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Purification, Characterization, and Primary Structure of a Novel Cell Wall Hydrolytic Amidase, CwhA, from *Achromobacter lyticus*

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A novel bacteriolytic enzyme CwhA (cell wall hydrolytic amidase) was purified by ion exchange and gel-filtration chromatographies from a commercial bacteriolytic preparation from *Achromobacter lyticus*. CwhA exhibited optimal pH at 8.5 and lysed $CHCl_3$-treated *Escherichia coli* more efficiently than *Micrococcus luteus*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pediococcus acidilactici*. The enzyme was inhibited by 1,10-phenanthroline strongly and by EDTA to a lesser extent, suggesting that it is probably a metalloenzyme. Amino acid composition and mass spectrometric analyses for the CwhA-derived *M. luteus* muropeptides revealed that CwhA is *N*-acetylmuramoyl-L-alanine amidase [EC 3.5.1.28]. The complete amino acid sequence of CwhA was established by a combination of Edman degradation and mass spectrometry for peptides obtained by *Achromobacter* protease I (API) digestion and cyanogen bromide (CNBr) cleavage. The enzyme consists of a single polypeptide chain of 177 amino acid residues with one disulfide bond, Cys114–Cys121. CwhA was found to be homologous to *N*-acetylmuramoyl-L-alanine amidase from bacteriophage T7 (BPT7). Its sequence identity with BPT7 is 35%, but the amino acid residues functioning as zinc ligands in BPT7 are absent in CwhA. These results suggest that CwhA is a new type of *N*-acetylmuramoyl-L-alanine amidase.

Key words: *N*-acetylmuramoyl-L-alanine amidase, *Achromobacter lyticus*, Achromopeptidase, primary structure, mass spectrometry.

Various peptidoglycan-degrading enzymes with different action mechanisms have been isolated from animals, plants, and microorganisms and characterized (*1*). These enzymes are able to hydrolyze particular bonds in cell wall peptidoglycan and are generally classified into three groups: muramidases, which hydrolyze the polysaccharide chain; endopeptidases, which cleave the peptide moiety; and *N*-acetylmuramoyl-L-alanine amidases, which split the linkage between polysaccharides and peptides (*1*). Through cell wall degradation, these hydrolytic enzymes may 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, having the potential for use as antimicrobial agents.

Achromopeptidase, a bacteriolytic preparation from *Achromobacter lyticus*, exhibits a higher bacteriolytic activity and a broader bacteriolytic spectrum than hen egg-white lysozyme, the most widely used and well studied bacteriolytic enzyme (*5*). Previously, we have demonstrated that Achromopeptidase contains two bacteriolytic proteases, α-(alp) and β-lytic proteases (blp), which hydrolyze the D-Ala-X peptide bond in the peptidoglycan and Gly-Gly peptide bonds in *Staphylococcus aureus* peptidoglycan (*6*, *7*). Cleavage of the D-Ala-X peptide bond, which invariably exists in bacterial cell wall peptidoglycan, has been proposed to contribute to the broader bacteriolytic spectrum of Achromopeptidase (*7*). Recently, we noticed that, in addition to alp and blp, several other enzymes that can lyse *Micrococcus luteus* and/or *S. aureus* also exist in Achromopeptidase. This prompted us to characterize these lytic enzymes in order to further our understanding of the mechanism of the high and broad bacteriolytic activity of Achromopeptidase. In this study, we purified an *M. luteus*–lysing enzyme from Achromopeptidase and characterized it as an *N*-acetylmuramoyl-L-alanine amidase. We also established the primary structure of the new enzyme and compared it with those of other homologous enzymes reported so far.

EXPERIMENTAL PROCEDURES

Materials—Crude Achromopeptidase, *Achromobacter* protease I (API), *Bacillus subtilis* α-amylase, cyanogen bromide (CNBr), diisopropylfluorophosphate (DFP), iodoacetic acid, and 1,10-phenanthroline were purchased from Wako Pure Chemical Industries. 1,7-Phenanthroline was from Aldrich. Spray-dried *M. luteus* cells, hen egg-white lysozyme, and CM-cellulofine (C-500) were obtained from Seikagaku Co. Sephacryl S-100 was purchased from Pharmacia Biotech. DNaseI, RNaseA, and trypsin were from Worthington Biochemical. *S. aureus* (IFO 13276), *Enterococcus*

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Abbreviations: alp, α-lytic protease; blp, β-lytic protease; API, *Achromobacter* protease I; BPT7, bacteriophage T7; TFA, trifluoroacetic acid; CM, carboxymethylated; RCM, reduced and carboxymethylated; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry.

faecalis (IFO 3971), and *Pediococcus acidilactici* (IFO 3385) were from the Institute for Fermentation, Osaka. All other chemicals were obtained from Wako Pure Chemical Industries and were of appropriate grade.

