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Complete Nucleotide Sequence of the Structural Gene for Alkaline Proteinase from *Pseudomonas aeruginosa* IFO 3455

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The DNA-encoding alkaline proteinase (AP) of *Pseudomonas aeruginosa* IFO 3455 was cloned, and its complete nucleotide sequence was determined. When the cloned gene was ligated to pUC18, the *Escherichia coli* expression vector, the gene-incorporated bacteria expressed high levels of both AP activity and AP antigens. The amino acid sequence deduced from the nucleotide sequence revealed that the mature AP consists of 467 amino acids with a relative molecular weight of 49, 507. The amino acid composition predicted from the DNA sequence was similar to the chemically determined composition of purified AP reported previously. The amino acid sequence analysis revealed that both the N-terminal side sequence of the purified AP and several internal lysyl peptide fragments were identical to the deduced amino acid sequences. The percent homology of amino acid sequences between AP and *Serratia* protease was about 55%. The zinc ligands and an active site of the AP were predicted by comparing the structure of the enzyme with of *Serratia* protease, thermolysin, *Bacillus subtilis* neutral protease, and *Pseudomonas* elastase.

Several extracellular products have been implicated in the pathogenicity of *Pseudomonas aeruginosa*. These include proteases, phospholipase, hemolysin, exotoxin A, and exoenzyme S (24, 32). *P. aeruginosa* can produce two or three proteases (21). One of them is alkaline proteinase (AP) (19, 20). Enzyme production was observed in a semisynthetic medium containing Ca as an essential component (22). AP can be regarded as a metalloproteinase, since it is inactivated by the addition of *o*-phenanthroline (25). However, its properties are completely different from those of *Pseudomonas* elastase, a known Zn-metalloproteinase (24). The chemically determined molecular weight was reported to be 48,400 (10), and the substrate specificity differed completely from those of Zn-metalloproteinases, including *Pseudomonas* elastase (23, 24, 26, 27). To determine more detailed characteristics, the entire amino acid sequence of AP was necessary.

We were recently able to clone the AP gene (1). In addition, Guzzo et al. (7) cloned the AP gene (*apr*), and they found the transposable promoter that secreted AP. In the present study, we were able to determine the entire sequence of *Pseudomonas* AP. The characteristics of the deduced AP amino acid sequence of the AP protein, as well as the amino acid homology of the active site of other Zn-metalloproteinases, were also studied.

MATERIALS AND METHODS

Bacterial strains and vectors. *P. aeruginosa* IFO 3455 was obtained from the Institute for Fermentation, Osaka, Japan. Both *Escherichia coli* HB101 and JM101 were used for DNA cloning. M13mp18 phages were used for DNA sequencing. *P. aeruginosa* IFO 3455 produced AP antigen and showed azocasein cleavage activity. *E. coli* strains used in the present study produced neither AP activity nor antigens that reacted with anti-AP antibody.

Preparation of inactive *Pseudomonas* AP. Crystal *Pseudomonas* AP was obtained from Nagase Biochemical Co., Fukuchiyama-shi, Kyoto, Japan. Inactive *Pseudomonas* AP was prepared by using the method described previously (9).

Amino acid sequence determination. For amino acid sequence analysis of peptide fragments, purified AP was digested with a lysyl endopeptidase (Wako Pure Chemical Industries) or trypsin (Biozyme Laboratories Ltd., New South Wales, Australia). The lysyl or tryptic peptides were separated and purified by reversed-phase high-pressure liquid chromatography. The N-terminal sequence of the purified AP, as well as the amino acid sequences of the internal lysyl peptides, was determined by automated Edman degradation with a model 477A gas-phase sequencer (Applied Biosystems, Foster City, Calif.). The resulting phenylthiohydantoin amino acids were analyzed with a model 120A phenylthiohydantoin analyzer (Applied Biosystems).

Construction and screening of the gene library. Chromosomal DNA was collected from *P. aeruginosa* IFO 3455 by using conventional methods (14). The method for the construction and screening of the library was described previously (1). In these clones, the AP activity was tested by a modified version of the method of Long et al. (12).

