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Chitinase Activity and Substrate Specificity of Endo-β-*N*-acetyl-glucosaminidase of *Staphylococcus aureus*, Strain M18

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A purified extracellular bacteriolytic endo-β-*N*-acetylglucosaminidase from *S. aureus* degrades colloidal chitin, oligosaccharides of chitin and glycolchitin. The pH optimum for bacteriolysis and degradation of purified cell walls is around pH 7, while the degradation of the chitin substrates is very low at this pH, but shows an optimum around pH 4.5. Degradation of the oligosaccharides of chitin followed by analysis by paper chromatography showed that mainly chitobiose was formed, and no free *N*-acetylglucosamine was detected, while digestion with an endo-β-*N*-acetylmuramidase (hen's egg white lysozyme) also gave higher oligosaccharides. Degradation of *M. lysodeikticus* cell walls and purified cell wall glycan, as well as staphylococcal peptidoglycan and the glycan part of this, showed that the glucosaminidase exclusively yields disaccharides with *N*-acetylglucosamine at the reducing end, while hen's egg white lysozyme gives a mixture of oligosaccharides with *N*-acetylmuramic acid at the reducing end.

Endo-β-*N*-acetylglucosaminidase has been shown to occur in *Streptococcus pyogenes*,[1] *Clostridium perfringens*,[2] *Staphylococcus epidermidis*,[3] *Staphylococcus aureus* strain Copenhagen [4] and strain HS968,[5] and has been purified from strain M18.[6] Recent studies showed that staphylococcal glucosaminidase is similar to hen's white lysozyme in several aspects. It is a basic protein, is more stable at acidic pH, and has a similar bacteriolytic spectrum and pH profile for bacteriolysis.[7,8] However, digestion of *N*-acetylmuramyl-L-alanine amidase "stripped" *Micrococcus lysodeikticus* wall peptidoglycan with glucosaminidase gives the disaccharide β-*N*-acetylmuramyl-1,4-*N*-acetylglucosamine (NAM-NAG) as the end product,[7] while lysozyme gives a mixture of oligomers of the disaccharide with *N*-acetylmuramic acid at the reducing end (NAG-NAM).[9]

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MATERIALS AND METHODS

Hen's egg white lysozyme (*N*-acetylmuramylglycanohydrolase, EC.3.2.1.17), lot 96B-8572, and chitin were purchased from Sigma Chem. Co., St. Louis, Mo. Glycolchitin (ethylene glycolchitin, mol.wt. 20 000 – 60 000), lot 4401, prepared according to Senju and Okimasu,[10] was obtained from Seikagaku Kogyo Co., Tokyo, Japan. The reference oligosaccharides were kindly donated by N.S. Sharon, U. Zehavi, and J.A. Rupley, and the *Myxobacter* enzyme [11] by R.S. Wolfe. All chemicals were of analytical grade.

Preparation of substrates. Colloidal chitin was prepared from crab and lobster shells dissolved in 11 N HCl at 4°.[12] Oligosaccharides of chitin were prepared by hydrolysis (11 N HCl; 2 h; 40°),[13] concentrated,[14] and purified. The purification was first made by chromatography on Sephadex G-50, and then on G-25 in the same column,[15] and finally on G-15 in a column of the same dimensions (2 × 20 cm). Each column was equilibrated with distilled water. Oligosaccharides of *M. lysodeikticus* cell walls, digested with glucosaminidase, were separated by G-50 and G-25 chromatography. The purified saccharides were lyophilized and chromatographed on Whatman No. 1 paper [17] with NAG-NAM and di- and trimers of the NAG-NAM disaccharide as reference substances in separate runs.