Purification of CwhA from Achromopeptidase—Crude Achromopeptidase (1 g) was dissolved in 400 ml of 10 mM Tris-HCl, pH 8.0, and centrifuged at 9,000 ×*g* for 10 min. The supernatant was applied to a CM-cellulofine column (2 × 25 cm) previously equilibrated with 10 mM Tris-HCl, pH 8.0, and elution was performed with a linear gradient of 0 to 0.4 M NaCl. The first bacteriolytic fraction was collected, concentrated by lyophilization, then subjected to gel-filtration on a Sephacryl S-100 column (2 × 100 cm) with 10 mM citrate buffer, pH 6.0, containing 0.2 M NaCl, followed by HPLC on a Cosmosil-packed column (5Diol-120, 7.5 × 600 mm) with 0.1 M ammonium acetate, pH 6.0. The bacteriolytic fraction was concentrated and checked for purity by electrophoresis. Lytic activity was assayed as described previously (*6*). Briefly, the spray-dried *M. luteus* cells were suspended in an appropriate volume of 10 mM Tris-HCl, pH 8.0, to yield an absorbance of 0.6 at 600 nm, and the decrease of absorbance at 600 nm was recorded.

Bacteriolytic Specificity, Optimal pH, Salt Concentration Dependence, and Inhibitors—*S. aureus*, *E. faecalis*, and *P. acidilactici* were cultured according to the supplier's instructions. When optical density at 600 nm reached 0.6, cells were harvested by centrifuging at 5,000 rpm for 10 min, washed twice with 10 mM Tris-HCl, pH 8.0, and boiled for 10 min. The heat-killed cells were lyophilized and stored at –80°C. $CHCl_3$-treated *Escherichia coli* (JM109) was prepared as described previously (*7*). Buffers used for pH optimum determination were 10 mM MOPSO for pH 6.5–7.6, 10 mM Tris-HCl for pH 7.4–8.8, 10 mM CHES for pH 8.6–9.6, and 10 mM CAPS for pH 9.8–10.7. The effect of salt concentration on lytic activity was examined with *M. luteus* in Tris-HCl, pH 8.0, and that of inhibitors was assayed after incubation with CwhA at room temperature for 1 h.

Determination of Cleavage Sites—*M. luteus* peptidoglycan was prepared as described previously except that the cells were disrupted by sonication (*8*). Peptidoglycan (1 mg/ml) was digested in 10 mM Tris-HCl buffer, pH 8.0, with CwhA (4 μg/ml) at 37°C for 16 h and boiled for 5 min. Part of this digest was further digested with lysozyme (4 μg/ml) or blp (4 μg /ml) under the conditions described above. The digest was centrifuged at 12,000 rpm for 10 min, and the supernatant was mixed with an equal volume of 0.5 M borate buffer, pH 9.0, and reduced with sodium borohydride (the same amount as that of muropeptides) at room temperature for 15 min. The excess borohydride was destroyed with 20% phosphoric acid, and the solution was adjusted to pH 3–4.

Muropeptides were separated by HPLC on a YMC ODS column (ODS-MB, S-5 120 Å 2.1 × 50 mm) as described previously (*7*). The column was first run with 0.1% trifluoroacetic acid (TFA), for 10 min, then elution was performed with a linear gradient (0 to 100%) of 15% methanol containing 0.1% TFA for 120 min at a flow rate of 0.2 ml/min. Elution was monitored at 215 nm, and eluted muropeptides were concentrated and subjected to amino acid composition and mass analyses.

Determination of Amino Acid Sequence—CwhA was reduced and *S*-carboxymethylated as described previously (*9*). The resulting reduced and *S*-carboxymethylated (RCM) protein was purified by HPLC on a Waters C4 column (μBondasphere 5μ 300 Å 3.9 × 150 mm) with a linear gradient from 0 to 80% acetonitrile containing 0.08% TFA for 60 min at a flow rate of 0.8 ml/min. RCM-CwhA was digested with API at 37°C for 12 h in 50 mM boric acid buffer, pH 9.0, containing 2 M urea at an enzyme–substrate ratio of 1 to 200 (mol/mol). CNBr cleavage was done in 70% formic acid containing 1% CNBr at room temperature for 14 h in the dark. Peptides were separated by HPLC on a Waters C4 column (μBondasphere 5μ 300 Å 3.9 × 150 mm) or a Senshu Par ODS column (PEGASIL-300 ODSII, 4.5 × 150 mm) with an increasing concentration of acetonitrile (80%/80 min) containing 0.08% TFA at a flow rate of 0.8 ml/min. Peptides were detected at 215 nm.

LC/ESI-MS of CwhA, CM-CwhA, RCM-CwhA, and the API Digest—ESI-MS was performed with a Finnigan MAT LCQ mass spectrometer equipped with a Finnigan MAT atmospheric pressure ionization interface operating in the electrospray ionization mode with a needle voltage of 4.3 kV and a heated capillary temperature of 220°C. A Michrom BioResources HPLC system (MAGIC 2002™) fitted with a PLRP-S column (8μ 300Å, 1.0 × 150 mm) was coupled directly to the mass spectrometer *via* an interface. CwhA, CM-CwhA, RCM-CwhA, and the API digest of RCM-CwhA were separately applied to the column equilibrated with water containing 2% acetonitrile and 0.02% TFA and eluted with an increasing concentration of acetonitrile containing 10% water and 0.018% TFA (60%/50 min) at a flow rate of 50 μl/min. The effluent was introduced into the mass spectrometer *via* the interface. The scan was made over 150–2,000 Da, and the total number of microscans was set at 2. A series of m/z values of the multiple charged peptides in each peak were deconvoluted to a given molecular mass.

