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Characterization of the Cloned Staphylococcal Peptidoglycan Hydrolase Gene Product

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Abstract: Cloned staphylococcal peptidoglycan hydrolase was used in determining the physiological characteristics of peptidoglycan hydrolase. This enzyme hydrolyzed the bacterial cell walls and released the N-terminal alanine, but not the reducing groups. This cloned gene product was localized in the cytoplasm of transformed *Escherichia coli*. Activity gels indicated the enzyme had an Mr of about 54,000, which was consistent with the deduced Mr from sequencing of the cloned gene. The activity bound to CM-cellulose but not DEAE-cellulose resin, indicating it as a basic protein. Enhanced enzyme activity in a low concentration of cations, and inhibited enzyme activity in a solution with dissolved phospholipids, suggested that the activity and the availability of this basic protein may be regulated between negatively charged and positively charged cellular molecules. The activity against boiled crude cell wall was much greater than against purifed cell wall, suggesting protein associated with crude cell wall may aid in the binding of the peptidoglycan hydrolase The cloned peptidoglycan hydrolase showed positive activity on whole cells of some lysostaphin-resistant coagulase-negative staphylococci. The cloned enzyme may be an alternative for lysostaphin for lysis of staphylococci.

Key words: amidase, lysis, peptidoglycan hydrolase, staphylococcal.

Autolysins (peptidoglycan hydrolases) are enzymes present in all walled eubacteria and have peptidoglycan as their substrate. Roles have been proposed for these enzymes in cell wall growth, cell separation, cell wall turnover, lysis initiated by antibiotics (Tomasz *et al.*, 1988), and pathogenicity (Berry *et al.*, 1989). In *Staphylococcus aureus*, amidase, glucosaminidase, and endopeptidase activities are present in crude cell walls retaining autolytic activity; among these, the major staphylococcal autolysin is amidase (Tipper, 1969). Different Mr for amidase and glucosaminidase have been reported (Valisena *et al.*, 1982; Sugai *et al.*, 1989).

An autolysin that has been studied in detail at the molecular level is the major amidase of *Streptococcus pneumoniae* (Garcia *et al.*, 1986). When the gene for this enzyme was insertionally deactivated, the only altered properties were an inhibition of lysis at the end of exponential growth, and an increased resistance to penicillin-induced lysis (Tomasz *et al.*, 1988), indicating redundancy in autolysin function.

Though there have been studies on autolysins in different genera of bacteria, most studies on the *S. aureus* autolysins are old and remain unclear in illustrating the importance of these enzymes in *S. aureus*. Because autolysins seem to play important roles in various aspects of staphylococcal metabolism, it is necessary to study them at the molecular level to achieve a better understanding of their roles in various physiological functions. It may be possible that cloned staphylococcal autolysin could be used for determination of the precise role of one autolysin. In this study, the staphylococcal peptidoglycan hydrolase, which was cloned and expressed in our previous experiment, was used to determine the physiological characteristics of peptidoglycan hydrolase (Jayaswal *et al.*, 1990).

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Materials and Methods

Bacterial strains, plasmids, and growth conditions

Escherichia coli strain HB101 containing the plasmid pBR322, or pRJ2-19 which has a staphylococcal peptidoglycan hydrolase gene 7.4 kb insert in pBR 322 (Jayaswal *et al.*, 1990) was grown with 1% (vol/vol) inoculation from 100-ml starter cultures, in 1 l of Luria-Bertani medium (LB, Bacto-tryptone, 10 g; Bacto-yeast, 5 g; NaCl, 10 g/l) in a 2 l flask overnight at 37℃ with shaking (200 rpm) with an appropriate antibiotic (ampicillin, 50 μg/ml). Using 1% (vol/vol) inoculation from 100 ml starter cultures, *Micrococcus luteus* and

Staphylococcus aureus PS47 were grown in 1 l of PYK medium (Bacto-peptone, 5 g; Bacto-yeast extract, 5 g; potassium phosphate dibasic 3 g/l) in a 2 l flask at 37℃ with shaking (200 rpm) to late exponential phase (A_{580} about 1.0), to isolate cells or cell walls.

Enzymes and chemicals

Polycations, phospholipids, lipoteichoic acid, lysostaphin, Triton X-100, antibiotics, dinitrophenyl (DNP)-alanine, DNP-serine, DNP-lysine, and DNP-glycine were purchased from Sigma Chemical Co. (St. Louis, USA).

Preparation of cell-free extract

E. coli cultures (1 l) were centrifuged (15,300×*g*, 10 min, 4℃), and the cells were resuspended in 15 ml of 0.05 M Tris-HCl buffer, pH 7.5. Cell free extracts were obtained by cell breakage using a French pressure cell operated at 16,000 psi. Unbroken cells and cell envelopes were removed by centrifugation (30,000×*g*, 30 min, 4℃).

