ELSEVIER

Carbohydrate Research 268 (1995) 143–149

CARBOHYDRATE RESEARCH

Note

The effect of *N*-substitution on the hydrolysis of chitosan by an endo-chitosanase ☆

Nongporn Hutadilok [1], Takako Mochimasu, Hiroki Hisamori, Ken-ichiro Hayashi, Hisashi Tachibana, Tomoko Ishii, Shigehiro Hirano *

Department of Agricultural Biochemistry and Biotechnology, Tottori University, Tottori 680, Japan

Received 17 December 1993; accepted in revised form 15 September 1994

Keywords: Kinetics of chitosanase; HE-chitosan; CM-chitosan; Chitosan; Chitin

Chitin is (1 → 4)-2-acetamido-2-deoxy-β-D-glucan, and its *N*- deacetylated derivative is chitosan. Chitinase (EC 3.2.1.14) and lysozyme (EC 3.2.1.17) catalyze the hydrolysis of 2-acetamido-2-deoxy-β-D-glucoside linkages, and chitosanase catalyzes the hydrolysis of 2-amino-2-deoxy-β-D-glucoside linkages in these polymers to give a series of the corresponding oligosaccharides [1]. Several chitosanases have been isolated from the culture media of microorganisms [2–7] including *Streptomyces griseus* [1], *Penicillium islandicum* [4], *Bacillus* [5–7], and *Pseudomonas* species [8]. A series of oligosaccharides having a D-glucosamine residue as the reducing end-group are produced in reactions by chitosanase [9]. Chitinase and lysozyme have been reported to have a similar broad specificity for both the chemical structure of the *N*-acyl groups of substrates and the degree of substitution (ds) [9–12]. Little is known, however, about the substrate specificity of chitosanase.

We now report the preparation of *O*-(2-hydroxyethyl)chitosan (HE-chitosan), and novel partially *N*-acylated derivatives of HE-chitosan, *O*-carboxymethylchitosan (CM-chitosan), and chitosan itself. We have made a kinetic study of their hydrolyses by an endo-chitosanase from *Bacillus pumilus* [7] with respect to the chemical structure and ds

☆ Part of this work was presented at the International Symposium on Chitin Enzymology, Senigallia, Ancona, Italy, May 10–12, 1993.

* Corresponding author.

[1] Present address: Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand.

SSDI 0008-6215(94)00301-7

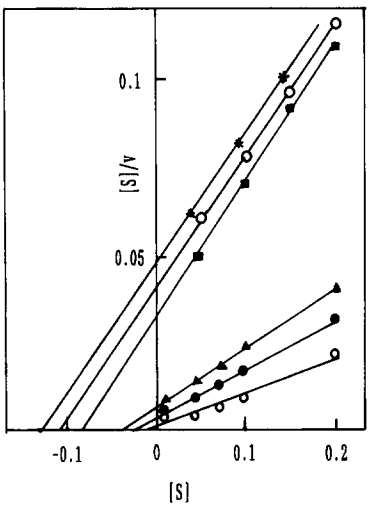

Fig. 1. Plots of [S] against [S]/v in the hydrolysis reactions of partially *N*-acylated derivatives of chitosan in homogeneous solutions by an endo-chitosanase from *Bacillus pumilus* at pH 5.0. *N*-Acetyl: ○, ds 0.05; ●, ds 0.16; ▲, ds 0.49; ■, ds 0.64. *N*-Butyryl: ⊙, ds 0.41. *N*-Propionyl: ∗, ds 0.56. [S], mg/mL; v, nM/min.

of the *N*-acyl group in homogeneous and heterogeneous conditions.

O-(2-Hydroxyethyl)chitin (HE-chitin) (ds 0.68 for *O*-hydroxyethyl, ds 1.0 for *N*-acetyl) [13] was *N*-deacetylated by treating with aqueous 15% NaOH at 90°C for 6 h to give HE-chitosan (ds 0.68 for *O*-hydroxyethyl) in 93% yield. CM-Chitosan (ds 0.69 for *O*-carboxymethyl) was prepared from CM-chitin as reported previously [9]. As shown in Table 1, a total of 38 partially *N*-acylated derivatives of chitosan, CM-chitosan, and HE-chitosan were prepared in 66–92% yields by the modified method as reported previously [9], [14]. These partially *N*-acylated derivatives showed absorptions at 1650 and 1550 cm^{-1} (C = O and NH of *N*-acyl) in their FTIR spectra, and their structures were confirmed by the elemental analysis data. The ds for *N*-acyl groups was calculated on the basis of the elemental analysis data. *N*-Acyl groups on the chains thus prepared have been reported to have a random distribution [15]. The partially *N*-acylated derivatives of chitosan were soluble at pH 5.0, but insoluble at pH 5.6 in 0.3 M acetate buffer. The partially *N*-acylated derivatives of CM-chitosan and HE-chitosan were soluble in these buffers.