The plasmids of AP-positive bacteria were collected and the appropriate DNA fragments were prepared with various restriction enzymes (Takara Shuzo Co., Ltd., Kyoto, Japan). For nucleotide sequence analysis, several smaller DNA fragments about 500 bp in length were made from the pAPDS2 clone as described previously (1). In addition, staggered deletions were prepared by unidirectional digestion by using the method of Henikoff (8). To determine the span of the AP structural gene, the AP activity of each *E. coli* clone, transformed with deleted DNA, was measured.

DNA sequencing. Various restriction fragments of the AP

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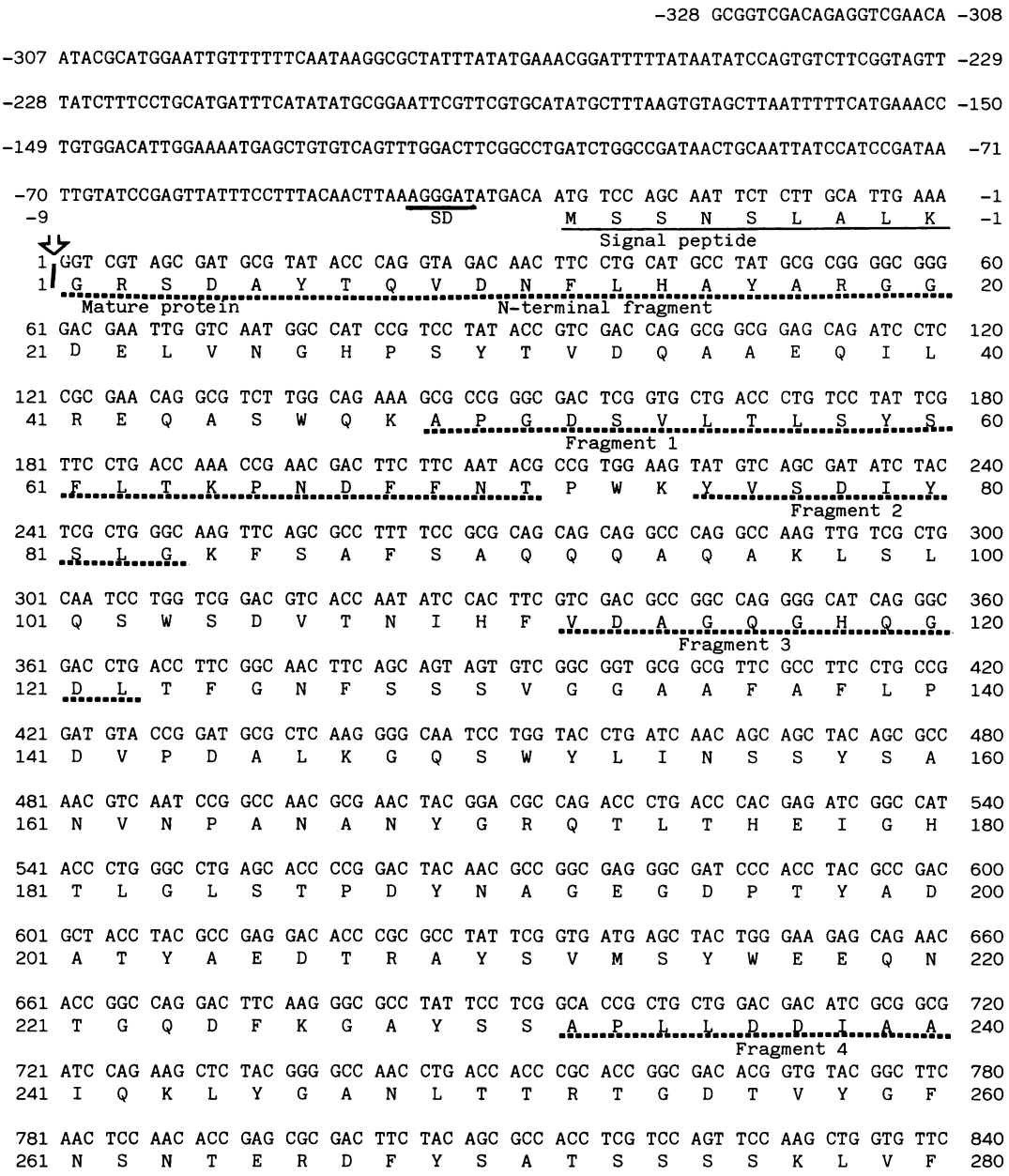