Amino Acid Composition and N-Terminal Amino Acid Sequence Analysis—The N-terminal sequence analysis was performed on a N-terminal protein sequencing system (HP G1005A, Hewlett Packard), 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.

RESULTS

Purification and Bacterial Specificity of CwhA—CwhA was purified from Achromopeptidase by a combination of ion exchange and gel-filtration chromatographies. Bacteriolytic enzymes were eluted as two major peaks on CM-cellulofine (Fig. 1A). The first peak was further purified on Sephacryl S-100 (Fig. 1B), and pure CwhA was obtained by HPLC (Fig. 1C). CwhA thus obtained was shown to be homogeneous by electrophoresis (Fig. 1C).

Since Achromopeptidase can lyse both Gram-positive and Gram-negative bacteria, the lytic activity of CwhA toward *M. luteus*, *S. aureus*, *E. faecalis*, *P. acidilactici*, *E. coli*, and $CHCl_3$-treated *E. coli* was measured and compared (Table I). Among the Gram-positive bacteria tested, CwhA lysed *M. luteus* most efficiently. *S. aureus*, *E. faecalis*, and *P. acidilactici* had sensitivities to CwhA of 30, 17, and 13% relative to *M. luteus*, respectively. Interestingly, CwhA lysed $CHCl_3$-treated *E. coli* 7.4 times faster than *M. luteus*, al-

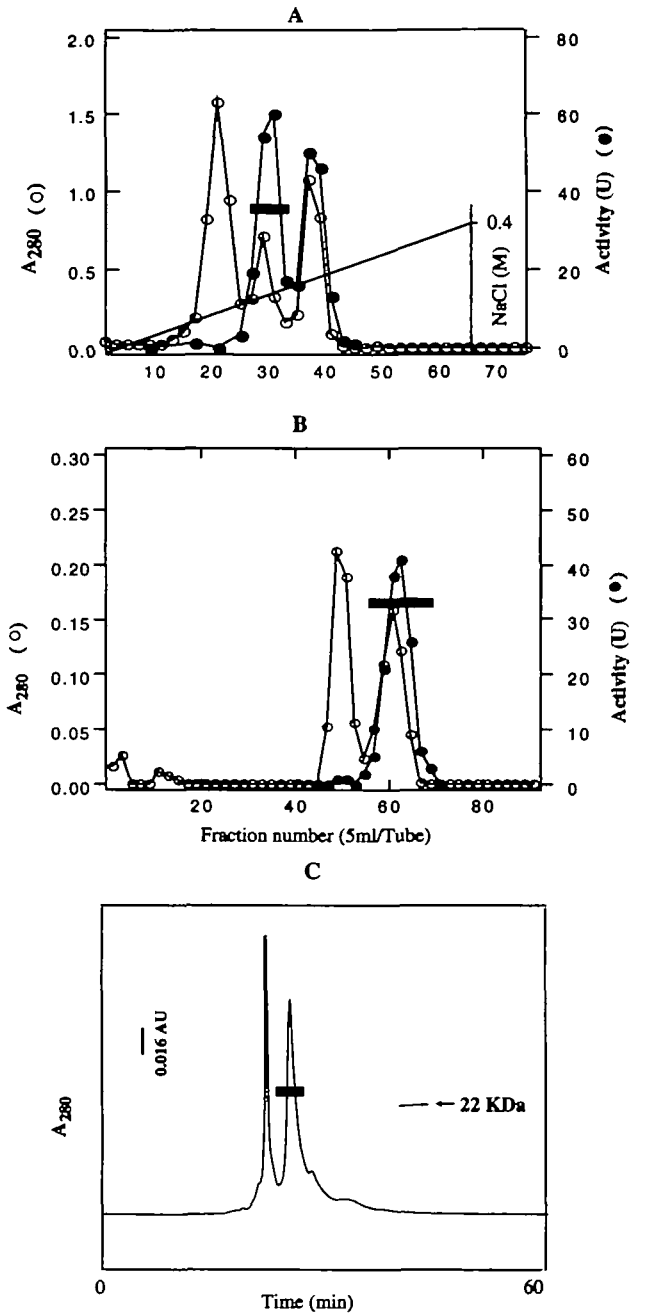

Fig. 1. **Purification of CwhA from Achromopeptidase.** (A) Ion-exchange chromatography on a CM-cellulofine column (2 × 25 cm). Elution was performed with an increasing NaCl concentration gradient. (B) Gel filtration on a Sephacryl S-100 column (2 × 100 cm) of the first bacteriolytic fraction separated by cation ion-exchange chromatography. (C) Purification of CwhA with HPLC. The horizontal bars indicate the fractions collected for the subsequent separation. The inset of (C) shows SDS-PAGE of the purified CwhA.

though intact *E. coli* was much less sensitive to CwhA.

