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Bacteriolytic Activity and Specificity of *Achromobacter* β-Lytic Protease

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***Achromobacter* β-lytic protease (blp), one of the bacteriolytic proteases secreted by *Achromobacter lyticus*, exhibited both peptidase and bacteriolytic activities at alkaline pH. The protease was strongly inhibited by 1,10-phenanthroline, and one zinc atom was detected in the molecule by ion-spray mass spectrometry. The zinc-protease specifically cleaved Gly-X bonds in peptides and possibly possessed subsites S2, S1, S1′, and S2′ for binding substrate [Schecter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157-162]. Blp lysed *Staphylococcus aureus* and *Micrococcus luteus* cells more efficiently than *Achromobacter* α-lytic protease (alp) and lysozyme, thus being responsible for the high bacteriolytic activity of *A. lyticus*. In the lysis of bacterial cell walls, blp hydrolyzed both the D-Ala-Gly/Ala bond at the linkage between the peptide subunit and the interpeptide and the Gly-Gly bond in the interpeptide bridge. These results indicate that blp is a highly active bacteriolytic enzyme with a broad bacteriolytic spectrum, which acts primarily by splitting the linkage between the peptide subunit and the interpeptide in the peptidoglycan.**

Key words: Achromopeptidase, β-lytic protease, mass spectrometry, specificity, staphylolysis.

Various peptidoglycan-degrading enzymes with different action mechanisms have been isolated from animals, plants, and microorganisms and characterized (*1*). These enzymes hydrolyze particular bonds in cell wall peptidoglycan and are generally classified into three groups: glycosidases, which hydrolyze the polysaccharide chain; endopeptidases, which cleave the peptide interbridge; and *N*-acetylmuramoyl-L-alanine amidases, which split the linkage between the polysaccharides and the peptides (*1*). Through cell wall degradation, these hydrolytic enzymes are thought to be involved in important biological processes such as cell turnover (*2*), cell separation (*3*), genetic transformation, formation of flagella, and sporulation (*4*). Some of these enzymes are bactericidal, and thus have potential for use as antimicrobial agents. Hen egg-white lysozyme [EC 3.2.1.17] is a typical enzyme used to lyse bacteria in laboratory and medical procedures, and its mechanism of enzymatic catalysis and protein structure have been extensively studied (*5*). However, certain Gram-positive bacteria such as *Staphylococcus aureus* and *Micrococcus caseolyticus* are resistant to lysozyme, limiting its use as an antimicrobial agent.

Achromopeptidase, a bacteriolytic preparation from *Achromobacter lyticus*, exhibits a higher bacteriolytic activity and a broader bacteriolytic spectrum than hen egg-white lysozyme. This bacterial preparation contains two bacteriolytic proteases that are very similar to α- and β-lytic proteases from *Lysobacter enzymogenes* (*6, 7*). Although *Lysobacter* β-lytic protease (blp) has been shown to degrade the *M. lysodeikticus* peptidoglycan, thereby increasing the detectable N- and C-terminal alanine in it (*8*), the details of its specificity for peptide and peptidoglycan substrates have not been investigated. Likewise, little is known about the lytic properties of *Achromobacter* blp. Accordingly, it is necessary to clarify the specificity of *Achromobacter* blp so as to elucidate the mechanism responsible for its broad bacteriolytic spectrum. Recently, we have successfully isolated active blp from Achromopeptidase (*7*), and this prompted us to investigate its lytic properties in a detail. Here, we describe the results of investigation into the bacteriolytic activity, peptide substrate specificity, and cleavage sites in *S. aureus* and *M. luteus* peptidoglycans of blp, and discuss the mechanisms of its potent bacteriolytic activity and broad bacteriolytic spectrum.

MATERIALS AND METHODS

Materials—All enzymes and reagents were obtained from the indicated sources. Lysozyme from hen egg-white (6×crystallized) and spray-dried *M. luteus* were purchased from Seikagaku. The synthetic peptides used for the substrate specificity experiment were from Peptide Institute, Osaka, or Bachem. Phosphoramidon, chymostatin, elastatinal, leupeptin, pepstatin, and ClAc-(OH)Leu-Ala-Gly-NH_2 were from Peptide Institute. Achromopeptidase,

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Abbreviations: alp, α-lytic protease; blp, β-lytic protease; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; TFA, trifluoroacetic acid; OP, 1,10-phenanthroline; MP, 1,7-phenanthroline; RP, reverse phase; PTH, phenylthiohydantoin; I_{50}, concentration of inhibitor that results in 50% loss of the original activity.

B. subtilis α-amylase, diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), and 1,10-phenanthroline were from Wako Pure Chemical Industries. 1,7-Phenanthroline was from Aldrich. *S. aureus* (IFO 13276) was from the Institute for Fermentation, Osaka. Mutanolysin and recombinant lysostaphin were from Sigma Chemical and DNaseI, RNaseA, and trypsin were from Worthington Biochemical.

Preparation of Cell and Peptidoglycan Substrate—Cells used for bacteriolytic activity assay were prepared as follows. *S. aureus* was grown in tryptic soy broth at 37°C until an optical density of 0.4 at 600 nm was obtained. Cells were harvested and washed twice with 10 mM Tris-HCl, pH 8, then boiled for 10 min, lyophilized and stored at −80°C. *Escherichia coli* (JM109) was cultured in LB medium at 37°C to an optical density of 0.6. Cells were washed twice with 10 mM Tris-HCl, pH 8, suspended in 50 mM Tris-HCl, pH 8, and treated with $CHCl_3$ [$CHCl_3$/cells: 3:50 (v/v)] at room temperature for 30 min with vortexing every 5 min. Finally, cells were removed from $CHCl_3$, washed twice with 50 mM Tris-HCl, pH 8, and stored at −80°C in 50 mM Tris-HCl, pH 8. Peptidoglycans of *S. aureus, M. luteus,* and *E. coli* were prepared as described previously (*9*) except that the cells were disrupted by sonication.