Assays. Bacteriolytic activity of lysozyme and glucosaminidase was assayed on whole cells of *Micrococcus lysodeikticus*.[6] All units (U) refer to this assay, and the specific activity of lysozyme is 900 U/mg, and of glucosaminidase 1300 U/mg. Chitinase activity was determined by three methods. (i) On glycolchitin by a viscosimetric method.[18] The decrease in viscosity in an incubation mixture containing either lysozyme or glucosaminidase and 3.75 mg/ml of glycol chitin in 0.1 M sodium acetate buffer, pH 4.5, was in both cases proportional to the enzyme concentration within a tenfold dilution range (approx. 0.05 – 0.5 mg/ml). The chitinase activity is expressed in Hultin units per ml (HU/ml).[18] The chitinase activity was also assayed on colloidal chitin by (ii) a turbidimetric method,[12] and (iii) by measuring the release of reducing sugar from this substrate.[19]

The release of reducing sugar from $(NAG)_5$ reduced with $NaHB_4$ [14] by the two glucosidases was also determined.[19] The results were compared with standards of free NAG. The separations upon column chromatography were also followed by determination of the release of reducing sugar.[19]

Other methods. Digestion of chitobiose: β-*N*-acetylglucosaminyl-1,4-*N*-acetylglucosamine, $(NAG)_2$, chitotriose $(NAG)_3$, chitotetraose $(NAG)_4$, chitopentaose $(NAG)_5$, and different chitin substrates were performed in 0.05 M ammonium acetate buffer at different pH and molarities. 800 U of the two enzymes were incubated separately with the substrate at 37° for 0, 2, and 8 h in a final volume of 20 ml. Experiments with lysozyme and glucosaminidase were always performed in parallel.

Portions of 5 ml were 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.[17] The spots on the paper chromatograms were located by fluorescence in UV-light after treatment in an alkaline solution (Na_3CO_3, 20 g; ethanol 600 ml; propanol 400 ml) and drying at 100° for 10 min.[17] The spots were eluted in water, and the amount of saccharide was estimated at 235 nm.[17,20]

Cell walls of *M. lysodeikticus* and *S. aureus* were prepared as earlier described,[7] and the staphylococcal walls were treated with cold trichloroacetic acid to obtain the peptidoglycan.[7] This was in some experiments digested with *Myxobacter* protease to obtain "stripped" cell wall glycan,[16] and then further digested with either of the two glucosidases. The incubation mixture contained 0.5 mg of enzyme and 200 mg of substrate, dissolved in 0.05 M ammonium acetate buffer, pH 6.5. After 10 min, 30 min, 2 h, and 6 h, samples were withdrawn and dialysed. The diffusible fraction (DF) was lyophilized and analysed by paper chromatography with reference substances.[17]

Spectrophotometry was performed in a Beckman DB-G instrument, equipped with an automatic recorder for determination of the bacteriolytic activity.[7]

RESULTS

Chitinase activity. Digestion of glycol chitin by staphylococcal glucosaminidase showed an optimum at pH 4.0 ± 0.5 in 0.05 M ammonium acetate

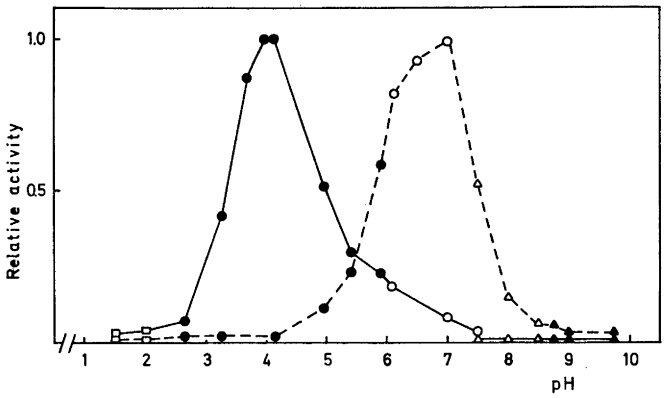

Fig. 1. Effect of pH on the enzymic activity of endo-β-*N*-acetylglucosaminidase (300 U/ml of bacteriolytic activity) assayed on whole cells of *M. lysodeikticus* (– – –) and on glycolchitin (———). Buffers of the final concentration of 0.1 M were used: glycine-HCl (□), sodium acetate (●), sodium phosphate (○), tris HCl (△), and glycine-NaOH (▲). The final pH of the incubation mixture was determined at 20° and plotted against the relative enzymic activity.

