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# Purification, Staphylolytic Activity, and Cleavage Sites of α-Lytic Protease from *Achromobacter lyticus*

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**α-Lytic protease (alp) was purified from a bacteriolytic agent, Achromopeptidase from *Achromobacter lyticus* M497-1, and has been shown to possess staphylolytic activity. Cleavage sites of this enzyme on the peptidoglycan of *Staphylococcus aureus* were determined by N-terminal amino acid sequence and amino acid composition analyses. Alp cleaved the *N*-acetylmuramoyl-L-alanine amide bond, the junction between the polysaccharide and peptide moieties, in addition to the D-Ala-Gly and Gly-Gly peptide bonds, implying that this enzyme recognizes the amino acid of D-configuration at the P1 site and possesses *N*-acetylmuramoyl-L-alanine amidase activity. However, alp could not cleave the D-Ala-Gly peptide bond in a synthetic peptide, suggesting that this hydrolytic activity of alp is peptidoglycan-specific. The results obtained from different consecutive actions of alp and glycosidase on *S. aureus* peptidoglycan indicate that the presence of polysaccharide in the peptidoglycan is necessary for the bacteriolytic activity of alp.**

**Key words: *N*-acetylmuramoyl-L-alanine amidase, *Achromobacter lyticus*, Achromopeptidase, α-lytic protease, *Staphylococcus aureus*.**

Various peptidoglycan-degrading enzymes such as autolysin have been found to lyse bacteria (*1*). These enzymes include muramidases that hydrolyze the polysaccharide chain, *N*-acetylmuramoyl-L-alanine amidases that split the linkage between polysaccharide and peptide subunits, and endopeptidases that cleave peptide bonds in the peptidoglycan. Hen egg-white lysozyme, a muramidase, is the most extensively studied bacteriolytic enzyme (*2*) and is widely used in laboratory and medical procedures. However, Gram-negative bacteria and some Gram-positive bacteria such as *Staphylococcus aureus* are resistant to the action of lysozyme, which limits the use of this enzyme as a potential antimicrobial agent for curing diseases caused by infection of pathogenic bacteria such as the methicillin-resistant strain of *S. aureus*, the most important nosocomial pathogen worldwide.

A bacteriolytic agent has been prepared from *Achromobacter lyticus* and is commercially available as Achromopeptidase. It has been reported that this agent exhibits a broader bacteriolytic spectrum and a higher bacteriolytic activity than lysozyme and has potential bacteriolytic activities toward several lysozyme-resistant pathogenic strains of *Staphylococcus*, *Streptococcus*, and *Clostridium*. We have previously found that Achromopeptidase contains two proteases quite similar to α- and β-lytic proteases from *Lysobacter enzymogenes* (*3*). α-Lytic protease (alp) is a mammalian-type serine protease, which hydrolyzes peptide bonds at the carboxyl side of small hydrophobic amino acids such as alanine and valine (*4*). However, the bacteriolytic mechanism of alp is unclear. The proteolytic activity of this lytic protease and the existence of alanine in peptidoglycans imply that alp may act on the peptide part of peptidoglycan to hydrolyze the Ala-X peptide bond. Since peptidoglycan is a complex macromolecule that contains both polysaccharide and amino acids of D-configuration, it is likely that a certain structural element (or elements) in the peptidoglycan participates in the alp hydrolysis of peptidoglycan. We investigated whether alp acts as an antimicrobial agent for *S. aureus* and which chemical bonds in the peptidoglycan are cleaved by alp. For this investigation, we purified alp from Achromopeptidase in the active form, investigated its staphylolytic activity, and characterized the major fragments derived from alp digestion of *S. aureus* peptidoglycan. The structural element in the peptidoglycan necessary for the lysis with alp was also identified.

## MATERIALS AND METHODS

*Materials*—Crude Achromopeptidase and α-amylase were purchased from Wako Pure Chemical Industries. Spray-dried *Micrococcus luteus* and CM-cellulofine (C-500) were obtained from Seikagaku. Sephacryl S-100 was purchased from Pharmacia Biotech. Mutanolysin, recombinant *S. simulans* lysostaphin (salt-free lyophilized powder) and *S. staphylolytic* lysostaphin (60% protein, minimum 500 units per mg protein) were from Sigma Chemical. DNaseI, RNaseA, chymotrypsin, and trypsin were from Worthington Biochemical Corporation. All other chemicals were obtained from Wako Pure Chemical Industries and were of appropriate grade. Trifluoroacetic acid, Suc-Ala-Ala-Ala-MCA and [D-Ala2, Met5]-enkephalin were from Peptide Institute, Osaka.

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Abbreviations: Alp: α-lytic protease; Blp: β-lytic protease; MCA: 4-methylcoumaryl-7-amide; TFA: trifluoroacetic acid; GlcNAc: *N*-acetylglucosamine; MurNAc: *N*-acetylmuramic acid.