The hydrolysis reaction of the partially *N*-acylated derivatives of chitosan by the chitosanase in homogeneous solution obeyed Michaelis–Menten kinetics. Fig. 1 shows plots of [S] (mg/mL) against [S] (mg/mL)/v (nM/min) (Eadie–Hofstee plot). The K_m and V_{max} values obtained by plotting [S] against [S]/v are in fairly good agreement with those obtained by plotting 1/[S] against 1/v (Lineweaver–Burk plot). The value of K_m (mg/mL) increased and V_{max} (nM/min) decreased with increasing ds for *N*-acyl group. In the enzymatic hydrolyses of *N*-acetyl (ds 0.64), *N*-propionyl (ds 0.41), and *N*-butyryl (ds 0.56) derivatives of chitosan, K_m increased with increasing the number of carbon atoms in the *N*-acyl groups, and V_{max} was almost unchanged. A similar result was observed with the enzymatic hydrolyses of *N*-acyl derivatives of both CM-chitosan and

Table 1
K_m and V_{max} obtained by plotting [S] against [S]/v for partially *N*-acylated derivatives of chitosan, CM-chitosan, and HE-chitosan in their hydrolysis reactions by an endo-chitosanase from *Bacillus pumilus* at pH 5.0

N-Acyl group	$[\alpha]_D^{20}$	K_m (mg/mL)			V_{max} (nM/min)		
(ds)	(°) [a]	Chitosan	CM-chitosan	HE-chitosan	Chitosan	CM-chitosan	HE-chitosan
Acetyl							
0.01	−10		0.025 ± 0.009			5.2 ± 1.1	
0.05	−12	0.012 ± 0.003			10 ± 1.3		
		(0.014 ± 0.03) [b]			(8.6 ± 0.6) [b]		
0.16	−39	0.026 ± 0.006			7.4 ± 0.6		
		(0.024 ± 0.003) [b]			(6.9 ± 0.7) [b]		
0.20	−30			0.010 ± 0.003			8.4 ± 0.2
0.22	−6		0.055 ± 0.004			3.4 ± 0.8	
			(0.050) [b]			(5.1) [b]	
0.49	−20	0.038 ± 0.005			6.1 ± 0.8		
		(0.066 ± 0.003) [b]			(5.0 ± 1.3) [b]		
0.64	−21	0.086 ± 0.010			2.7 ± 0.7		
		(0.1 ± 0.01) [b]			(3.0 ± 0.9) [b]		
	−9		0.17 ± 0.03			2.4 ± 0.7	
			(0.18) [b]			(3.6) [b]	
0.83	−9		1.3 ± 0.1			1.1 ± 0.4	
			(1.25) [b]			(1.6) [b]	
Propionyl							
0.05	−10		0.034 ± 0.010			3.7 ± 0.2	
0.15	−25		0.031 ± 0.006			2.5 ± 0.7	
0.21	−21	0.027 ± 0.005			7.7 ± 0.7		
	−20			0.011 ± 0.004			7.7 ± 1.7
0.30	−30	0.044 ± 0.008			6.5 ± 0.3		
0.40	−10		0.080 ± 0.008			1.4 ± 0.6	
0.41	−20	0.10 ± 0.01			2.6 ± 0.4		
0.42	−20			0.020 ± 0.004			5.9 ± 0.8
0.55	−20			0.026 ± 0.006			4.2 ± 0.1
0.68	−30			0.033 ± 0.007			4.4 ± 0.5
Butyryl							
0.05	−15		0.064 ± 0.004			3.8 ± 0.3	
0.15	−12		0.10 ± 0.02			3.7 ± 0.2	
0.11	−35	0.033 ± 0.003			8.4 ± 0.2		
0.25	−30	0.054 ± 0.010			6.4 ± 0.8		
0.27	−10			0.013 ± 0.005			7.4 ± 0.1
0.42	−18		0.33 ± 0.08			2.3 ± 0.4	
			(0.31) [b]			(3.5) [b]	
0.54	−10			0.016 ± 0.002			5.9 ± 0.1
0.56	−31	0.13 ± 0.02			2.8 ± 0.5		
0.58	−20			0.26 ± 0.01			5.5 ± 0.8
0.73	−20			0.31 ± 0.002			4.2 ± 1.2

