagao, *Agric. Biol. Chem.*, 52 (1988) 2111–2112.
6 R. Trujillo, *Carbohydr. Res.*, 7 (1968) 483–485.
7 S. Hirano, *Methods Enzymol.*, 161B (1988) 408–410.
8 R. W. Jeanloz and E. Forchielli, *Helv. Chim. Acta*, 33 (1950) 1690–1697.
9 S. T. Horowitz, S. Roseman, and H. J. Blumenthal, *J. Am. Chem. Soc.*, 79 (1957) 5046–5049.
10 M. L. Wolfrom and T. M. Shen Han, *J. Am. Chem. Soc.*, 81 (1959) 1764–1766.
11 S. Hirano, Y. Ohe, and H. Ono, *Carbohydr. Res.*, 47 (1976) 315–320.
12 S. Hirano, S. Tsuneyasu, and Y. Kondo, *Agric. Biol. Chem.*, 45 (1981) 1335–1339.
13 S. Hirano, R. Yamaguchi, N. Fukui, and M. Iwata, *Carbohydr. Res.*, 201 (1990) 145–149.
14 T. Imoto and K. Yagishita, *Agric. Biol. Chem.*, 35 (1971) 1154–1156.