Bacteriolytic and Peptidase Activity Assay—Blp was prepared and the bacteriolytic activity was assayed as described previously (*7*). Briefly, the spray-dried *M. luteus* cells, heat-killed *S. aureus* cells, or $CHCl_3$-treated *E. coli* cells were suspended in an appropriate volume of 10 mM Tris-HCl, pH 8, to yield an absorbance of 0.6 at 600 nm, and the decrease of absorbance at 600 nm was recorded. The peptidase activity was assayed by analyzing the hydrolysis of the peptide substrate neuromedin B (GNLW-ATGHFM) by reverse-phase HPLC. Hydrolysis was conducted at an E/S ratio of 1:400 (mol/mol) in 10 mM Tris-HCl, pH 8, at room temperature for 20 min. One unit of peptidase activity was arbitrarily designated as the amount of blp which yielded a 0.1% decrease in the peak area of the peptide substrate in 20 min.

Optimal pH and Effect of Salt Concentration—The pH optimum and salt concentration dependence of the bacteriolytic and the peptidase activity of blp were determined in the following buffers. For the pH optimum, 10 mM phosphate buffer of pH 6-7, 10 mM Tris-HCl of pH 8-9, and 10 mM NaOH-glycine buffer of pH 10-11 were used. The salt concentration dependence experiment was performed in Tris-HCl, pH 8.

Inhibition Experiments—After preincubation of blp with each inhibitor in 50 mM Tris-HCl, pH 8, at room temperature for 30 min, the residual peptidase activity was measured with neuromedin B at an E/S ratio of 1:400 (mol/mol) for 20 min. The apparent number of 1,10-phenanthroline molecules (n) required for inhibition was calculated according to the following derivation from the equilibrium equation for an enzyme-inhibitor interaction as described previously (*10*).

$$\log[(v_0/v_i)-1] = n \log[\mathrm{I}] - \log K_{app}$$

where v_0 is the rate in the absence of inhibitor, and v_i is the rate at [I]. A plot of $\log[(v_0/v_i)-1]$ *vs.* $\log$ [I] gives n from the slope, pI_{50} from the x-intercept, and pK_{app} from the y-intercept.

Enzymatic Digestion—To determine the peptide substrate specificity of blp, the synthetic peptides were digested with blp at an E/S ratio of 1:400 (mol/mol) in 10 mM Tris-HCl, pH 8, at 37°C for 8 h, and the digests were separated by RP-HPLC on a C18 column. The cleavage sites were determined from the amino acid compositions of the separated fragments. For the specificity toward peptidoglycan, the peptidoglycan (0.5 mg/ml) prepared from *S. aureus, M. luteus*, or *E. coli* was first digested with blp (0.5 μg/ml) in 20 mM Tris-HCl, pH 7.5, at 37°C for 12 h and boiled for 10 min, then half of the sample was further digested with mutanolysin (10 μg/ml), a muramidase from *Streptomyces globisporus*, or hen egg-white lysozyme (1 μg/ml) at 37°C for 12 h and boiled for 10 min. The digests thus obtained were centrifuged at 12,000 rpm for 10 min, and the supernatant was mixed with an equal volume of 0.5 M borate buffer, pH 9, and reduced with sodium borohydride (the same amount of muropeptides) at room temperature for 15 min. Excess borohydride was destroyed with phosphoric acid and the sample was adjusted to pH 3-4.

HPLC Separation—Separation of peptides and muropeptides was performed with a Hitachi HPLC system at room temperature. Peptides were usually separated on a Waters C18 column (μBondasphere 5μ C18-300 Å, 3.9 mm × 150 mm) with a linear gradient (0 to 80% for 40 min) of acetonitrile containing 0.08% TFA at a flow rate of 0.8 ml/min. Muropeptides were separated on a YMC ODS column (ODS-MB, S-5 120 Å, 2.1 mm × 50 mm) as described previously (*7*). The column was first run with 0.1% TFA, and 10 min after injection, the elution was started with a linear gradient (0 to 100% for 120 min) of 15% methanol containing 0.1% TFA at a flow rate of 0.2 ml/min. The eluted peptides and muropeptides were detected at 215 nm.

Amino Acid Composition and N-Terminal Amino Acid Sequence Analysis—N-terminal sequence analysis was performed on a 470A Applied Biosystems protein sequencer, and the amino acid composition was determined with a Hitachi L8500S automatic amino acid analyzer. Peptides and muropeptides were hydrolyzed in constant boiling HCl (5.7 N) in evacuated tubes at 110°C for 24 h.

Mass Spectrometry—Native blp was desalted on a Fast Desalting column (PC 3.2/10 Pharmacia) in 10 mM NH_4OAc, and the denatured blp was prepared by introducing native blp into a Waters C4 column (μBondasphere 5μ C4-300 Å, 3.9 mm × 150 mm) and eluting with a linear gradient (0 to 80% for 60 min) of acetonitrile containing 0.08% TFA at a flow rate of 0.8 ml/min. The molecular masses of the intact and the denatured blp were measured by introducing each sample into an ion-spray triple quadrupole mass spectrometer (API III, Sciex) through a fused silica tube (100 μm i.d.) at a flow rate of 2 μl/min.

RESULTS

Molecular Mass—The molecular mass was measured by ion spray mass spectrometry. Molecular masses of the active and the acetonitrile-inactivated blp were determined to be 19,347.0 and 19,284.2 Da, respectively. The latter value is consistent with the theoretical one, 19,283.0 Da, calculated from the amino acid sequence deduced from the nucleotide sequence of the cloned blp gene (*6*). The mass difference between the theoretical and the experimental value for native blp is 64.0 Da.

TABLE I. **Effect of proteinase inhibitors on blp.** Blp was preincubated with inhibitors in 50 mM Tris-HCl, pH 8, at room temperature for 30 min. The reaction was started by adding substrate neuromedium B and allowed to proceed at room temperature for 20 min. The residual peptidase activity was measured by analyzing the hydrolysis of neuromedin B with RP-HPLC.

