Purification and Characterization of a Novel Zinc-Proteinase from Cultures of Aeromonas hydrophila*

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While searching for an enzyme capable of breaking ε-(γ-Glu)-Lys isopeptide bonds cross-linking protein chains, we purified a metallo-proteinase which mimics the action of an isopeptidase on the γ-chain dimers of cross-linked fibrin. The enzyme is present in the growth medium of the bacterium Aeromonas hydrophila, isolated from the intestinal tract of the leech Hirudo medicinalis. It is a 19-kDa protein which specifically hydrolyzes the Gly-Ala peptide bond within the Gly-Gly-Ala sequence, located near the cross-link site in the γ-chain dimer of fibrin. Substrate specificity studies with a number of synthetic peptides suggest that the enzyme prefers Gly-Gly or acetyl-Gly in the P₁ and P₂ positions, respectively. Beating one atom of zinc and inhibited by 1,10-phenanthroline, but not by EDTA. Iodoacetate, leupeptin, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, pepstatin, and α-macroglobulin have no effect on enzyme activity. Disulfide reducing reagents, such as dithiothreitol or 2-mercaptoethanol, inactivate the enzyme completely. The partial amino-terminal sequence shows 46% identity with a zinc metallo-proteinase from a strain of Lysobacter enzymogenes and 69% identity with the LasA protein from Pseudomonas aeruginosa.

An isopeptidase capable of breaking ε-(γ-Glu)-Lys isopeptide bond cross-linking protein chains has been sought for a number of years. Such an enzyme could be used to identify the protein chains, extracellular or intracellular, which are cross-linked by transglutamminase and occur in cell and tissue extracts in highly polymerized form detectable at the top of polyacrylamide gels (1). Moreover, an isopeptidase could be therapeutically important if sufficiently specific for cross-linked fibrin since it could act synergistically with plasmin in clot dissolution, especially in the stationary phase of growth at pH 7.4. The cells and culture fluid were chilled on bacterization of the A. hydrophila sp. was grown on buffered tryptone yeast extract broth (tryptone, 8 g/liter; yeast extract, 5 g/liter; and NaH₂PO₄, 10 g/liter, adjusted to pH 7.0) for 16-18 h at 30°C. During purification, AhP activity was monitored using a fibrin assay described below. Optimal activity is obtained in the stationary phase of growth at pH 7. By adjusting pH 7.10 for 16–18 h at 30°C. During purification, AhP activity was monitored using a fibrin assay described below. Optimal activity is obtained at pH 7.4. The cells and culture fluid were chilled and centrifuged to remove the bacteria. A concentrated EDTA solution, pH 7.4, was added to make the final concentration 20 mM; this reduced the total proteolytic activity in the supernatant sufficiently to proceed with measurement of AhP activity. The supernatant was concentrated 20-fold in an Amicon CH2 ultrafiltration system with a hydroxylated γ-glutamyl-p-nitroanilide (γ-Glu-pNA) and appeared to convert the γ-chain dimer of cross-linked fibrin into the monomer. Although we were able to confirm the presence of a γ-Glu-pNA hydrolyzing activity in the saliva and salivary gland extracts of starved leeches, we found no fibrin isopeptidase activity. However, by using enrichment techniques, we isolated from the intestinal extract of the leech a bacterium which secretes an enzyme into its culture medium that appears to convert the γ-chain dimer of fibrin into the γ-monomer. This bacterium was identified as a strain of Aeromonas hydrophila. The enzyme hitherto referred to as A. hydrophila proteinase (AhP) was found not to be an isopeptidase, but rather a metallo-proteinase, which specifically cleaves the Gly-Ala peptide bond located within the sequence Gly-Gly-Ala, near the cross-link site of the γ-chain dimer. Another metallo-proteinase with analogous fibrinolytic specificity has been found in puffadder snake venom (6–8). Preliminary reports of the purification and some of the properties of AhP have appeared elsewhere (40, 41). In the present paper a detailed purification procedure and characterization of the enzyme are presented.