buffer (Fig. 1). The specific activity for degradation of glycolchitin was 25 HU/mg for glucosaminidase, and 4700 HU/mg for lysozyme in this buffer. It was used for all experiments with chitin oligosaccharides, since the increase of reducing power upon digestion of reduced $(NAG)_5$ was also maximum at this pH for both enzymes, and was inhibited at higher ionic strengths. An increase in reducing power, corresponding to a release of 5 μg/min for glucosaminidase and 6 μg/min for the lysozyme, was calculated from an incubation mixture containing 0.1 mg of each enzyme in 1 ml buffer.

Digestion of $(NAG)_4$ by staphylococcal glucosaminidase showed the same optimum, and analysis by paper chromatography revealed that $(NAG)_2$ was detected as the only end product. After degradation of 10 mg of $(NAG)_6$ for 2 h at 37° by 0.5 mg of glucosaminidase, only one spot corresponding to $(NAG)_2$ (R_F 0.65) was visible in UV-light. Elution showed that more than 90 % was hydrolyzed, while the same amount of lysozyme hydrolyzed only 60 % with recovery of the product as $(NAG)_2$ 25 %, $(NAG)_3$ 5 %, and $(NAG)_4$ 10 %, but no free NAG was found. In both cases, less than 20 % was hydrolyzed after 6 h at pH 7.0. Hydrolysis of chitopentaose by the glucosaminidase gave $(NAG)_2$ and $(NAG)_3$, but no free NAG. Chitobiose $(NAG)_2$ was not further degraded by either of the two enzymes.

No higher mol.wt. saccharides were found upon incubation of 2 mg $(NAG)_4$, 10 mg $(NAG)_2$ with glucosaminidase when 2 mg extra NAG added. A spot with an R_F corresponding to $(NAG)_6$ (R_F 0.06) was found in the control with muramidase, as well as a very faint spot, probably containing $(NAG)_3$ (R_F 0.43).

Digestion of M. lysodeikticus *cell walls and staphylococcal peptidoglycan.* While digestion of cell walls with lysozyme gave di-, tetra-, and hexasaccharides of the NAG-NAM structure, practically only the disaccharide (NAM-NAG)

was found in the glucosaminidase digests (Table 1). Only traces of saccharides of higher molecular weights were visible upon paper chromatography of a 30 min and a 40 h digest. Digestion of "stripped" glycan of the staphylococcal peptidoglycan gave similar results, and proceeded at the same rate, as estimated by the rate of release of reducing sugar in the soluble fraction.

Table 1.

I. *M. lysodeikticus* cell walls digested with lysozyme or staphylococcal endo-β-*N*-acetylglucosaminidase.

	Lysozyme %	Glucosaminidase %
Nondiffusible fraction (NDF)	58	64
Diffusible fraction (DF)	42	36
Disaccharide	6	10
Tetrasaccharide	1.5	0.5
Hexasaccharide	0.6	0.5

II. *M. lysodeikticus* cell walls digested with *Myxobacter* protease before incubation with lysozyme or glucosaminidase.

	Lysozyme %	Glucosaminidase %
NDF	12	10
DF	88	90
Disaccharide	11	19
Tetrasaccharide	3.2	< 0.5
Hexasaccharide	2.8	< 0.5

III. Staphylococcal peptidoglycan digested with *Myxobacter* protease before incubation with lysozyme or glucosaminidase.

