Carbohydrate Research, 237 (1992) 325–332
Elsevier Science Publishers B.V., Amsterdam

Note

Unusual susceptibility of chitosan to enzymic hydrolysis

David Pantaleone, Manssur Yalpani and Mark Scollar
The NutraSweet Co., 601 East Kensington Road, Mount Prospect, Illinois, 60056 (USA)

(Received April 20th, 1992; accepted in revised form June 24th, 1992)

Chitosan and its *N*-acetylated analogue, chitin, are among the most abundant glycans in nature. The cationic charge of chitosan is unique among glycans, and its biological and physicochemical properties have been extensively studied in recent years[1]. Chitosan plays a regulatory role in eucaryotic organisms, in which it can either activate or suppress specific genes[2]. The aminopolymer and its oligomeric degradation products exhibit also immuno-potentiating and bacteriostatic activities[3–5]. With the emergence of these and other potential biomedical and food applications for chito-oligosaccharides, the development of viable processes for the hydrolysis of chitosan is attracting growing interest. Enzymic processes are generally preferable over chemical reactions, since the hydolysis course and product distribution are subject to more facile control. Chitosan hydrolyses with chitosanases and chitinases derived from various sources, including *Bacillus* sp. and *Serratia marcescens*, have been examined[6–8]. Chitinases are ubiquitous in plants, their pH optima are broad (pH 3–9 for chitin), and their substrate specificity is narrow, as they accept chitin but not cellulose[9]. Similarly, chitosanases have reported pH optima of pH 4.0–6.8, and narrow substrate specificity[10]. Chitinases and chitosanases are presently research reagents that are unavailable in bulk quantities for commercial applications. Of particular interest to us were, therefore, recent reports on the chitosanolytic activity of pectinases[11] and cellulases, for which optimal activities were found at near neutral pH and at 60°C[12]. As part of our interest in carbohydrate-based materials, we report herein our findings on the susceptibility of chitosan to a broad range of commercial enzyme preparations.

We focused initially on the chitosanolytic activities of cellulases, for which we employed a series of commercial cellulase preparations at a relatively high enzyme (1%) to chitosan addition level. The hydrolyses of low viscosity grade chitosan were performed at pH 4.5 and ambient temperature for 24 h. The treatments were

Correspondence to: M. Yalpani, The NutraSweet Co., 601 East Kensington Road, Mount Prospect, IL, 60056, USA.

monitored by HPLC-SEC to determine the generation of low degree of polymerization (dp) species, and qualitatively by neutralization of the cellulase-treated solutions to detect high molecular weight species by precipitation. Three of the six cellulase preparations tested caused significant chitosan hydrolysis, whereas the others resulted in either moderate or only insignificant hydrolysis as indicated by the absence of low dp species and the formation of precipitates upon neutralization. The three most active cellulases (derived from *Aspergillus niger*, *Trichoderma viride*, and *T. reesei*), were found to result in a 99% reduction of the viscosity of a solution of the native polymer ($\eta_{initial}$ 720 cps). The other cellulases caused either no or only modest hydrolysis.

These observations prompted us to examine other enzyme classes. In an initial screening experiment, we incubated low-viscosity chitosan solutions with a range of 13 different enzyme preparations, including six glycanases, six proteases and a tannase, using again relatively high concentrations (1% enzyme additions) at pH 4.5 as well as at pH 5.5. The course of the hydrolyses was followed by HPLC-SEC and viscosity measurements. Pectinase and hemicellulase treatments were found to effect viscosity reductions of 93–99%, and treatments with amylase, dextranase, three proteases and tannase resulted in substantial hydrolysis. Some enzyme preparations, e.g., a protease (Alcalase) and a hemicellulase (Gammanase), also displayed sensitivity to pH environments, being active at the lower, and inactive at the higher pH value. Overall, the efficient hydrolysis of chitosan by such an extensive array of enzymes was unexpected.

