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An examination of the unusual susceptibilities of aminoglycans to enzymatic hydrolysis

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ABSTRACT

The hydrolytic susceptibilities of aminoglycans, including chitosan, chitin, water-soluble chitin, chitin azure, and α-(1 → 4)-poly(galactosamine), to a series of commercial enzyme preparations were examined. An unexpectedly large number of enzyme preparations gave rise to varying degrees of aminoglycan hydrolysis. Remarkably, several of these enzyme preparations displayed lytic activities towards chitosan that equaled or surpassed those of established catalysts with chitosanolytic activities, such as chitinase and lysozyme. Thus, based on their dose–response profiles, a number of proteases, such as pepsin, bromelain, ficin, and pancreatin, were more efficient catalysts for chitosan hydrolysis than a commercial chitinase (*Serratia marcescens*) and lysozyme preparation. For a cellulase, hemicellulase, lipase, and protease evidence was obtained that strongly suggested the absence of a common lytic agent. Thus, different profiles were observed when the lytic activities of these enzyme preparations were examined in terms of their pH and temperature optima, susceptibilities to substrate concentration and the degree of substrate *N*-acetylation, and their molecular weight fractions. Similarly, distinctions in hydrolytic efficacy emerged for several enzyme preparations, when chitosan solutions were subjected to two simultaneous or sequential enzyme treatments. Chitosan hydrolysis was also observed upon treatment with human salivary preparations. Preparative-scale hydrolyses of chitosan were performed with papain and hemicellulase preparations at pH 3 and 40°C. The results demonstrate the feasibility of hydrolyzing chitosan, chitin and other aminoglycans with several low-cost enzymes.

INTRODUCTION

Chitosan is in many respects unique among glycans, and its biological and physicochemical properties have been extensively studied in recent years[1]. The aminopolymer and its oligomeric degradation products exhibit biological activities[2–4]. With the emergence of various potential biomedical and food applica-

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tions for chito-oligosaccharides, the development of viable processes for the hydrolysis of chitosan is attracting growing interest. Enzymatic processes have advantages over chemical reactions, since the hydrolysis course and product distribution are subject to more facile control. Chitosan hydrolyses with chitosanases and chitinases derived from various sources have been examined[5–7]. Chitinases are ubiquitous[8], their pH optima are generally broad, and their substrate specificity is narrow[9]. Similarly, chitosanases have reported pH optima of 4.0–6.8, and narrow substrate specificity[10].

For commercial applications, the utility of chitinases and chitosanases is limited as they presently constitute costly research reagents that are unavailable in bulk quantities. In view of this, and prompted by indications of the chitosanolytic activity of pectinases[11] and cellulases[12], we have initiated investigations on the susceptibility of glycosaminoglycans to enzymatic hydrolysis[13].

In a preliminary report, we described the unexpected chitosanolytic activities of a substantial number of commercial enzyme preparations, including glycanases, proteases, lipases, and others[13]. Among the glycanases, several cellulases, as well as an amylase, dextranase, hemicellulase, and pectinase were found to effect chitosan hydrolysis at ambient temperatures and relatively low pH values (pH 3.3–4.5), as manifested by 93–99% viscosity reductions of dilute glycan solutions. In examining several of the more active preparations under these conditions, we found higher specific activities for two glycanases (Cellulase TV and Hemicellulase), a lipase (Lipase AIE), and a protease (papain), than for a chitinase (of *Serratia marcescens*). Further evidence for the efficient activity of glycanases has subsequently also been demonstrated by Muraki and co-workers[14], who prepared multigram quantities of chito-oligosaccharides with a degree of polymerization (dp) of 6–8, using a cellulase of *Trichoderma viride*. Their hydrolyses were conducted over several hours at elevated temperature (50°C) and pH 5.6.

The availability of a broad spectrum of enzyme preparations as potential alternatives to the presently identified chitinases and chitosanases opens up prospects for viable, large-scale preparations of chito-oligosaccharides. On the other hand, the observation of lytic activity for the extensive array of enzyme preparations, normally unknown for chitosanolytic activity, is highly unusual and intriguing, particularly in view of the narrow substrate specificity of established chitosanases[9]. Since the commercial enzyme preparations we employed were not further purified, the presence of minor enzyme contaminations was a likely contributor to the observed lytic activities. However, this would still be a surprising finding, in light of the higher specific activities of the four enzyme preparations in comparison to that of a chitinase (*S. marcescens*). A common contaminant in all of the enzyme preparations seemed implausible, since (*i*) they were derived from a broad spectrum of bacterial, fungal, mammalian, and plant origins, and (*ii*) no or minimal lytic activities were observed for about one-fourth of the catalysts examined, including several derived from microorganisms that are otherwise associated with chitosanolytic activities.

We report herein on the susceptibility of chitosan and other aminoglycans to a range of enzyme preparations. We also describe our efforts aimed at elucidating possible mechanisms for the observed lytic activities.

RESULTS AND DISCUSSION

In a comparative experiment, we examined the activities of several commercial enzyme preparations together with catalysts with established chitosanolytic activities. Dilute aqueous acetic acid solutions of high-viscosity grade chitosan were separately treated with equal amounts (on a weight basis) of a number of proteases, including pepsin, bromelain, and ficin, as well as with a chitinase (*Serratia marcescens*) and lysozyme. The proteases reduced the chitosan solution viscosity by 89–98%, whereas lysozyme resulted in a 77% reduction in viscosity. Based on these initial findings, we determined the dose response profiles of these enzyme preparations. The results of these experiments (Figs. 1a and b) clearly indicated that, based on the low dosage required for chitosan hydrolysis, the proteases bromelain and pepsin were more efficient catalysts than both a commercial chitinase and lysozyme preparations. Ficin, on the other hand, required doses similar to chitinase (*S. marcescens*). These findings agree with dose–response results for another protease preparation, i.e., papain, as well as those of cellulase, hemicellulase, and lipase preparations, which effectively hydrolyzed chitosan at lower enzyme-to-substrate ratios than a chitinase[13].