Inhibitor	Concentration	Inhibition (%)
1,10-Phenanthroline	10 mM	90
2-Mercaptomethanol	10 mM	66
EDTA	10 mM	2
Diisopropyl fluorophosphate	5 mM	0
Phenylmethylsulfonyl fluoride	5 mM	0
Iodoacetate	5 mM	0
Phosphoramidon	0.5 mM	0
Cl-Ac-(OH)Leu-Ala-Gly-NH_2	0.5 mM	0
Chymostatin	90 μM	0
Elastatinal	90 μM	0
Leupeptin	90 μM	0
Pepstatin	90 μM	0

Cl-1Ac-(OH)Leu: *N*-chloroacetyl-*N*-hydroxy-leucyl.

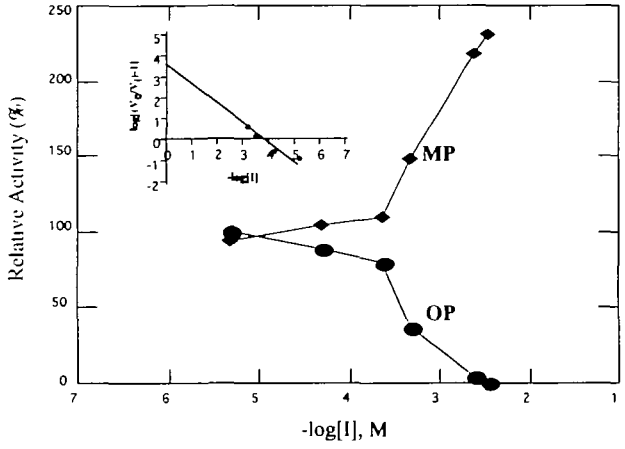

Fig. 1. **Inhibition of peptidase activity of blp by OP and MP.** OP and MP were preincubated with blp in 50 mM Tris-HCl, pH 8, at room temperature for 1 h, and the reaction was started by adding substrate neuromedin B and continued for 45 min. Peptidase activity was assayed by measuring the digestion of neuromedin B with RP-HPLC. Inset is the plot of $\log[(v_0/v_i)-1]$ *versus* $\log[I]$. OP: 1,10-phenanthroline. MP: 1,7-phenanthroline.

Inhibition of Hydrolytic Activity with Protease Inhibitors—Inhibitors of different types of proteases were tested against *Achromobacter* blp (Table I). The peptidase activity was not inhibited by typical serine protease inhibitors, DFP and PMSF. Blp was also resistant to chymostatin and elastatinal. Neither the cysteine protease inhibitors iodoacetate and leupeptin nor the aspartic protease inhibitor pepstatin inhibited the peptidase activity. However, blp was strongly inhibited by 1,10-phenanthroline (OP), a metal chelator, and by 2-mercaptoethanol at a concentra-

TABLE II. **Cleavage sites and relative activity of blp for peptide substrates.** The substrates were incubated with blp at room temperature for 8 h (specificity) or 20 min (relative activity) and analyzed by RP-HPLC. For the nitroanilide substrate, the release of nitroanilide was monitored at 420 nm.

substrate	Relative activity(%)
WAG—G—NASGE	100
PyrVPQWAVG—HFM-$CONH_2$	97
GNLWATG—HFM-$CONH_2$	73
DYMG—WMDF-$CONH_2$	60
PyrADPNKFYG—LM-$CONH_2$	47
KFIG—LM-$CONH_2$	44
PyrQRLG—NQWAVG—HLM-$CONH_2$	16
WAG—G—DASGE	6
RPPG—FSPFR	2
PyrHWSYG—LRPG-$CONH_2$	2
YAG—FM-$CONH_2$	0
PyrG—LPPRPKIPP	0
Boc—AAG—*p*NA	0

Boc: *t*-butyloxycarbonyl. Pyr L-pyroglutamyl. *p*NA. *p*-nitroanilide. ↓ major cleavage site. ↓ minor cleavage site. *A* represents D-alanine.

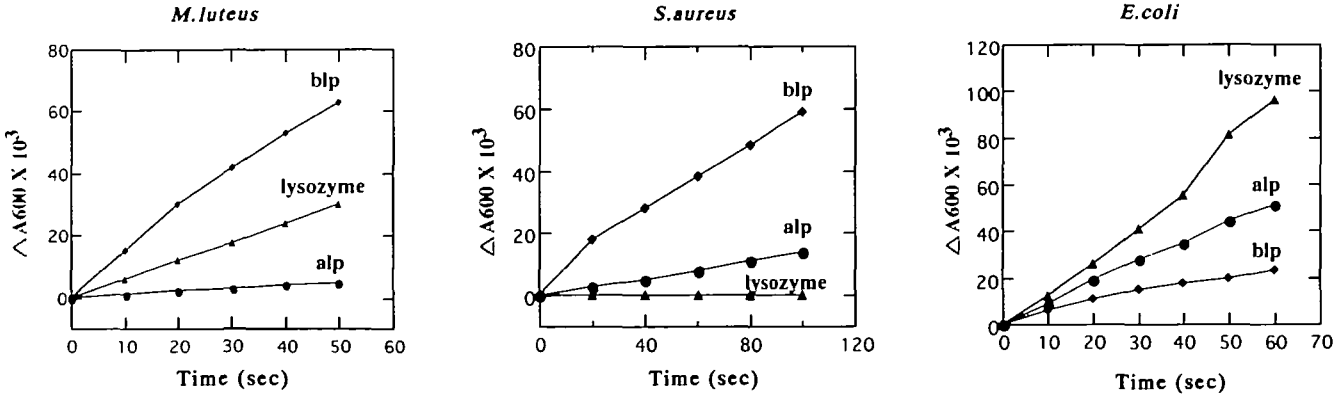

Fig. 2. **Relative activity of alp, blp, and lysozyme on *M. luteus*, *S. aureus*, and *E. coli*.** The bacteriolytic activity was measured in 10 mM Tris-HCl, pH 8. The amount of enzyme used was 3 μg for *M. luteus*, 0.5 μg for *S. aureus*, and 3 μg for *E. coli*.

tion of 10 mM, though EDTA and phosphoramidon, a metalloproteinase inhibitor, had no effect on the peptidase activity (Table I). The inhibition mechanism by OP was further studied together with 1,7-phenanthroline (MP), a nonchelating isomer of OP (Fig. 1). When blp was preincubated with various concentrations of OP or MP for 1 h, the protease activity was decreased by OP in a dose-dependent manner. In contrast, the peptidase activity was increased by MP. The pI_{50} and the number of OP molecules bound to blp were 3.6 and 0.82, respectively (Fig. 1), indicating that OP acts as a modest inhibitor of blp by binding to it in a 1:1 molar ratio.