Experimental Procedures

Enrichment Culture Isolation of Bacterium—Leeches (BioPharm) were fed once with rabbit blood and kept at 16°C for 4–6 weeks without further feeding. Salivary gland excretions were collected according to the method of Bigli et al. (9) with minor modifications. Salivary excretions, salivary glands, and intestinal contents were used as inocula for enrichment cultures on insoluble fibrin (see "Fibrin assay of AhP") or on a variety of isopeptide-containing substrates in minimal salts under a variety of conditions. Clot-dissolving cultures were further investigated, and a bacterium was isolated which produced an isopeptidase-like effect on fibrin in the presence of EDTA. The bacterium isolated is a Gram-negative rod which was identified as a member of the species A. hydrophila by the American Type Culture Collection and will be referred to as A. hydrophila sp.

Purification of AhP—A. hydrophila sp. was grown on buffered tryptone yeast extract broth (tryptone, 8 g/liter; yeast extract, 5 g/liter; and NaH₂PO₄, 10 g/liter, adjusted to pH 7.0) for 16–18 h at 30°C. During purification, AhP activity was monitored using a fibrin assay described below. Optimal activity is obtained in the stationary phase of growth at pH 7.4. The cells and culture fluid were chilled and centrifuged to remove the bacteria. A concentrated EDTA solution, pH 7.4, was added to make the final concentration 20 mM; this reduced the total proteolytic activity in the supernatant sufficiently to proceed with measurement of AhP activity. The supernatant was concentrated 20-fold in an Amicon CH2 ultrafiltration system with a

1 The abbreviations used are: pNA, p-nitroanilide; Ac, N-acetyl; AhP, A. hydrophila proteinase; LeP, L. enzymogenes proteinase; Nph, p-nitrophenylalanine; PAGE, polyacrylamide gel electrophoresis; Tfa, trifluoroacetyl group; Suc, N-α-succinyl; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HPLC, high performance liquid chromatography; PEI, polyethyleneimine.

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SIY10 membrane, and the protein was precipitated with ammonium sulfate added to 80% of saturation. The precipitate was resuspended in a minimal volume of 20 mM HEPES, 0.1 M NaCl, 20 mM EDTA, pH 7.4, dialyzed against the same buffer and applied onto a Bakerbond WP-PEI equilibrated in 20 mM HEPES, 0.1 M NaCl, pH 7.4 (buffer A). AhP was not retained on the column, but a major peak of protein was eluted in the void volume. The AhP fraction obtained from the PEI column was dialyzed for 1-3 h at 4°C against 20 mM HEPES, 30 mM NaCl, pH 6.8 (buffer B); longer dialysis at this point resulted in some loss of AhP activity. The dialyzed material was loaded onto a CM-cellulose column equilibrated in the same buffer. AhP was eluted with a 50 mM NaCl, pH 6.8-4.0, 0.4 M NaCl, pH 7.4 gradient in 20 mM HEPES. The gradient was monitored with a Markson conductance meter (model 1096). In some studies (as indicated) gelatin-agaroze chromatography (10) was also employed to remove minor contaminants. The protein was adsorbed on a column of immobilized gelatin (Pierce Chemical Co.) in 0.05 M Tris, 0.02 M NaH2PO4, 0.15 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4, and AhP was eluted with 1 M urea in the same buffer. The final product was dialyzed against buffer A, or precipitated with ammonium sulfate (80%) and then dialyzed against buffer A and stored frozen.

Polyacrylamide Gel Electrophoresis (PAGE) — SDS-PAGE was performed according to Laemmli (11). Proteins were stained with a silver stain reagent (13). Proteins were stained with a silver stain reagent (13).