Table 1 (continued)

N-Acyl group (ds)	$[\alpha]_D^{20}$ (°) [a]	K_m (mg/mL)			V_{max} (nM/min)		
		Chitosan	CM-chitosan	HE-chitosan	Chitosan	CM-chitosan	HE-chitosan
Pentanoyl							
0.05	−18		0.083 ± 0.005			3.8 ± 0.5	
0.15	−20		0.25 ± 0.08			2.5 ± 0.3	
0.19	−20			0.015 ± 0.004			8.0 ± 0.7
0.49	−20			0.020 ± 0.003			7.1 ± 0.1
0.54	−30			0.021 ± 0.003			6.0 ± 0.4
0.60	−30			0.030 ± 0.004			4.6 ± 0.3
Hexanoyl							
0.26	−40			0.013 ± 0.002			6.3 ± 0.6
0.40	−30			0.017 ± 0.002			5.2 ± 0.4
0.46	−30			0.022 ± 0.003			3.1 ± 0.2

[a] Measured at *c* 1, aq 2% AcOH for *N*-acyl derivatives of chitosan and HE-chitosan, and at *c* 1, aq 0.005% NaOH for *N*-acyl derivatives of CM-chitosan.
[b] Obtained by plotting 1/[S] against 1/v.

HE-chitosan (Table 1). These data strongly indicate that aliphatic *N*-acyl derivatives of chitosan act as competitive inhibitors at nearly the same ds for all *N*-acyl groups. In the enzymatic hydrolysis at nearly the same ds for *N*-acyl group, the K_m value increased in the following sequence: chitosan > CM-chitosan > HE-chitosan, and the V_{max} value increased in the following sequence: HE-chitosan > chitosan > CM-chitosan. These data indicate that chitosanase is more active towards HE-chitosan than chitosan and CM-chitosan at the initial stage of the enzymatic reaction.

On the other hand, the enzymatic hydrolyses in a heterogeneous suspension differed distinctly from those in the homogeneous solution. Partially *N*-acylated derivatives hav-

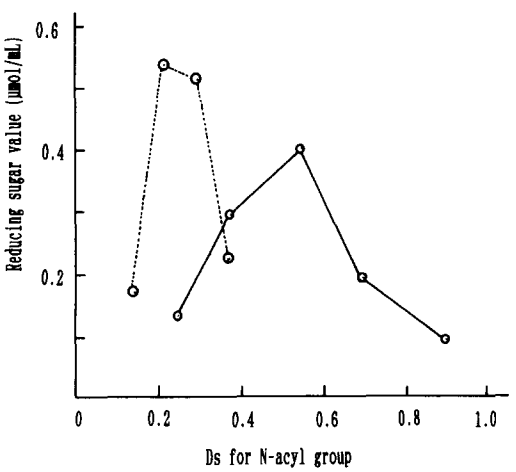

Fig. 2. Hydrolyses of partially *N*-acetylated (—) and *N*-propionylated (---) derivatives of chitosan by the endo-chitosanase in heterogeneous suspensions at pH 5.6. Reducing-sugar values (μmol of D-glucosamine per mL) were analyzed after incubation at 50°C for 30 min (see text for details).

ing ds 0.49 for *N*-acetyl and ds 0.30 for *N*-propionyl were hydrolyzed faster than the derivatives having other ds values for the corresponding *N*-acyl group (Fig. 2). The hydrolysis rate decreased with increasing size of the *N*-acyl groups. The enzymatic reactions in the heterogeneous suspension seem to be affected by the hydrophilic and swelling properties of the substrates in water. Their hydrophilicity depends mainly on the structure of the *N*-acyl groups, and their ds and distribution. Partially *N*-deacetylated chitin or partially *N*-acetylated chitosan (ds ~ 0.5) are apparently soluble in aqueous solutions [16,17], resulting in an increase in the enzymatic reaction rate.

