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Chitinase Activity and Substrate Specificity of Three Bacteriolytic Endo-β-*N*-acetylmuramidases and one Endo-β-*N*-acetylglucosaminidase

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Purified lysozymes from fig and papaya plants, hen's egg white, and human urine, a *Streptomyces* chitinase and an endo-β-*N*-acetylglucosaminidase from *Cl. perfringens* were assayed for chitinase activity on glycol chitin, chitin-azur, chitosan-azur, chitopentaose, and for bacteriolytic activity on whole cells, cell walls, and cell wall glycan of *Micrococcus lysodeikticus*. The release of reducing sugar from colloidal chitin and cell walls was also determined. The specific activities on the different substrates were compared. All enzymes except the *Streptomyces* enzyme and an endo-β-*N*-acetylglucosaminidase from *Clostridium perfringens* degraded both chitin $(NAG)_n$ and cell wall glycan $(NAG\text{-}NAM)_n$. The enzyme from hen's egg white and human urine and the endo-β-*N*-acetylglucosaminidase from *S. aureus*, strain M18, had comparatively higher specific activities on the bacterial cell walls, while the plant enzymes degraded chitin and glycol chitin better and should thus be regarded primarily as chitin glycanohydrolases (E. C. 3.2.1.14) and classified accordingly and not as endo-β-*N*-acetylmuramidases (E. C. 3.2.1). *Streptomyces* chitinase did not hydrolyse cell walls and is thus a strict chitinase with no action on the *N*-acetylglucosamininyl-*N*-acetylmuramic acid polymer of the bacterial cell wall glycan.

Endo-β-*N*-acetylmuramidases or lysozymes (E. C. 3.2.1) have been isolated from a great variety of sources: mammalian tissues, birds, insects, plants, and microorganisms.[1–3] These degrade the glycan of the bacterial cell walls to oligosaccharides of the *N*-acetylglucosaminyl-*N*-acetylmuramic acid (NAG-NAM) structure, while endo-β-*N*-acetylglucosaminidases give saccharides with NAG at the reducing end (NAM-NAG). An enzyme of the latter type was recently shown to be producted by strains of *Staphylococcus aureus* and *albus*,[4–7] *Streptococcus pyogenes*,[8] and *Clostridium perfringens*.[9] Only one enzyme with this specificity has yet been purified and characterized for substrate specificity [4] and enzymatic properties.[10] The ability of hen's egg white lysozyme to degrade chitin, *i.e.* poly-NAG or (NAG_n), was first reported by Berger and Weiser [11]

and glycol chitin by Hamaguchi and Funatsu.[12] Fig and papaya lysozymes [13,14] as well as one enzyme (muramidase I) from *Helix pomatia* degrade chitin, while a second enzyme (muramidase II) does not.[15] It seems as if some muramidases (E. C. 3.2.1) and some chitinases, *i.e.* chitin glucanohydrolases (E.C. 3.2.1.14), tend to overlap in their specificities.[15,16] This investigation was performed to compare the specific activities of bacteriolysis, chitin degradation, and release of reducing sugar from bacterial cell walls and colloidal chitin for lysozymes from hen's egg white, human urine, fig, papaya latex, a glucosaminidase from *S. aureus*, strain M18, and one from *Cl. perfringens*, and a chitinase from *Streptomyces*.

MATERIALS AND METHODS

Hen's egg white lysozyme (E. C. 3.2.1.17, lot 96B – 8572), lyophilized whole cells of *M. lysodeikticus*, and chitin were purchased from Sigma Chem. Co., St. Louis, Mo., USA, and ethylene glycol chitin, mol. wt. 20 000 – 60 000 (glycolchitin), from Seikagaku Kogyo Co., Tokoy, Japan. Endo-β-*N*-acetylglucosaminidase from *Cl. perfringens* was a contaminant of phospholipase C from Worthington Biochem. Corp., Freehold, N. J., USA (lot 8 B A). Chitin-azur and chitosan-azur were purchased from Calbiochem, Los Angeles, Calif., USA. *Streptomyces* chitinase was purchased from Mann Res. Labs., New York, USA. Human urine lysozyme [17] was kindly donated by Dr. E. F. Osserman, and fig [13] and papaya [14] lysozymes from Drs. J. B. Howard and A. N. Glaser. *Myxobacter* AL protease was generously supplied by R. S. Wolfe. All chemicals were of analytical grade.