Reverse-phase High Performance Liquid Chromatography (HPLC) — A Waters HPLC system consisting of two model 510 pumps, a Lambda-Max model 481 LC Gradient Controller, a Lambda-Max model 481 LC Spectrophotometer, and a model 745 Data Module. A 300 Ǻ/5 μm Vydac or Waters Delta-Pak C18 analytical column (0.46 × 15 cm or 0.39 × 15 cm, respectively) was employed with a 30-min linear CH3CN gradient (10–95%) containing 0.1% trifluoroacetic acid. The flow rate was 0.5 ml/min. Absorbance of thiol was usually monitored at 214 nm or occasionally at 278 nm for peptides containing nitrophenylalanine.

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Amino Acid Analysis — Protein/peptide samples were hydrolyzed under vacuum in a nitrogen atmosphere for 24 h at 110°C for 24 h or its vapor at 110°C. Methanesulfonic acid (4 M), containing 0.2% 3-(2-aminoethyl) indole (Pierce), was used for tryptophan analysis. The acid hydrolysates were analyzed using the PICO-TAG system (Waters Associates) and the standard ninhydrin system (Beckman).

Amino Acid Sequence Determination — AhP, prepared as described above including gelatin-agaroze chromatography, was further purified on a Vydac C4 reverse-phase column (4.6 mm × 15 cm) to remove some minor contaminants. The sample was eluted with a 50 mM sodium borate, pH 9.2, and the free amino content was determined with TNBS according to Snyder and Sobocinski (16) with a minor modification. The absorbance of the trinitrophenyl derivatives was measured on a microplate reader ( Molecular Devices) at 405 nm. The linearity and precision of the assay was tested by changing the time of the reaction and the concentration of the enzyme. In most of the assays, no more than 10% of substrate was allowed to hydrolyze. If the extent of hydrolysis was more than 10% (but less than 20%), the substrate concentration was corrected for the loss during the reaction (17).

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was developed with a linear acetonitrile gradient containing tri-fluoroacetic acid (0.1%, v/v). Homogeneity of these peptides was assessed by analytical reverse-phase HPLC (Vydac CIS column) using similar gradients, and purity was found to be 95% or higher. Amino acid analysis of purified peptides gave the expected amino acid ratios (±5%).

Other Methods and Analyses—Zinc content was determined by Drs. B. L. Vallee and R. Shapiro, Harvard Medical School, Boston, using electrothermal atomic absorption spectroscopy.

Protein concentration was determined using BCA reagent (Pierce).