Encouraged by these findings, we incubated solutions of chitosan with an even broader selection of enzymes from various bacterial, fungal, plant, and mammalian sources, using more stringent pH environments (pH 3.3 and 3.6), and high-viscosity chitosan instead of the low-viscosity grade. The lytic activities were followed viscometrically as the most convenient means of screening. As shown in Table I, the results again indicated that a majority of the enzyme preparations tested mediated chitosan hydrolysis, even under these low pH conditions. Some 17 enzyme preparations effected viscosity reductions of between 66–100%, and 12 others produced viscosity reductions in the 20–60% range. However, nine enzyme preparations displayed no or only marginal hydrolytic activity, producing viscosity reduction in the range of 0–15% under these conditions (Table I). The glycanases and, with one exception, the lipases represented the most active groups of enzymes. Three proteases, particularly papain and a fungal protease, and tannase displayed also high lytic activities, while most of the marginal activities were observed for the remaining proteases.

Although the experiment was not designed to provide pH optima for the individual enzymes, it was unexpected to observe substantial lytic activity for such a broad range of enzyme preparations at the low pH values. As mentioned above, chitosanolytic activities of chitinases and chitosanases in relatively low pH media have previously been described[9,10], whereas a pH optimum of 5.6–6.0 was recently reported for cellulase and chitosan as substrate[12].

TABLE I

Susceptibility of chitosan to selected enzyme treatments

Enzyme	Source (Supplier [a])	pH	Viscosity reduction (%)
Glycanases			
Cellulase TV	*Trichoderma viride* (E)	3.6	99
Cellulase 300P	*Trichoderma reesei* (B)	3.6	87
Cellulase AP	*Aspergillus niger* (E)	3.6	99
Biocellulase AC20	*Aspergillus niger* (F)	3.6	71
Hemicellulase	*Aspergillus oryzae* (E)	3.6	99
Bioxylanase	*Trichoderma reesei* (F)	3.6	68
Pectinase G	*Aspergillus niger* (E)	3.6	99
Pectinex 3XL	*Aspergillus niger* (A)	3.6	87
Dextranase 50L	*Penicillium lilacinum* (A)	3.6	76
Fungamyl 1600S	*Aspergillus oryzae* (A)	3.6	79
Carbohydrases			
Lactozyme	*Kluyveromyces fragilis* (A)	3.6	0
Proteases			
Papain	*Carica papya* (E)	3.6	98
Acid-stable protease	*Rhizopus* sp. (E)	3.6	55
Fungal protease 31,000	*Aspergillus oryzae* (C)	3.6	96
Protease M	*Aspergillus oryzae* (E)	3.3	36
Protease N	*Bacillus subtilis* (E)	3.6	10
		3.3	0
Protease 2A	*Aspergillus oryzae* (E)	3.3	27
Prozyme 6	*Aspergillus oryzae* (E)	3.3	81
Peptidase A	*Aspergillus oryzae* (E)	3.3	20
HT Proteolytic 200	*Bacillus subtilis* (C)	3.6	7
Protease S	*Bacillus* sp. (E)	3.3	0
Newlase A	*Aspergillus niger* (E)	3.3	22
Proleather	*Bacillus subtilis* (E)	3.3	7
Fungal protease	*Aspergillus oryzae* (F)	3.3	32
Fungal protease S202	*Aspergillus oryzae* (I)	3.3	35
Bacterial protease S203	*Bacillus* sp. (I)	3.3	8
Rhozyme P41	*Aspergillus oryzae* (B)	3.3	25
Rhozyme P11	*Aspergillus oryzae* (B)	3.3	37
Rhozyme P64	*Bacillus licheniformis* (B)	3.3	56
Thermoase	*Bacillus thermoproteolyticus* (H)	3.6	53
Alcalase 2.4L	*Bacillus licheniformis* (A)	3.3	10
Neutrase 0.5L	*Bacillus subtilis* (A)	3.3	8
Lipases			
Lipase MAP-10	*Mucor* sp. (E)	3.6	35
Lipase AIE	*Aspergillus niger* (E)	3.6	100
PPLipase	Porcine pancreas (D)	3.6	84
Lipase 3TBU	*Aspergillus oryzae* (B)	3.6	15
Microbial lipase	*Aspergillus niger* (I)	3.6	100
Other			
Tannase SP292	*Aspergillus oryzae* (A)	3.6	74

[a] A, Novo Chemical Co., Inc.; B, Genencor International, Inc.; C, Miles Laboratories, Inc.; D, Sigma Chemical Co.; E, Amano International Enzyme Co., Inc.; F, Quest International; G, Crescent Chemicals, Inc.; H, Daiwa Kasei KK; I, Enzyme Development Co.