Bacteriolytic activity was assayed on whole cells of *M. lysodeikticus* suspended in 0.05 M sodium acetate buffer, pH 6.5, as previously described.[4] The specific bacteriolytic activity of the lysozymes were: hen's egg white, 900 U/mg; human urine lysozyme, 700 U/mg; fig, 15 U/mg; papaya, 12 U/mg; staphylococcal glucosaminidase, 1300 U/mg. The same amount in milligrams of chitinase from *Streptomyces* did not lyse bacterial cells.

Chitinase activity was determined by three methods: (i) on glycolchitin by a viscosimetric method,[18,19] on colloidal chitin by (ii) a turbidimetric method,[20] and (iii) by measuring the release of reducing sugar (*N*-acetylhexosamine μmol/min).[21]

In the turbidimetric assays the incubation mixture, which contained 60 mg of colloidal chitin suspended in 0.1 M sodium acetate buffer, pH 4.5, and 1 mg of enzyme in a final volume of 10 ml, was incubated under agitation in a water bath at 37°. Samples were withdrawn after different time intervals (5 – 60 min) and centrifuged (3500 *g*, 10 min). The reducing sugar in the supernatants was determined [21] and expressed as μmol *N*-acetylhexosamine released per minute per milligram of enzyme.

Each enzyme was assayed in several concentrations for different intervals of time (15 min to 8 h). The activities were determined and the specific activities were calculated from the linear part of the plotted curve. Chitinase activity on glycolchitin is defined in Hultin units (HU).[18] The accuracy in the viscosimetric assays and the measurement of the release of reducing sugar was $\pm$ 20 %.

Degradation of oligosaccharides of chitin. Each sample of enzyme (0.1 mg) was incubated at 37° for 30 min with 10 mg of chitopentaose $(NAG)_5$ or chitobiose $(NAG)_2$ in 10 ml of 0.1 M ammonium acetate buffer, pH 5.0. The product was freeze-dried and chromatographed on Whatman No. 1 paper in a solvent of butanol:acetic acid:water (25:6:25 v/v) in the presence of pyridine.[22] The spots on the chromatograms were located by fluorescence in UV-light after treatment in an alkaline solution and drying at 100° for 10 min.[22] The spots were eluted and the amount of saccharide was estimated at 235 nm. Controls of NAG, $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, and $(NAG)_5$ were always run in parallel.

Degradation of bacterial peptidoglycan and glycan from Micrococcus lysodeikticus. Cell walls were prepared as recently described.[4] Incubation of walls with *Myxobacter* protease was performed to prepare "stripped" glycan from the cell wall peptidoglycan.[23] The incubation mixture contained 60 mg of cell wall peptidoglycan (or 20 mg cell wall glycan) suspended in 0.05 M sodium acetate buffer, pH 4.5 (or pH 6.5).

Spectrophotometry was performed in a Beckman DB-G instrument equipped with an automatic recorder for determination of the bacteriolytic activity.

Table 1.

Enzyme	Degradation of glycolchitin		Degradation of colloidal chitin	Bacteriolytic activity [a]		Degradation of cell wall peptidoglycan		Degradation of cell wall glycan	
	pH-Optimum	Specific activity (HU/mg)	Release of reducing sugar μg/(min mg)	Specific activity		Release of reducing sugar μmol/(min mg)		Release of reducing sugar μmol/(min mg)	
				pH 4.5	pH 6.5	pH 4.5	pH 6.5	pH 4.5	pH 6.5
Hen's egg white lysozyme	(4.8)	4 700	7	20	900	4	28	<1	25
Human urine lysozyme	(4.2)	4 200	5	20	700	1	21	<1	15
Papaya latex lysozyme	(5.4)	18 800	12	<10	10	<1	<1	<1	<1
Fig lysozyme	(3.6)	16 500	11	<10	15	<1	<1	<1	<1
Staphylococcus aureus glucosaminidase [b]	(4.2)	25	2	15	1 300	2	33	<1	30

[a] Estimated on whole cells of *M. lysodeikticus* suspended in 0.05 M sodium acetate buffer pH 4.5 or pH 6.5.