RESULTS

Purification of AhP—AhP activity was initially measured using a fibrin assay (Fig. 1) as described under “Experimental Procedures.” Later purifications were followed with the Azocoll and fibrin assays. Our early observations that AhP activity is not affected by 5–20 mM EDTA allowed us to use this chelator during initial steps of AhP purification. EDTA (20 mM) reduced the proteolytic activity in the bacterial media. An Amicon CH2 ultrafiltration system with a 10-kDa cut-off membrane was very useful, not only for concentrating the culture fluids, but also for the removal of small molecular contaminants. Subsequent purification was accomplished by anion-exchange chromatography on a Bakerbond WP-PEI column (Fig. 2), cation-exchange (CM-cellulose) chromatography (Fig. 3), and occasionally (see “Discussion”) gelatin-agarose chromatography. When concentrated culture fluid was applied onto the Bakerbond WP-PEI column (Fig. 2), the non-adsorbed material, which was eluted with increased salt concentration, contained several fractions which completely hydrolyzed fibrin clots even in the presence of EDTA. However, these fractions, assayed in the presence of 20 mM EDTA, showed 60–80% of the Azocoll hydrolysis activity measured in the absence of EDTA. Only this material was used for further purification. The retained material, which was eluted with increased salt concentration, contained several fractions which completely hydrolyzed fibrin clots even in the presence of EDTA. However, these fractions, assayed in the presence of EDTA, showed only 3–25% of the Azocoll hydrolysis activity in the absence of EDTA. Interestingly, DEAE-cellulose did not effect a satisfactory separation of AhP from other proteases. The non-adsorbed fraction from the anion-exchange column was briefly dialyzed to change the buffer and applied to CM-cellulose (Fig. 3). The two ion-exchange columns (WP-PEI and CM-cellulose) removed most of the contaminating proteins. The final yield of AhP was 2–4 mg of active enzyme/liter of culture medium. Specific activity was highest after CM-cellulose (Fig. 3) with 400–500 or 150–200 units/mg protein, measured in the fibrin or Azocoll assay, respectively. Specific activities of AhP at earlier steps of purification could not be measured with accuracy because contaminating proteinases, even in the presence of EDTA, interfered with the fibrin and Azocoll assays. Gelatin-agarose purification reduced specific activity by approximately 50% which may be due to tight binding of AhP to the affinity column and subsequent use of urea for elution. This step was employed in early stages of our work in order to assess whether the apparent isopeptidase activity of the partially purified enzyme could be distinguished from proteolytic activity of this preparation on Azocoll and on the α- and β-chains of cross-linked fibrin. We also employed Superose 12 (Pharmacia), Bakerbond CBX (J. T. Baker Chemical Co.) and propyl aspartamide (The Nest Group) columns for the same purpose (data not shown). The fact that several different chromatographic procedures were unable to separate the above activities from each other was an early indication that AhP is a proteinase rather than an isopeptidase. The gelatin-agarose purification step was included nevertheless in the preparation of the enzyme samples used for sequencing. Unless otherwise indicated, the CM-cellulose chromatography was the final step in purification of AhP. The purified enzyme migrated as a single band in SDS-polyacrylamide gel assay for AhP action on cross-linked fibrin. The same batch of fibrinogen and Factor XIII was used to prepare all samples. Lane 1, fibrinogen and Factor XIII also used for fibrin in lanes 2–5; lane 2, uncross-linked fibrin clotted in the presence of 5 mM EDTA; lane 3, cross-linked fibrin clotted in the presence of 10 mM CaCl₂; lanes 4 and 5, cross-linked fibrin incubated for 20 and 60 min, respectively, with 1 unit of AhP (for details, see “Experimental Procedures”). Identification of bands on the left corresponds to the fibrinogen chains in lane 1; identification of bands on the right corresponds to the fibrin chains in lanes 2–5. Lane 1, Aα-doublet, Bβ- and γ-chains of fibrinogen. Lane 2, α-doublet, β- and γ-chains of uncross-linked fibrin. Lane 3, α-doublet, β-chain, and γ-γ-dimer of cross-linked fibrin. Lanes 4 and 5, reduced amounts of α-doublet and γ-γ-dimer, and appearance of γ', the pseudo-γ-monomer generated by the action of AhP on cross-linked fibrin. Factor XIII, XIIIa, and AhP are too low in concentration to be visible in this gel. Note that AhP also splits the α-chains; one of the breakdown products is seen at the bottom of lane 5.

Physicochemical Properties—The molecular weight of AhP
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FIG. 2. Purification of AhP by Bakerbond WP-PEI chromatography. A 2.5 × 20-cm column of WP-PEI was equilibrated with 20 mM HEPES, 0.1 mM NaCl, pH 7.4 (buffer A), and 38 ml of dialyzed enzyme (see "Experimental Procedures") applied. The column was washed with buffer A, and developed with a linear gradient from buffer A to 2 M sodium acetate in buffer A starting at fraction 90. The flow-through material (pooled fractions, 17-60) displayed significant fibrinolytic activity (more than 0.1 AhP unit/ml, see "Experimental Procedures"). The other non-adsorbed fractions showed little or no AhP activity. The adsorbed fractions could not be assayed for fibrinolytic activity due to interference caused by other proteases (see "Results").