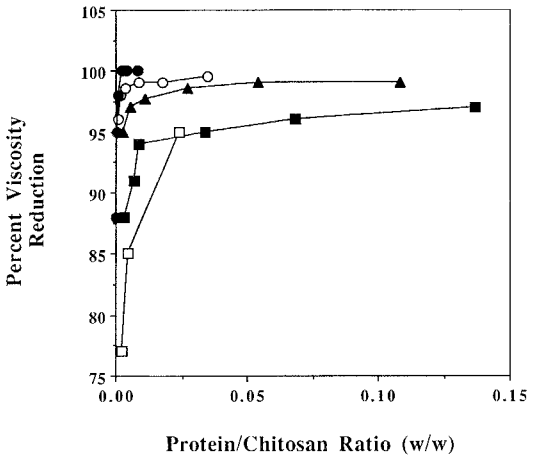

Fig. 1. Percent viscosity reduction of a chitosan solution (5% aq HOAc, pH 3.6) as a function of protein : chitosan ratios (w/w) after 18-h enzyme treatments at 25°C with: (▲) Cellulase TV; (●) Lipase AIE; (■) papain; (○) hemicellulase; and (□) chitinase. Viscosity of untreated 0.5% chitosan solution: 432 cps.

From Table I, we selected two glycanases (Cellulase TV, hemicellulase), a lipase (Lipase AIE) and a protease (papain) as representatives of the most active enzyme preparations for further investigations. The dose response was established for these enzyme preparations and compared to that of a chitinase (*Serratia marcescens*), based on viscosity reductions of a high-viscosity chitosan solution (0.5%, pH 3.6). To account for the varying inert filler ratios in the commercial preparations, all enzyme levels were expressed on a protein weight basis. Fig. 1 illustrates viscosity reductions of 94 and 95% with papain and chitinase at protein : chitosan ratios of 0.09 and 0.024, respectively. Lipase, cellulase, and hemicellulase treatments produced identical viscosity losses at substantially lower protein : chitosan ratios (0.0004–0.003).

To obtain a measure of the hydrolysis rates, we then followed viscometrically the activities of chitinase and the same four selected enzyme preparations at pH 3.3 as a function of time for up to ~ 20 h. The resulting viscosity profiles are depicted in Fig. 2. Specific enzyme activities during the initial ten minutes of hydrolysis were determined from plots of the ratio of the specific viscosities at time zero over the specific viscosity after different reaction times versus time, as illustrated in Fig. 3. It is evident from the slopes of the curves in Fig. 3, that the fastest hydrolysis rate was associated with papain under the experimental conditions employed here. All four enzyme preparations tested displayed more rapid lytic activity than chitinase. Specific activities were determined from the slopes obtained from Fig. 3 and the respective protein concentrations employed (Table II). Table II shows that papain displayed the highest specific acitivity, followed sequentially by hemicellulase, lipase, cellulase and chitinase. It may be noted here

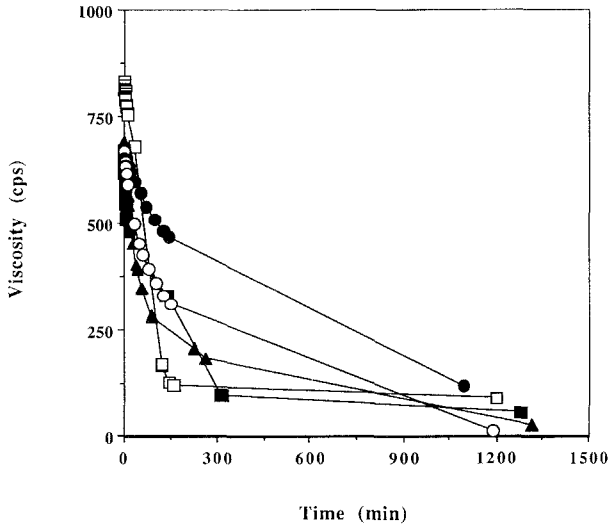

Fig. 2. Viscosities of chitosan solutions as a function of time following treatment with enzymes at pH 3.3 and 20.5°C; (▲) Cellulase TV; (●) Lipase AIE; (■) papain; (○) hemicellulase; and (□) chitinase.

that under different experimental conditions, intermediate specific activities were found for chitinase from *S. marcescens* in comparison to those from other sources[6].