[b] *Streptomyces* chitinase was a crude product with an activity of 4 800 HU/mg on glycolchitin and a pH optimum of 4.2. This enzyme did not degrade bacterial whole cells, cell wall peptidoglycan, or cell wall glycan under the assay conditions described on p. 654.

RESULTS

Chitinase activity. The viscosimetric assay on glycol chitin was found to be sensitive and reproducible. So was the determination of reducing sugar within each experiment, but the results varied very much for different batches of colloidal chitin. Chitin prepared from cuttlefish was found to be more easily degraded than chitin from crab and lobster shells. Determination of reducing sugar released from colloidal chitin gave reproducible results within each experiment but the results obtained varied between different batches. Chitin from cuttlefish was the best substrate also for these studies. Deacetylated chitin (chitosan) was not degraded by any of the enzymes investigated, neither when measured as decrease in turbidity of colloidal chitin nor as release of reducing sugar from the substrate (Table 1).

Streptomyces chitinase was a crude product with an activity of 4800 HU/mg on glycolchitin and a pH optimum of 4.2. This enzyme did not degrade bacterial whole cells, cell wall peptidoglycan, or cell wall glycan under any of the assay conditions used.

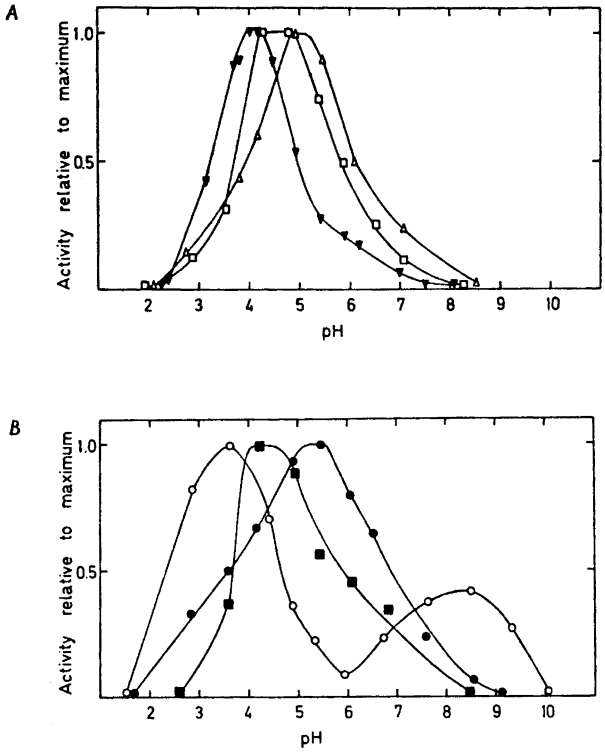

Fig. 1. A and *B.* pH Activity profile of hen's egg white lysozyme (△), human lysozyme (○), fig lysozyme (□), papaya lysozyme (●), staphylococcal glucosamidase (▼), and chitinase from *Streptomyces* (■) on glycolchitin (for assay procedure, see Materials and Methods).

Effect of pH on enzyme activity. Lysozymes purified from fig, papaya latex, hen's egg white, human urine, and endo-β-*N*-acetylglucosaminidase purified from *Staphylococcus aureus*, strain M18, as well as an enzyme with the same specificity from *Clostridium perfringens*, and chitinase from *Streptomyces* were assayed by the viscosimetric method at different pH and ionic strengths. All enzymes except the glucosaminidase from *Clostridium perfringens* degraded glycolchitin, and all six showed the highest activities at an acidic pH (pH 4.5 ± 1.0). Human and hen's egg white lysozyme and the staphylococcal glucosaminidase showed rather narrow pH profiles ($1-2$ pH units) shown in Fig. 1A, while the fig and papaya lysozymes and the chitinase from *Streptomyces* showed broader profiles ($2-3$ pH units) (Fig. 1B). The double pH optimum for the fig enzyme was quite reproducible in this assay system as well as in the reducing sugar assay. Neither was a difference noticed when the viscosimetric assay was performed in buffers of different ionic strengths ($0.05-1$ M final concentration).

