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THE EFFECTS OF *N*-SUBSTITUTION OF CHITOSAN AND THE PHYSICAL FORM OF THE PRODUCTS ON THE RATE OF HYDROLYSIS BY CHITINASE FROM *Streptomyces griseus*

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ABSTRACT

N-Formyl, *N*-chloroacetyl, *N*-glycyl, *N*-isobutyryl, and *N*-pentanoyl derivatives of chitosan have been prepared. *N*-Acetylchitosan was the derivative most susceptible to chitinase from *Streptomyces griseus* and lysozyme from chicken egg-white, but the susceptibility was not restrictive. The relative rates of hydrolysis by chitinase with respect to R in the RCONH group were $CH_3 > CH_3CH_2 > H > CH_3CH_2CH_2 > (CH_3)_2CH > NH_2CH_2 > ClCH_2$. Neither enzyme hydrolysed chitosan or its *N*-methylene, *N*-benzylidene, *N*-benzoyl, *N*-nicotinyl, and *N*-fatty acyl (C_5–C_{18}) derivatives, and lysozyme did not hydrolyse *N*-butyrylchitosan. *N*-Acetylhexanoylchitosans, which had d.s. ratios of $\sim 0.7 : \sim 0.3$ and $\sim 0.3 : \sim 0.7$, were hydrolysed at ~ 0.75 and ~ 0.04 of the rate of *N*-acetylchitosan (powder) by chitinase. *O*-Acylation of *N*-acylchitosans caused a decrease in the rates of hydrolysis by chitinase. *N*-Acetylchitosan gels were hydrolysed at 8–13 times the rate for crab-shell chitin. These results indicate that not only *N*- and *O*-substituents but also the physical form of the substrates influence the rates of hydrolysis by these enzymes.

INTRODUCTION

Chitinase (EC 3.2.1.14) and chitobiase (EC 3.2.1.29)[1] are widely distributed in bacteria, fungi, some animals, and fishes, and the enzymes degrade chitin [a (1→4)-linked 2-acetamido-2-deoxy-β-D-glucan][2] into 2-acetamido-2-deoxy-D-glucose by their consecutive reactions. These enzymes are secreted by the epidermis of nematodes during the hatching process[3] and by the epidermis of arthropods at the time of moulting[4]. In spite of these important functions, the substrate specificity of the enzymes is little understood[5,6], because suitably modified chitins have not been available. Recently, we found a mild method for gel formation by *N*-substitution of chitosan [a (1→4)-linked 2-amino-2-deoxy-β-D-glucan][7–9], which is applicable to the preparation of a series of *N*-substituted chitosans.

We now report on the effect of *N*-substitution of chitosan and the physical form of the products on the rate of hydrolysis by chitinase from *Streptomyces griseus* and by lysozyme (EC 3.2.1.17) from chicken egg-white.

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RESULTS AND DISCUSSION

N-Substitution of the amino groups of chitosan afforded *N*-benzylidene[8], *N*-methylene[9], *N*-fatty acyl[7], *N*-benzoyl[7], and *N*-nicotinyl[10] derivatives, and several novel products, namely, *N*-formyl, *N*-chloroacetyl, *N*-glycyl, *N*-isobutyryl, and *N*-pentanoyl derivatives. *N*-Glycylchitosan was prepared by treating *N*-chloroacetylchitosan with ammonia at room temperature. These structures were confirmed by elemental analyses, by i.r. spectra, and, in part, by p.m.r. spectra. All of the derivatives were insoluble in the buffer solutions. Experiments were performed with mechanical shaking, using each of the derivatives suspended in the buffer solutions.

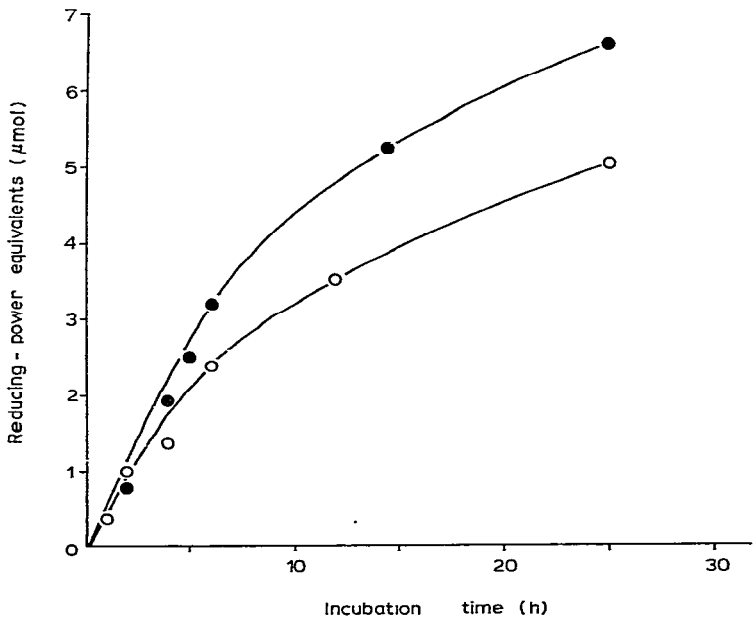

Fig. 1. Hydrolysis of *N*-acetylchitosan by chitinase from *Streptomyces griseus* and by lysozyme from chicken egg-white. Chitinase, —●—; lysozyme, —○—. See text for experimental details.