Peptide Substrate Specificity—A series of glycine-containing peptides were sensitive to blp and the cleavage sites were investigated (Table II). The peptide substrates used are composed of 5 to 14 residues and include 18 typical protein amino acids. The cleavage sites in each peptide were determined by analyzing the amino acid compositions of all the peptide fragments separated by RP-HPLC. Of 13 peptides tested, 10 were hydrolyzed to different extents, and the hydrolysis took place only at the Gly-X bond (Table II). Blp favored peptides with hydrophobic and polar amino acids flanking the Gly-X peptide bond and hardly cleaved or did not cleave peptides with a charged amino acid or proline in the vicinity of the scissile bond (Table II). The hydrolytic susceptibility of these peptides indicates that blp possesses at least S2, S1, S1′, and S2′ subsites for substrate binding. Blp did not hydrolyze Boc-Ala-Ala-Gly-*p*NA, suggesting that the protease lacks universal amidase activity.

Bacteriolytic Activity on M. luteus, S. aureus, and E. coli—Lysis of two Gram-positive bacteria, *M. luteus* and *S. aureus*, and one Gram-negative bacterium, *E. coli*, by blp was tested and compared with that of alp and lysozyme. Blp lysed *M. luteus*, *S. aureus*, and *E. coli* (Fig. 2). *M. luteus* and *S. aureus* were much more sensitive to blp than to alp and lysozyme. Blp lysed *M. luteus* 60 and 3 times faster than alp and lysozyme, respectively, and *S. aureus* 6 times faster than alp. Blp and alp also lysed $CHCl_3$-treated *E. coli* cells, though the former enzyme was the least potent of the three lytic enzymes tested. The bacteriolytic activities of alp and blp toward *E. coli* were 1/2 and 1/5 of that of lysozyme, respectively (Fig. 2). Blp also lysed intact *S. aureus* cells significantly. The rate of lysis was comparable to that for the heat-killed *S. aureus* cells, and the intact *S. aureus* cell suspension was clarified about 50 times faster with blp than with the recombinant lysostaphin, a staphylolytic enzyme from *S. simulans*, in 10 mM Tris-HCl, pH 8.

Optimal pH and Salt Concentration Dependence—Blp was active toward both the peptide substrate and the bacterial substrates in the range of pH 7 to 10.5 and favored alkaline media for lysis (Fig. 3A). With *M. luteus* and *S. aureus*, blp exhibited a higher bacteriolytic activity at pH 10 than at pH 7-9, another optimum (Fig. 3A). A similar

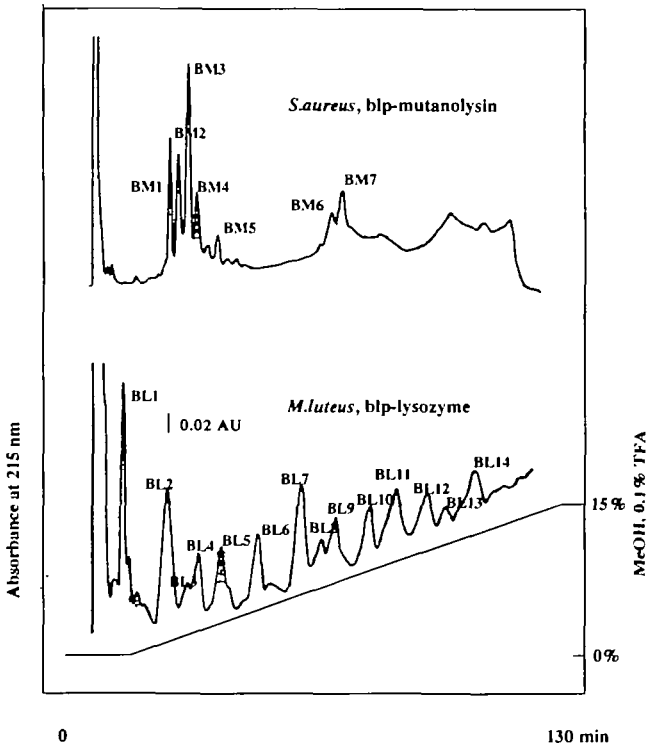

Fig. 4. **HPLC of blp-mutanolysin-derived muropeptides of *S. aureus* and blp-lysozyme-derived muropeptides of *M. luteus*.** The muropeptides subjected to the N-terminal amino acid sequencing and amino acid composition analyses are numbered. Conditions for chromatographies, see text.