*Purification of alp from Achromopeptidase*—Crude Achromopeptidase was dissolved in 200 ml of 10 mM citrate buffer, pH 6 and centrifuged at 10,000×*g* for 10 min. The supernatant was applied to a CM-cellulofine column (2×25 cm) equilibrated with 10 mM citrate buffer, pH 6, and the elution was performed with a linear gradient to 0.5 M NaCl. The effluent was monitored at 280 nm, and the bacteriolytic fractions were collected and concentrated by lyophilization. Then, the lyophilizate was subjected to gel filtration chromatography on a Sephacryl S-100 column (2×100 cm) with 10 mM citrate buffer, pH 6, containing 0.2 M NaCl. All the eluted peaks were assayed for both bacteriolytic and Suc-Ala-Ala-Ala-MCA-hydrolyzing activity in 10 mM Tris-HCl buffer, pH 8. The turbidity of the *M. luteus* cell suspension was recorded at 600 nm for bacteriolytic activity assay, and the increase of the fluorescence intensity of Suc-Ala-Ala-Ala-MCA was measured at 440 nm (excitation at 380 nm) for peptidase activity assay. The purity of the purified bacteriolytic enzyme was checked by SDS-PAGE (15%) and RP-HPLC on a C4 column.

*Mass Spectrometry*—The molecular mass of alp was measured accurately by mass spectrometry using an ion-spray triple quadrupole mass spectrometer (API III, Sciex). The sample was prepared by passing alp through a C4 column (μBondasphere 5μ C4 300 Å, 3.9×150 mm) using acetonitrile as the eluent and introduced into the mass spectrometer through a fused silica tube (100 μm i.d.) at a flow rate of 2 μl/min.

*Staphylolytic Activity Assay*—*S. aureus* (ATCC 6563) was grown in tryptic soy broth at 37°C with aeration to an optical density of 0.7 at 600 nm. Cells were centrifuged at 7,000 rpm for 10 min and washed twice with 10 mM Tris-HCl buffer, pH 8. A portion of the cells was killed at 120°C for 10 min and lyophilized. Staphylolytic activity was assayed by measuring the rate of lysis of the heat-killed *S. aureus* cell suspension. Lyophilized cells were suspended in 10 mM Tris-HCl buffer, pH 8, to an optical density of 0.4 at 600 nm, and the decrease of absorbance was monitored at 600 nm at room temperature.

*Preparation and Digestion of Peptidoglycan and Separation of Muropeptides*—Peptidoglycan from *S. aureus* was prepared by the method described previously (*5*) except that the cells were disrupted by sonication. Peptidoglycan (0.5 mg/ml) was digested in 20 mM Tris-HCl buffer, pH 7.5, with alp (0.4 μg/ml) at 37°C for 16 h and boiled for 5 min, and then half of the sample was further digested with mutanolysin (10 μg/ml), a muramidase of *Streptomyces globisporus*, at 37°C for 16 h and boiled for 5 min. The two digests thus obtained were centrifuged at 12,000 rpm for 10 min. The supernatant was concentrated to an appropriate volume by lyophilization, mixed with an equal volume of 0.5 M borate buffer, pH 9, and reduced with sodium borohydride (same amount as that of muropeptides) at room temperature for 15 min. Excess borohydride was destroyed with 20% phosphoric acid and the samples were adjusted to pH 3-4. In another experiment, peptidoglycan was first digested with mutanolysin then half of the digest was further digested with alp. Peptidoglycan was also digested with alp or mutanolysin alone under the conditions described above.

Muropeptides were separated on a 2.1×50 mm reversed-phase column (ODS-MB, S-5, 120 Å, YMC-Pack) with a linear gradient of 0-15% (v/v) methanol in 0.1% trifluoroacetic acid (0 to 100% for 120 min) at a flow rate of 0.2 ml/min. Elution was started 10 min after injection of the sample and monitored at 215 nm. Eluted muropeptides were concentrated and subjected to amino acid composition and N-terminal amino acid sequence analyses.

*Determination of Cleavage Sites of alp*—Amino acid composition analysis was performed with a Hitachi L8500S automatic amino acid analyzer, and the N-terminal amino acid sequence was determined on a 470A Applied Biosystems protein sequencer. The structures of the eluted muropeptides were deduced by comparing the data from amino acid composition and N-terminal amino acid sequence analyses for isolated muropeptide fragments. The proposed cleavage sites of alp were assigned by comparing the deduced structures of the alp-derived muropeptides with the known structure of *S. aureus* peptidoglycan.