Cell wall preparation

Crude cell walls (CCW). Three liters of *S. aureus* PS47 exponential-phase culture (A_{580} of about 1.0) were resuspended in 100 ml of cold distilled water and mixed with 100 ml of glass beads (0.10~0.11 mm in diameter, VWR Scientific). The cells were broken using a Bead-Beater (Biospec Products, Bartlesville, USA) for 10 min by repeating 1-min breaking and 1-min resting for 20 min in the cold room. The mixture was scraped out onto a coarse grade fritted filter on a Buchner flask connected to a vacuum aspirator. The filtrate was centrifuged (13,800 g, 10 min, 4℃) to deposit cell walls. The white part of the pellet, CCW, was suspended in water, leaving the yellow part (unbroken cells) behind. CCWs were washed four times with cold distilled water, lyophilized, and stored at −20℃ with desiccation.

Purified cell walls (PCW). CCW were resuspended in water and heated for 10 min in boiling water to destroy autolytic activity. They were harvested by centrifuging and resuspended in 200 ml of 0.05 M Tris-HCl, pH 7.5 containing 5 mM $MgCl_2$, DNase (5 μg/ml) and RNase (5 μg/ml). The mixture was shaken at 37℃ gently (100 rpm) for 1 h, trypsin (200 μg/ml) was then added, and shaking was continued for 4 h. The PCW were washed 5 times with cold distilled water, lyophilized, and stored at −20℃ with desiccation.

Peptidoglycan (PG). To obtain PG, the PCW were resuspended in 10% (wt/vol) trichloroacetic acid, heated at 60℃ for 90 min, and washed 5 times with cold distilled water, lyophilized, and stored at −20℃ with desiccation.

Assay of peptidoglycan hydrolase activity

Samples (cell free extracts, column fractions) were increased up to 3.7 ml by adding 0.05 M Tris-HCl (pH 7.5), followed by addition of 0.3 ml boiled CCW (10 mg/ml). The sample was incubated at 30℃, and the A_{580} was measured at intervals. The rate of enzyme activity was determined from the initial rate and expressed as $\Delta A_{580}/min/mg \times 10^4$.

Studies of the bond(s) cleaved in peptidoglycan

Release of amino and reducing groups. Three ml of PCW (10 mg/ml) isolated from strain PS47 grown in PYK containing 5% NaCl were mixed with cell free extract (200 μg of protein) from strain HB101 containing pRJ2-19 or pBR322 and incubated at 37℃; 0.4 ml samples were removed at various intervals and diluted with 2.8 ml of water, and the A_{580} was measured to observe the turbidity change. Samples of supernatant were assayed for the appearance of amino groups with 1-fluoro-2,4-dinitrobenzene (FDNB) (Ghuysen *et al.*, 1966). Supernatant (0.1 ml), 0.1 ml of 2% (wt/vol) potassium tetraborate, and 20 μl of FDNB (130 μl FDNB/10 ml 100% ethanol) were mixed and heated at 60℃ for 30 min in a water bath. The cooled mixture was added to 3.8 ml of 2 M HCl, and the A_{420} was determined. Reducing groups were determined as described by Thompson and Shockman (1969). One ml of carbonate-cyanide (5.3 g sodium carbonate+0.65 g KCN/l), and 1 ml of ferricyanide (0.5 g of potassium ferricyanide/l) were added to samples and heated for 15 min in a boiling water bath. After cooling, 5 ml of ferric iron solution (1.5 g ferric ammonium sulfate+1 g of SDS in 1 l of 0.1 M sulfuric acid) were added, and color was allowed to develop for 15 min. The A_{690} of the samples was then determined.

Identification of released N-terminal amino acid

One hundred mg of NaOH-treated PCW were resuspended in 10 ml of 0.05 M Tris-HCl, pH 7.5 and divided into 2 tubes. Eight hundred ul of cell-free extract from HB101 containing pRJ2-19 (7.6 mg protein/ml) were added to one tube. The A_{580} was measured by diluting 0.4 ml of sample with 3.6 ml of 0.05 M Tris-HCl, pH 7.5 at various time intervals. Samples were boiled for 10 min and microfuged. Ten % (wt/vol) potassium tetraborate solution was added to the supernatant which contains 10~50 millimicromoles of each N-terminal groups to a final concentration of 1% (wt/vol), 500 μl of FDNB (130 μl FDNB in 10 ml of 100% ethanol) were added, and the mixture was incubated at 60℃ for 30 min. The mixture was acidified with 2.5 ml of concentrated HCl, and the small amount of free dinitrophenyl (DNP)-derivatives was extracted three times with 5 ml of ether and discarded. The resid-

ual ether was evaporated by incubation in a 60℃ water bath. Then, the samples were placed in ampules, which were sealed, and hydrolyzed at 105℃ for 6 h. The N-terminal amino acids were extracted 3 times with 5 ml of ether and the ether extracts were evaporated in a shaking water bath at 37℃ overnight. The residue was dissolved in 400 μl of 0.05 M NH_4OH, and the remaining residue was redissolved in 3 ml of ether, evaporated overnight, and dissolved in 200 μl of 0.05 M NH_4OH; the samples were frozen until used. These extracted DNP-amino acids were analyzed by chromatography. Silica gel G plates were stored in a desiccator and activated for 2 h at 105℃ just prior to use. The plate was first developed with solvent A (n-butanol-1% ammonia) at room temperature, and after drying was developed with solvent B (chloroform-methanol-acetic acid, 85 : 14 : 1) at 4℃. The tracings of DNP-amino acids on the chromatogram were compared with those of DNP-alanine and DNP-lysine.