1. Experimental

General methods.—FTIR spectra (KBr) were recorded on a Jasco FTIR 5300 spectrometer, specific rotations on a Jasco Dip-181 polarimeter, and ^{1}H NMR and ^{13}C NMR spectra (D_2O) on a Jeol JNM-GX 270 FT NMR spectrometer. Elemental analyses were carried out at the Micro-analytical center of Kyoto University, Kyoto. Weight-average molecular weights were estimated by GPC with dextran standards (Shodex Co., Ltd., Tokyo).

Materials.—An endo-chitosanase (640–960 U/g of protein, mol wt 30 000; optimum pH 5.5–6.5; optimum temperature 30–50°C, pI 9.3) from *Bacillus pumilus* BN-262 was a commercial product from Wako Pure Chemical Industries, Ltd., Osaka [7]. Crab shell chitosan {ds 0.05 for *N*-acetyl; $[\alpha]_D^{20}$ −6.7° (*c* 0.5, aq 2% AcOH); mol wt 0.83×10^6} was a product of Katakurachikkarin Co., Ltd., Tokyo. CM-chitosan {ds 0.69 for *O*-carboxymethyl; $[\alpha]_D^{22}$ −4° (*c* 1, aq 0.05% NaOH); mol wt 1.2×10^6} was prepared as described previously [9].

HE-chitosan.—A solution of 1% HE-chitin {1.0 g, ds 0.68 for *O*-hydroxyethyl, ds 1.0 for NAc, $[\alpha]_D^{16}$ −8°(*c* 0.7, H_2O)} [15] in 100 mL of aq 20% NaOH containing $NaBH_4$ (0.01 g) was heated at 90°C for 6 h, and the mixture was neutralized with dil HCl and dialyzed against distilled water for 2 days by changing with fresh distilled water several times. The dialyzed solution was filtered, the filtrate was concentrated in vacuo to a solution of 3–4% concentration, and 3 vol of EtOH were added to give the title product as a precipitate (0.65 g, 73% yield); $[\alpha]_D^{16}$ −6°(*c* 1, aq 2% AcOH). The product was soluble in water and aqueous AcOH, and had ν_{max}^{KBr} 2929 (C–H) and 1670 cm^{-1}(NH_2), but no absorption for NAc at 1650 and 1550 cm^{-1}. An acetyl-methyl proton signal was not detected at $\delta \sim 2$ in the ^{1}H NMR spectrum. ^{13}C NMR data (D_2O): δ 100.0 (C-1), 79.5 (C-4), 77.5 (C-5), 76.3 (C-3), 74.9 (-CH_2OH), 72.6 (-OCH_2-), 63.1 (C-6), 58.5 (C-2). Anal. Calcd for $[C_6H_{10}NO_4(C_2H_5O)_{0.68}(H)_{0.32} \cdot 0.86H_2O]_n$: C, 42.79; H, 7.48; N, 6.78. Found: C, 42.89; H, 7.61; N, 6.43.

Partially N-*acylated derivatives.*—Samples (0.2 g) of chitosan, CM-chitosan and HE-chitosan were separately dissolved in 30 mL of 1:2 aq 2% AcOH–MeOH, the corresponding carboxylic anhydride (0.1–1.0 mol equiv to GlcN) was added with stirring at room temperature [9], [14], and the mixture was kept at room temperature overnight. To each solution was added $\sim$ 150 mL of EtOH to give a precipitate. The precipitate was filtered, washed with EtOH several times, and again dissolved in aq 0.2% NaOH (20–25 mL). After removing a small amount of insoluble materials, 3 vol of EtOH were added. The precipitate thus produced was collected by centrifugation at 4 200*g* for 30 min to

give the corresponding *N*-acyl derivative in 66–92% yields. The ds for *N*-acyl groups was calculated from the elemental analysis data.