## RESULTS

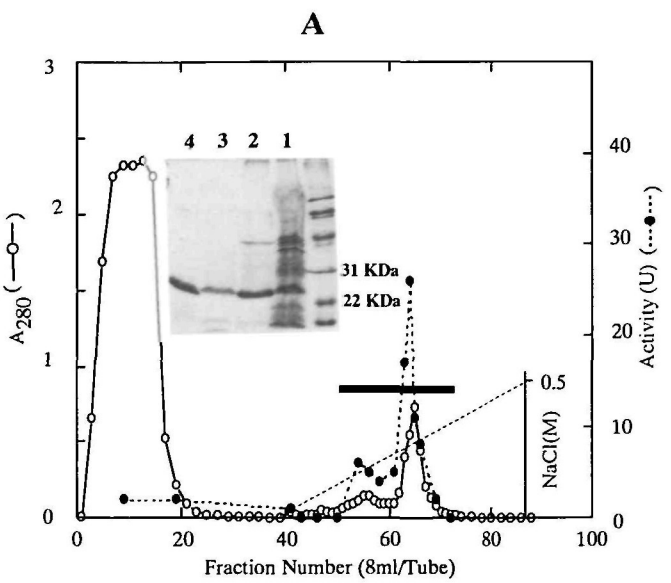
*Purification and Staphylolytic Activity of alp from Achromopeptidase*—A two-step separation procedure for intact alp was developed to purify it from a commercial preparation, Achromopeptidase (Table I). Bacteriolytic enzymes in this crude preparation were eluted as two main peaks from a CM-Cellulofine column (Fig. 1A). These active fractions were well separated from other proteins and were collected as one fraction for subsequent complete resolution on a Sephacryl S-100 column (Fig. 1B). The first bacteriolytic peak, apparently homogeneous as assessed by SDS-PAGE (15%) (Fig. 1A) and RP-HPLC (data not shown), also possessed Suc-Ala-Ala-Ala-MCA-hydrolyzing activity and was assigned as alp by analysis of both N-terminal amino acid sequence and amino acid composition (Table II). The second bacteriolytic peak had no Suc-Ala-Ala-Ala-MCA-hydrolyzing activity, and was similarly identified as β-lytic protease (Table II).

Although corrections were not made for decomposed and incompletely hydrolyzed amino acids during hydrolysis, the amino acid contents of *A. lyticus* alp and blp were shown to be very similar to those of *L. enzymogenes* alp and blp (Table II). The molecular mass of *Achromobacter* alp was determined by ion-spray mass spectrometry to be 19,909.58 Da, which is 56.44 Da larger than that calculated for *Lysobacter* alp (19,853.14 Da). The numerical value of this mass difference is equivalent to one glycyl residue (57.05 Da), suggesting that *Achromobacter* alp differs from its *Lysobacter* counterpart by only a single glycine residue in the amino acid sequence.

Since Achromopeptidase, consisting of alp and blp, can lyse *S. aureus*, the lytic activity of alp was tested with heat-killed *S. aureus* cells and compared with that of lysostaphin, a staphylolytic enzyme from *S. simulans*. It

TABLE I. **Purification of alp and blp from Achromopeptidase.** Protein quantification was conducted by the method of Bradford (*12*).

| Step | Protein (mg) | Lytic activity | | Yield (%) |
|---|---|---|---|---|
| | | Total (U) | Specific (U/mg) | |
| Commercial Achromopeptidase preparation | 156 | $1.8\times10^6$ | $1.2\times10^4$ | 100 |
| CM-Cellulofine | 8.8 | $2.4\times10^5$ | $2.7\times10^4$ | 13 |
| Sephacryl S-100 alp | 6.6 | $3.1\times10^4$ | $4.7\times10^3$ | 1.7 |
| blp | 1.8 | $5.8\times10^4$ | $3.3\times10^4$ | 3.2 |



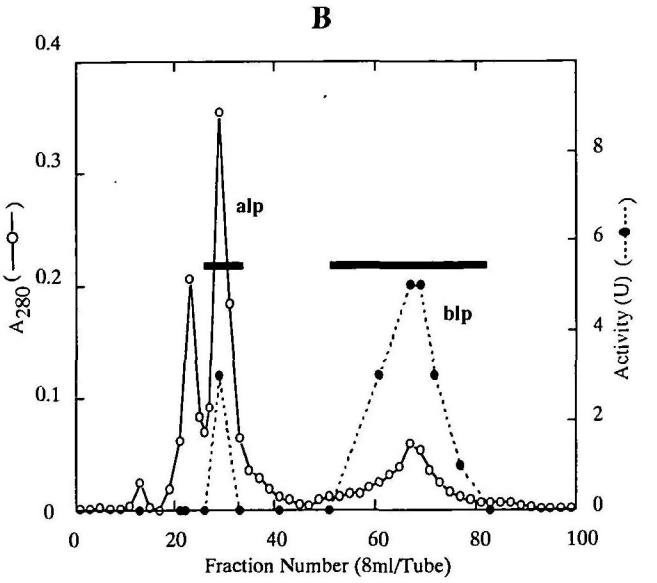




Fig. 1. **Purification of alp and blp from Achromopeptidase.** (A) Ion-exchange chromatography on a CM-Cellulofine column (2×25 cm). Elution was performed with an increasing NaCl concentration gradient. (B) Gel filtration on a Sephacryl S-100 column (2×100 cm) of bacteriolytic fractions separated by cation ion-exchange chromatography. The horizontal bars indicated the fractions collected for the subsequent separation. Presented in the inset of (A) is the SDS-PAGE of samples at individual separation steps: lane 1, crude Achromopeptidase; lane 2, CM-Cellulofine; lane 3, alp after Sephacryl S-100 gel filtration; lane 4, blp after Sephacryl S-100 gel filtration.