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Localization of the cloned peptidoglycan hydrolase activity in *E. coli*. *E. coli* periplasm, cytoplasm, and cell envelope fractions were obtained by the process as described by Nossal and Heppel (1966). Fifty ml of *E. coli* HB101 containing pRJ2-19 was grown to A_{580} 0.5, harvested, suspended in 20 ml of plasmolysis buffer (100 mM Tris-HCl, pH 8 containing 1mM EDTA and 20% (wt/vol) sucrose), and incubated at room temperature for 10 min. The cells were pelleted by centrifugation (15,300×*g*, 5 min, 4℃), resuspended in 10 ml of cold distilled water, and incubated on ice for 10 min. The cells were pelleted and the supernatant (periplasmic fraction) was saved. The cell pellet was resuspended in 20 ml of 0.05 M potassium phosphate buffer, pH 7.2. Cells were broken using a French pressure cell, and unbroken cells were removed by centrifugation (8,000×*g*, 5 min, 4℃). The supernatant was

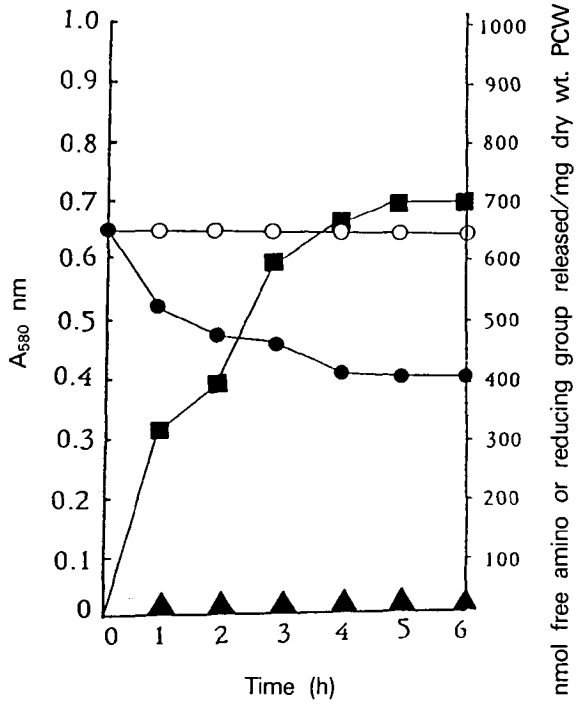

Fig. 1. Release of free amino group from PCW in the presence peptidoglycan hydrolase-containing cell free extract. PCW (3 ml, 10 mg/ml, isolated from PS47 grown in PYK+5% NaCl) and 900 μl cell-free extract from HB101 containing pRJ2-19 (320 μg/ml) were incubated at 37℃ and diluted (1 : 8) with 0.05 M phosphate buffer (pH 7.2) at various intervals. ○, A_{580} PCW plus cell free extract from pBR 322; ●, A_{580} PCW plus cell free extract from pRJ2-19; ■, release of free amino groups from PCW in the presence of cell free extract from pRJ2-19; ▲, release of reducing groups from PCW in the presence of cell free extract from pRJ2-19.

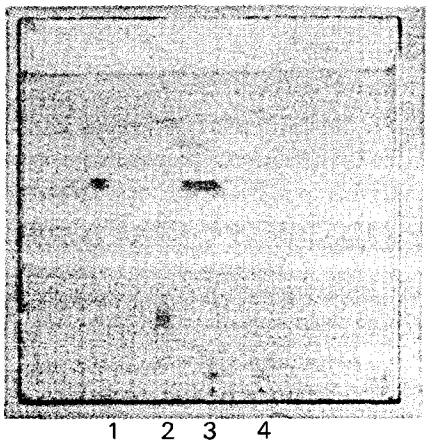

Fig. 2. Identification of DNP-amino acids by silica gel thin-layer chromatography. After dinitrophenylation and acid hydrolysis, DNP-amino acids were extracted by ether and analyzed by chromatography. The plate was first developed with solvent A (n-butanol-1% ammonia) at room temperature, and after drying was developed with solvent B (chloroform-methanol-acetic acid, 85 : 14 : 1) at 4℃. The figure shows the tracings of DNP-amino acids on the chromatogram. 1 : DNP-alanine; 2 : DNP-lysine; 3 : PCW plus cell free extract from pRJ2-19; 4 : PCW plus cell free extract from pBR322.

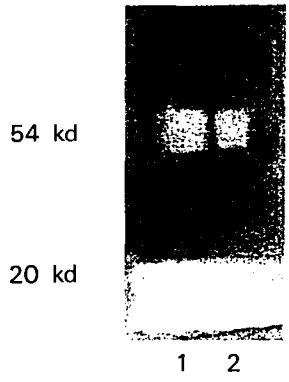

Fig. 3. Lytic activity of partially purified peptidoglycan hydrolase on activity gels. Samples (1 : cell free extract of HB101 containing pRJ2-19; 2 : sample eluted from CM-cellulose column) were prepared and subjected to SDS-PAGE and renaturing SDS-PAGE.

centrifuged (105,000×*g*, 1 h, 4℃) to separate the cytoplasmic and cell envelope fractions.