Optimal pH, Influence of Salt Concentration and Inhibitors—CwhA was active in the alkaline range with optimal pH at 8.5 (Fig. 2A) and sensitive to salt concentration (Fig. 2B). Lytic activity in 30 mM Tris-HCl was only 30% of that in 10 mM solution, and CwhA was almost inactive in 70 mM Tris-HCl (Fig. 2B). CwhA also lost its lytic activity in 10 mM Tris-HCl containing 100 mM NaCl. DFP and

TABLE I. **Bacterial specificity of CwhA.** Bacteriolytic activity was measured in 10 mM Tris-HCl, pH 8.0, and the activity for *M. luteus* was taken as 100.

Bacterial	Relative activity (%)
Gram-positive	
Micrococcus lutes	100
Staphylococcus aureus	30
Enterococcus faecalis	17
Pediococcus acidilactici	13
Gram-negative	
Escherichia coli	8
Escherichia coli ($CHCl_3$-treated)	740

iodoacetic acid did not affect lytic activity, indicating that serine and cysteine residues are not involved in catalysis (Table II). Upon incubation with either EDTA or 1,10-phenanthroline, the enzyme lost 25 and 94% of the lytic activity, respectively. The lytic activity was reduced to 47% by treatment with DTT, suggesting the importance of disulfide bond in catalysis. The metal ions zinc, calcium, and magnesium had no effect on lytic activity.

Peptidoglycan Specificity—Peptidoglycan consists of glycan chains and short peptide chains that are cross-linked so as to constitute the overall structure of a network surrounding the cell (Fig. 3). The linear chain structure of the glycan part consists of alternating molecules of *N*-acetylglucosamine and its 3-*O*-D-lactyl ether derivative, muramic acid. The peptide part is composed of two parts: an obligatory peptide subunit consisting of the primary chain linked to muramic acid, and an optional peptide bridge involved in the formation of cross-links in peptidoglycans. The peptide part contains amino acids of D-configuration that are usually not present in proteins. In addition to the α-amino and α-carboxyl group, the γ-carboxyl group of D-glutamic acid, and the ε-amino group of lysine also participate in amide bond formation in peptidoglycan (Fig. 3). Peptidoglycan-degrading enzymes are classified as muramidases, endopeptidases, or *N*-acetylmuramoyl-L-alanine amidases, according to their cleavage sites.

To identify the CwhA-sensitive chemical bonds in the peptidoglycan, *M. luteus* peptidoglycan was digested under three different conditions with lytic enzymes and chromatographically analyzed. The digests were: (i) CwhA only; (ii) CwhA, then lysozyme (hereafter noted as CwhA plus lysozyme); and (iii) CwhA, then blp, a D-Ala-X bond specific bacteriolytic endopeptidase from *A. lyticus* (CwhA plus blp). The chromatographic pattern of the CwhA plus lysozyme digest was identical to that of the CwhA only digest, showing a total of five peaks, designated as C1-C5 (Fig. 4). Digestion with CwhA followed by blp increased the number of peaks to nine (Cb1–Cb9) and significantly increased the amount of fragments eluted in peaks corresponding to peaks 1 and 2 in the digest with CwhA only (Fig. 4). These results suggested that the lytic enzyme purified here is a muramidase or *N*-acetylmuramoyl-L-alanine amidase, but not an endopeptidase. Each muropeptide (C1–C5 and Cb1–Cb9) was analyzed for both amino acid composition and molecular mass (Table III). Amino acid composition analysis showed that Lys, Ala, Glu, and Gly in a molar ratio of 1:2–2.4:1:1 were common constituents of these muropeptides, and that none contained glucosamine. Molecular masses of muropeptides C-1 and Cb-1 were determined to be about 474 Da, which corresponds to the peptide subunit A-E (G)-K-A (Fig. 3). The molecular masses of the other

muropeptides, except for C-3 and Cb-4, which contain an additional alanine, are well consistent with those of dimer, trimer, and tetramer of the peptide subunit (Table III). These results indicated that all the isolated peptides were from *M. luteus* peptidoglycan and that none contained glucosamine. Previously, we reported that blp specifically hydrolyzes the D-Ala-Ala bond in *M. luteus* peptidoglycan (*7*). The action of blp on the CwhA-degraded *M. luteus* peptidoglycan produced severalfold more muropeptides C1 and C2, indicating that the D-Ala-Ala bond was insensitive to CwhA. Hence, the lytic enzyme purified here primarily hydrolyzes the *N*-acetylmuramoyl-L-alanyl amide bond and is an *N*-acetylmuramoyl-L-alanyl amidase (Fig. 3).

Primary Structure—HPLC-purified RCM-CwhA was

TABLE II. **Inhibition of CwhA by chemical compounds.** CwhA was preincubated with a number of compounds at room temperature for 60 min. Lytic activity was measured with *M. luteus* in 10 mM Tris-HCl, pH 8.0.