TABLE II. **N-Terminal sequences and amino acid compositions of alp and blp.**

N-terminal amino acid sequences

| | 1 | 10 | 20 |
|---|---|---|---|
| *Achromobacter* alp: | AN I VGG I EYS I NNAS I XS VGF S VTR | | |
| *Lysobacter* alp: | AN I VGG I EVS I NNAS I CS VGF S VTR | | |
| | 1 | 10 | 20 |
| *Achromobacter* blp: | S P NGLL QF P F P RGAS WHVGGAHTNT | | |
| *Lysobacter* blp: | S P NGLL QF P F P RGAS WHVGGAHTNT | | |

Amino acid compositions

| Amino acid | alp (mol/mol of protein) | | blp (mol/mol of protein) | |
|---|---|---|---|---|
| | *A. lyticus* | *L. enzymogenes* | *A. lyticus* | *L. enzymogenes* |
| Asp | 14.9 | 15[a,b] | 22 (22)[b] | 22[a] |
| Thr | 16.3 | 18 | 12.9 (14) | 14 |
| Ser | 17.8 | 20 | 19.5 (21) | 21 |
| Glu | 12.6 | 13 | 10.6 (10) | 10 |
| Pro | 4.0 | 4 | 9.2 (8) | 8 |
| Gly | 31.5 | 32 | 24.8 (25) | 25 |
| Ala | 24 | 24 | 13 (13) | 13 |
| Cys/2 | [c] | 6 | [c] (4) | 4 |
| Val | 17.3 | 19 | 4.7 (5) | 5 |
| Met | 1.4 | 2 | 3.5 (4) | 4 |
| Ile | 7.3 | 8 | 3.7 (4) | 4 |
| Leu | 9.5 | 10 | 9.2 (9) | 9 |
| Tyr | 5.0 | 4 | 13 (13) | 13 |
| Phe | 4.6 | 6 | 6 (6) | 6 |
| Lys | 2.0 | 2 | 3 (3) | 3 |
| His | 1.0 | 1 | 7.8 (8) | 8 |
| Arg | 11.2 | 12 | 4.8 (5) | 5 |
| Trp[d] | 1.1 | 2 | 2.2 (4) | 4 |

[a]Calculated from the published sequences (National Biomedical Research Foundation protein sequence database. alp: TRYXB4; blp: LYYXB4). [b]Calculated from the amino acid sequence deduced from the cloned alp gene of *L. enzymogenes* (*13, 14*) or the blp gene of *A. lyticus* (*3*). [c]Not determined. [d]Not corrected for decomposition during hydrolysis.

was found that alp significantly decreased the turbidity of *S. aureus* cell suspensions (Fig. 2), indicating that this protease, as an active component of bacteriolytic Achromopeptidase, is responsible for the lysis of this Gram-positive bacterium. Both alp and lysostaphin showed high activities in the range of pH 7 to 8. More precisely, the staphylolytic activity of alp was about 4 times higher than that of lysostaphin at pH 8. An unexpectedly higher staphylolytic activity was observed for these two enzymes above pH 10 (Fig. 2). Moreover, the lytic activity of alp toward intact *S. aureus* cells was also investigated. Alp lysed intact cells at the same rate as the heat-killed cells at pH 8 (data not shown).

*Structures of alp-Derived Muropeptides*—To determine which chemical bonds in the peptidoglycan are hydrolyzed by alp and which part of the peptidoglycan is necessary for the actual lysis of alp, the following four digests were prepared and analyzed. *S. aureus* peptidoglycan was digested with: (1) mutanolysin, a staphylolytic muramidase, only; (2) alp only; (3) mutanolysin, then alp (hereafter noted as mutanolysin plus alp); and (4) alp, then mutanolysin (alp plus mutanolysin). Muropeptides were separated by the method of Glauner (*6*) with slight modifications. In the modified method, trifluoroacetic acid was used in place of phosphate, which enabled us to skip the subsequent time-consuming desalting step. When the four digests were separately chromatographed, no peaks were detected for the digests with mutanolysin and mutanolysin plus alp (Fig. 3). The digests with alp and alp plus mutanolysin gave six essentially identical peaks (AM1 to AM6) on the chromatogram (Fig. 3). Peaks AM1 to AM4 were eluted soon after the void volume and AM5 and AM6 were eluted much later (Fig. 3).

Each alp-derived muropeptide thus obtained was analyzed for both amino acid composition and N-terminal amino acid sequence (Table III). All the muropeptides contained four common amino acids in a molar ratio of Lys, Ala, Glx, and Gly of 1:1:2-3:2-6. Surprisingly, gluco-