FIG. 3. Purification of AhP by CM-cellulose chromatography. The column (2.5 × 20 cm) was equilibrated with 20 mM HEPES, 30 mM NaCl, pH 6.8. The flow-through material (fractions, 17-60) eluted from the column illustrated in Fig. 2 was dialyzed against the equilibrating buffer and applied to the CM-cellulose column. The column was developed with a gradient from the equilibrating buffer to 20 mM HEPES, 0.4 M NaCl, pH 7.4, starting at fraction 32 as indicated by the dashed line. The Azocoll hydrolysis assay was carried out on an aliquot of each fraction, but note that the absorbance readings are one fraction before the actual collected fractions. The fibrinolytic assay (not shown) displayed the same profile as the Azocoll hydrolysis assay.

was measured by SDS-PAGE in 15% gels under reducing conditions (Fig. 4) with the apparent molecular weight being 19,000. The enzyme was also electrophoresed in the absence of 2-mercaptoethanol (Fig. 4). Under these conditions the migration distance of AhP was slightly greater (probably because the protein maintains a more compact structure when the disulfide bridges are intact), and the band was broader. AhP seems to have little or no carbohydrate as the enzyme was not retained on a ConA-Sepharose column, and its molecular weight as measured by SDS-PAGE agrees well with our gene sequence data (178 amino acid residues). Metal analysis by atomic absorption spectrophotometry showed that there is 1 mol of zinc/mol of enzyme (0.94 gram-atoms of zinc/mol, average of two measurements). However, AhP was not adsorbed on a zinc chelating column (Boehringer Mannheim).

The amino acid composition of AhP and the sequence of the first 40 amino acids of AhP are presented in Table I and Fig. 5, respectively. The enzyme contains a large proportion of aromatic amino acid residues and a small fraction of lysine and cysteine residues. The partial amino-terminal sequence shows 46% identity with a zinc metallo-proteinase from a strain of *Lysobacter enzymogenes* (former *Myxobacter*) (23-25) and 69% identity with the LasA protein (LasA) from *Pseudomonas aeruginosa* (26, 27) (Fig. 5).

**Enzymatic Properties**—The optimal pH for AhP activity, assessed with the substrate, Suc-Gly-Gly-Nph-CONH₂, was 7.5. The enzyme was stable in 4 M urea, 5% methanol, 2% acetic acid, 2% dimethyl sulfoxide, 4.5 M ammonium sulfate, and 2 M NaCl. Stability in methanol, acetone, and dimethyl sulfoxide was tested by preincubating the enzyme and the reagent for 30 min and then carrying out the fibrinolytic assay in the presence of the reagent. Stability to urea, ammonium sulfate, and NaCl was tested by preincubating in the presence of the reagent for 1 h and then dialyzing against the assay buffer.
Fig. 4. SDS-polyacrylamide gel electrophoresis of purified AhP. Lanes 2 and 3 were analyzed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions, respectively, in 15% acrylamide gels. Lanes 1 and 4 show molecular weight standards (Bio-Rad). The arrows on the right indicate the molecular masses of the standards in kDa. Proteins were stained with Coomassie Blue R-250.

Table I

| Amino acid       | Mol % |
|------------------|-------|
| Asx              | 11.70 |
| Glx              | 7.00  |
| Ser              | 10.90 |
| Gly              | 12.70 |
| His              | 2.69  |
| Arg              | 5.18  |
| Thr              | 6.14  |
| Ala              | 8.00  |
| Pro              | 4.00  |
| Tyr              | 9.00  |
| Val              | 6.00  |
| Met              | 0.46  |
| Ile              | 2.10  |
| Leu              | 4.97  |
| Phe              | 2.80  |
| Lys              | 0.62  |
| Cys*             | 1.73  |
| Trp*             | 3.90  |
| Total            | 100.00|

*PICO-TAG method was used for all amino acids except cystine.

**Determined as cystine using standard minhydin method.

*Acid hydrolysis was performed in the presence of 4 M methanesulfonic acid (Pierce).