We then compared the activities of enzymes with established chitosanolytic activities, including lysozyme and chitinases from different sources (*S. marcescens* and a cloned chitinase) and with differing degrees of purity, with those of two other commercial enzyme preparations (hemicellulase and Lipase AIE) that we had previously identified as effective catalysts. The effects of treating 0.5%

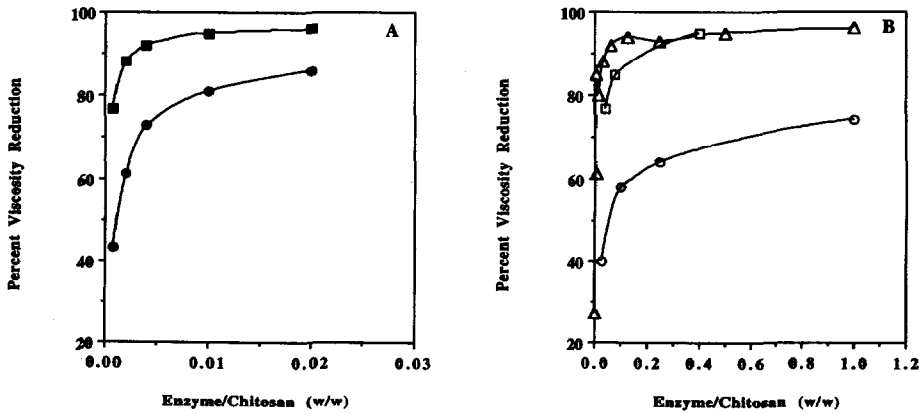

Fig. 1. Dose–response comparison for chitosan hydrolysis by selected proteases, a chitinase and lysozyme. (A) ■, pepsin; ●, bromelain. (B) ○, Lysozyme; △, ficin; and □, chitinase (*S. marcescens*).

TABLE I

Comparison of single and combined hydrolytic treatments of chitosan

Enzyme treatment (μg /g chitosan)	Dosage (μmol/mL)	Viscosity reduction (%)	Increase in reducing sugar (equiv)
Lysozyme	1,724	71.7	0.407
Hemiccellulase	361	80.4	0.599
Lipase AIE	102	54.3	0.316
Chitinase [a]	3,000	95.2	0.667
Cloned Chitinase [b]	542	7.5	0.099
Cloned Chitinase [c]	51	5.1	0.053
Lysozyme and Hemicellulase	2,085	89.5	0.668
Lysozyme and Lipase AIE	1,826	81.9	0.620
Lysozyme and Chitinase	4,724	95.2	0.665

[a] *S. marcescens.* [b] Crude preparation. [c] Purified preparation.

high-viscosity chitosan solutions in acetate buffer at pH 3.3 with these enzyme preparations for 24 h were monitored both viscometrically and by reducing sugar assay (see Table I). The results of these independent activity measurements revealed a close correlation between the assays, with viscosity reductions being accurately reflected by increases in liberated reducing sugars. Furthermore, the cloned chitinase, both in its crude and purified form, gave rise to only insignificant hydrolysis (i.e., $< 10\%$ viscosity reductions) under these conditions, whereas the chitinase from *S. marcescens* and lysozyme gave rise to 95 and 72% viscosity reductions, respectively. This is possibly ascribable to the absence of one or more lytic components (e.g., with endo activity) in the cloned preparations. It is noteworthy here, that the enzyme-to-substrate ratio of the crude cloned chitinase was about one-fifth that of the chitinase from *S. marcescens* and about one-third that of lysozyme. The hemicellulase and lipase treatments resulted by comparison in 80 and 54% viscosity reductions, respectively. The dosages for these two preparations represented only 3 and 12%, respectively, that of the microbial chitinase and about 6 and 21%, respectively, that of lysozyme. Thus, based on the enzyme-to-substrate ratios employed, the hemicellulase and lipase preparations were substantially more effective hydrolytic catalysts than either the chitinases or lysozyme under these experimental conditions.

We also found that human saliva preparations were very effective in hydrolyzing chitosan, as indicated in Fig. 2. Although the degree of chitosan depolymerization during the first 48 h of the treatment did not match those of some of the enzyme preparations described above, viscosity reductions of over 80% were eventually achieved after ~ 7 days. Chitosanolytic and chitinolytic activity in saliva has been previously described[15,16], but, to the best of our knowledge, not in mammalian species. Among the constituents of human saliva[17–19] is lysozyme in relatively high preponderance, together with other carbohydrases. The observed chitosanolytic activities are therefore not surprising. The somewhat attenuated lytic potency of

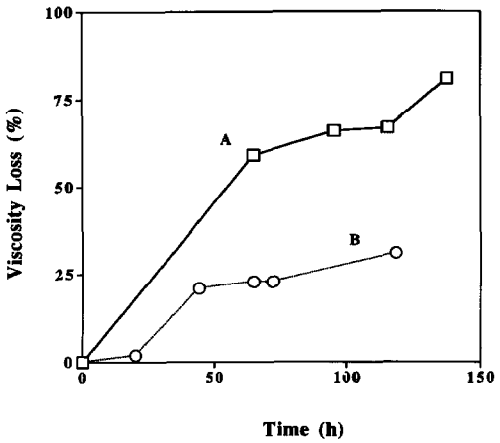

Fig. 2. Viscosity–time profile of chitosan solutions [A, 1% and B, 2%] treated with human salivary preparations at ambient temperature.

the human salivary preparation we observed here may in part be due to the acidic conditions we employed, as the highest reported activity for lysozyme from human saliva was at pH 9.0 at low ionic strength (*I* 0.03, ref 20). It is noteworthy that egg-white lysozyme can mediate transglycosylation at a higher rate than hydrolysis under certain conditions.[21]

Lytic activities as a function of molecular weight, pH, and temperature.—In order to provide further support for differences among the active components, four representative enzyme preparations (Cellulase TV, hemicellulase, Lipase AIE, and papain) with high lytic activities were subjected to a fractionation study in which different molecular weight fractions were monitored for lytic activity by viscometry[22]. The results obtained[22] indicated different fractionation patterns for all four enzyme preparations. This suggests that the lytic activities are associated with different molecular weight species for these enzyme preparations. Based on these results, it appears unlikely that identical chitinase or chitosanase impurities were present in all of the above enzyme preparations.