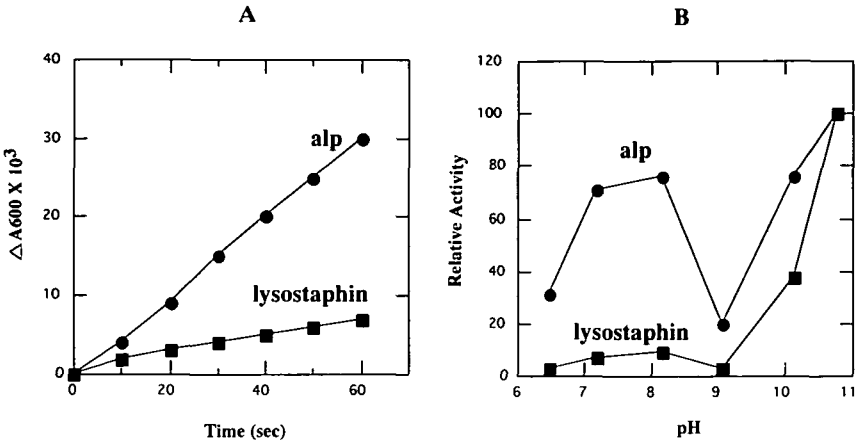



Fig. 2. **Staphylolytic activity and optimum pH of alp.** (A) The staphylolytic activity of alp (3 μg) was measured in 10 mM Tris-HCl buffer, pH 8, and compared with that of lysostaphin (3 μg). (B) The optimum pH was determined using the following buffers: 10 mM sodium phosphate buffer, pH 6-7; 10 mM Tris-HCl, pH 8-9; 10 mM NaOH-glycine, pH 10-11.

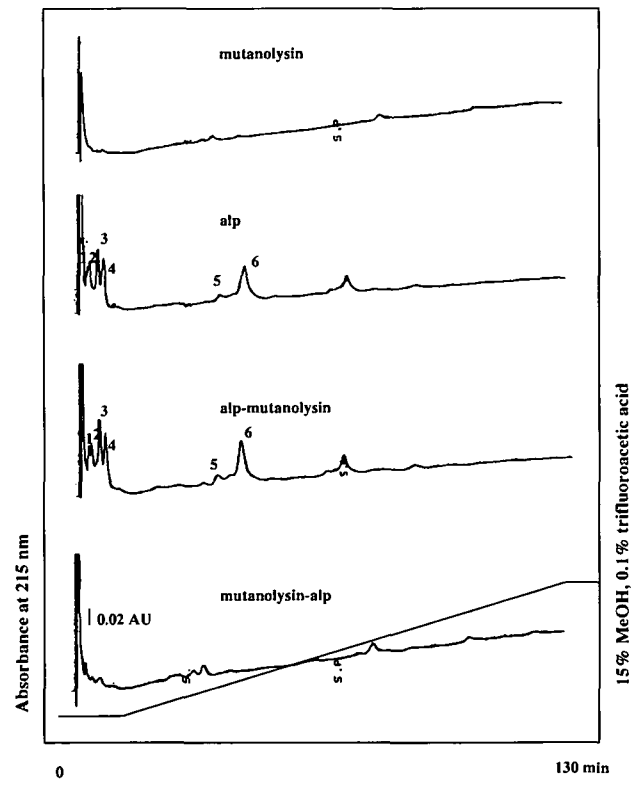



Fig. 3. **Separation of alp-derived muropeptides from *S. aureus*.** The digestion was conducted in 20 mM Tris-HCl, pH 7.5, at 37°C for 16 h. The numbered peaks were subjected to N-terminal amino acid sequence and amino acid composition analyses.

TABLE III. **Amino acid compositions of alp-mutanolysin-derived muropeptides.** Molar ratio of amino acids and glucosamine was normalized to lysine.

| Peak | Molar ratio of amino acids and glucosamine | | | | | N-terminal amino acid sequences |
|---|---|---|---|---|---|---|
| | Lys | Glx | Gly | Ala | GlcN | |
| AM1 | 1.0(1)[a] | 1.3(1) | 3.9(4) | 2.0(2) | ND | Gly-Gly;Ala |
| AM2 | 1.0(1) | 1.2(1) | 2.2(2) | 2.3(2) | ND | Gly;Ala |
| AM3 | 1.0(1) | 1.2(1) | 5.1(5) | 2.3(2) | ND | Gly-Gly-Gly-Gly-Gly;Ala |
| AM4 | 1.0(1) | 1.2(1) | 5.2(5) | 2.9(3) | ND | Gly-Gly-Gly-Gly-Gly;Ala |
| AM5 | 1.0(1) | 1.2(1) | 3.8(4) | 2.2(2) | ND | Gly;Ala |
| AM6 | 1.0(1) | 1.2(1) | 5.2(5) | 2.3(2) | ND | Gly-Gly-Gly-Gly-Gly;Ala |

ND, not detected; [a] integral value.

samine was not detected in any of these alp-derived muropeptides. N-terminal amino acid sequence analysis was performed to ascertain the length of the interpeptide attached to the ε-amino group of lysine and the cleavage sites of the peptidoglycan. The analysis revealed that all isolated muropeptides except AM5 and AM6 had two N-termini, Gly and Ala, in an equal molar ratio, but the length of interpeptide chains attached to the ε-amino group of lysine was in the range of 1-5. Muropeptides AM2 and AM5 have only one Gly attached to the ε-amino group of lysine, while AM1 has two Gly. Muropeptides AM3, AM4, and AM6 have the full-length interpeptide consisting of the pentaglycine chain. The molar ratio of Ala to Gly at the N-terminus in AM5 and AM6 was 2:1. Based on the results of amino acid composition and N-terminal amino acid sequence analyses, the structures of muropeptides AM1-6 were assigned (Fig. 4). For every muropeptide, the length of the oligoglycine interpeptide linked to D-Ala was estimated by subtracting the number of consecutive glycines detected by Edman degradation from the total number of glycines determined by amino acid composition analysis. This estimation showed that the number of glycines linking to D-Ala was two in AM1 and AM5, one in AM2, and zero in AM3 and AM6. The molar ratio of PTH-Ala to PTH-Gly at the first cycle of Edman degradation indicated that AM1, AM2, AM3, and AM4 were monomers, and AM5 and AM6 were dimers.