Fig. 5. NH₂-terminal sequences of AhP, L. enyzmogenes proteinase (LeP) and LasA protein (LasA) from P. aeruginosa. X, indicates an unidentified residue. Solid line indicates identical residues; colon indicates residues with a similarity index of 0.5 or greater; and one dot indicates residues with a similarity index of 0.1 or greater. Values for similarities are from Gribskov and Burgess (39).

and 40 °C, respectively, and complete inactivation after 15 s of boiling in the same buffer. The enzyme activity was not inhibited by serine proteinase inhibitors such as diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and leupeptin, nor the cysteine protease inhibitors iodoacetate and leupeptin (Table II). Pepstatin, an aspartic proteinase inhibitor, and α₂-macroglobulin, a nonspecific proteinase inhibitor, also had no effect on enzyme activity. However, the enzyme was inhibited by the metal chelators 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid, and the sulfhydryl-containing compounds diithiothreitol and 2-mercaptoethanol. 1,7-Phenanthroline, which is not a chelator, and phosphoramidon, which inhibits some metallo-proteinases, had only a marginal inhibitory effect on AhP activity. Phosphoramidon had some effect at millimolar concentrations (Table II); however, the effective concentration of this compound is usually
that the split of the cross-linked y-chains occurred after the isopeptide-linked chains with the same molecular weight and the electrophoretic mobility of the hydrolysis product with be due to the presence of proteolytic contaminants. Additional 6. It can be noted that this kind of cleavage results in two amino acid content as the y-chain monomer (Fig. 6). The primary sequence was the predicted NHz-terminal sequence of sequencing, revealed a primary derived product of the y-dimer, when subjected to a few cycles of dissociation this conclusion when we found that the AhP hydrolysis of a chain which in SDS-PAGE had a mobility exactly equal to that of the monomer. Other hydrolytic effects on the polymer, and on the α- and β-chains were at first judged to be due to the presence of proteolytic contaminants. Additional results such as cross-reactivity of the product of γ-dimer hydrolysis with anti-γ-chain antibodies and the identity of the electrophoretic mobility of the hydrolysis product with that of the γ-chain suggested that AhP was indeed an ε-(γ-Glu)-Lys cleaving isopeptidase. However, we began to question this conclusion when we found that the AhP hydrolysis product of the γ-dimer, just like the dimer itself, contained one ε-(γ-Glu)-Lys cross-link/chain. Furthermore, the AhP-derived product of the γ-dimer, when subjected to a few cycles of sequencing, revealed a primary Tyg-Val-Ala-Thr-Arg-Asp-Aaα and a secondary Ala-Xxx-Gln-Ala-Gly sequence. The primary sequence was the predicted NH2-terminal sequence of the γ-chain. The secondary sequence, however, indicated that the split of the cross-linked γ-chains occurred after the 2nd Gly residue in the Gly-Gly-Ala sequence, immediately adjacent to the Glu-Lys isopeptide bonds of the γ-dimer (Fig. 6). It can be noted that this kind of cleavage results in two isopeptide-linked chains with the same molecular weight and amino acid content as the γ-chain monomer (Fig. 6). This cleavage site was confirmed using a synthetic fragment (F1) of the γ-chain of fibrin, Ac-Glu-Gly-Gln-Gln-His-His-Leu-

**TABLE II**

| Inhibitor                  | AhP inhibition (%) |
|----------------------------|--------------------|
| Dithiothreitol (10 mM)     | 100                |
| 2-Mercaptoethanol (20 mM)  | 100                |
| Iodocetate (5 mM)          | 0                  |
| Leupeptin (30 μM)          | 0                  |
| Phenylmethylsulfonyl fluoride (5 mM) | 0            |
| Disopropylfluorophosphate (5 mM) | 0            |
| EDTA (5 mM)                | 0                  |
| 1,7-Phenanthroline (1 mM)  | 6                  |
| 1,10-Phenanthroline (1 mM) | 99                 |
| 8-Hydroxyquinoline-5-sulfonic acid (1 mM) | 86             |
| Phosphoramidon (0.5 mM)    | 30*                |
| Phosphoramidon (1 mM)      | 50                 |
| Pepstatin (1 μM)           | 0                  |
| α2-Macroglobulin           | 0b                 |

* Effective concentration of phosphoramidon is usually in the micromolar range.

b) AhP was not retained on immobilized α2-macroglobulin (see "Experimental Procedures').