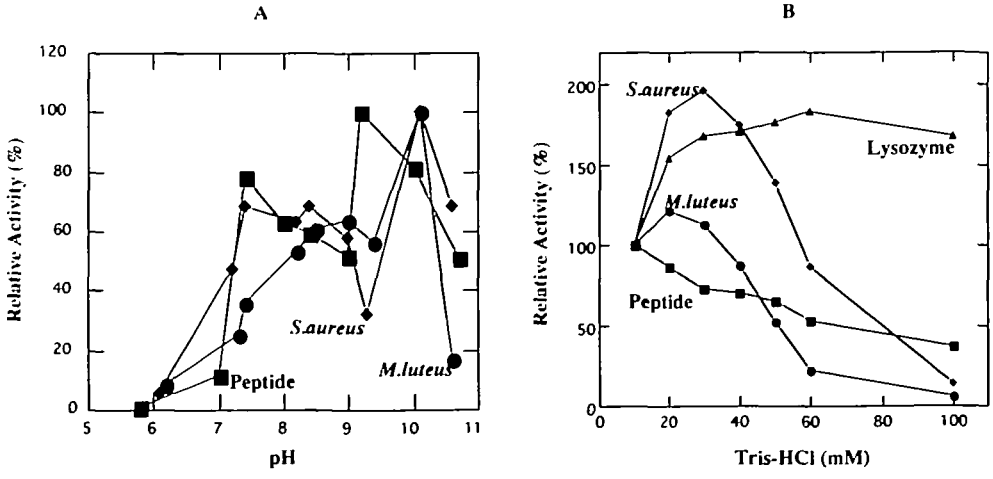

Fig. 3. **pH and salt concentration dependence of blp in bacteriolysis and proteolysis.** (A) *S. aureus* cells, *M. luteus* cells, and neuromedin B were the substrates used for the lytic and the peptidase activity assay. The optimal pH was determined using the following buffers: 10 mM sodium phosphate, pH 6–7; 10 mM Tris-HCl, pH 8–9; 10 mM NaOH-glycine, pH 10–11. The highest activity for each individual substrate was taken as 100%. (B) *M. luteus* was used as the substrate in the salt concentration dependence experiment and the activity in 10 mM Tris-HCl was taken as 100%.

pH-profile, though partly shifted to lower pH, was also recorded for the peptidase activity when assayed with neuromedin B. The lytic activity of blp was sensitive to the change of salt concentration up to about 100 mM: it increased until about 35 mM Tris-HCl and then decreased. However, the peptidase activity was much less sensitive to salt concentration than lytic activity. The influence of salt concentration on the lysis with lysozyme was different from that in the case of blp. Lysozyme retained the enhanced lytic activity in the range of 30-100 mM of buffer concentration (Fig. 3B).

Blp-Sensitive Sites in the Peptidoglycan—*S. aureus* and *M. luteus* peptidoglycans were incubated with blp alone or subsequently with mutanolysin or lysozyme, to identify the sites sensitive to the action of blp in each peptidoglycan. When the digest of *S. aureus* peptidoglycan with blp alone was analyzed by RP-HPLC, soluble muropeptide was not detected (data not shown). Subsequent incubation with

TABLE III. **Amino acid compositions and N-terminal sequences of blp-derived muropeptides of *S. aureus* and *M. luteus*.** Molar ratio of amino acids and glucosamine was normalized to lysine. The integral value is shown in parentheses. BM means blp-mutanolysin-digested muropeptides of *S. aureus*, and BL means blp-lysozyme-digested muropeptides of *M. luteus*.

Peak	Molar ratio of amino acid and glucosamine					N-terminal amino acid sequence
	Lys	Glx	Gly	Ala	GlcN	
BM1	1.0 (1)	1.2 (1)	1.6 (2)	2.1 (2)	0.7 (1)	Gly
BM2	1.0 (1)	1.1 (1)	3.0 (3)	2.2 (2)	0.7 (1)	Gly-Gly
BM3	1.0 (1)	1.2 (1)	4.7 (5)	2.3 (2)	0.7 (1)	Gly-Gly-Gly-Gly-Gly
BM4	1.0 (1)	1.2 (1)	4.6 (5)	2.8 (3)	0.7 (1)	Gly-Gly-Gly-Gly-Gly
BM5	1.0 (1)	1.1 (1)	3.2 (3)	2.3 (2)	0.8 (1)	Gly-Gly-Gly-Gly-Gly; Gly
BM6	1.0 (1)	1.2 (1)	4.0 (4)	2.3 (2)	1.0 (1)	Gly-Gly-Gly-Gly-Gly; Gly
BM7	1.0 (1)	1.2 (1)	3.6 (4)	2.2 (2)	0.8 (1)	Gly-Gly-Gly-Gly-Gly; Gly-Gly
BL1	1.0 (1)	1.8 (2)	1.6 (2)	2.1 (2)	28.4 (28)	Ala-X-Gly-X
BL2	1.0 (1)	1.1 (1)	1.0 (1)	2.0 (2)	0.6 (1)	Ala-X-Gly-X
BL3	1.0 (1)	1.2 (1)	1.2 (1)	2.6 (3)	1.0 (1)	Ala-X-Gly-X
BL4	1.0 (1)	1.1 (1)	1.1 (1)	2.1 (2)	0.3 (1)	Ala-X-Gly-X
BL5	1.0 (1)	1.3 (1)	1.2 (1)	2.2 (2)	8.6 (9)	Ala-X-Gly-X
BL6	1.0 (1)	1.1 (1)	1.1 (1)	2.1 (2)	1.7 (2)	Ala-X-Gly-X
BL7	1.0 (1)	1.2 (1)	1.0 (1)	2.1 (2)	1.6 (2)	Ala-X-Gly-X
BL8	1.0 (1)	1.2 (1)	1.3 (1)	2.1 (2)	0.7 (1)	Ala-X-Gly-X
BL9	1.0 (1)	1.3 (1)	1.3 (1)	2.0 (2)	1.4 (2)	Ala-X-Gly-X
BL10	1.0 (1)	1.2 (1)	1.1 (1)	2.1 (2)	2.3 (2)	Ala-X-Gly-X
BL11	1.0 (1)	1.1 (1)	1.0 (1)	2.1 (2)	1.1 (1)	Ala-X-Gly-X
BL12	1.0 (1)	1.2 (1)	1.0 (1)	2.1 (2)	1.6 (2)	Ala-X-Gly-X
BL13	1.0 (1)	1.2 (1)	1.2 (1)	2.1 (2)	1.5 (2)	Ala-X-Gly-X
BL14	1.0 (1)	1.1 (1)	1.0 (1)	2.1 (2)	1.2 (1)	Ala-X-Gly-X

X: unknown.