*Cleavage Sites of alp*—A comparison of the deduced structures of alp-derived muropeptides with the known structure of *S. aureus* peptidoglycan revealed that the following chemical bonds in the peptidoglycan were sensitive to alp: (1) the amide bond of *N*-acetylmuramoyl-L-alanine at the junction between the polysaccharide and the peptide subunits, (2) the D-Ala-Gly peptide bond, the linkage between the peptide subunit and the interpeptide, and (3) the Gly-Gly peptide bond in the interpeptide (Fig. 5). The peptide bonds in peptide subunits and another linkage, Lys(ε)-Gly, between the peptide subunit and the interpeptide could not be hydrolyzed by alp. These results indicate that alp possesses both peptidase activity and *N*-acetylmuramoyl-L-alanine amidase activity and that the presence of the polysaccharide moiety is necessary for these two activities (Fig. 3).

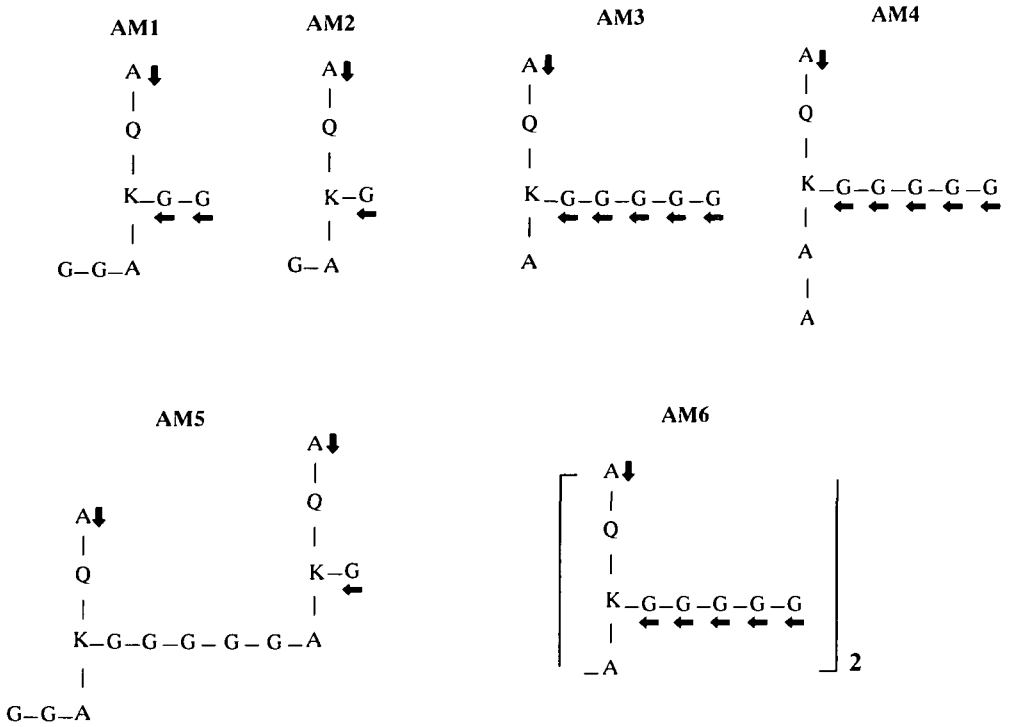



Fig. 4. **Structures of alp-derived muropeptides from *S. aureus*.** Structures were assigned according to the results of N-terminal amino acid sequence and amino acid composition analyses of isolated muropeptides. The arrow indicates the detectable N-terminal amino acid sequence.