Compounds	Concentration (mM)	Relative activity (%)
Blank		100
Zn^{2+}	5	114
Ca^{2+}	5	109
Mg^{2+}	5	109
1,10-Phenanthroline	5	6
1,7-Phenanthroline	5	125
EDTA	10	75
DTT	5	47
ICH_2COOH	5	102
DFP	5	124

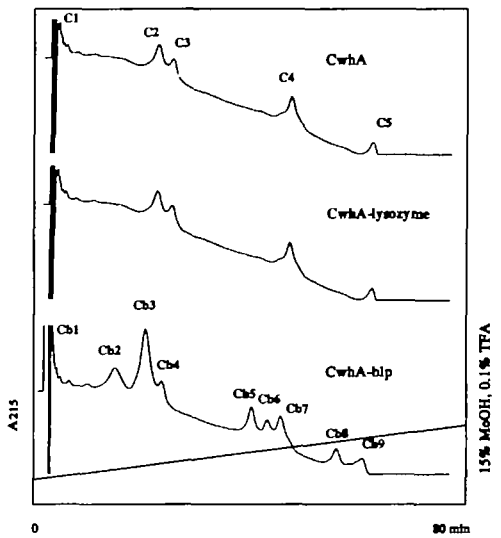

Fig. 4. **HPLC separation of *M. luteus* muropeptides.** The numbered peaks were subjected to amino acid composition and mass analyses. blp: *Achromobacter* β-lytic protease.

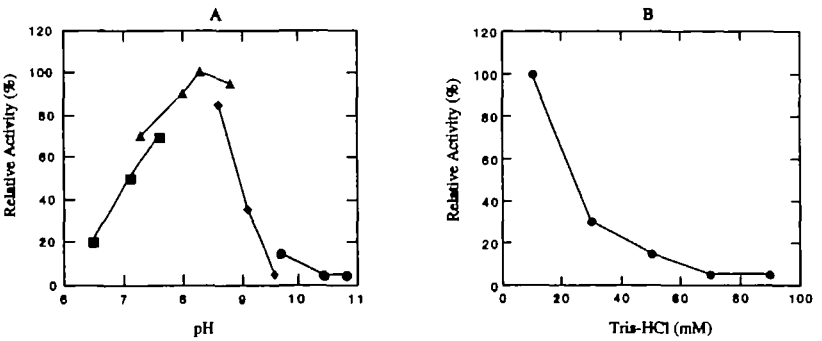

Fig. 2. **pH-profile and influence of salt concentration.** (A) The optimal pH was determined using the following buffers: 10 mM MOPSO, pH 6.5–7.6; 10 mM Tris-HCl, pH 7.4–8.8; 10 mM CHES, pH 8.6–9.6; and 10 mM CAPS, pH 9.8–10.7. (B) The influence of salt concentration on lytic activity was investigated in Tris-HCl, pH 8.0.

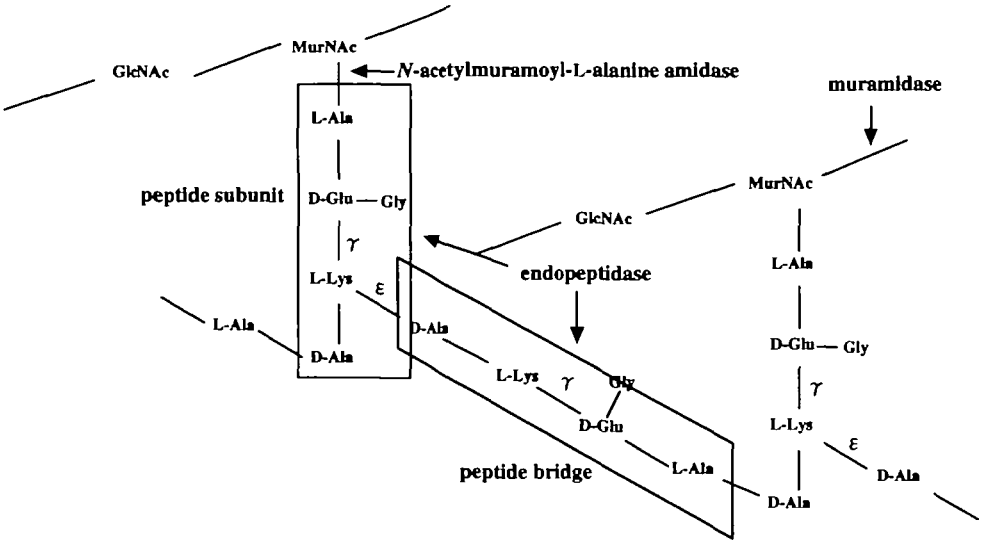

Fig. 3. **Structure of *M. luteus* peptidoglycan.** Bacterial peptidoglycan is composed of glycan strands, peptide subunits, and peptide bridges. The primary structure of the peptide subunit is presented as A-E (G)-K-A in the text. Isopeptide bonds formed by the γ-carboxyl group of D-glutamic acid and the ε-amino group of lysine are indicated by γ and ε, respectively. Arrows indicate the chemical bonds sensitive to peptidoglycan-degrading enzymes. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid.