Renaturing gel electrophoresis (activity gels). To check the lytic activity of protein bands (Leclerc & Asselin, 1989), SDS-PAGE was carried out in a gel containing 0.4% PCW and 0.2% sodium dodecyl sulfate. After complete running, the substrate-containing gel was renatured overnight with 0.05 M Tris-HCl, pH 7.5 containing 1% Triton X-100 with gentle shaking at 37℃. After renaturation, the gel was stained in 0.1 % methylene blue in 0.01% KOH for 3 h with gentle shaking, and then destained in distilled water (Foster, 1992).

Influence of various cations and phospholipids on peptidoglycan hydrolase activity. The effects of 0~150 mM monovalent cations (NaCl, KCl, LiCl), divalent cations ($MgCl_2$, $CaCl_2$), 0~10 mM polycations (lysyl-lysine, streptomycin, poly-L-lysine), 0~100 μM lipoteichoic acid, phosphatidyl glycerol, and cardiolipin on peptidoglycan hydrolase activity were determined. Cardiolipin and phosphatidyl glycerol were supplied with methanol and chloroform-methanol (98 : 2) solutions respectively, by the manufacturer. These compounds were diluted in methanol for use in the assays. Methanol was present at a final concentration of 10% (vol/vol) in the samples and the control. Lipoteichoic acid was resuspended in 0.05 M Tris-HCl buffer, pH 7.2 and used in the assays.

Results and Discussion

The *S. aureus* peptidoglycan hydrolase activity was originally cloned as a 7.4-kb insert in PBR322, yielding pRJ2-19 (Jayaswal *et al.*, 1990). This cloned staphylococcal peptidoglycan hydrolase was used in the present experiment. Four common types of autolysins are recognized : (i) a lysozyme-like enzyme hydrolyzing N-acetylmuramyl-1, 4-β-N-acetylglucosamine bonds (muramidase); (ii) a β-N-acetylglucosaminidase liberating the free reducing groups of N-acetylglucosamine (glucosaminidase); (iii) a β-N-acetylmuramyl-L-alanine amidase (amidase) hydrolyzing the bond between N-acetylmuramic acid and L-alanine, and (iv) peptidases hydrolyzing the stem or bridge peptides (Rogers *et al.*, 1980). To determine the specificity of the cloned enzyme, recombinant cell free extract was added to PCW. The rates of lysis and release of NH_2 groups increased markedly, however there was no increase in the rate of liberation

Table 1. Distribution of peptidoglycan hydrolase activity in cell fraction from *E. coli* containing pRJ2-19

Fraction	Total mg protein and (% distribution)	Peptidoglycan hydrolase activity		
		Specific activity[a]	Total units	% Distribution
Periplasm	0.44 (9.7)	2	0.9	1.6
Cytoplasm	3.11 (68.7)	16.8	52.3	94.7
Cell envelope	1.97 (21.5)	2.1	4.1	3.7

[a] A_{580}/min/mg protein×10^4.

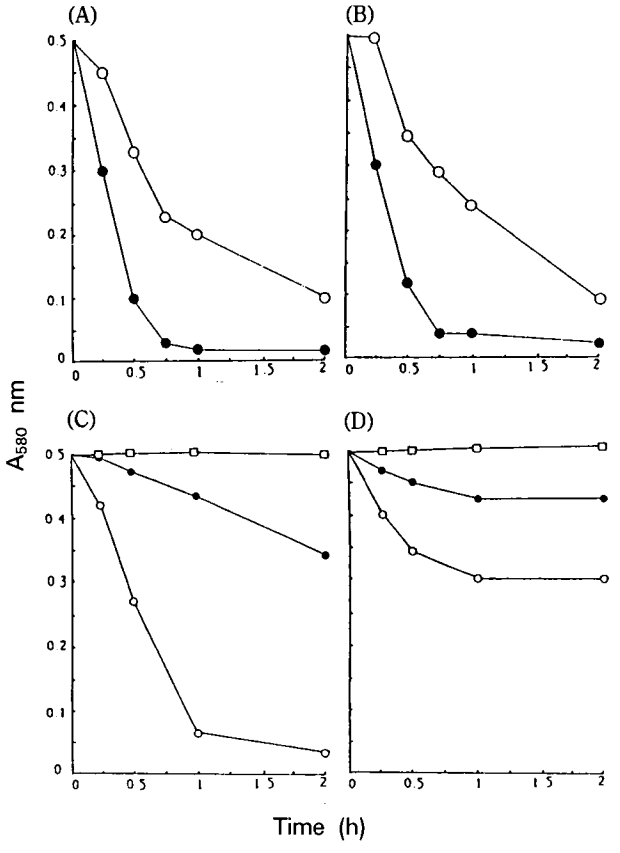

Fig. 4. Activity of the cloned peptidoglycan hydrolase and lysostaphin on whole cells. Bacteria were grown overnight and resuspended in 5 ml of 0.05 M Tris-HCl and 0.05 M Tris-HCl containing 0.05 M NaCl to an A_{580} of 0.5~0.6. One hundred μl of cloned peptidoglycan hydrolase (protein concentration, 48 mg/ml) were added to 5 ml of 0.05 M Tris-HCl. 0.1 ml lysostaphin (50 μg/ml), were added to 5 ml of 0.05 M Tris-HCl containing 0.15 M NaCl. Turbidity changes were measured at various intervals. □, cell free extract from HB101 containing pBR322; ○, cloned peptidoglycan hydrolase; ●, lysostaphin. Strains; A: *S. aureus* PS 47; B: *S. capitis* 27840^T; C: *S. hominis*; D: *S. xylosus*.