**Fig. 6.** Proteolysis of the γ-chain dimer of fibrin by AhP. Partial sequence of γ-chain dimer at the cross-link site. Vertical lines represent ε-(γ-Glu)-Lys isopeptide bonds. AhP splits between G and A at the G-G-A sequence. The double scission of the dimer results in a molecule consisting of a large and a small chain cross-linked by one ε-(γ-Glu)-Lys bond equaling the γ-chain in molecular weight.
strates. The \( k_{cat}/K_m \) value for Ac-Gly-Nhp-CONH\(_2\) was 240 M\(^{-1}\) s\(^{-1}\). The kinetic analysis of Suc-Gly-Gly-Nhp-CONH\(_2\) was complicated by the fact that, unexpectedly, two peptide bonds were hydrolyzed by AhP (Table III). First-order kinetics was still observed but only an apparent \( k_{cat}/K_m \) constant could be obtained in such a case. However, additional HPLC analysis of the reaction products (ratio) at equilibrium allowed us to calculate the individual \( k_{cat}/K_m \) values for each peptide bond hydrolyzed. These values were 170 M\(^{-1}\) s\(^{-1}\) and 1030 M\(^{-1}\) s\(^{-1}\) for the Gly-Gly and Gly-Nhp peptide bonds, respectively.

The separate kinetic constants, \( k_{cat} \) and \( K_m \), were determined for Ac-Gly-Nhp-CONH\(_2\), pH 7.5, using the TNBS assay. The \( k_{cat} \) and \( K_m \) constants were 3.4 s\(^{-1}\) and 17 mM, respectively. (The \( K_m \) value was about 12 mM at pH 7.0.) An accurate measurement of \( k_{cat} \) and \( K_m \) in this instance was difficult, because the \( K_m \) value is so high that the substrate solubility limited the maximal substrate concentration to about 2.5 \( K_m \), and the enzymatic reaction could not be monitored continuously when coupled with the TNBS assay. In addition, we would like to emphasize that the enzyme concentration in stock solutions was measured as the protein concentration by amino acid analysis. The calculated \( k_{cat}/K_m \) value, 200 M\(^{-1}\) s\(^{-1}\), pH 7.5, based on the TNBS assay, agrees reasonably well with the value 240 M\(^{-1}\) s\(^{-1}\), pH 7.0, shown above. The pH optimum studies showed that AhP activity at pH 7.0 is only 20% less than that at pH 7.5. The overall \( K_m \) value for Suc-Gly-Gly-Nhp-CONH\(_2\), pH 7.0, was 1.05 and 1.24 mM in the chromophoric and TNBS assays, respectively.

**DISCUSSION**

A novel bacterial proteinase was isolated from the culture medium of *A. hydrophila* sp. Since AhP contains one atom of zinc and is inhibited by 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid, but not by other protease class-specific inhibitors, we conclude that the enzyme is a zinc-dependent metallo-proteinase. The partial NH\(_2\)-terminal sequence shows 46% identity with a zinc metallo-proteinase (\( \beta \)-like protease) isolated from a strain of *L. enzymogenes* (23) and 69% identity with the LasA protein from *P. aeruginosa* (26). LasA, which is probably a selective proteinase, enhances the elastolytic activity of *P. aeruginosa* elastase (27). However, the biochemical activity of LasA is not known. It is interesting to note that both the Lysobacter enzyme and LasA do not have a typical His-Glu-Xxx-Glu-Xxx/His consensus sequence (32, 33), or its reversal (34), which is a zinc binding motif frequently found in zinc-proteinases.