Our investigation has demonstrated the hydrolytic susceptibility of chitosan to a wide range of enzyme preparations, including glycanases, lipases, proteases and a tannase derived from bacterial, fungal, mammalian and plant sources. The study extends the scope of enzymatic processes, and reaffirms recent reports of cellulase- and pectinase-mediated chitosan hydrolyses[11,12]. The observation of lytic activity

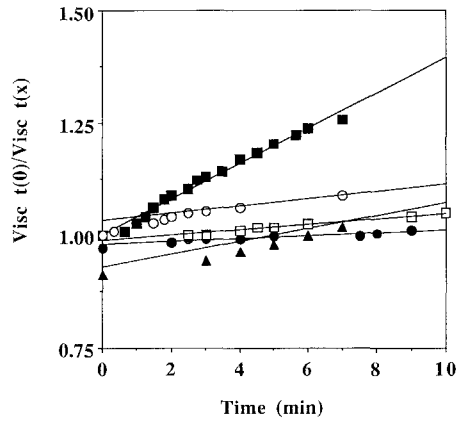

Fig. 3. Viscosity changes of chitosan solutions following treatment with enzymes at pH 3.3 and 20.5°C expressed as the ratio of the viscosities at times 0 and x ($Visc_{t(0)}/Visc_{t(x)}$), as a function of time for the first 10 min of the hydrolyses; for symbols see Fig. 2.

TABLE II

Comparison of specific activities for selected enzymes

Enzyme [a]	Slope [b] ($\times 10^2$) ($\eta_{sp\,t(0)}/\eta_{sp\,t(x)}$ min^{-1})	Protein used (mg)	Specific activity ($\eta_{sp\,t(0)}/\eta_{sp\,t(x)}$ min^{-1} mg^{-1})
Cellulase TV (E)	1.54	0.0594	0.260
Lipase AIE (E)	34.76	0.0064	0.543
Papain (E)	3.937	0.0197	1.998
Hemicellulase (E)	1.24	0.0192	0.647
Chitinase (D)	65.11	0.0750	0.087

[a] Suppliers; E, Amano; D, Sigma. [b] Obtained from curves in Fig. 3.

for the extensive array of enzyme preparations, normally unknown for chitosanolytic activity, is highly unusual, particularly in view of the narrow substrate specificity of established chitosanases[10]. There are, to the best of our knowledge, no previous reports of such ubiquitous chitosanase activities of enzymes derived from various sources. Since the commercial enzyme preparations we used were impure, the presence of minor enzyme contaminations is a likely contributor to the observed lytic activities. However, this would still be a surprising finding, in the light of the higher specific activities of the four enzyme preparations in comparison to that of chitinase. A common contaminant in all of the enzyme preparations seems implausable, since no or minimal lytic activities were observed for about a fourth of the catalysts examined. Clearly, the nature of the enzyme or enzymes responsible for the chitosan hydrolysis still needs to be elaborated. It also remains to be determined whether the activities are catalyzed by a true chitosanase or, for instance, a cellulase with broad specificity. We are extending our studies to chitosans with varying degrees of *N*-acetylation, as well as to chitin substrates.

We believe that, regardless of the underlying catalytic mechanisms, these findings are of interest, because they open up prospects for industrially viable modifications of chitosan with inexpensive enzymes. Among the potential applications of these enzyme preparations are assays for the degree of chitosan *N*-acetylation, the treatment of seafood-derived wastes, the production of feedstock chemicals for fermentation and other processes, and biocontrol agents in agricultural applications, in addition to various pharmaceutical uses.