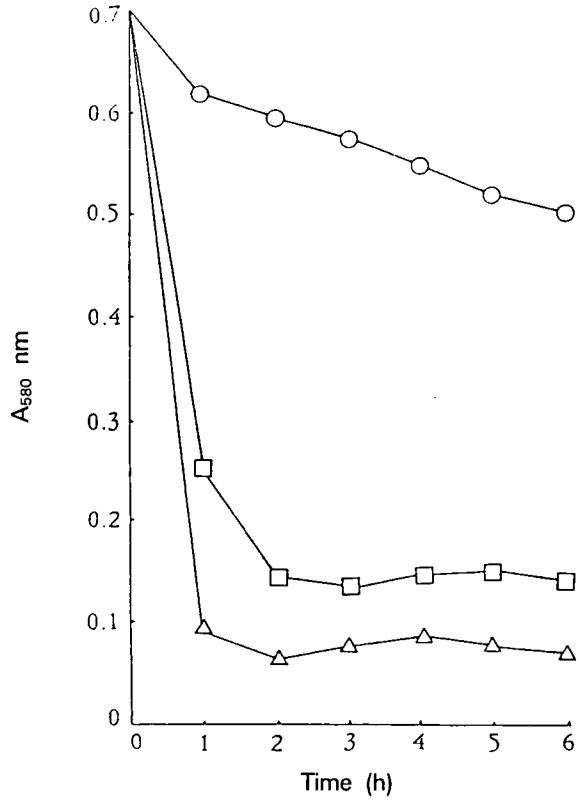

Fig. 5. Susceptibilities of boiled CCW, PCW, and PG to peptidoglycan hydrolase. Four ml of each cell wall preparation (1.2 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.2 were mixed with 100 μl of cell free extract (pRJ2-19, 7.6 μg/μl), and turbidity changes were measured at various intervals. □, boiled CCW; ○, PCW; △, PG.

of reducing groups (Fig. 1). This strongly indicates that the cloned gene does not encode glucosaminidase activity. The N-terminal amino acid(s) released by the cloned peptidoglycan hydrolase was identified by reaction of the supernatant of digested NaOH treated-PCW with 1-fluoro-2,4-dinitrobenzene (FDNB), followed by acid hydrolysis and thin-layer chromatography of the dinitrophenyl amino acids (Fig. 2). The major N-terminal amino acid released by the cloned peptidoglycan hydrolase was clearly identified as DNP-alanine, suggesting that a gene encoding amidase had been cloned. A lytic band appeared on activity gels with an approximate Mr of 51~54,000 which was consistent with the deduced Mr from sequencing of the cloned gene (Fig. 3.) (Wang *et al.*, 1991). Sometimes lower Mr lytic bands could be seen between 20,000 and the dye front. The bands may represent protein processing or degradation of the 54,000 protein. As a prelude to attempts to purify the peptidoglycan hydrolase, its distribution among the periplasmic, cell envelope, and cytoplasmic fractions of *E. coli* HB101 containing pRJ2-19 was determined (Table 1). The protein distribution among the different cell fractions was similar to that reported by Diaz *et al.* (1989). The overwhelming majority of the activity was located in the cytoplasm. Consequently, this cytoplasmic fraction was used as the starting point for purification attempts. Enzyme activity of cell free extracts from *E. coli* HB101 with and without pBR322 was determined; no activity was observed (data not shown).

Lysostaphin is used extensively to digest staphylococcal cell walls for the preparation of cell membranes (Madiraju *et al.*, 1987) and cell-free extracts, and for the isolation of chromosomal and plasmid DNA (Novick, 1991). Most staphylococci investigated were sensitive to lysostaphin (Fig. 4A, B.). However, some coagulase-negative staphylococci are considerably less susceptible to lysostaphin than *S. aureus* because of peptidoglycan cross bridges containing serine and glycine