AhP seems to be selective for the Gly-Gly-Xxx sequence, which contains an N-Suc-glycine residue with a hydrophobic side chain, is a recognition sequence located internally in polypeptides, where the proteolytic cleavage occurs after the 2nd Gly residue. However, a wider variety of substrates needs to be tested to confirm this conclusion. The specificity suggested above might explain why \( \alpha \)-macroglobulins (a general proteinase inhibitor) did not inhibit (bind) AhP as there is no Gly-Gly sequence in the bait region of the five \( \alpha \)-macroglobulins (35) that have been characterized. Unfortunately, to our knowledge, there are no sequence data available for bovine \( \alpha \)-macroglobulins, which was used in this study. Smaller peptides containing N-Suc-Gly and especially N-Ac-Gly were also effectively hydrolyzed (after the Gly residue) by AhP. The unbranched Ac-Gly and Suc-Gly residues may resemble the Gly-Gly fragment in longer peptides and thus fit well to the “binding groove” in the enzyme.

While Phe and Nhp were preferred in the \( P_i \) and \( P_s \) positions in synthetic oligopeptides, the \( \gamma \)-chain dimer of fibrin which contains a Gly-Gly-Ala-Lys-N\( \gamma \)-Glu sequence in the cross-link region is a very good substrate for AhP. The Gly-Ala peptide bond was rapidly split producing two “pseudo-\( \gamma \)-monomers” (Fig. 6). Purves and Purves (8) observed similar sequence specificity for a puffadder venom metallo-proteinase which was inhibited by EDTA. Furthermore, it appears that Gly-Gly-Xxx, where Xxx is an amino acid residue with a hydrophobic side chain, is a recognition sequence for the proteolytic processing of a number of viral and cellular proteins (36).

Interestingly, the \( \gamma \)-chain in fibrinogen does not appear to be a substrate for AhP. Fibrinogen, in contrast to fibrin, has an unsubstituted Lys residue in the Gly-Gly-Ala-Lys sequence at the cross-link site. The enzyme splits Suc-Gly-Gly-Nhp-Lys-CONH\(_2\) (0.3 mM) with a free \( \epsilon \)-amino group of the Lys residue in the \( P_s \) position at about one-quarter of the rate that it splits the analogous substrate containing an N-Suc-Gly residue in the same position (data not shown). This difference in rate alone cannot explain the resistance of the \( \gamma \)-chain of fibrinogen to hydrolysis by AhP. We think that
the specificity which AHP exhibits for the γ-chain of fibrin is probably due to a conformational change occurring in the fibrinogen to fibrin transformation. That such a conformational change indeed occurs was shown by Donovan and Mihalyi (37) in their scanning calorimetric study of fibrinogen cloting and by Rowbotham et al. (38), who raised a monoclonal antibody to fibrin which does not cross-react with fibrinogen.

We were not able to detect significant AHP activity in Escherichia coli, mouse liver or brain, more than 100 bacteria obtained by various enrichment techniques from soil samples, and three other strains of A. hydrophila from the American Type Culture Collection. The absence of AHP activity in other strains of A. hydrophyla sp. suggests that its production by the leech endosymbiont is an evolutionary adaptation to the bacterium’s nutritional environment. We observed that the supplementation of the tPA-plasminogen clot lysis system with AHP significantly increases the lysis rate of cross-linked fibrin (41). Its role in the bacterium and in the puffadder venom may be that of accelerating the lysis of cross-linked fibrin.

The preliminary kinetic data, generated for some synthetic substrates, showed moderate $k_{cat}/K_m$ values of 140–1000 M$^{-1}$ s$^{-1}$. The $k_{cat}/K_m$ value for Suc-Gly-Gly-Nph-CONH$_2$ was about 5-fold higher than that for a shorter substrate, Ac-Gly-Nph-CONH$_2$.

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