Additional evidence for differences in lytic activity among these four enzyme preparations were noted with respect to their pH optima. The cellulase preparation displayed a pH optimum of 4.5, whereas the papain, Lipase AIE and hemicellulase preparations revealed pH optima of 3.0–4.0, 3.0, and 3.5, respectively. It is noteworthy, that in comparison to these relatively narrow optima, the reported pH optima for most, but not all[23], established chitosanases are broad (pH ~ 4.0–6.5)[11,24,25].

Similar observations were made, when the temperature dependence of the same enzyme preparations were examined. The data in Fig. 3 indicate that after hydrolysis for 3.5 h the lytic activity of papain remained high and essentially constant in the region between 30 and 60°C, whereas it increased for hemicellulase and Lipase AIE, but decreased for Cellulase TV with increasing temperature.

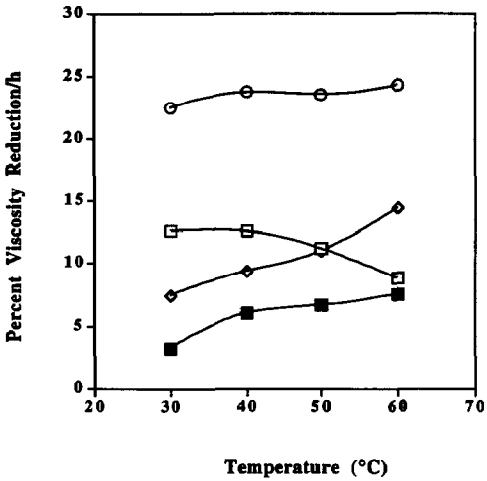

Fig. 3. Percent viscosity reduction per h for a 1% chitosan solution after enzyme treatment for 3.5 h as a function of temperature. Enzymes tested were: □, Cellulase TV; ◇, hemicellulase; ○, papain; and ■, Lipase AIE.

Table II summarizes the reducing sugar results obtained after 20 h hydrolysis in the temperature regime of 40–60°C. At this point, the amount of liberated reducing sugar remained essentially constant for papain and Lipase AIE, whereas it decreased about 30 and 60% for hemicellulase and cellulase, respectively. Thus, the temperature studies also failed to indicate identical lytic profiles for the four selected enzyme preparations.

TABLE II

Influence of temperature on liberated reducing sugar from chitosan for selected enzymes [a]

Enzyme preparation	Temperature (°C)	Reducing sugar (ΔGlcNAc/equiv)
Cellulase TV	40	0.22
	50	0.16
	60	0.06
Lipase AIE	40	0.20
	50	0.22
	60	0.18
Hemicellulase	40	0.31
	50	0.28
	60	0.23
Papain	40	0.31
	50	0.31
	60	0.27

[a] After 20-h hydrolysis.

TABLE III

Influence of chitosan concentration on enzymatic hydrolysis

Enzyme preparation	Specific activity [a]		
Chitosan concentration	2%	1%	0.5%
Cellulase TV	0.64	6.13	15.83
Hemicellulase	1.34	53.54	73.80
Lipase AIE	0.00	148.56	286.20
Papain	0.36	4.86	4.77

[a] μmol GlcNAc equiv/h/mg protein.

Lytic activities as a function of chitosan concentration.—Chitosan solutions at several concentrations [0.5, 1.0, and 2.0% (w/v)] were treated with different enzyme preparations. Cellulase TV, hemicellulase, Lipase AIE, and papain preparations were added, followed by incubation at ambient temperature. Aliquots of the reaction mixtures were removed after 2 h and analyzed for reducing sugar content. Enzymatic treatments of the most concentrated (2%) chitosan solution resulted in no detectable release of reducing sugar for lipase, whereas low specific activities were noted for hemicellulase, Cellulase TV, and papain (see Table III). For the lower concentration region, it is interesting to note that lipase displayed the highest specific activity, whereas papain was apparently unaffected by substrate concentrations. The other two enzyme preparations revealed substantially reduced (~ 30–50%) specific activities when the solution concentration was increased from 0.5 to 1.0%.

The reasons for the observed reductions in specific activities with increasing substrate concentration are not fully clear at this point. Among the potential contributing factors are inactivation of the lytic catalysts by high solution viscosities and inhibition by substrate or hydrolysis products. Thus, Price and Storck[24] reported reaction inhibition for a chitinase from *Streptomyces* sp No. 6 at chitosan concentrations above 0.5 mg/mL. Fenton and Eveleigh[25] noted chitinase (from *Penicillium islandicum*) inactivation by certain chito-oligomers produced. On the other hand, no inhibitory effects of hydrolyzate products were described for a chitinase from *Streptomyces* sp S-84 (ref. 26).

Overall, the four enzyme preparations tested here displayed dissimilar dependencies on substrate concentrations, providing further suggestive evidence against a common lytic agent.