Fig. 1 shows the hydrolyses of *N*-acetylchitosan (powder) by chitinase and by lysozyme. The increase of reducing-sugar values was almost proportional to the incubation time, for up to 3 h by chitinase and up to 6 h by lysozyme. The relative rates of hydrolysis were compared on the basis of increase in the reducing-sugar values for a 2-h incubation by chitinase and for a 5-h incubation by lysozyme (Table I).

The *N*-acetylated group of substrates was the most susceptible to chitinase and lysozyme, but the susceptibility was not restrictive, and these enzymes hydrolysed the other *N*-acylchitosans. The relative rates of hydrolysis by chitinase with respect to the nature of R in the RCONH group were $CH_3 > CH_3CH_2 > H > CH_3CH_2CH_2 > (CH_3)_2CH > NH_2CH_2 > ClCH_2$. Neither enzyme hydrolysed chitosan and its

TABLE I

THE EFFECTS OF *N*-SUBSTITUTION OF CHITOSAN AND THE PHYSICAL FORM OF THE PRODUCTS ON THE RATES OF HYDROLYSIS BY CHITINASE FROM *Streptomyces griseus* AND BY LYSOZYME FROM CHICKEN EGG-WHITE[a]

Chitosan	*Increase in reducing-sugar values*[b]	*Relative rates*[c]
N-Acetyl (gels)	3.09 (1.53)	13 (8.1)
N-Acetyl (xerogels)	1.98 (2.02)	8.3 (11)
N-Acetylhexanoyl (d.s. ratio ~0.7 : ~0.3)	1.49	6.2
Partially *O*-acetylated *N*-acetyl[d]	0.63	2.6
N-Propionyl	0.34 (0.18)	1.5 (1.0)
Natural chitin (crab shells)	0.24 (0.19)	1.0 (1.0)
N-Formyl	0.19	0.8
N-Butyryl	0.12 (0.00)	0.5 (0.0)
N-Isobutyryl	0.09	0.4
N-Glycyl	0.07	0.3
N-Acetylhexanoyl (d.s. ratio ~0.3 : ~0.7)	0.07	0.3
N-Chloroacetyl	0.03	0.1

[a]The partially *O*-chloroacetylated *N*-chloroacetyl (d.s. for *O*-acyl was not determined), *N*-pentanoyl, *N*-hexanoyl, *N*-lauroyl, *N*-stearoyl, *N*-benzoyl, *N*-nicotinyl, *N*-methylene, and *N*-benzylidene derivatives and chitosan were not hydrolysed by either enzyme. [b]μMol/2 h for chitinase and μmol/5 h for lysozyme, respectively. The values increased by lysozyme are shown in parentheses. [c]On the basis of the hydrolysis rate for natural chitin. [d]D.s. for *O*-acetyl ~0.9/GlcNAc.

N-methylene, *N*-benzylidene, *N*-benzoyl, *N*-nicotinyl, and *N*-fatty acyl (C_5–C_{18}) derivatives, and lysozyme did not hydrolyse *N*-butyrylchitosan. Karrer and White[6] reported that snail chitinase hydrolysed *N*-acetylchitosan but not the *N*-formyl, *N*-propionyl, and *N*-butyryl derivatives. Hara and Matsushima[11] reported that lysozyme from chicken egg-white did not hydrolyse *N*-propionylchitosan. The inconsistency with the present results may reflect the origins of the enzymes and the different degree of substitution (d.s.) per hexosaminyl residue for *N*-acyl groups: the d.s. values were ~1.0 in the present study and <1.0 in the reported studies[6,11], which indicate the presence of some free amino groups. Chitosan (free amino groups) and its *N*-benzylidene[8] and *N*-methylene[9] derivatives were not hydrolysed by chitinase (Table I).