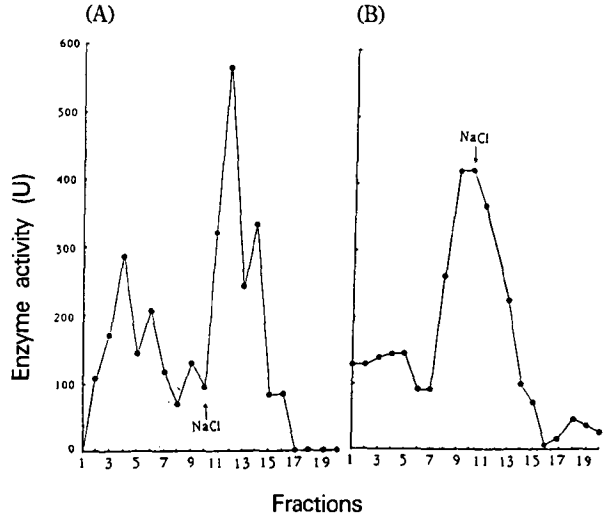

Fig. 6. a) CM- b) DEAE-cellulose chromatography. Twenty five ml of cell free extract (enzyme activity, 100, ΔA_{580}/min/mg$\times 10^4$) were applied to a 100 ml CM column (diameter, 3.7 cm; height, 8.5 cm) and eluted with 200 ml of 0.01 M potassium phosphate buffer (fractions 1~10) followed by 200 ml of a 0~1 M NaCl gradient in 0.01 M potassium phosphate buffer (fractions 11~20). The volume of each fraction was 20 ml.

residues, rather than just glycine residues as in *S. aureus* (Schleifer, 1983). Because the cloned peptidoglycan hydrolase (amidase) hydrolyzes peptidoglycan at a different site from lysostaphin (glycyl glycine endoepeptidase), its ability to lyse coagulase-negative staphylococci was tested. The cloned peptidoglycan hydrolase showed good activity on whole cells of some coagulase-negative staphylococci, *S. hominis* and *S. xylosus* (Fig. 4C, D). The cloned enzyme may be an alternative for lysostaphin for lysis of staphylococci.

The activities of cell free extract from HB101 containing pRJ2-19 on various cell wall preparations were compared. The activity against boiled CCW was much greater than against PCW (Fig. 5). In order to prepare PCW, CCW were treated with RNase, DNase, and trypsin. Trypsin (200 μg/ml) treatment of boiled CCW suspension (5 mg/ml) produced cell walls with lower susceptibility to the cloned peptidoglycan hydrolase (Data not shown). This implies that protein associated with CCW (Wilkinson *et al.*, 1978) may aid in the binding of the peptidoglycan hydrolase to CCW. Peptidoglycan is produced by hot trichloroacetic acid treatment of PCW. The treatment is relatively harsh, breaking covalent bonds, and possibly attacking various funtional groups in cell walls (Schleifer, 1975; Wilkinson *et al.*, 1981). Partial chemical degradation may explain theincreased susceptibility of PG to the peptidoglycan hydrolase. Similar results to those in Fig. 5 were obtained when cell wall preparations were made from cells grown in the presence of 1 M NaCl (Data not shown).

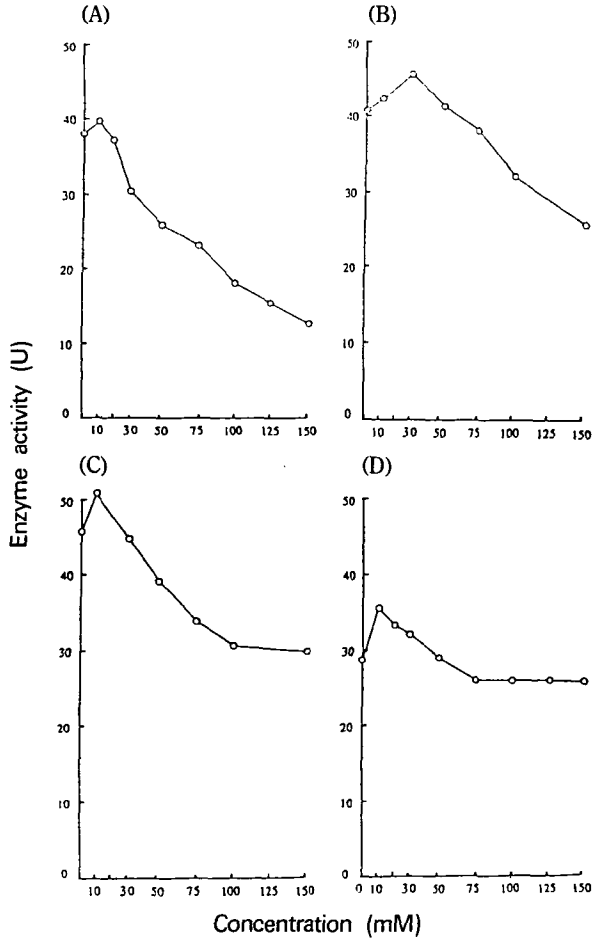

Fig. 7. Influence of monovalent and divalent cations on peptidoglycan hydrolase activity. Boiled CCW (3 mg) were resuspended in 4 ml of 0.05 M Tris buffer (pH 7.4) containing various concentrations of cationic compound, 40 μl of cell free extract (protein concentration, 48 mg/ml) were added, and enzyme activity was determined by turbidity measurements. Cations: A, NaCl; B, KCl; C, $MgCl_2$; D, $CaCl_2$. Enzyme activity, 100, ΔA_{580}/min/mg$\times 10^4$.