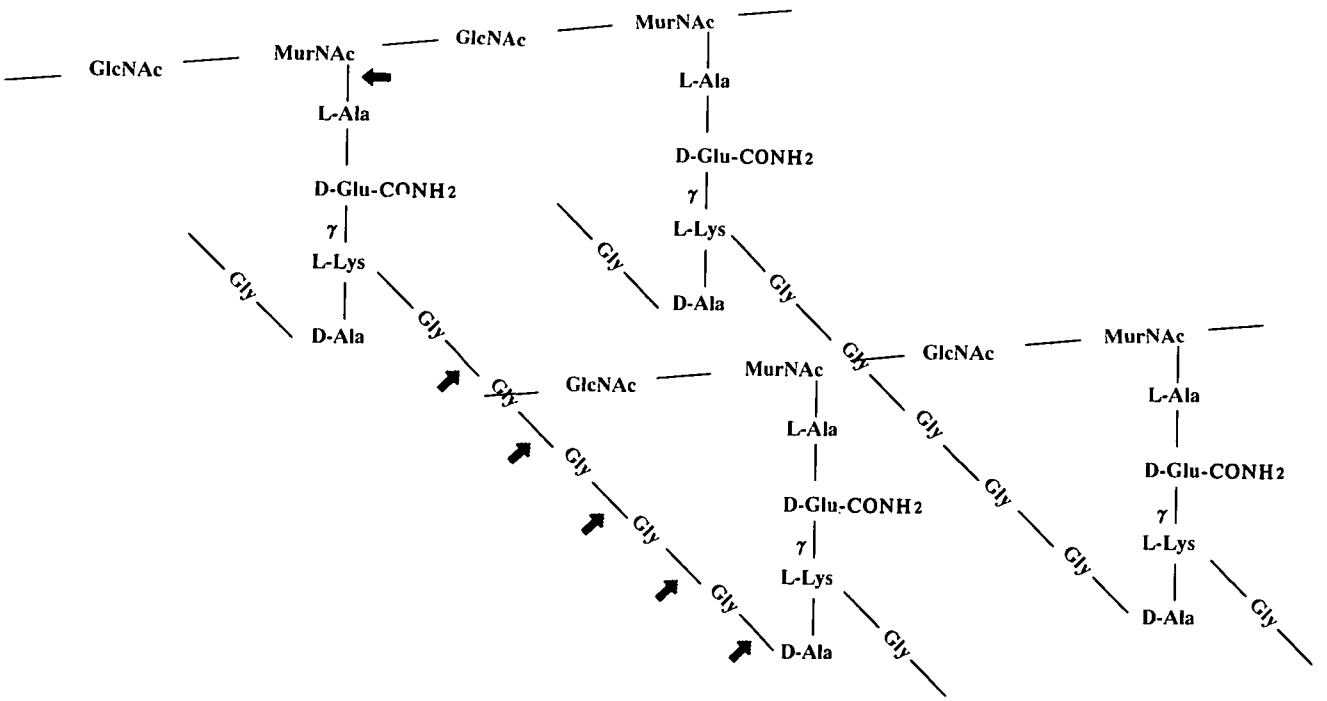



Fig. 5. **Cleavage sites of alp on peptidoglycan of *S. aureus*.** The arrow indicates cleavage. GlcNAc: *N*-acetylglucosamine. MurNAc: *N*-acetylmuramic acid.

## DISCUSSION

Previously, we have found that Achromopeptidase is composed of two bacteriolytic enzymes, which are very similar to $\alpha$- and $\beta$-lytic proteases from *L. enzymogenes* with respect to their N-terminal amino acid sequences and amino acid compositions. However, it was difficult to isolate the two lytic enzymes individually in the active form (*3*). In this investigation, these two bacteriolytic enzymes were successfully separated on a Sephacryl column. Since both enzymes have a similar molecular mass of 20 kDa, their

separation on Sephacryl was possibly due to the weaker affinity of blp to a matrix of Sephacryl, as blp was unusually retarded (Fig. 1B). The finding that alp exhibits lytic activity toward both *M. luteus* and *S. aureus* indicates that this enzyme really acts as an active component of Achromopeptidase, although it is less active than blp (data not shown). In addition to alp and blp, *A. lyticus* also secretes a lysyl bond-specific serine protease (API) (*7*), of which the primary structure is identical with that of a lysyl bond-specific serine protease from *L. enzymogenes* [Sakiyama *et al.*, poster presentation (T77) at the 5th Symposium of the Protein Society]. It is suggested that *A. lyticus* and *L. enzymogenes* are closely related genera and that the three proteases, alp, blp, and the lysyl bond-specific serine protease, efficiently collaborate to effect bacteriolysis.

All ribosomally synthesized proteins and peptides are usually composed only of amino acids of L-configuration, and very few peptides are thought to be susceptible to post-translational modification toward a D-amino acid counterpart at a given site (*8*). It is believed that proteases evolved to recognize only amino acids of L-configuration. Alp is also a mammalian-type serine protease that prefers hydrophobic L-amino acids such as Ala, Met, and Val at the P1 subsite. Nevertheless, the structure of peptidoglycan containing D-Ala can be recognized by alp, implying that the glycan moiety may play a critical role in the bacteriolytic function of this lytic enzyme. To clarify this point, *S. aureus* peptidoglycan was chosen as a substrate for lysis since the structure of peptidoglycan is known. Chemical analyses revealed hydrolysis products derived by cleavage at the *N*-acetylmuramoyl-L-alanine amide bond, the D-Ala-Gly, and the Gly-Gly peptide bonds in the peptidoglycan, demonstrating that alp possesses both *N*-acetylmuramoyl-L-alanine amidase activity and peptidase activity for the D-alanyl bond. However, the amide bond between the ε-amino group of lysine and the carboxyl group of glycine of all the tested muropeptides was insensitive to the action of alp, as glycine was detected at the first cycle of Edman degradation. The sensitivity of D-Ala-Gly and Gly-Gly peptide bonds to alp was almost the same under the present conditions, as judged from the observation that the muropeptides AM1, AM2, AM4, and AM5 were formed simultaneously with muropeptides AM3 and AM6 that lack the D-Ala-Gly bonds.