N-Acetyl groups in the heterogeneously *N*-acylated chitosans gave the higher rates of enzymic hydrolysis. *N*-Acetylhexanoylchitosans, which had d.s. ratios of ~0.7 : ~0.3 and ~0.3 : ~0.7 for acetyl–hexanoyl groups, were hydrolysed at ~0.75 and ~0.04 of the rates for *N*-acetylchitosan (powder) with chitinase. The distribution of the *N*-acetyl groups is unknown[11].

O-Acylation of *N*-acylchitosans caused a decrease in the rates of hydrolysis by chitinase: a partially *O*-acetylated derivative (d.s. ~0.9/GlcNAc) of *N*-acetylchitosan was hydrolysed at ~0.33 of the rate for *N*-acetylchitosan (powder).

N-Acetylchitosan gels and the powdered xerogels were hydrolysed eight to thirteen times faster than natural chitin. These results indicate that not only *N*- and *O*-substitution but also the physical form of the substrates affect the rates of enzymic hydrolysis. *N*-Acetylchitosan gels were formed by filling water droplets in micropores surrounded with thin, membraneous walls. The walls were constructed of lamellae that were produced from meshy frameworks of microfibrils, and the fibrils were formed by assemblies of *N*-acetylchitosan chains[13]. The pore dimensions were 30–50 × 80–300 μm, and the micropores were separated from each other by the membranous walls. The molecular weight of chitobiase is unknown, but chitinase (mol. wt. ~30,000)[14] and lysozyme (mol.wt. 14,307) can enter the micropores of *N*-acetylchitosan gels and move almost freely through the membraneous walls. The enzymes attack not only the surface but also the interior of the chitin microfibrils. By contrast, natural chitin has no micropores and is constructed of lamellae in which many chitin microfibrils are regularly arranged within a small space, much more compactly than in *N*-acetylchitosan xerogels[15]. The enzymes cannot enter the microfibrils, and therefore attack only the surface of chitin molecules. *N*-Acetylchitosan gels are new substrates for chitinase and lysozyme.

EXPERIMENTAL

Methods. — Specific rotations were measured with a JASCO Dip-181 polarimeter, using a cell of path-length 10 cm. P.m.r. spectra (60 MHz) were recorded with a Hitachi R-24 spectrometer and i.r. spectra with a Hitachi 215 grating spectrophotometer. P.c. was performed by the descending method on Whatman No. 1 filter paper with 6:4:3 (v/v) 1-butanol–pyridine–water.

Enzymic hydrolyses. — (*a*) *Chitinase.* To a suspension of substrate (10 mg) in 1.0 ml of buffer (0.05M citric acid–0.1M Na_2HPO_4, pH 6.0) was added 4.0 ml of chitinase solution (0.32 mg of chitinase/ml of buffer solution). The mixture was incubated in a test tube at 37° with mechanical shaking.

(*b*) *Lysozyme.* To a suspension of substrate (20 mg) in 4.0 ml of buffer (0.05M citric acid–0.1M Na_2HPO_4, pH 5.2) was added 1.0 ml of lysozyme solution (1.0 mg of lysozyme/ml of buffer solution), and the mixture was incubated in a test tube at 40° with mechanical shaking.

Aliquots were withdrawn from the supernatant solutions at appropriate intervals, and the increase of reducing-sugar value was analysed by a modified Schales' method[16] and calculated as μmol of 2-acetamido-2-deoxy-D-glucose.

Materials. — Chitosan, $[\alpha]_D^{12}$ −10.3° (*c* 1.3, 10% acetic acid), was prepared as previously described[9]; the p.m.r. spectrum (9:1 D_2O–DCO_2D) contained no signals for NAc at ~2 p.p.m.

Chitinase from *Streptomyces griseus* (Sigma, 3 units/mg), lysozyme from chicken egg-white (Sigma, Grade III, 57,200 units/mg), and chitin from crab shells (Nakarai Chemicals, extra-pure grade) were commercial products.

N-*Formylchitosan.* — Chitosan (0.16 g) was dissolved in formic acid (15 ml)

at room temperature, and acetic anhydride (3 ml) was added. The mixture was kept at room temperature overnight and then poured into ice–water (~30 ml). The solution was dialysed against running water overnight, and ethanol (100 ml) was then added to the dialysate, to afford precipitates that were collected by centrifugation. The precipitates were washed with ethanol followed by ether, to give the title derivative (0.17 g, 84%), $[\alpha]_D^{18}$ −20° (*c* 0.75, 20% formic acid); ν_{max}^{KBr} 1680 and 1550 cm^{-1} (C=O and NH of *N*-acyl), with no absorptions at 1800–1700 and 1300–1200 cm^{-1} (C=O and C–O of *O*-acyl); p.m.r. data (1:4 DCO_2D–D_2O): δ 8.2 (formyl protons) and 5.5–3.0 (sugar-ring protons), with no signals at ~2 p.p.m. (Ac).