Effects of simultaneous and sequential enzyme treatments.—In view of the differential hydrolytic profiles described above, additional experiments were conducted to investigate potential synergistic or complementary activities, by using combined treatments of lysozyme with either chitinase (*S. marcesens*), Hemicellulase, or Lipase AIE. As indicated in Table I, synergy was apparent when lysozyme was used together with either hemicellulase or lipase, since the final viscosity values were lower than those of the individual enzyme preparations alone. These

TABLE IV

Effects of sequential hydrolytic treatments of chitosan solutions with two enzyme preparations

No.	Hydrolytic treatment (Enzyme concentration, μg enzyme/g chitosan)		Reducing sugar content (μmol GlcNAc equivalents)			Viscosity reduction (%)
	Initial	Second	After initial treatment	After second treatment	Difference (Δμmol)	
1	Lysozyme (21.55)	Hemicellulase (144.2)	0	0.200	0.200	52.5
2	Lysozyme (86.20)	Hemicellulase (144.2)	0.014	0.266	0.252	74.2
3	Lysozyme (215.5)	Lipase AIE (40.80)	0.040	0.175	0.135	62.5
4	Lysozyme (862.0)	Lipase AIE (40.80)	0.134	0.233	0.099	73.3
5	Hemicellulase (144.2)	Lysozyme (862.0)	0.116	0.236	0.120	81.5
6	Lipase AIE (40.80)	Lysozyme (862.0)	0.135	0.206	0.071	68.6

observations seem to be therefore indicative of different modes of action for the lytic principles involved. On the other hand, the simultaneous lysozyme and chitinase treatment afforded the same endpoint viscosity as the chitinase hydrolysis. Based on these rheological observations, as well as the reducing sugar assays, the course of chitosan hydrolysis seemed to be predominantly influenced by the chitinase activity, rather than by that of lysozyme.

In a second approach, chitosan solutions (1.0 or 0.5% HV) were first treated with an enzyme preparation for 24 h at ambient temperature. An aliquot of the reaction mixture was then removed to determine the reducing sugar content and the enzyme in the remaining solution was inactivated by microwave irradiation. A second enzyme preparation was then added to the hydrolyzed chitosan solution, followed by another 24-h incubation. Following this treatment, the extent of hydrolysis was determined by viscosity and reducing sugar measurements. The results of these experiments are summarized in Table IV. In comparing reactions 1 and 2 with an equal dose of hemicellulase for the second treatment, it is apparant that reaction 2 with the higher initial lysozyme treatment showed a greater viscosity reduction and a more significant difference in reducing sugar content. For reactions 5 and 6 with an equal lysozyme dose in the second treatment, an initial treatment with hemicellulase (reaction 5) was found to be more effective in reducing viscosity and producing a higher level of reducing sugar equivalents than a treatment with Lipase AIE (reaction 6). However, for reactions 3 and 4, the reverse trend was noted for equal dosage second treatments with Lipase AIE. The initial high-lysozyme dose (reaction 4) afforded a higher viscosity reduction, while the lower lysozyme dosage (reaction 3) yielded a greater difference of reducing

TABLE V

Comparison of hydrolytic activities of enzyme preparations as a function of substrate *N*-acetylation for chitosan and water-soluble chitin

Enzyme preparation	Initial viscosity reduction rates [Percent viscosity reduction/s/mg protein] [a]				
	Chitosan				Water-soluble chitin
	Percent free amine				
	70	80	90	100	
Bromelain	1.98	–	0.82	0	2.40
Cellulase TV	0.17	0.07	0.11	0	0.47
Papain	2.47	3.67	1.68	0	3.30
PPLipase	0.25	–	–	0	0

[a] pH 3.0, ambient temperature.

sugar equivalents. In all cases, these sequential treatments demonstrated that the end result was directly dependent on the initial treatment. Thus, if a specific viscosity reduction is desired, such digestions may be achieved by appropriate selection of the initial enzyme treatment.

Lytic activities as a function of the degree of N-*acetylation.*—Further support for heterogeneities in the lytic activities of the various enzyme preparations were obtained from studies with chitosan substrates of varying degrees of residual *N*-acetyl substitution. In addition, a partially *N*-deacetylated (~ 50%), water-soluble chitin sample was examined. The results, listed in Table V, indicated again different substrate specificities for each of the four selected enzyme preparations. Higher specific activities (percent viscosity reduction/s/mg protein; pH 3.0, 25°C) were generally observed for higher levels of residual *N*-acetylation. An exception was noted for PPLipase, which hydrolyzed the 30% acetylated chitosan sample, but not the chitin preparation. Fully *N*-deacetylated chitosan constituted no substrate for any of these enzyme preparations.

These findings are in qualitative agreement with similar studies of chitosanases by other workers. Thus, Yabuki[27] reported an 80% deacetylated chitosan to be the preferred substrate for a chitosanase from *Bacillus circulans*. Chitosans with degrees of *N*-acetylation of 0.4–0.8 were noted to be most rapidly hydrolyzed by chitinases of *Streptomyces* spp.[28]. A fungal chitosanase (*Penicillium islandicum*) hydrolyzes chitosan with 33–70% deacetylation[25]. Other microbial chitinases, including those elaborated by *Serratia marcescens* and *Aeromonas hydrophila*, are known to display greater hydrolysis rates for substrates with decreasing degrees of *N*-acetylation, being much less active for chitosan with 7–10% acetylation[5]. It has also been generally noted that fully deacetylated chitosan is not susceptible to enzymatic hydrolysis[29].

Enzymatic treatment of chitin and α-1,4-poly(galactosamine).—In an attempt to hydrolyze chitin, we treated aqueous suspensions with the above four enzyme preparations. Although the water-soluble chitin described above was hydrolyzed by

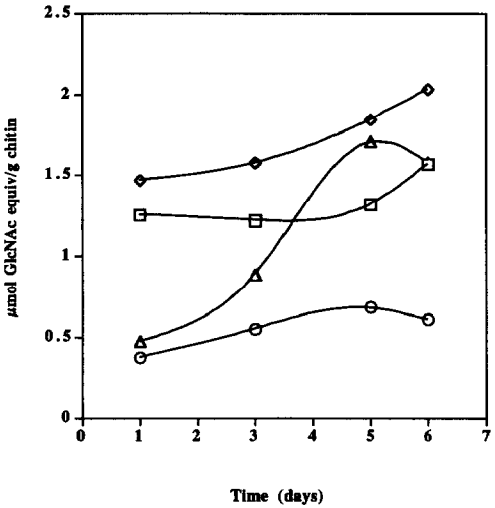

Fig. 4. Time-course of chitin hydrolysis for different enzymes: □, Lipase AIE; ◇, hemicellulase; △, papain; and ○, Cellulase TV. Chitin samples were pretreated with aqueous acetic acid (see Experimental section).