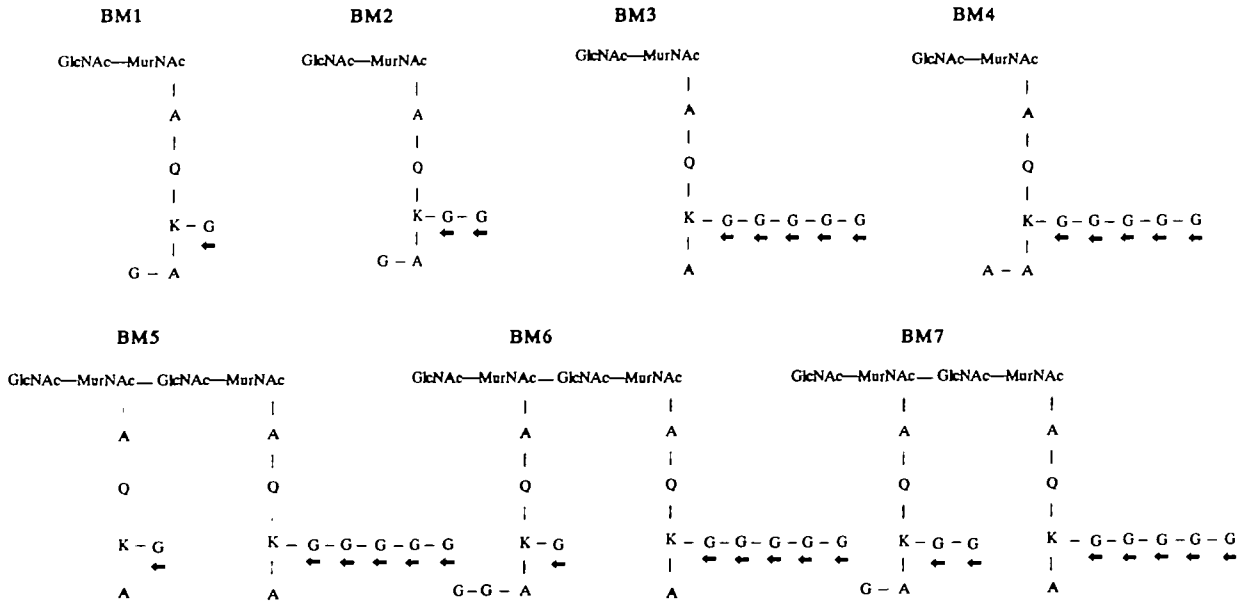

Fig. 5. **Structures of blp-mutanolysin-derived muropeptides of *S. aureus*.** The structures of muropeptides were deduced by analyzing the N-terminal amino acid sequences and amino acid compositions. The length of oligoglycine linked to D-Ala was estimated by subtracting the number of consecutive glycine residues detected by Edman degradation, which are indicated by arrows, from the total number of glycine residues estimated by amino acid composition analysis. For BM5, BM6, and BM7, only one of the possible structures is shown, as the number and position of glycine residues linked to D-Ala were not determined.

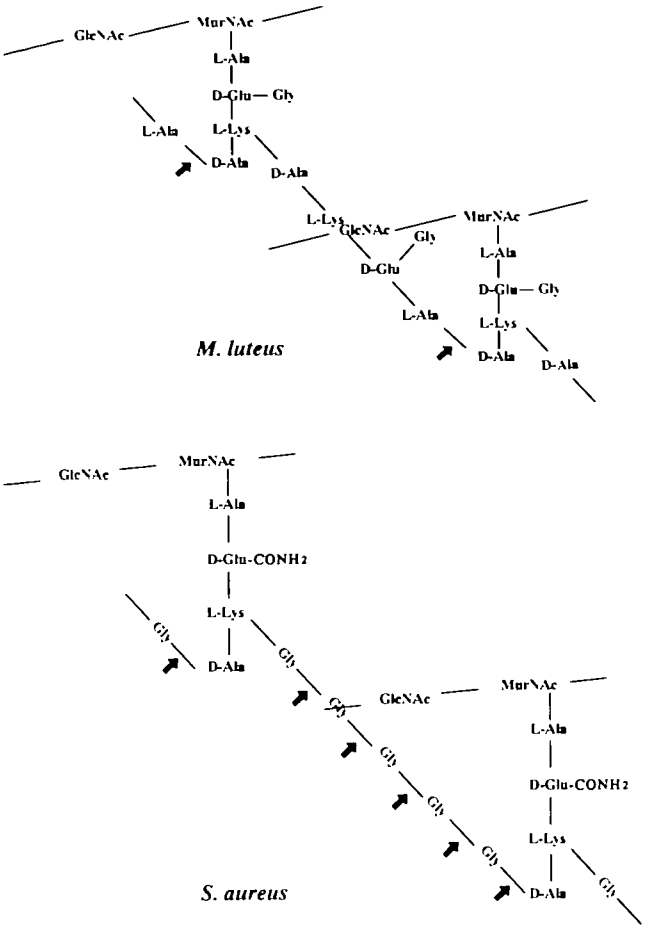

Fig. 6. **Cleavage sites of blp on *S. aureus* and *M. luteus* peptidoglycan.** The arrows indicate the peptide bonds sensitive to blp. GlcNAc: *N*-acetylglucosamine. MurNAc: *N*-acetylmuramic acid.

mutanolysin produced a number of detectable muropeptide fragments (Fig. 4), indicating that blp hydrolyzed the peptidoglycan at the peptide moiety but not at the polysaccharide moiety. Amino acid composition analyses showed that all the blp-mutanolysin-derived *S. aureus* muropeptides, BM1-7, were composed of Lys, Glx, Gly, Ala, and GlcN (Table III), which are the known constituents of *S. aureus* peptidoglycan. The N-terminal amino acid sequence analysis revealed that BM1-7 have glycine as their N-termini and BM3-7 each retain the intact interpeptide chain (Table III). Only glycine was detectable as PTH-derivative up to the 5th cycle of Edman degradation. However, in the second cycle for BM5 and 6 and the third cycle for BM7, an unexpectedly low amount of PTH-glycine was recovered (70, 53, and 60%, respectively, of that observed in the previous cycle). We interpreted this low recovery as indicating that BM5, 6, and 7 have a dimeric structure with two interpeptides of different length (Fig. 5). This is compatible with the results of amino acid composition analysis, which showed the ratio of glycine to lysine in BM5, 6, and 7 to be smaller than that in BM3 and 4. Based on the analyses, the structures of the isolated muropeptides were reasonably assigned (Fig. 5). Comparison of the proposed structures with the known structure of *S. aureus* peptidoglycan led to the conclusion that blp cleaves the Gly-Gly and the D-Ala-Gly bonds in peptidoglycan (Fig. 6).