	Lysozyme %	Glucosaminidase %
NDF	22	20
DF	78	80
Disaccharide	7	9
Tetrasaccharide	2.5	< 0.5
Hexasaccharide	1.5	< 0.5

The cell walls and peptidoglycan were digested twice for 4 h at 37° with 0.5 mg of lysozyme or glucosaminidase in 0.05 M ammonium acetate buffer, pH 6.5, and dialyzed for 24 h against water. The diffusate, *i.e.* the diffusible fraction (DF), and the dialysis residue, *i.e.* the non-diffusible fraction (NDF), from both digestions were pooled, respectively. After lyophilization, the recovery of NDF and DF was determined by dry weight. DF was analysed by paper chromatography.

DISCUSSION

Several muramidases degrade the glycan of bacterial cell walls $(NAG\text{-}NAM)_n$ as well as chitin $(NAG)_n$, oligosaccharides of chitin, and derivatives such as glycolchitin,[17,21–24] while others, *e.g.* that from *Helix pomatia*,[25] do not split $(NAG)_n$. Recent studies showed that enzymes from fig [22] and papaya [23] plants have a much higher specific activity as chitinases than as muramidases. Human and hen's egg white lysozymes, however, are more active on the bacterial substrate.[26] This is probably the first time that a bacteriolytic glucosaminidase is shown to have chitinase activity as well. However, staphylococcal glucos-

aminidase is an enzyme of the second type, *i.e.* more active on bacterial cell walls than on chitin.

It is remarkable that the enzyme degrades glycol chitin (Fig. 1) and $(NAG)_5$ at acidic pH, while the activity is very low at neutrality where the rate of bacteriolysis is highest.[8] Egg white lysozyme was also found to have a lower pH optimum on chitin saccharides than on bacterial cells, which is in agreement with earlier studies.[27]

Both enzymes are endoenzymes, having a proposed random action on the NAG and NAG-NAM-polymers. Lysozyme shows specificity for certain bonds in the oligosaccharides of chitin,[28] which is probably also true for the glucosaminidase, since more $(NAG)_2$ than $(NAG)_3$ was obtained upon degradation of $(NAG)_6$ by lysozyme, while only traces of NAG_3 were detected in digests of the glucosaminidase. Another observation was that no NAG_3 or NAG_6 was found on the paper chromatograms after incubation of NAG_2 and NAG_4 with glucosaminidase, as in the case of lysozyme.

Digestion of oligosaccharides of chitin or peptidoglycan showed that the muramidase gave several end products, as already reported,[17,29] while the staphylococcal enzyme only gave disaccharides, as reported for the glucosaminidase from *S. epidermidis*.[30] This differs in other aspects from the *S. aureus*, M18 enzyme, however.[7,8] These results are consistent with the fact that probably no transglycosylation occurs, as reported for lysozyme and certain glucosidases.[20,29] However, to completely exclude this possibility, more sensitive methods should be used, *e.g.* incubation of NAG and ^{3}H-NAG_2, followed by autoradiography after separation of the oligosaccharides by chromatography or electrophoresis on paper.[20] Enzymatic degradation of "stripped" glycan of *M. lysodeikticus* and *S. aureus* with glycosaminidase gave less than a few percent of higher oligosaccharides (Table 1). The tetrasaccharide from a digest after treatment with lysozyme was purified by Sephadex G-25 and G-50 chromatography. More than 90 % hydrolyzed to NAM and NAG-NAM-NAG by the glucosaminidase. On the other hand, incubation with lysozyme gave the disaccharide (NAG-NAM), but still a spot corresponding to undigested tetramer was observed after 24 h, in accordance with earlier observations.[17,20,27,29]

Inhibition experiments with oligosaccharides of cell walls and chitin confirmed that lysozyme was inhibited by concentrations of these,[29] which did not inhibit either the bacteriolysis or release of reducing sugar from cell walls, colloidal chitin or glycol chitin (unpublished data). This also shows that the endo-β-*N*-acetylglucosaminidase is not inhibited by end products to the same extent as hen's egg white lysozyme.[29]

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