EXPERIMENTAL

Polymer and enzyme sources.—Chitosan (Seacure low- and high-viscosity grades, with ~ 15% *N*-acetyl content, as specified by the supplier) was purchased from Protan Laboratories, Inc. (Redmond, WA). The enzymes were obtained from Novo Chemical Co., Inc. (Danbury, CT), Genencor International, Inc. (San Francisco, CA), Miles Laboratories, Inc. (Elkhart, IN), Sigma Chemical Co. (St. Louis, MO), Amano International Enzyme Co., Inc. (Troy, VI), Quest International (Lexington,

KY), Enzyme Development Co. (New York, NY), Crescent Chemicals, Inc. (Westbury, NY), and Daiwa Kasei KK (Osaka, Japan).

Substrate preparation.—Stock solutions of chitosan (1.0 or 2.5% (w/v) for low-viscosity (LV) grade, 0.5 or 1.0% (w/v) for high-viscosity (HV) grade) were prepared in either 5% (w/v) aqueous acetic acid or in acetate buffer, pH 4.0. All solution concentrations are reported as % (w/v), unless otherwise stated, and the pH was carefully adjusted to the desired values.

Enzymatic hydrolyses.—For the cellulase treatments, the enzyme preparations (250 mg) were added to the stock solution (25 mL, pH 4.5), and the incubation was performed with stirring at ambient temperature for 24 h. The pH was then adjusted to pH 7–9, to precipitate the chitosan. The precipitates were collected by centrifugation and lyophilization. For the samples which did not form a precipitate, the enzymes were first destroyed by briefly autoclaving (5 min) the samples, and subsequently removed by processing with an Amicon pressure cell with a PM 30 membrane (30 000 MW cutoff). The hydrolyses were monitored either by HPLC-SEC after 0.5, 2.5, 6 and 24 h, or by viscosity determinations. Food-grade proteases were exhaustively dialyzed against potassium phosphate (10 mM, pH 7.0, 0.02% NaN_3), lyophilized and then reconstituted with distilled water (5 mg solid/mL). Solutions of HV chitosan (0.5%, 10 mL) were incubated overnight with 20 μL of each enzyme with stirring at 20°C. Viscosities were measured, and the data was expressed as percent viscosity reduction based on an untreated chitosan control solution.

Dose–response profiles were obtained for chitinase (*Serratia marcescens*, Sigma), Cellulase TV, hemicellulase, papain and Lipase AIE. Solutions of HV chitosan (25 mL) were mixed with different amounts of the enzymes in distilled water (2 mL), with enzyme-to-chitosan ratios of 0.04–1.6, and incubated overnight. Hydrolysis rates were determined by monitoring the reaction viscometrically. The solid enzymes (20 mg) were dissolved in distilled water (0.5 mL), and various aliquots, generally 20 μL, of this stock solution were used to initiate the reaction. Incubations were performed with HV chitosan solutions (0.5%, 10 mL), and the viscosity was monitored over time. Final protein concentrations in the above reactions were 5.93, 0.64, 1.97, 1.91 and 7.5 μg/mL, for Cellulase TV, hemicellulase, papain, Lipase AIE, and chitinase, respectively.

Analyses.—Molecular weight determinations were conducted by HPLC, using a series of three Waters Ultrahydrogel analytical columns (2000, 500, and 120) with sample concentrations of 1.5 mg/mL and 7:3 acetonitrile–water as the mobile phase. Fractionation of the chitosan hydrolysates was performed on a Waters μ-Bondapak amine column and an Erma Optical Works RI detector, using chitopentaose and a chitooligosaccharide mixture (dp 1–5) as calibration standards (Seigaku Kogyo Co). Viscosity determinations were performed at 20.5°C with a Brookfield DV-II instrument, using spindle #21 at 50 rpm. Protein analysis was performed using the dye-binding method[13] adapted to a Molecular Devices Thermomax microtiter plate reader. Specific enzyme activities during the initial hydrol-

ysis periods were determined from plots of the ratio of the specific viscosities at time zero over the specific viscosity after different reaction times ($Visc_{t(0)}/Visc_{t(x)}$) versus time, according to methods previously developed for endo $(1 \rightarrow 4)$-β-D-glycanases[14].

ACKNOWLEDGEMENTS

We thank Mr. Mike Hsiao for his skillfull technical assistance and Dr. Nigel Grinter for useful comments.

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