Anal. Calc. for $(C_7H_{11}NO_5 \cdot 0.37\ H_2O)_n$: C, 42.94; H, 6.04; N, 7.15. Found: C, 42.95; H, 6.16; N, 7.08.

The derivative was originally prepared by boiling under reflux a mixture of chitosan and formic acid at elevated temperatures[6].

N-*Chloroacetylchitosan.* — Chitosan (0.32 g) was dissolved in chloroacetic acid (15 g) at 60–70°, chloroacetic anhydride (0.7 g) was added, and the mixture was kept at this temperature for 30 min and then at room temperature overnight. The resulting, solid mass was suspended in cold water (~100 ml) and stirred, to afford an amorphous precipitate that was collected by centrifugation. The precipitate was resuspended in distilled water (~100 ml), filtered off, and washed with water, ethanol, and then ether, to afford a partially *O*-chloroacetylated *N*-chloroacetylchitosan (0.52 g); ν_{max}^{KBr} 1760 and 1180 (C=O and C–O of *O*-acyl), 1670 and 1550 (C=O and NH of *N*-acyl), and 780 cm^{-1} (C–Cl). A suspension of the product in 0.05M NaOH (~100 ml) was kept at room temperature overnight. The precipitate was filtered off, washed with water, ethanol, and ether, and dried over P_2O_5 in a desiccator *in vacuo*, to afford the title derivative (0.36 g, 75%); ν_{max}^{KBr} 1670 and 1550 (C=O and NH of *N*-acyl), and 780 (C–Cl), with no absorptions at 1800–1700 and 1300–1170 cm^{-1} (*O*-acyl).

Anal. Calc. for $(C_8H_{12}ClNO_5 \cdot 0.30\ H_2O)_n$: C, 39.52; H, 5.23; Cl, 14.59; N, 5.77. Found: C, 39.53; H, 5.26; Cl, 14.29; N, 5.62.

N-*Glycylchitosan.* — A suspension of *N*-chloroacetylchitosan (0.24 g) in 40 ml of aqueous ammonia (sp. gr. 0.90) was stirred at room temperature for 4 days. Water (~40 ml) was added to the suspension, and the precipitate was filtered off, washed with water, ethanol, and ether, and dried, to afford the title derivative (0.19 g, 86%); ν_{max}^{KBr} 1660 and 1550 cm^{-1} (C=O and NH of *N*-acyl).

Anal. Calc. for $[(C_8H_{12}ClNO_5)_{0.21}(C_8H_{14}N_2O_5)_{0.79} \cdot 0.40\ H_2O]_n$: C, 41.87; H, 6.32; Cl, 3.24; N, 10.93. Found: C, 41.93; H, 6.25; Cl, 3.02; N, 10.57.

The derivative was hydrolysed with 6M HCl at 105° for 24 h, and p.c. examination revealed only two spots, R_F 0.05 and 0.16, corresponding to authentic glycine and 2-amino-2-deoxy-D-glucose, respectively.

N-*Isobutyrylchitosan.* — This derivative was prepared from chitosan and isobutyric anhydride by our procedure[7]; yield, 98%; ν_{max}^{KBr} ~2900 (CH), 1750 and 1240 cm^{-1} (C=O and NH of *N*-acyl).

Anal. Calc. for $(C_{10}H_{17}NO_5 \cdot 0.47\ H_2O)_n$: C, 50.11; H, 7.54; N, 5.84. Found: C, 49.91; H, 7.76; N, 5.92.

N-*Pentanoylchitosan.* — This derivative was prepared from chitosan and pentanoic anhydride by our procedure[7]; yield, 96%; ν_{max}^{KBr} ~2900 (CH), 1750 and 1250 cm^{-1} (C=O and NH of *N*-acyl).

Anal. Calc. for $(C_{11}H_{19}NO_5 \cdot 0.53\ H_2O)_n$: C, 51.75; H, 8.13; N, 5.49. Found: C, 51.60; H, 8.36; N, 5.32.

N-*Acetylhexanoylchitosans.* — The derivatives[17] were prepared by treatment of chitosan with acetic anhydride (0.5 mol/GlcN) and then with hexanoic anhydride (1.5 mol/GlcN), to afford *N*-acetylhexanoylchitosan (d.s. ratio ~0.3 : ~0.7 for *N*-acetyl–hexanoyl), and by treatment of chitosan with hexanoic anhydride (0.5 mol/GlcN) and then with acetic anhydride (1.5 mol/GlcN), to afford *N*-acetylhexanoylchitosan (d.s. ratio ~0.7 : ~0.3 for *N*-acetyl–hexanoyl).

The other derivatives were prepared as described previously[6-8].

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