Effect of ionic strength. Each enzyme was investigated at different ion concentrations in the buffer in which optimum activity was achieved. All the enzymes showed less than $70-80$ % of the activity in the standard 0.1 M buffer system in the same buffer of $0.2-0.5$ M final concentrations.

Degradation of oligosaccharides of chitin. Each enzyme was incubated with chitopentaose $(NAG)_5$ and chitobiose $(NAG)_2$ in separate experiments. The product was separated by paper chromatography. All enzymes except the one from *Clostridium perfringens* hydrolyzed more than 50 % of the amount $(NAG)_5$ to $(NAG)_2$ and $(NAG)_3$, while none degraded $(NAG)_2$. Only a trace of free NAG was found in each of the $(NAG)_5$ hydrolysates. Less than 5 % of the same amount of $(NAG)_5$ in 0.1 M ammonium acetate buffer, pH 7.2, was

Table 2. Survey of the lytic activity of purified hexosamidases against various micro-organisms [a]

Micro-organism	Hen's egg white lysozyme (U/mg)	*Staphylococcus aureus* glucosaminidase (U/mg)	Human urine lysozyme (U/mg)	Papaya latex lysozyme (U/mg)	Fig lysozyme (U/mg)
M. lysodeikticus N. C. T. C. 2665	900	1300	700	10	15
B. subtilis 168	950	1850	1100	20	25
B. megaterium KM	850	3200	950	20	25
Sarcina lutea R 262	800	1900	650	10	15
M. mucoruber	700	1900	400	5	10
S. epidermidis	70	1700	40	< 1	< 1
S. aureus, 3528	0	550	< 1	< 1	< 1
Lactobacillus acidophilus	120	350	< 1	< 1	< 1
E. coli B	< 1	< 1	< 1	< 1	< 1

[a] Whole cells were used and controls without addition of enzyme were run. In no case was the autolytic activity so significant that heat treated cells had to be used.

hydrolyzed by the same amount of each enzyme, except for the fig (15 %) and the *Streptomyces* enzymes (20 %).

Degradation of bacterial cell walls. Each enzyme was assayed for bacteriolytic activity under standardized conditions in 0.5 M sodium acetate buffer at pH 6.5 and pH 4.5, and the specific activity in units per milligram was calculated (Table 1). These assays were performed with whole cells of *M. lysodeikticus* as the test organism. The staphylococcal glucosaminidase was also active on whole cells of *Staphylococcus aureus*, strain 3528 (375 U/mg), while these cells were completely resistant to the action of the other enzymes in Table 2 (< 1 U/mg). *Bacillus cereus* and *Streptococcus pyogenes* group A type 18, and *Escherichia coli B*, *Mycobacterium tuberculosis BCG*, *Bordetella pertussis*, *Micrococcus aureus*, and *Listeria monocytogenes* were all highly resistant organisms and the cell walls were not degraded, while *B. megaterium* KM and *Sarcina lutea* R 262 whole cells were approximately as sensitive to all enzymes as was *M. lysodeikticus*. These studies show that the bacteriolytic spectra of all four hexosaminidases of Tables 1 and 2 are similar and none of the cells from the bacterial species investigated were sensitive to degradation by the chitinase from *Streptomyces*. The crude glucosaminidase from *Cl. perfringens* only lysed peptidoglycan of *E. coli* and did not release significant quantities of reducing sugar from cells or peptidoglycan of the other species mentioned above.

Degradation of the cell wall peptidoglycans of *M. lysodeikticus* and *S. aureus* 3528 were measured both turbidimetrically and as the soluble reducing sugar released from the insoluble substrate. The staphylococcal glucosaminidase, hen's egg white lysozyme, and human urine lysozyme showed the highest specific activities in all assays, and correlation between bacteriolytic activity and release of reducing sugar existed. However, the initial rate of bacteriolysis with staphylococcal glucosaminidase is more rapid than the release of reducing sugar as compared with egg white lysozyme. This gives further evidence to the hypothesis that both enzymes possess a true endo-action on the glycan polymer probably by attacking the glycosidic bonds at random. The staphylococcal substrate was in each case more resistant than the corresponding *M. lysodeikticus* one.

Degradation of purified cell wall glycan. These experiments showed that all four enzymes in Table 1 are neither inhibited nor stimulated in action by peptide substitution on the 3-*O*-lactyl group of the muramic acid, since reducing sugar is released at a high rate both from the glycan and peptidoglycan substrates.