three of the four enzyme preparations examined, no indications of hydrolysis were obtained for insoluble chitin under these conditions. This lack of hydrolytic susceptibility was not unexpected, as it presumably reflects the intractable nature of chitin. Previous studies have encountered a similar obstacle for chitinases and resolved the problem by using colloidal or otherwise pretreated chitin preparations as substrate. A fungal chitinase has, for instance, been reported to rapidly hydrolyze regenerated chitin[23]. As an alternative to the moderately laborious preparation of colloidal[30] or partially deacetylated chitin, we suspended chitin powder in aqueous acetic acid for several days and generated a homogeneous suspension. The preswollen chitin thus obtained was then directly treated with the Cellulase TV, hemicellulase, Lipase AIE, and papain preparations for several days at ambient temperatures. Based on reducing sugar analysis, we found (see Fig. 4) that hydrolysis did indeed occur, albeit at a much slower rate than for the chitosan solutions described above.

In a related experiment, a dye-modified chitin, chitin azure[31], was suspended in dilute aqueous acetic acid and also subjected to enzymatic treatments at room temperature for 24 h. The progress of the hydrolysis was monitored by measuring the released dye spectrophotometrically. The results of these experiments are summarized in Table VI. The gradual hydrolysis of the substrate is again evident. Thus, chitin hydrolysis could be achieved with commercial enzyme preparations under both of the above conditions.

In order to further probe the scope of the lytic activities of the commercial enzyme preparations, we treated a dilute aqueous acetic acid solution of α-(1 →

TABLE VI

Enzymatic degradation of dye-modified chitin [a]

Treatment	Dose (μg enzyme)	Absorbance			
		Observed A_{575}	Increase ΔA_{575}	Observed A_{575}	Increase ΔA_{575}
Time period		17 h		45 h	
Control [b]	–	0.056	–	0.068	–
Cellulase TV	150.3	0.068	0.012	0.089	0.021
Hemicellulase	100.4	0.057	0.001	0.081	0.013
Lipase AIE	21.1	0.062	0.006	0.082	0.014
Papain	213.1	0.063	0.007	0.075	0.007

[a] Chitin–azure. [b] Water.

4)-poly(galactosamine) with Cellulase TV and papain under conditions similar to those described above. Reducing sugar analysis was performed after a 48-h incubation, and the hydrolysis results were expressed in terms of micromolar equivalents of *N*-acetylglucosamine per gram of polymer. For Cellulase TV and papain treatments, 50.35 and 40.52 μmol GalNAc equiv/g polymer above the control (no enzyme treatment) were observed, respectively. These results indicate that α-(1 → 4)-poly(galactosamine) is a better substrate for Cellulase TV than for papain under these conditions. The findings demonstrate that the hydrolytic activities of the enzyme preparations examined also apply to several representatives of the aminoglycan family.

An additional experiment was also conducted with xanthan as a representative of nonaminated glycans. Treatment of aqueous xanthan solutions (1.5%, pH 6.22) with Cellulase TV, hemicellulase, Lipase AIE, and papain for 5 days (at ambient temperature and similar dosages as employed above) did not result in any noticeable hydrolysis as indicated by viscosity measurements. Although this experiment does not warrant general conclusions about the hydrolytic susceptibilities of other nonaminated glycans, it provides an indication that, at least under these conditions, none of the enzyme preparations tested displayed activity for xanthan. Investigations of other glycans are in progress, the results of which will be reported elsewhere.

Preparative-scale hydrolyses of chitosan.—We also applied the potent chitosanolytic activities of two commercial enzyme preparations for larger scale preparations of chito-oligosaccharides. Thus, a 2% chitosan solution in aqueous acetic acid was treated with either papain or hemicellulase preparations for 2–3 h at pH 3 and 40°C. Additional enzyme was added and, after the glycan concentration was raised to 4% by dissolving chitosan in the partially hydrolyzed solution, the treatment was continued for ~ 2–3 h. This step was repeated once more, so that effectively a 6% chitosan solution was hydrolyzed overall. The effects of these treatments are summarized in Table VII. We also included for comparison the characteristics of a chemically-hydrolyzed, commercial shrimp chitosan sample.

TABLE VII

Effects of preparative-scale enzyme treatments of chitosan

Sample	Increase in reducing sugar equiv [a] (μmol GlcNAc equiv/g chitosan)	Viscosity [b] (mPa·s)	Viscosity Reduction (%)
Chitosan [c]		1260	
Acid-hydrolyzed chitosan [d]	16.61	16	99
Papain-treated chitosan	0.50	28	98
Hemicellulase-treated chitosan	1.88	118	91

[a] Determined from 0.5% (w/v) solutions. [b] Determined from 2.0% (w/v) solutions. [c] Low viscosity chitosan (Seacure LV). [d] Shrimp chitosan.

The viscosity reduction data in Table VII indicated similar apparent hydrolytic endpoints for the chemical and enzymatic treatments. On the other hand, the reducing sugar assay revealed similar results for both enzymatic hydrolysates, which were distinctly lower than that of the acid hydrolyzate sample. This suggests that the enzymatic treatments produce hydrolyzate patterns that are distinct from those derived by chemical hydrolysis.

