Short Communication

Effects of the *N*-Substitution and Physical Form of Chitosan and the Modification of its Nonreducing End Groups on the Rates of Hydrolysis by Chitinase from *Streptomyces griseus*

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Chitin, (1→4)-2-acetamido-2-deoxy-β-D-glucan, is an unutilized natural resource. Chitinase (EC 3.2.1.14) and chitobiase (EC 3.2.1.29)¹⁾ hydrolyze chitin into 2-acetamido-2-deoxy-D-glucose by consecutive reactions. These enzymes are widely distributed in bacteria, fungi, fish, several other animals, and elsewhere. In spite of the wide distribution, little is known^{2,3)} about the substrate specificity because a suitable method for the modification of chitin had not been found. Recently we found a mild method for the gel formation of chitosan by *N*-substitution,^{4,5)} and the method was applicable to the modification of chitin.

We now report the effects of the *N*-substitution and physical form of chitosan and the modification of its nonreducing end groups on the rates of hydrolysis by chitinase from *Streptomyces griseus*.

Several novel chitin derivatives (Table I) were prepared by substituting the acetamidomethyl (CH₃) group with H (*N*-formyl), ClCH₂ (*N*-monochloroacetyl), N₂NCH₂ (*N*-glycyl), (CH₃)₂CH₂ (*N*-iso-butyl) or CH₃(CH₂)₂CH₂ (*N*-pentanoyl). Their structures were confirmed by elemental analyses, by IR spectra and in part by PMR spectra. The detailed methods for the preparation of these novel derivatives will be reported elsewhere. The other *N*-acyl derivatives examined in the present study were prepared by the method reported pre-

TABLE I. THE HYDROLYSES OF VARIOUS CHITOSAN DERIVATIVES BY CHITINASE FROM *Streptomyces griseus*^a

Chitosan	Reducing sugar value increase ^b	Relative rate ^b
<i>N</i> -Acetyl, oxidized with NaIO ₄	3.69	15
<i>N</i> -Acetyl (gels)	3.09	13
<i>N</i> -Acetyl, oxidized with NaIO ₄ and then reduced with NaBH ₄	2.04	8.5
<i>N</i> -Acetyl	1.98	8.3
<i>N</i> -Acetylhexanoyl (d.s. = ~0.7: ~0.3, acetyl-hexanoyl)	1.59	6.2
Partially <i>O</i> -acetylated ^d		
<i>N</i> -acetyl	0.63	2.6
<i>N</i> -Propionyl	0.34	1.5
Natural chitin (crab shell)	0.24	1.0
<i>N</i> -Formyl	0.19	0.8
<i>N</i> -Butyryl	0.12	0.5
<i>N</i> -Isobutyryl	0.09	0.4
<i>N</i> -Glycyl ^e	0.07	0.3
<i>N</i> -Acetylhexanoyl (d.s. = ~0.3: ~0.7, acetyl-hexanoyl)	0.07	0.3
<i>N</i> -Monochloroacetyl	0.03	0.1

^a Partially *O*-monochloroacetylated *N*-monochloroacetyl (d.s. for *O*-acyl was not determined) and *N*-pentanoyl, *N*-hexanoyl, *N*-lauroyl, *N*-stearoyl, *N*-benzoyl, *N*-nicotinyl, *N*-methylene, *N*-benzylidene, and chitosan were not hydrolyzed by chitinase.

^b μmol/2 hr.

^b On the basis of the hydrolysis rate (=1.0) of natural chitin.

^c d.s. for *O*-acetyl = ~0.9/GlcNAc.

^e d.s. for *N*-glycyl = ~0.8/GlcN.

viously.^{4,5)} All the derivatives were insoluble in the buffer solutions.

A substrate (10 mg) to be examined was suspended in 1.0 ml of a buffer solution (0.05 M citric acid-0.1 M Na₂HPO₄, pH 6.0). To the suspension was added 4.0 ml of chitinase solution (0.32 mg/ml of the buffer solution). The mixture was incubated in a test tube at 37°C with mechanical shaking. Aliquots were withdrawn from the supernatant of the reaction mixture at appropriate time intervals, and the increase of the reducing sugar value was analyzed by a modified version of Schales' method⁶⁾ and calculated as μmol of 2-acetamido-2-deoxy-D-glucose.

Chitinase from *Streptomyces griseus* (3

units/mg, Sigma), which was used in the present study, hydrolyzed chitin into 2-acetamido-2-deoxy-D-glucose. The enzyme preparation may contain both chitinase and chitobiase. The increase of the reducing sugar value was almost proportional to the incubation time for up to 3 hr, and the relative rate of hydrolysis was compared with the reducing sugar value increase for 2 hr.

Chitinase was the most active on the acetamido groups of substrates, but the reaction was not restrictive. The relative rates of hydrolysis with respect to the substituents of the acetamido-methyl group were $\text{CH}_3- > \text{CH}_3\text{CH}_2- > \text{H}- > \text{CH}_3\text{CH}_2\text{CH}_2- > (\text{CH}_3)_2\text{CH}- > \text{NH}_2\text{CH}_2- > \text{ClCH}_2-$ (Table I). The enzyme did not hydrolyze chitosan or *N*-methylene-, *N*-benzylidene-, *N*-benzoyl-, *N*-nicotinyl- and *N*-fatty acyl($\text{C}_5\text{--C}_{18}$)-chitosans. Karrer and White³⁾ reported that snail chitinase did not hydrolyze *N*-formyl-, *N*-propionyl- or *N*-butyryl-chitosan. The inconsistency compared with the present results may be due to the different source of enzyme and to the different degree of substitution (d.s.) per hexosaminy residue for *N*-acyl groups: The d.s. values were <1.0 in the reported study³⁾ which indicates the presence of some free amino groups.

N-Acetylchitosan gels and the powdered xerogels were hydrolyzed eight to thirteen times as fast as natural chitin (crab shell). These results indicate that not only *N*-substitution but also the physical form of substrates affects the rates of enzymic hydrolysis. *N*-Acetylchitosan gels are formed by introducing solvent droplets into their microporous ultrastructures.⁷⁾ Chitinase ($M_r \sim 30,000$)⁸⁾ can enter the micropores and move almost freely through the membranous walls of the micropores.⁹⁾ On the contrary, natural chitin has no micropores in the ultrastructure, and the enzyme can not enter.

N-Acetylchitosan, after oxidation with NaIO_4 , was hydrolyzed by chitinase fifteen times as fast as natural chitin, but *N*-acetylchitosan, after oxidation with NaIO_4 followed by reduction with NaBH_4 , was hydrolyzed at almost the same rate as untreated *N*-acetylchitosan (Table I). The oxidation of a hexosaminy residue at the nonreducing end affords a product having two aldehyde groups. These groups may accelerate the formation of the enzyme-substrate complex, a more detailed study on the mechanism is under way in our laboratory.

The most striking feature of the present study is that *N*-acetylchitosan, in which the nonreducing end group was oxidized, is the most hydrolyzable and the rate was fifteen times faster than for natural chitin. The present study also assessed the novel potential utilization of chitin wastes as a novel form of substrate in chitin and lysozyme studies and as a digestable feedstuff as a carbon and nitrogen source for chickens and fish who secrete these enzymes.

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