submitted to automated Edman degradation. At each cycle, a single PTH-amino acid was detected, and the N-terminal 50 amino acid sequence was determined. Thereby we concluded that the protein was pure enough for sequence analysis. RCM-CwhA was digested with API, and eight peptides (K1-K7-2) were separated completely by HPLC (Fig. 5). These K peptides, numbered in order from the N-terminus of CwhA, were each subjected to N-terminal sequencing. Peptide K1, the N-terminal 58-residue peptide, was directly sequenced up to the 50th position, and peptides K2, K3, K5, and K6 were sequenced completely (Fig. 6). In K4, the 3rd and 10th residues from the N-terminus were identified as PTH-CM-Cys. Peptide K7 was detected as two fragments: peptide K7-2 is an 18-residue ipeptide ending at

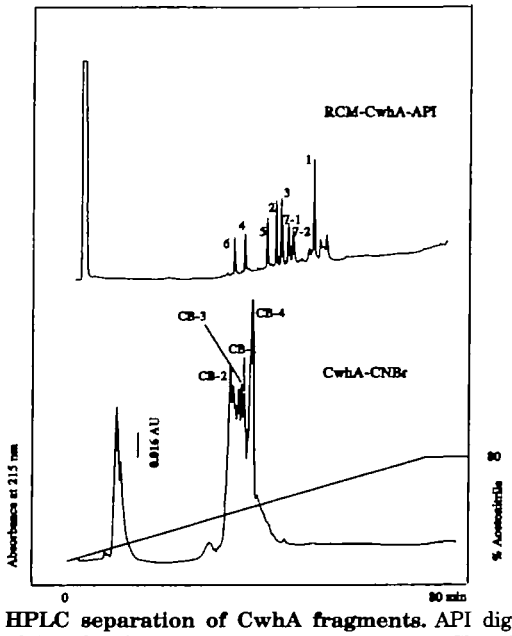

Fig. 5. **HPLC separation of CwhA fragments.** API digests of RCM-CwhA (RCM-CwhA-API) were separated on a Shenshu Par ODS column and CNBr-treated CwhA (CwhA-CNBr) on a Waters C4 column. Peaks were numbered in order from the N-terminus and subjected to N-terminal sequence analysis.

TABLE IV. **LC/MS analyses of CwhA, CM-CwhA, RCM-CwhA, and API peptides of RCM-CwhA.** CM-CwhA and RCM-CwhA were prepared by alkylating CwhA with iodoacetic acid in the absence of DTT and in the persence of DTT, respectively. Theoretical mass value of CwhA is calculated from the amino acid sequence determined in this experiment, in which one disulfide bond is assumed to form between Cys114 and Cys121. Theoretical mass values of API peptides are based on the amino acid sequence determined by Edman degradation. CM, carboxymethylated; RCM, reduced-carboxylmethylated.

Peptide	Residues	Theoretical mass (Da)	Observed mass (Da)	Deviation (%)
CwhA	1 to 177	19,395.9	19,393.4	−0.013
CM-CwhA	1 to 177		19,395.6	
RCM-CwhA	1 to 177	19,513.4	19,511.5	−0.01
K1	1 to 58	6,265.0	6,267.2	+0.035
K2	59 to 77	2,285.1	2,284.9	−0.009
K3	78 to 111	3,674.8	3,675.4	+0.016
K4	112 to 126	1,737.8	1,737.8	0
K5	127 to 143	1,887.0	1,887.7	+0.037
K6	144 to 159	1,731.8	1,731.6	−0.012
K7-1			2,033.7	
K7-2	160 to 177	2,032.9	2,033.7	+0.039

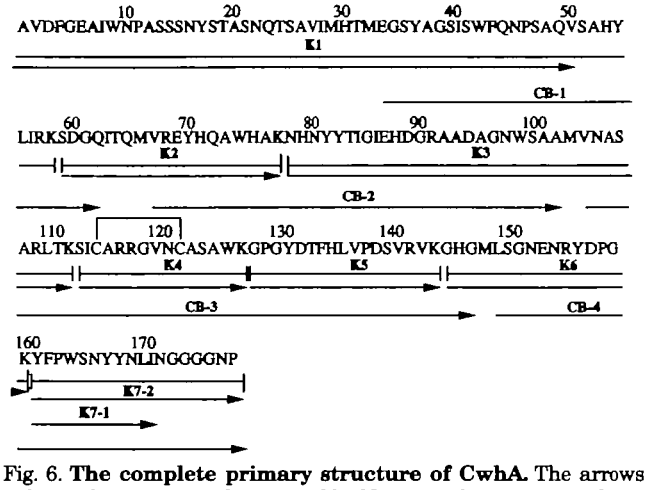

Fig. 6. **The complete primary structure of CwhA.** The arrows indicate the sequences determined by N-terminal sequence analysis. K, API-derived fragments; CB, CNBr-derived fragments. For amino acid sequence accession number, see the text.

TABLE III. **Amino acid compositions and molecular masses of muropeptides derived from CwhA and CwhA-blp digestion of *M. luteus* peptidoglycan.** Molar ratio of amino acid was normalized to lysine. blp: *Achromobacter* β-lytic protease.