In structural determinations of peptidoglycan, hydrolytic enzymes of different specificities are often required to generate relatively small fragments from the peptidoglycan for subsequent analyses. Mutanolysin from *S. globisporus* and *Staphylococcus* lysostaphin are such enzymes, and they were used in this study to facilitate the specificity determination of alp. The alp-muramidase digestion, in which *S. aureus* peptidoglycan was digested first with alp and then with mutanolysin, showed a chromatogram of hydrolysis products very similar to that recorded for alp digestion only. This indicates that alp is an *N*-acetylmuramoyl-L-alanine amidase and peptidase, but not a muramidase. Conversely, muramidase-alp digestion in which peptidoglycan was incubated with mutanolysin prior to alp digestion did not produce any additional fragments, showing that alp hydrolyzed neither D-Ala-Gly nor Gly-Gly peptide bonds. It is likely that the degradation of the polysaccharide chain by mutanolysin causes scissile bonds to become inaccessible to alp. This idea is supported

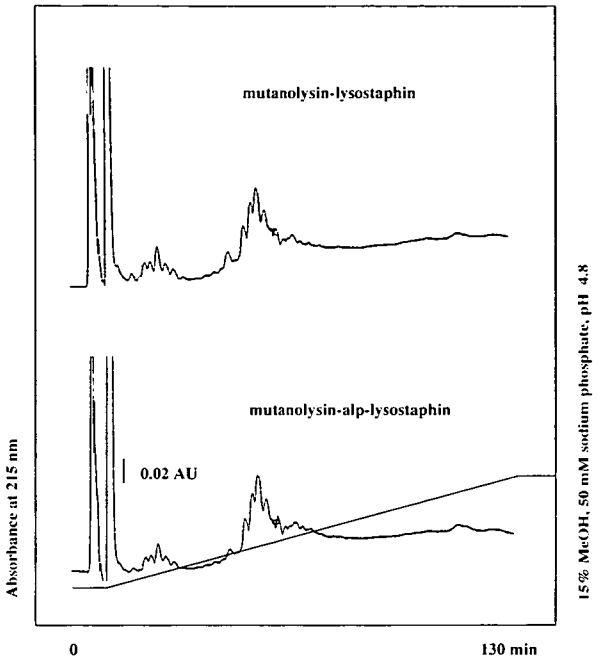



Fig. 6. **Separation of mutanolysin-lysostaphin and mutanolysin-alp-lysostaphin-derived *S. aureus* muropeptides.** Enzymatic digestion and separation were conducted as described in "MATERIALS AND METHODS" except that elution was done with a linear gradient of 0–15% (v/v) methanol in 50 mM sodium phosphate, pH 4.8 (0–100% for 120 min) at a flow rate of 0.6 ml/min. *S. staphylolyticus* lysostaphin (10 μg/ml) was used in this digestion.

by the detection of dimeric alp-derived muropeptides in which Gly-Gly and D-Ala-Gly peptide bonds are insensitive or only slightly sensitive to alp (Fig. 4). The possibility that the degradation of peptidoglycan with mutanolysin prior to alp altered the specificity of alp was excluded by the results obtained in Fig. 6, in which mutanolysin-lysostaphin and mutanolysin-alp-lysostaphin digestions showed the same chromatogram. To confirm the involvement of glycan in alp-catalyzed fragmentation, a D-Ala-containing peptide, Tyr-D-Ala-Gly-Phe-Met, was incubated with alp, but the intact peptide was recovered (data not shown). Consequently, the ability of alp to hydrolyze the D-Ala-Gly bond in the peptidoglycan is closely associated with the presence of the polysaccharide, although the mode of interaction between the glycan strand and alp is not known.

In addition to *A. lyticus* alp, peptidase LasA from *Pseudomonas aeruginosa* (*9, 10*) and lysostaphin of *S. simulans* (*11*) have been reported to exhibit staphylolytic activity *via* Gly-Gly peptide bond cleavage in the peptidoglycan. Alp differs critically from LasA and lysostaphin in that the *A. lyticus* enzyme cleaves the D-Ala-Gly peptide bond in addition to the Gly-Gly peptide bond. Alp was also shown to have an activity 4 times higher than that of *S. simulans* lysostaphin, which is probably attributable to the presence of multiple cleavage sites for alp in *S. aureus* peptidoglycan. Since the D-Ala-X peptide bond is present in the peptidoglycan of either Gram-positive or Gram-negative bacteria, alp must lyse these bacteria by hydrolyzing this particular peptide bond, if the site of the sensitive bond is accessible. The ability to lyse Gram-positive bacteria such as *S. aureus* and the peptidoglycan substrate speci-

ficity demonstrated in this study may lead to some practical applications for alp in inhibiting growth of antibiotic-resistant bacteria, the determination of the peptidoglycan structure and the preparation of useful muropeptides.

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