The chromatogram of blp-lysozyme-digested muropeptides from *M. luteus* was more complex than that recorded in the case of *S. aureus* (Fig. 4). All muropeptides separated were composed of Lys, Glx, Gly, Ala, and GlcN (Table III) and could be tentatively classified into five groups according to the molar ratio of Ala and GlcN: group 1, BL1; group 2, BL5; group 3, BL3; group 4, BL6, BL7, BL9, BL10, BL12, and BL13; and group 5, BL2, BL4, BL8, BL11, and BL14. Edman degradation revealed that all these muropeptides have the same N-terminal amino acid sequence, Ala-X-Gly (Table III). Comparison of this sequence with the proposed structure of *M. luteus* peptidoglycan revealed that peptide bond was cleaved at the amino side of L-Ala. The glycine residue detected in the third cycle was the one covalently bound to the α-carboxyl group of D-glutamic acid, which was freed from the parent muropeptide during the second cycle of Edman degradation. Since the peptide subunit and the interpeptide have the same structure, two possibilities emerge as cleavage sites: the D-Ala-Ala bond between the peptide subunit and the interpeptide, and the *N*-acetylmuramoyl-L-alanine amide bond. Detection of glucosamine in all of the muropeptides led us to conclude that the cleavage site of blp was the D-Ala-Ala bond (Fig. 6). The different retention times of the muropeptides, which have both the same molar ratio of constituent amino acids and the same N-terminal sequence, suggested that muropeptides produced by blp-lysozyme digestion are a mixture of fragments with different oligomeric structures.

DISCUSSION

Mass spectrometry is a very sensitive tool for determining the molecular mass of a protein. The molecular mass of active blp estimated by this technique was 19,347.0 Da, and the calculated one as a Zn-containing protein based on the deduced amino acid sequence was 19,348.4 Da. Since ion-spray mass spectrometry can generally determine the molecular mass of a medium-sized protein with $\pm 0.01\%$ accuracy, the limit of molecular mass deviation for blp is calculated to be 1.9 Da. Therefore, the molecular mass difference (1.4 Da) between the above two values is within the limit of deviation of the mass spectrometry used. Considering the high amino acid identity between the two proteases (96%), we conclude that *Achromobacter* blp, like *Lysobacter* blp, contains one zinc atom per molecule. This conclusion was supported by the inhibition experiment with OP. In regard to this chelating agent, its isomer, MP, behaved rather as an activator, although the reason for this is unknown. Tolerance of the intrinsic zinc on the ionization suggests that zinc is held more tightly in blp than in *Streptomyces griseus* aminopeptidase (*11*). The inhibition of blp by 2-mercaptoethanol indicates that at least one disulfide bond is exposed and is necessary for the maintenance of the tertiary structure.

The unique structure of peptidoglycan, which contains polysaccharide and amino acid in D-configuration, implies that some of the amino acid residues responsible for binding peptidoglycan should be different from those for peptides. To prove this, the optimal pH and the salt concentration dependence were investigated for both pep-

tidase and bacteriolytic activity. The pH-profile for peptidase activity was similar to that for bacteriolytic activity (Fig. 3A). Both activities exhibited two plateaus separated at pH 9: the low one flanking pH 8 and the high one at pH 10. This suggests that the amino acid residue(s) responsible for catalysis may be the same for the two substrates. However, salt concentration exhibited different effects on peptidase and lytic activities (Fig. 3B): it invariably decreased the peptidase activity but increased the lytic activity first then decreased it. Moreover, the bacteriolytic activity was influenced much more significantly than peptidase activity. For example, in 100 mM Tris-HCl solution, the peptidase activity decreased to about 40% of that in 10 mM solution while the bacteriolytic activity decreased to near 0 (Fig. 3B). Peptidoglycan is a complex polymer that has a network-like structure. Thus, pH and moderate salt concentration seem unlikely to induce a significant structural change in it, and this was well reflected by the insensitivity of lysozyme to high salt concentration (under 100 mM) (Fig. 3B). Accordingly, the difference in the salt concentration dependence of the peptidase and the bacteriolytic activity suggests that some of the amino acid residues involved in peptidoglycan binding differs from those for peptide substrate. It also suggests that ionic interaction is probably the main driving force for blp to bind with peptidoglycan.

Bacteriolytic activity of blp was investigated using two typical Gram-positive strains, *M. luteus* and *S. aureus*, and one Gram-negative strain, *E. coli*. Like Achromopeptidase, both alp and blp were able to lyse the two Gram-positive bacteria. Blp exhibited higher bacteriolytic activity toward Gram-positive strains than both alp and lysozyme (Fig. 2). Though alp and blp can also lyse $CHCl_3$-treated *E. coli*, their activities are lower than that of lysozyme. This difference is attributable to the difference in the cleavage sites of these three enzymes and the low degree of cross-linkage of *E. coli* peptidoglycan. Both alp and blp show similar pH and ion-strength dependence in that they function most efficiently at alkaline pH and their activities are decreased by high concentration of salt (*7*). From the results described above, it is concluded that the high bacteriolytic activity of Achromopeptidase is mainly attributable to blp in the case of the Gram-positive strains.