Compared to previous enzymatic methods, our procedure offers the advantage of minimizing solution volumes and viscosities for subsequent workup, while permitting the processing of relatively large glycan quantities. In a typical experiment, a one-liter solution containing 60–70 g of chitosan could readily be processed in a day, resulting, after enzyme inactivation and lyophilization, in essentially quantitative hydrolysis of the glycan. By comparison, the procedure of Muraki and co-workers[14] employed a lower chitosan concentration (~ 30 g/L) and a cellulase (*Trichoderma viride* at 0.2 g enzyme/g chitosan) at 50°C for similar reaction periods.

Since the study of Muraki and co-workers[14] was conducted at pH 5.6, we performed an additional set of experiments to examine the influence of pH on the hydrolytic efficiencies. Chitosan solutions at pH 2.8 and 5.6 were treated with several enzyme preparations, and the hydrolyses were monitored by reducing sugar assay. The results obtained after 22 h are summarized in Table VIII. The data indicated an overall similar performance of the enzyme preparations at both pH values, although some differences in the levels of reducing sugar released per protein equivalent were observed for Cellulase TV, ficin, and papain. Matching observations were also made when reducing sugar results were obtained after 4 h of hydrolysis (data not shown). It may be noted that the pH 5.6 data were derived from less concentrated chitosan solutions due to solubility limitations at this pH. Some of the differences in reducing sugar levels, e.g., for Cellulase TV, may therefore be merely a reflection of concentration effects described above. Thus, our choice of lower pH values imposed no limitations on the hydrolytic efficiencies

TABLE VIII

Influence of pH on liberated reducing sugar levels from chitosan for selected enzymes

Enzyme preparation	Reducing sugar equiv liberated per enzyme equiv [a] ($\times 10^{-3}$ mM GlcNAc equiv/mg protein)		
pH	2.8		5.6
Concentration	0.5%	1.0%	0.12
Bromelain	23.22	22.52	28.65
Cellulase TV	2.61	1.07	6.17
Ficin	10.99	14.69	25.74
Hemicellulase	15.69	14.89	10.58
Lipase AIE	44.19	53.91	41.23
Papain	1.84	2.31	6.95

[a] Normalized to amounts of enzyme used, after 22-h hydrolysis.

of the enzyme preparations. It also offers a practical advantage in that these chitosan solutions are more conveniently prepared than those at pH 5.6.

At the scales we have employed here, the estimated cost of the enzyme preparations would be quite acceptable for both papain and hemicellulase (estimated at ca. \$0.10/g enzyme). The fact that both are used in many large-scale commercial processes is reflected in their low cost, making them attractive catalysts for this type of application.

For a comparison of the approximate catalyst costs for processes with established chitinases, we can consider the recent study of Kuzuhara and co-workers[30] that employed chitinases for preparative-scale hydrolyses of chitin. These workers employed a chitinase from *Streptomyces griseus* for the preparation of *N,N'*-diacetylchitobiose. Using a chitin concentration of ~ 14 g/L, they obtained 198 g of purified disaccharide product from 2.1 kg of colloidal chitin (with a water content of ~ 85%) after more than 10 days of processing. The enzyme:substrate dosage in their study was about equivalent to ours. Thus, from a commercial perspective, a chitinase-based process would clearly be associated with substantially higher costs (estimated at ca. \$700/g chitinase), while affording lower chito-oligosaccharide yields after significantly longer processing periods. It may noted that, in contrast to the chitinase study[30], our preparative hydrolyses were not aimed at obtaining maximum yields of a particular chito-oligosaccharide fraction, nor were they optimized for the amounts of enzyme preparation employed. Assuming that both types of catalysts can afford the same desired hydrolysis product(s), the above comparison provides therefore an approximate indication of the potential economic advantages involved in using commodity enzymes for any large-scale application.

CONCLUSIONS

Our investigation has demonstrated the hydrolytic susceptibility of several aminoglycans to a range of enzyme preparations. This study was not primarily

aimed at optimizing the preparation of any specific aminoglycan hydrolysis products. Instead, we were interested in using alternative catalysts from a practical perspective. Nevertheless, the observations of hydrolytic activities for a broad spectrum of enzyme preparations, as well as a human salivary preparation, are intriguing. It is remarkable that several of these enzyme preparations displayed lytic activities towards chitosan that equaled or surpassed those of chitinase and lysozyme preparations.

The experimental evidence obtained for the cellulase, hemicellulase, lipase, and protease preparations, strongly suggests the absence of a common lytic principle. It appears highly unlikely that the lytic activities of *all* these enzyme preparations originated from a single source, based on the distinct lytic profiles in terms of their pH and temperature optima, specific activities, susceptibilities to substrate concentration and the degree of substrate *N*-acetylation, and their molecular weight fractions. Similarly, distinctions in hydrolytic efficacy emerged for several enzyme preparations when chitosan solutions were subjected to two simultaneous or sequential enzyme treatments. These lytic activities can be readily exploited for preparative-scale hydrolyses of chitosan, as exemplified here for papain and hemicellulase preparations. The results demonstrate the feasibility of hydrolyzing chitosan, chitin, and other aminoglycans with several low-cost enzymes.

The distinct lytic patterns displayed by the various enzyme preparations should also facilitate the tailoring of hydrolysis products by appropriate choice of catalysts and experimental conditions. An illustration of this was provided in the experiments with consecutive enzyme treatments. Further work is, of course, required to characterize the individual hydrolyzate patterns for the various enzyme preparations and to resolve the question about the lytic principles involved.