In the CM-cellulose column (Fig. 6A), there was an increase in peptidoglycan hydrolase activity in the first fraction of gradient elution (#11) as compared to the last fraction of washing with 0.01 M potassium phosphate buffer, pH 7.2 (#10). Cloned peptidoglycan hydrolase had an affinity for the CM cellulose column, but was eluted by an NaCl gradient. The cloned peptidoglycan hydrolase did not bind to the DEAE cellulose column (Fig. 6B), and most of the activity was recovered by washing with a 0.01 M potassium phosphate buffer. The NaCl gradient did not elute any further activity. These results confirm the indications from batch chromatography (Data not shown) that the enzyme is basic. The effect of pH on peptidoglycan hydrolase activity was determined with 0.05 M Tris buffer at various pH values. The pH optimum of this enzyme was 6.9~7.1 (Data not shown).

Influence of various cations and phospholipids on

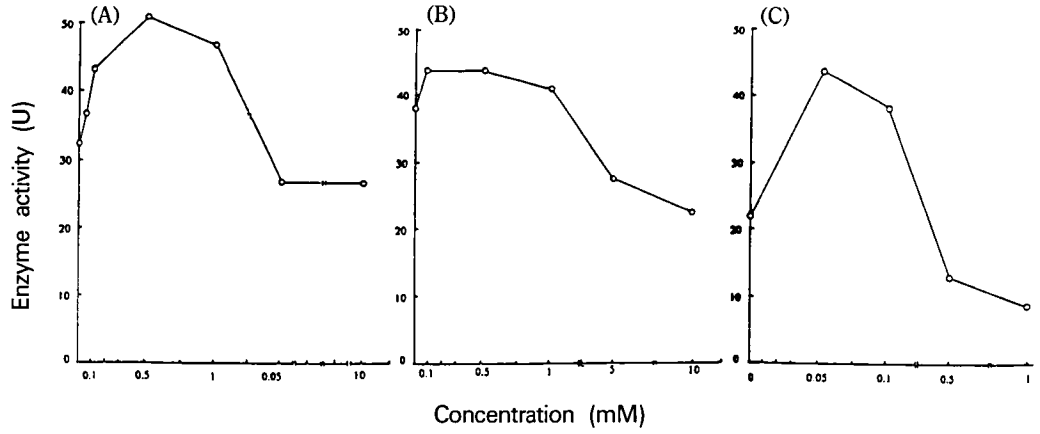

Fig. 8. Influence of organic cations on peptidoglycan hydrolase activity. Determination of peptidoglycan hydrolase activity is described in the legend to Fig. 7. Organic cations: A, *Streptomycin*; B, Lysyl-lysine; C, Poly-L-lysine. Enzyme activity, 100, ΔA_{580}/min/mg$\times 10^4$

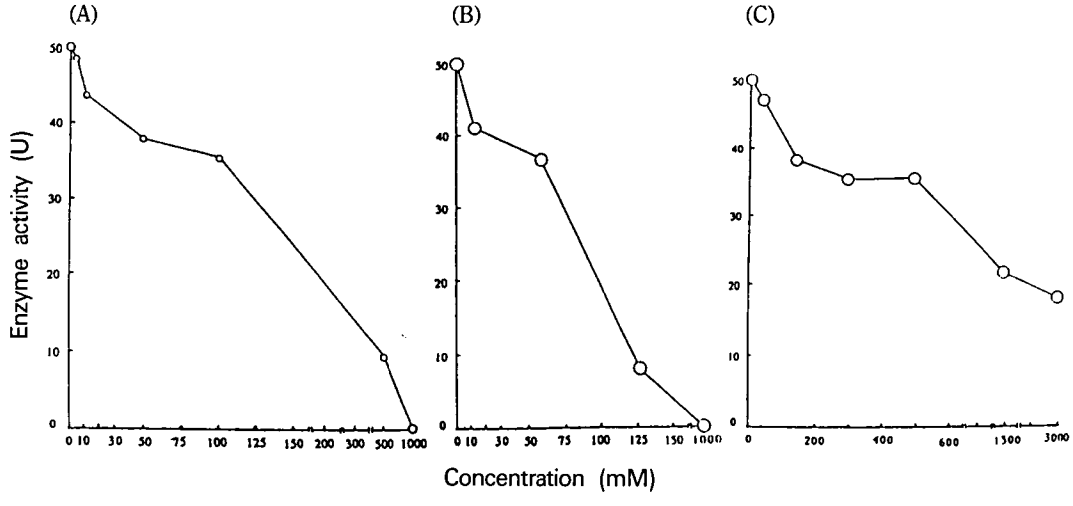

Fig. 9. Influence of cardiolipin, phosphatidyl glycerol and lipoteichoic acid on peptidoglycan hydrolase activity. Determination of peptidoglycan hydrolase activity is described in the legend to Fig. 7. A, Cardiolipin; B, Phosphatidyl glycerol; C, Lipoteichoic acid. Enzyme activity, 100, ΔA_{580}/min/mg$\times 10^4$