Blp specifically targeted the D-Ala-X peptide bond in the peptidoglycans and cleaved the interpeptide bridge in *S. aureus* peptidoglycan (Fig. 6). The hydrolytic cleavages at these two sites are thought to take place simultaneously, because muropeptides with glycine attached to D-alanine of the peptide subunit and muropeptides containing the full-length interpeptide were both obtained (Fig. 5). Pentaglycine itself is not a sensitive substrate for blp since half of the muropeptides analyzed contained it. Unlike alp (*7*), blp exhibited bacteriolytic activity toward peptidoglycan that had been degraded by mutanolysin (data not shown), implying that blp probably recognizes peptidoglycan in a manner different from alp. *M. luteus* peptidoglycan is quite different in structure from that of *S. aureus*; its peptide subunit and the interpeptide have the same structure, and the extent of linkage between the polysaccharide and the peptide subunit is only 50%. This low extent of linkage can explain why the ratio of GlcN to Lys in some of the blp-lysozyme-digested muropeptides is larger than unity. Despite these structural differences, its cleavage site is the same D-Ala-X bond as that of *S. aureus* peptidoglycan. Although Gram-negative bacteria have an outer membrane to prevent the direct access of bacteriolytic enzymes to peptidoglycan, cleavage sites of blp on the *E. coli* peptidoglycan were also investigated. Chromatograms of mutanolysin-digested and blp-mutanolysin-digested *E. coli* peptidoglycan were very similar, and the amino acid compositions of the isolated fragments were essentially identical (data not shown). Thus, it was difficult to identify novel blp-derived muropeptides. It is known that the extent of linkage between the peptide subunit and the interpeptide bridge of *E. coli* peptidoglycan is about 10%, and even though this linkage is cleaved by blp, the amount of blp-derived muropeptides must be too low to be detected due to the preexistence of a large amount of muropeptides. The basic structure of peptidoglycan is similar in Gram-positive and Gram-negative bacteria: a heteropolymer built out of glycan strands cross-linked through short peptides called peptide subunits and interpeptide bridges (*12*). The composition and structure of glycan strands and peptide subunits seem to be fairly constant among bacteria, while the interpeptide bridges show great variation among Gram-positives but are uniform among Gram-negatives (*12*). The cross-link between the interpeptide bridge and the peptide subunit of most known Gram-positives has been demonstrated to be D-Ala-Ala, D-Ala-Gly, or D-Ala-Ser (*12, 13*). This is consistent with the peptidoglycan substrate specificity of blp determined in this paper. Accordingly, the D-Ala-X specificity no doubt contributes to the broad bacteriolytic spectrum and probably to the high bacteriolytic activity of blp.

TABLE IV. **Characteristics of bacteriolytic endopeptidases.**

	blp	LasA	AhP	alp	Lysostaphin
Type of protease	Zinc	Zinc	Zinc	Serine	Zinc
Molecular mass (kDa)	20	20	20	20	27
Amino acid sequence homology[a] (%)	100	46	52[b]	30	28
Peptide specificity	Gly-X	Gly-Gly	Gly-X	Broad, but small side-chain preferable	Gly-Gly
Peptidoglycan specificity	D-Ala-X, Gly-Gly	Gly-Gly	Unknown	D-Ala-X, Gly-Gly, *N*-acetylmuramoyl-L-alanyl	Gly-Gly
Bacteriolytic spectrum	Broad	Staphylolytic	Unknown	Broad	Staphylolytic
Biosynthesis	Preproenzyme	Preproenzyme	Unknown	Preproenzyme	Preproenzyme

blp: *Achromobacter* β-lytic protease. LasA: *P. aeruginosa* LasA protein (*13, 15*). AhP: *A. hydrophila* proteinase (*19*). alp: *Achromobacter* and *Lysobacter* α-lytic protease (*7, 21, 22*). Lysostaphin: *S. simulans* lysostaphin (*16-18*). a: sequence was aligned to that of *Achromobacter* blp. b: the N-terminal 40 amino acid residues only.

Blp exhibits strict specificity for glycine. It prefers hydrophobic and polar amino acids such as leucine, phenylalanine, tryptophan, histidine, and asparagine at the P1′ site (Table II), in which it is similar in specificity to thermolysin. However, the P1 site is restricted to glycine. This fact indicates that unlike thermolysin-type metalloprotease, in which substrate specificity is determined by the P1′ site, the specificity of blp is determined much more by P1 than by the P1′ site. The cleavage of D-Ala-Gly and D-Ala-Ala bonds in peptidoglycan by blp suggests that blp may recognize amino acid of D-configuration in peptides. However, no cleavage of the D-Ala-Gly bond was observed in a synthetic peptide (Table II), indicating that blp could not recognize D-amino acid in peptide. Accordingly, it appears that the glycan part of peptidoglycan may facilitate cleavage of the D-Ala-X bond in peptidoglycan by interacting with blp directly or by altering the conformation of the D-Ala-X bond to one sensitive to blp.

Lysobacter blp, which has 96% sequence identity with *Achromobacter* blp (*6*), has been shown to cleave dipeptides Gly-Phe and Gly-Leu and contain a zinc atom (*14*), which is consistent with the substrate specificity and metal content of *Achromobacter* blp determined in this paper. Several other bacteriolytic endoproteases including *Achromobacter* alp (*7*), LasA of *P. aeruginosa* (*15*), and lysostaphin of *S. simulans* (*16*) have also been reported to be of staphylolytic. All these endoproteases, except for alp, are zinc-proteases with a molecular mass of about 20 kDa, specifically hydrolyze Gly-X or Gly-Gly peptide bonds in peptides, and exhibit staphylolytic activity by cleaving Gly-Gly peptide bonds in peptidoglycan (Table IV) (*15-18*). Although its bacteriolytic activity has not been investigated, it can be predicted that AhP of *A. hydrophila* (*19*) is staphylolytic and may possess a broad bacteriolytic spectrum, because it exhibits very similar peptide specificity to blp and shares about 50% amino acid identity with blp (Table IV). From their amino acid similarity, blp, LasA of *P. aeruginosa* (*15*) and AhP of *A. hydrophila* (*19*) have been proposed to form a novel zinc protease family of β-lytic endopeptidases (*20*). In this study, we demonstrated that *Achromobacter* blp differs from the other staphylolytic endoproteases that hydrolyze the Gly-Gly peptide bond in peptidoglycan in that it exhibits a broader bacteriolytic spectrum by cleaving the D-Ala-X peptide bond in peptidoglycan (Table IV). The higher bacteriolytic activity and specificity of blp demonstrated in this investigation suggest the potential of blp for use as an antimicrobial agent and in the preparation of useful muropeptides and the determination of peptidoglycan structures.

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