EXPERIMENTAL

Polymer and enzyme sources.—Chitosan [Seacure low (LV), medium (MV), and high-viscosity (HV) grades, with ~ 15% *N*-acetyl content, as specified by supplier] was purchased from Vanson (Redmond, WA). Another low-viscosity grade shrimp chitosan (8 mPa · s, 7.7% *N*-acetyl content, Lot no. VNS-461) was obtained from the same source. Chitin powder was obtained from Burlington Chemicals (Framingham, NJ), chitin azure from Sigma Chemical Co. (St. Louis, MO), and partially deacetylated chitin was obtained from Ajinomoto U.S.A. (Teaneck, NJ). α-(1 → 4)-Poly(galactosamine) and deacetylated (70–100%) chitosan samples were purchased from Funakoshi Pharmaceutical Co. (Tokyo). The following enzymes were obtained from Sigma Chemical Co. (St. Louis, MO): PPLipase, lysozyme, ficin, pepsin, pancreatin, and chitinase (*S. marcescens*); from Amano International Enzyme Co., Inc. (Troy, VI): Lipase AIE, Cellulase TV, hemicellulase, and papain; from V-Labs, Inc. (Covington, LA): crude and purified cloned chitinase (*V. parahemolyticus*); and from Miles Laboratories, Inc. (Elkart, IN): bromelain.

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) aq AcOH or in acetate buffer pH 3.0 or 4.0. A 0.12% (w/v) solution of HV chitosan was prepared in acetate buffer, pH 5.6. All solution concentrations are reported as % (w/v), unless otherwise stated, and the pH was carefully adjusted to the desired values. Chitin powder (100 g) was suspended in dil aq AcOH (pH 2.5, 1.5 L) for 4 days at 4°C prior to hydrolytic treatments. α-(1 → 4)-Poly(galactosamine) (0.5%) and xanthan (1.5%) solutions were prepared in dil aq AcOH and in water, respectively.

Analyses and enzymatic hydrolyses.—Details of the rheological and some of the enzymatic methods employed have been described elsewhere[13]. Specific activities are defined based on viscosity reduction or reducing sugar production as percent viscosity reduction/s/mg protein and μmol GlcNAc equiv/h/mg protein, respectively, unless stated otherwise.

Dosage studies.—Enzymes were incubated at ambient temperatures overnight with chitosan at different ratios. Viscosities were determined as previously described[13], and the percent viscosity reduction was plotted vs. the enzyme : chitosan (w/w) ratio.

Reducing sugar assay.—The modified Schales method[32] as described by Imoto and Yagashita[33] was used with *N*-acetyl-D-glucosamine as standard.

Protein assay.—Protein analysis was performed using the dye-binding method of Bradford[34] adapted to a Molecular Devices Thermomax microtiter plate reader.

Correlation of viscosity reduction with reducing sugar production.—Chitosan (1% HV) was incubated with lysozyme (50 mg/mL solution) at various doses at ambient temperature. After 24 h, viscosity and reducing sugar equivalents were measured as previously described[13].

Enzyme fractionation.—Four enzymes, cellulase, papain, bromelain, and PPLipase, were dissolved at 2.5 mg/mL water and fractionated using Amicon Centricon concentrators with 30K and 10K MWCOs. After concentration to more than 90% by volume, both supernatant fluid and retentate were mixed with an equal volume of buffer and assayed for viscosity reduction.

Hydrolysis of chitosan with human saliva preparations.—Solutions of high-viscosity chitosan (1 or 2%) in 3 N AcOH (50 mL, pH 2.8) were treated with 100 μL of human saliva at ambient temperature. The course of hydrolysis was monitored viscometrically.

pH studies.—For pH optima determinations chitosan solutions (1% HV in 5% AcOH) were adjusted to different pH values with NaOH, followed by incubation with enzymes (10 mg solid/g chitosan) at 25°C. Half-unit increments were prepared and tested for viscosity reductions at ambient temperature as previously stated. Comparison of hydrolytic efficiencies at pH 2.8 and 5.6 was carried out using HV chitosan at 1.0 and 0.12%, respectively. Aliquots (20 μL) of selected enzymes (50 mg solid/mL in distilled water) were incubated with vigorous mixing using an orbital shaker (200 rpm) at 30°C. Samples were analyzed for liberated

reducing sugar after 4 and 22 h, and the results were normalized to protein content.

Temperature optima.—Chitosan solutions (2% in 3 N AcOH) were incubated with enzymes (5 mg/g chitosan) at 30–60°C in 10°C increments. After incubation for 3.5 h, viscosities were measured and the initial hydrolysis rates were determined.

Enzymatic hydrolysis of chitosan at different concentrations.—Chitosan solutions (15 mL) at 0.5, 1.0, and 2.0% (w/v) in AcOH were treated with several enzyme preparations. Cellulase TV, hemicellulase, Lipase AIE, or papain were respectively added (30 μL), followed by incubation at ambient temperature with stirring. Samples (0.5 mL) were removed after 2 h and analyzed for reducing sugars as previously described. Data was expressed as μmol GlcNAc equiv/h/mg protein in each 15-mL reaction. Reactions at 2% showed insignificant levels of hydrolysis.

Combined enzyme treatments.—Chitosan solutions (1.0 or 0.5% HV) were treated with 20 μL of enzyme (50 mg/mL) for 24 h at ambient temperature. An aliquot was removed for reducing sugar assay, followed by enzyme inactivation by microwave irradiation on full power for 40 s. Upon cooling, a second enzyme dose was added, followed by another 24 h incubation. Following this treatment, viscosities and reducing sugars were determined as previously stated. Another type of combination study was carried out by incubation of chitosan solutions with mixtures of enzyme preparations.

Chitin hydrolysis.—Chitin suspensions (11.0 g), preswollen as described above, were diluted with water (5 mL) and treated with 50 μL of the respective enzyme preparations at ambient temperatures for several days. The mixtures were placed on an orbital shaker (150 rpm) to ensure efficient mixing. Aliquots were removed after appropriate times and analyzed by reducing sugar assay.

Hydrolysis of dyed-chitin.—Chitin azure (25 mg) was added to 5.0 mL of 3 N AcOH in a screw cap vial. To this suspension was added 50 μL of either enzyme preparation or water, followed by shaking at room temperature overnight. A 1.0-mL aliquot was removed, centrifuged, and the absorbance at 575 nm was determined.