Peak	Molar ratio of amino acid					Observed mass (Da)	Deduced structure
	A	E	G	K	GlcN		
C-1	2.0	1.0	2.5	1	ND	474.2 (474.5)*	A-E(G)-K-A
C-2	2.0	1.0	1.0	1	ND	930.6 (931.0)	[A-E(G)-K-A]$_2$ A-K-E(G)-A
C-3	2.4	1.0	1.0	1	ND	1,001.6 (1,002.1)	A-E(G)-K-A-A
C-4	2.0	1.0	1.0	1	ND	1,387.1 (1,387.5)	[A-E(G)-K-A]$_3$
C-5	2.0	1.0	1.1	1	ND	1,843.6 (1,844.0)	[A-E(G)-K-A]$_4$
Cb-1	2.1	1.3	1.4	1	ND	474.1 (474.5)	A-E(G)-K-A
Cb-2	2.0	1.0	1.0	1	ND	930.6 (931.6)	[A-E(G)-K-A]$_2$
Cb-3	2.0	1.0	1.0	1	ND	930.5 (931.0)	[A-E(G)-K-A]$_2$
Cb-4	2.4	0.9	1.0	1	ND	1,001.4 (1,002.1)	same as that of C3
Cb-5	2.0	1.0	1.0	1	ND	1,386.9 (1,387.5)	[A-E(G)-K-A]$_3$
Cb-6	2.0	1.0	1.0	1	ND	1,387.4 (1,387.5)	[A-E(G)-K-A]$_3$
Cb-7	2.1	1.0	1.0	1	ND	1,387.0 (1,387.5)	[A-E(G)-K-A]$_3$
Cb-8	2.0	1.0	1.0	1	ND	1,387.5 (1,387.5)	[A-E(G)-K-A]$_3$
Cb-9	2.0	1.0	1.0	1	ND	1,844.1 (1,844.0)	[A-E(G)-K-A]$_4$

ND, not detcted; a, thoretical mass; GlcN, *N*-acetylglucosamine.

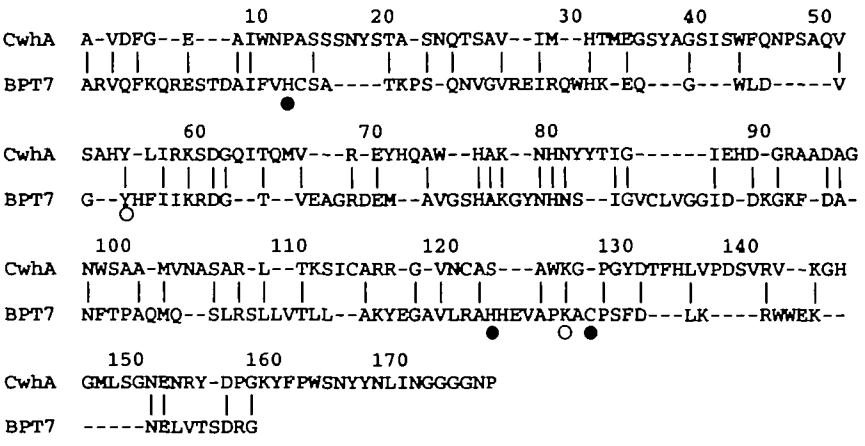

Fig. 7. **Amino acid identity between CwhA and BPT7.** ●: amino acids as ligands for zinc in BPT7. ○: amino acids important for catalysis in BPT7. BPT7: *N*-acetylmuramoyl-L-alanine amidase from bacteriophage T7.

proline, and peptide K7-1 is an 11-residue peptide ending at isoleucine. Peptide K7-1 was identical to the N-terminal 11 amino acid sequence of peptide K7-2 (Fig. 6).

The state of half-cystine in CwhA was determined by mass analyses for intact CwhA, iodoacetate-treated CwhA, and RCM-CwhA (Table IV). Intact CwhA and iodoacetate-treated CwhA had almost the same molecular mass, whereas that of RCM-CwhA was 19,511.5 Da, corresponding to intact CwhA plus two carboxymethyl groups. It was thus concluded that two half-cystine residues in peptide 4 are linked to form a disulfide bond.

To align all the API-derived peptides into a single peptide chain, CwhA was fragmented with CNBr, fractionated (Fig. 5), and sequenced (Fig. 6). Peptide CB-1 was sequenced up to the 30th residue, by which peptide K1 was connected to peptide K2. Peptide CB-2 overlapped peptide for peptides K2 and K3. Peptide CB-3, completely sequenced, spanned peptides K3, K4, K5, and K6. Peptide CB-4 connected peptide K7 to peptide K6. The amino acid sequences of these API-derived peptides were verified by measuring their molecular masses (Table IV). Peptide7-1 had the same molecular mass as peptide7-2, indicating that peptide7-1 was derived from peptide7-2 through the α→β rearrangement of the Asn-Gly peptide bond (*10*).