peptidoglycan hydrolase was determined. Concentrations of NaCl of 30 mM and above were progressively more inhibitory on peptidoglycan hydrolase activity (Fig. 7A). Some stimulation of activity occurred with KCl up to 30 mM, but higher ionic strength proved inhibitory (Fig. 7B). LiCl concentrations up to 50 mM were stimulatory, and marked inhibition was observed only with high (500 and 1,000 mM) LiCl concentrations (Data not shown). Similarly, low concentrations of $MgCl_2$ (Fig. 7C) and $CaCl_2$ (Fig. 7D) were slightly stimulatory, and higher concentrations were somewhat inhibitory ($MgCl_2$), or had little effect ($CaCl_2$). The effects of the organic cations, streptomycin (Fig. 8A), lysyl-lysine (Fig. 8B), and poly-L-lysine (Fig. 8C) on peptidoglycan hydrolase activity were determined. In all cases, low concentrations of the cations were stimulatory and high concentrations were inhibitory. Autolysins are potentially dangerous enzymes to the cell and must be strictly regulated to avoid damage to the cell envelope, which would result in lysis of the protoplast and cell death. In my studies, the activity of cloned peptidoglycan hydrolase was modulated by basic peptides *in vitro*. Low concentrations of cationic compounds increased the cloned peptidoglycan hydrolase activity, although concentrations higher than a certain level were inhibitory. Low concentrations may prevent the positively charged peptidoglycan hydrolase from being complexed with negatively charged teichoic acid, thereby increasing the activity of the enzyme on the substrate. In contrast, high concentrations of cationic compounds may interfere with access of the enzyme to its substrate, thereby decreasing enzyme activity. The cloned peptidoglycan hydrolase activity was inhibited by cardiolipin (Fig. 9A), phophatidyl glycerol (Fig. 9B), and lipoteichoic acid (Fig.

9C). Polyanionic lipoteichoic acids have been discussed as potential regulators of autolytic activity (Rogers *et al.*, 1984). Phospholipids and lipoteichoic acid are negatively charged because of phosphate groups in these molecules. The positively charged peptidoglycan hydrolase may bind to the negatively charged compounds, thereby interfering with access to the substrate. Above results suggest that the activity and the availability of this basic protein may be regulated between negatively charged and positively charged molecules. Because bacterial peptidoglycan hydrolases are important enzymes involved in various bacterial metabolisms, understanding the regulatory mechanisms of these enzymes may help the control of bacterial growth.

References

Berry, A. M., Lock, R. A., Hansman, D. and Paton, J. C. (1989) *Infect. Immun.* **57**, 2324.

Diaz, E., Garcia E., Ascaso C., Mendez E., Lopez R. and Garcia, J. L. (1989) *J. Biol. Chem.* **264**, 1238.

Foster, S. J. (1992) *J. Bacteriol.* **174**, 464.

Garcia, P., Garcia, J. L. Garcia, E. and Lopez, R. (1986) *Gene* **43**, 265.

Ghuysen, J. M., Tipper, D. J. and Strominger, J. L. (1966) *Methods Enzymol.* **8**, 685.

Jayaswal, R. K., Lee, Y. I. and Wilkinson, B. J. (1990) *J. Bacteriol.* **172**, 5783.

Leclerc, D. and Asselin, A. (1989) *Cana. J. Microbiol.* **35**, 749.

Madiraju, M. V. V. S., Brunner, D. P. and Wilkinson, B. J. (1987) *Antimicro. Agents Chemothera.* **31**, 1727.

Nossal, N. G. and Heppel, L. A. (1966) *J. Biol. Chem.* **241**, 3055.

Novick, R. P. (1991) *Methods Enzymol.* **204**, 587.

Rogers, J. J. *et al.* (1980) in Microbial cell walls and membranes, pp. 437-460, Chapman and Hall, London.

Rogers, H. J., Taylor C., Rayter, S. and Ward, J. B. (1984) *J. Gen. Microbiol.* **130**, 2395.

Schleifer, K. H. (1975) *Z. Immunitaetsforsch. Exp. Klin. Immunol.* **149**, 104.

Schleifer, K. H. (1983) in The Cell Envelope in *Staphylococci* and Staphylococcal Infections (Easmon, C. S. F. and Adlam, C., eds.) pp. 387-428 Academic Press, Inc., London.

Sugai, M., Koike, H., Hong, Y., Miyake, Y., Nogami, R. and Suginaka, H. (1989) *FEMS Mocrobiol. Lett.* **61**, 267.

Thompson, J. S. and Shockman, G. D. (1969) *Anal. Biochem.* **22**, 260.

Tipper, D. J. (1969) *J. Bacteriol.* **97**, 837.

Tomasz, A., Moreillon, P. and Pozzi, G. (1988) *J. Bacteriol.* **117**, 5931.

Valisena, S., Varaldo, P. E. and Satta, G. (1982) *J. Bacteriol.* **151**, 636.

Wang, X., Wilkinson, B. J. and Jayaswal, R. K. (1991) *Gene.* 105.

Wilkinson, B. J., Dorian, K. J. and Sabath, L. D. (1978) *J. Bacteriol.* **136**, 976.

Wilkinson, B. J., Kim, Y. and Peterson, P. K. (1981) *Infect. Immu.* **32**, 216