Enzymatic hydrolysis of xanthan.—Aqueous xanthan solutions (pH 6.22) were treated with 50 μL of Cellulase TV, hemicellulase, Lipase AIE, and papain for five days at ambient temperature. Viscosity measurements were used to monitor differences between the treated samples and a control (50 μL water).

Preparative-scale enzymatic hydrolysis of chitosan.—Chitosan (20 g in 1 L of 3 N AcOH) was placed in a 2-L beaker and heated to 40°C. The final pH was maintained between pH 2.85 and 3.0. The enzyme preparation, either papain or hemicellulase, was added as a solution (50 mg/mL in distilled water) at a dose of 5 mg enzyme/g chitosan. After $\sim$ 2 h incubation, an additional 20 g chitosan was added and allowed to dissolve. Another dose of enzyme was added, followed by stirring for another 2-h period. This protocol was repeated a second time, thereby elevating the final chitosan concentration to 6%. After an overnight incubation,

the solution was heated to boiling to inactivate the enzyme, filtered through two layers of cheesecloth, and lyophilized. The dried powder was analyzed for reducing sugar content and viscosity.

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REFERENCES

1 P. Sandford, and G.P. Hutchings, in M. Yalpani (Ed.), *Industrial Polysaccharides: Genetic Engineering, Structure / Property Relations and Applications*, Elsevier, Amsterdam, 1987, pp 363–376.
2 K. Suzuki, Y. Okawa, H. Hasimoto, S. Suzuki, and M. Suzuki, *Microb. Immun.*, 28 (1984) 903–912.
3 A.M. Papineau, D.G. Hoover, D. Knorr, and D.F. Farkas, *Food Biotechnol.*, 5 (1991) 45–57.
4 M. Yalpani, F. Johnson, and L.E. Robinson, in C.H. Brine, P.A. Sandford and J.P. Zikakis (Eds.), *Advances in Chitin and Chitosan*, Elsevier Applied Science, Amsterdam, 1992, pp 543–548.
5 A. Ohtakara, M. Izume, and M. Mitsutomi, *Agric. Biol. Chem.*, 52 (1988) 3181–3182.
6 S. Joshi, M. Kozlowski, M. Srulovicz, and R.W. Davies, in M. Yalpani (Ed.), *Industrial Polysaccharides: Genetic Engineering, Structure / Property Relations* and *Applications*, Elsevier, Amsterdam, 1987, pp 95–99.
7 M. Poulicek and C. Jeauniaux, *Biochem. System. Ecol.*, 19 (1991) 385–394.
8 J.P. Zikakis (Ed.), *Chitin, Chitosan and Related Enzymes*, Academic Press, London, 1984.
9 Y. Tominaga and Y. Tsujisaka, *Biochim. Biophys. Acta*, 410 (1975) 145–155.
10 Nakano Sumise KK, *Jpn. Pat. Appl.*, (1990) JP 1 291 799.
12 M. Yoshida, *Bio / Technology*, 8 (1990) 617.
13 D. Pantaleone, M. Yalpani, and M. Scollar, *Carhohydr. Res.*, 237 (1992) 325–332.
14 E. Muraki, F. Yaku, and H. Kojima, *Carbohydr. Res.*, 239 (1993) 227–237.
15 S. Masih, *Proc. Indian Natl. Sci. Acad., Part B*, 39 (1973) 598–603; *Chem. Abstr.* 82, 95573u.
16 M.S. Grisley and P.R. Boyle, *Comp. Biochem. Physiol. B: Comp. Biochem.*, 95 (1990) 311–316.
17 J.A. Beeley, *J. Chromatogr.*, 569 (1991) 261–280.
18 W.R. Den Tandt and J. Jaeken, *Methods Enzymatic Analysis*, 3rd ed. Vol. 3, 1983, pp 35–38.
19 P. Audy, J. Grenier, and A. Asselin, *Comp. Biochem. Physiol. B Comp. Biochem.*, 92 (1989) 523–528; *Geigy Scientific Tables*, Vol. 1, p 121.
20 E.N. Vasstrand and H.B. Jensen, *Scand. J. Dent. Res.*, 88 (1990) 219–228.
21 A. Masaki, T. Fukamizo, A. Ohtakara, T. Torikata, K. Hayashi, and T. Imoto, *J. Biochem.*, 90 (1981) 527–533.
22 D. Pantaleone and M. Yalpani, in M. Yalpani (Ed.), *Carbohydrates and Carbohydrate Polymers*, ATL Press, Mount Prospect, 1993, pp 44–51.
23 C.H. Ulhoa and J.F. Peberdy, *Enz. Microb. Technol.*, 14 (1992) 236–240.
24 J.S. Price and R. Storck, *J. Bacteriol.*, 124 (1975) 1574–1585.
25 D.M. Fenton and D.E. Eveleigh, *J. Gen. Microbiol.*, 126 (1981) 151–165.
26 H. Ueno, K. Miyashita, Y. Sawada, and Y. Oba, *J. Gen. Appl. Microbiol.*, 36 (1990) 377–392.
27 M. Yabuki, in G. Skjåk-Brœk, T. Anthonsen, and P. Sandford (Eds.), *Chitin and Chitosan, Sources, Chemistry, Biochemistry, Physical Properties and Applications*, Elsevier Applied Science, London, 1989, pp 197–206.
28 S. Hirano, H. Tsuchida, and N. Nagao, *Biomaterials*, 10 (1989) 574–576.
29 S. Aiba, *Int. J. Biol. Macromol.*, 14 (1992) 225–228.
30 H. Terayama, S. Takahashi, and H. Kuzuhara, *J. Carbohydr. Chem.*, 12 (1993) 81–93.
31 R.H. Hackman and M. Goldberg, *Anal. Biochem.*, 8 (1964) 397–401.
32 O. Schales and S.S. Schales, *Arch. Biochem. Biophys.*, 8 (1945) 285–292.
33 T. Imoto and K. Yagashita, *Agric. Biol. Chem.*, 35 (1971) 1154–1156.
34 M.M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.