From the results described above, the complete amino acid sequence of CwhA was established (Fig. 6). The protein consists of a single peptide chain of 177 amino acid residues in which a disulfide bond is formed between Cys 114 and 121. The molecular mass (19,393.4 Da) of CwhA calculated based on the established sequence is consistent with the measured value of 19,395.9 Da.

DISCUSSION

We previonsly demonstrated that Achromopeptidase contains two bacteriolytic proteases, alp and blp (*11*). Like these lytic partners, CwhA is also active in the alkaline pH range and is optimized at pH 8.5. Its lytic activity decreases as salt concentration increases. However, CwhA has a significantly different bacteriolytic specificity from blp, the major bacteriolytic agent in Achromopeptidase (*7*). In fact, CwhA lysed $CHCl_3$-treated *E. coli* more efficiently than *M. luteus* and *S. aureus*, the Gram-positive species. In addition, CwhA decreased the turbidity of viable *E. coli*. It can be noted that *A. lyticus* synthesizes at least three different lytic enzymes to cope with various substrates for effective lysis.

In structural determination of peptidoglycan, hydrolytic enzymes with different specificities are required to generate relatively small fragments from the peptidoglycan for subsequent analyses. In this study, we chose the CwhA-sensitive *M. luteus* peptidoglycan as a substrate and used lysozyme and blp to facilitate the specificity determination of CwhA. From the HPLC chromatograms, amino acid composition, and mass analyses of muropeptides derived from CwhA, CwhA plus lysozyme, and CwhA plus blp digest, we concluded that CwhA is an *N*-acetylmuramoyl-L-alanine amidase. Generally, cleavage at a given site in peptidoglycan would not produce HPLC-detectable muropeptide of low-molecular mass, if the network of peptidoglycan were perfectly formed. It has been estimated that the extent of linkage between polysaccharide and peptide subunits in *M. luteus* peptidoglycan is about 50%, but the extent of linkage at other sites is unknown. In our experiment, peptide subunits and their oligomers were both detected in the CwhA digest, thus indicating that a portion of D-Ala-Ala and D-Ala-Lys (ε) bonds are not formed in *M. luteus* peptidoglycan (Table III). The complexity of bacterial peptidoglycan structure as evidenced by this study is that muropeptides Cb2 and Cb3, Cb5 and Cb6, and Cb7 and Cb8 have the same molecular masses, though eluted with different retention times (Fig. 4). As the peptide subunit and interbridge of *M. luteus* peptidoglycan have the same structure, this difference may arise from structural isomerization and/or different configurations (D or L) of constituent amino acids.

A homology search conducted with FASTA (*12*) revealed a higher score with a related enzyme BPT7, the only *N*-acetylmuramoyl-L-alanine amidase with known tertiary structure (*13*). The amino acid sequence identity between CwhA and BPT7 is 35%, and the identical amino acids are distributed rather evenly along the polypeptide chain (Fig. 7). Two regions, His54–Gly62 and His76–Gly86, are relatively well conserved between the two enzymes, suggesting that these regions probably play an important role. In the three-dimensional structure, BPT7 contains one Zn^{2+}, which is liganded to His17, His122, and Cys130. Cheng *et al.* have suggested that Lys128 and Tyr46 in BPT7 may play critical roles in catalysis, by interacting with the substrate carbonyl group and by promoting nucleophilic attack by an activated water molecule, respectively (*13*). Although the three

ligands of Zn^{2+} in BPT7 are not directly conserved in CwhA, Tyr53, and Lys126 in CwhA are closely aligned with Tyr46 and Lys128 in BPT7. These tyrosine and lysine residues in CwhA may play the same roles as those in BPT7 play. Since CwhA does not contain the known conserved motif of hydrolytic enzymes and is specifically inhibited by 1,10-phenanthroline, it is certain that it is a metalloenzyme, although the ligands of the metal ion may be different from those in BPT7. CwhA is probably a new type of enzyme in the *N*-acetylmuramoyl-L-alanine amidase family.

It is interesting that the C-terminal sequence of CwhA, GGGNP, is similar to its counterpart in API, GGGTP (*14*). API is synthesized as a proenzyme bearing both N- and C-terminal extensions; and the N-terminal propeptide, but not the C-terminal extension, is necessary for maturation of active API (*15*). The role of the C-terminal extension is not known. One possibility is that it participates in translocation of activated API across the outer membrane of *A. lyticus* and is then released from the active API by a Pro-Asn bond–specific processing enzyme. The close similarity of the two C-terminal pentapeptides from CwhA and API suggests that the precursors of CwhA and API may undergo the same post-translational processing at their C-termini.

So far, three lytic enzymes, alp, blp, and CwhA, with different bacterial and peptidoglycan specificities have been identified for *A. lyticus*. Since we have detected at least two more staphylolytic enzymes in crude Achromopeptidase (data not shown), *A. lyticus* may utilize a set of lytic enzyme with different specificities for different purposes, including cell separation, cell wall turnover, and degrading exogenous peptidoglycan as nutrients.

The amino acid sequence of CwhA has been submitted to the SWISS-PROT data bank and assigned the accession number P81717.

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