Enzymatic hydrolysis.—(*a*) *In homogeneous solution.* To a solution (2 mL) of each substrate at several concentrations (0.01–3.0 mg/mL) in 0.1 M acetate buffer (pH 5.0) was added 20 μL of the chitosanase solution (4.5 mg/mL of the same buffer). The mixture was incubated at 40°C for 10 min. The reaction was stopped by addition of 0.05% Na_2CO_3–KCN solution (2.0 mL), the precipitate produced was removed by centrifugation at 1 500*g* for 10 min, and the reducing-sugar value in the supernatant solution was analyzed by the Park–Johnson method [18]. The increase in the reducing-sugar value for each substrate concentration is expressed as nmol of D-glucosamine · HCl per mL. K_m (mg/mL) and V_{max} (nM/min) were calculated by plotting [S] (mg/mL) against [S] (mg/mL)/v (nM/min) (Eadie–Hofstee plot) and by plotting 1/[S] against 1/v (Lineweaver–Burk plot) on a computer, and an average value of three experiments is shown.

(*b*) *In heterogeneous suspension.* To a solution of the chitosanase (0.02 mg) in 100 μL of 0.3 M acetate buffer (pH 5.6) was added a suspension of partially *N*-acylated chitosan (10 mg) in 1.95 mL of the same buffer. The mixture was incubated at 50°C for 30 min. The reaction was stopped by adding 1.25 mL of 0.05 M $NaCO_3$, and the precipitate was removed by centrifugation at 1 500*g* for 15 min at 4°C. The reducing-sugar value (μmol/mL) as D-glucosamine · HCl in the supernatant solution was analyzed by a modification [19] of the method of Schales and Schales.

Acknowledgements

This work was supported by grants from the Japan Society for the Promotion of Science, Tokyo (N.H.), the Japan Science Society, Tokyo (T.M.), the New Energy and Industrial Technology Development Organization, and the Research Institute of Innovative Technology for the Earth, Kyoto (S.H.).

References

[1] M. Mitsutomi and A. Ohtakara, in C.J. Brine, P.A. Sandford, and J.P. Zikakis (Eds.), *Advances in Chitin and Chitosan*, Elsevier, Amsterdam, 1992, pp 304–313.
[2] A. Hedges and R.S. Wolfe, *J. Bacteriol.*, 120 (1974) 844–853.
[3] J.S. Price and R. Storck, *J. Bacteriol.*, 124 (1975) 1574–1585.
[4] D.M. Fenton and D.E. Eveleigh, *J. Gen. Microbiol.*, 126 (1981) 151–165.
[5] M. Izume, S. Nagae, H. Kawagishi, M. Mitsutomi, and A. Ohtakara, *Biosci. Biotechnol. Biochem.*, 56 (1992) 448–453.
[6] Y. Tominaga and Y. Tsujisaka, *Biochim. Biophys. Acta*, 410 (1975) 145–155.
[7] K. Uotani, H. Takebe, T. Matsunobu, K. Nakagawa, and A. Sato, *Abstr. Pap. Presented Ann. Meet. Agric. Chem. Soc. Jpn.*, 1987, pp 454–455.
[8] K. Yoshihara, J. Hosokawa, T. Kubo, M.M. Nishiyama, and Y. Koba, *Biosci. Biotechnol. Biochem.*, 56 (1992) 972–973.
[9] S. Hirano, K. Hayashi, and K. Hirochi, *Carbohydr. Res.*, 225 (1992) 175–178.
[10] S. Hirano, H. Tsuchida, and N. Nagao, *Biomaterials*, 10 (1989) 574–576.
[11] M. Mitsutomi, A. Ohtakara, T. Fukamizo, and S. Goto, *Agric. Biol. Chem.*, 54 (1980) 871–877.

[12] S. Aiba, *Int. J. Biol. Macromol.*, 15 (1993) 241–245.
[13] S. Hirano, *Methods Enzymol.*, 161 (1988) 408–410.
[14] S. Hirano, O. Miura, and R. Yamaguchi, *Agric. Biol. Chem.*, 41 (1977) 1755–1759.
[15] S. Aiba, *Int. J. Biol. Macromol.*, 13 (1991) 40–44.
[16] T. Sannan, K. Kurita, and Y. Iwakura, *Macromol. Chem.*, 177 (1976) 3589–3600.
[17] T. Sannan, K. Kurita, and Y. Iwakura, *Macromol. Chem.*, 176 (1975) 1191–1195.
[18] J.T. Park and M.J. Johnson, *J. Biol. Chem.*, 181 (1949) 149–151.
[19] T. Imoto and K. Yagishita, *Agric. Biol. Chem.*, 35 (1971) 1154–1156.