DISCUSSION

Berger and Weisser [11] showed for the first time that egg white lysozyme degrades chitin besides bacterial cell walls. It has more recently been found that other endo-β-*N*-acetylmuramidases (E. C. 3.2.1.17) possess both muramidase and chitinase activity,[13,14] while one from *Helix pomatia* only degrades bacterial cell walls.[15] Egg white lysozyme has an even broader substrate specificity, since glycosidic linkages at glucose, 2-deoxyglucose [26] and 2-amino-2-deoxyglucose are split.[25] However, Neuberger and Wilson earlier indicated the implication of the acetoamido group in the binding and catalysis of substrates by this enzyme.[26]

Degradation of derivatives of chitin, *e.g.* carboxymethylchitin [27] and glycolchitin [12,18] by different lysozymes has been investigated by viscosimetric assays. While degradation of the bacterial cell wall by human and egg white lysozyme is optimal around a neutral pH, degradation of glycolchitin, chitin, and oligosaccharides of chitin is most rapid at an acidic pH.

As far as is known to the authors no systematic investigation of the susceptibility of chitin derivatives to different muramidases has been performed. This study was made to compare the activity of these enzymes on bacterial cell walls, chitin, and derivatives of chitin. For comparison a purified bacteriolytic endo-β-*N*-acetylglucosaminidase from *Staphylococcus aureus* [4] and a crude enzyme with the same specificity from *Clostridium perfringens* [9] were included in this study. These two enzymes degrade the β-*N*-acetylmuramyl-1→4-*N*-acetylglucosamine (NAM-NAG) polymer of the cell wall to yield disaccharides of the NAM-NAG structure, while muramidases give NAG-NAM saccharides, *i.e.* *N*-acetylmuramic acid, at the reducing end.[4]

All enzymes investigated except the *Streptomyces* chitinase and the glucosaminidase from *Clostridium perfringens* degraded *Micrococcus lysodeikticus* whole cells, chitin, and glycolchitin. The *Streptomyces* enzyme has no bacteriolytic activity, while the glucosaminidase of *Cl. perfringens* degrades cell walls of *Escherichia coli* [9] but neither cell walls of *M. lysodeikticus* nor the different chitin substrates. This enzyme is now being purified and the experiments will be repeated with a greater quantity of purified enzyme.

From the results reported in this paper it seems probable that the lysozyme from fig and papaya latex, and probably similar enzymes from other plants, should primarily be regarded as chitinases and not as lysozymes or muramidases. It is shown in Table 1 that both the papaya and fig enzymes have much higher specific activities on glycolchitin than on bacterial cell walls, while on the other hand the enzymes from the animal kingdom as well as the staphylococcal glucosaminidase are more active on the bacterial substrates. The animal enzymes are probably involved in the host defence against bacterial invasion [2] or the metabolism of the bacterial cell wall,[28] while the plant enzymes probably have another physiological significance.[29,30]

Several assays have been developed for the studies on chitinases.[1] The turbidimetric assay using colloidal chitin as substrate was not a very sensitive method and was not very reproducible for different batches of substrate. Chitin prepared in the laboratory from cuttlefish was more easily degraded than chitin from crab and lobster shells. This is in accordance with the observation that *Sepia* chitin is more satisfactory than substrates prepared from shrimps and lobsters (R. C. W. Berkeley, personal communication). The staphylococcal enzyme did not degrade a colloidal substrate prepared from commercial chitin, probably due to impurities, since the enzyme is extremely sensitive to several heavy metal ions.[10] However, the assay based on the decrease in viscosity upon degradation of glycolchitin was a much more sensitive assay which also gave quite reproducible results for two different batches of substrate for all the enzymes investigated.

Chitin-azur and chitosan-azur have recently become commercially available and are recommended by the manufacturer for rapid and convenient assays of chitinase activity. The sensitivity of the first substrate to degradation

of all enzymes was very low or negligible, while chitosan-azur was highly resistant to all enzymes investigated. Work is now in progress to prepare another substrate suitable for simple colorimetric estimations based on the findings on Procion Red G dyed chitin reported by Hackman and Goldberg.[31]

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