FIG. 1. DNA sequence and deduced amino acid sequence of AP. Both strands were sequenced by using several independent, overlapping clones. A putative ribosome-binding site (Shine-Dalgarno-like sequence) is underlined. The start site of the mature product is amino acid 1. The N terminus of the mature protein was identified on the basis of its homology to the N-terminal sequence of chemically determined amino acids of the AP fragment. Dashed lines indicate chemically identified peptides. Numbers written on both sides of the lines indicate the positions of both nucleotides and amino acids. A palindromelike structure or a putative transcription terminator is indicated by arrows.

gene were subcloned into M13mp18 (44). Sequencing reactions were carried out by dideoxy chain termination (34) with ^{32}P-labeled nucleotide. The region of the structural gene was sequenced by using universal or synthetic oligonucleotides primers. The oligonucleotides were synthesized by use of an automatic DNA synthesizer (model 381A; Applied Biosystems).

Hydropathy analysis. Hydropathy analysis was performed by using a method described previously (11).

Analysis of amino acid homology. The program of homology analysis was as described in the Genetyx manual (Software Development Co., Tokyo).

RESULTS

DNA sequencing. As described previously (1), the AP101 clone was further subcloned into a smaller DNA fragment into *E. coli* (pAPDS2). Several smaller DNA fragments about 500 bp in length were made from the pAPDS2 clone for DNA sequencing. In addition, staggered deletions of about 300 bp each were also prepared by unidirectional digestion with exonuclease III. The nucleotide sequence of the AP gene and the deduced primary structure of the protein encoded by this gene are shown in Fig. 1. The entire *Pseudomonas* AP reading frame was sequenced on both

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841 TCG GTG TGG GAC GCC GGC GGC AAC GAC ACC CTG GAC TTC TCC GGC TTC AGC CAG AAC CAG  900
281  S   V   W   D   A   G   G   N   D   T   L   D   F   S   G   F   S   Q   N   Q  300

901 AAG ATC AAC CTC AAC GAG AAG GCG CTG TCC GAT GTC GGC GGG TTG AAG GGC AAT GTG TCG  960
301  K   I   N   L   N   E   K   A   L   S   D   V   G   G   L   K   G   N   V   S  320

961 ATC GCT GCC GGG GTC ACC GTG GAA AAC GCC ATC GGC GGC TCG GGT AGC GAC CTG TTG TAC 1020
321  I   A   A   G   V   T   V   E   N   A   I   G   G   S   G   S   D   L   L   Y  340

1021 GGC AAC GAC GTG GCC AAC GTG CTC AAG GGC GGC GCC GGC AAC GAC ATC CTC TAC GGC GGC 1080
341  G   N   D   V   A   N   V   L   K   G   G   A   G   N   D   I   L   Y   G   G  360

1081 CTC GGC GCG GAC CAG TTG TGG GGC GGC GCG GGG GCC GAC ACC TTC GTC TAC GCG ATA TCG 1140
361  L   G   A   D   Q   L   W   G   G   A   G   A   D   T   F   V   Y   A   I   S  380

1141 CCG AGT CCT CCG CGC GCG CCG GAT ACC CTG CGC GAC TTC GTC AGC GGC CAG GAC AAG ATC 1200
381  P   S   P   P   R   A   P   D   T   L   R   D   F   V   S   G   Q   D   K   I  400

1201 GAC CTG TCC GGG CTG GAT GCC TTC GTC AAC GGC GGG CTG GTG CTG CAA TAC GTC GAC GCC 1260
401  D   L   S   G   L   D   A   F   V   N   G   G   L   V   L   Q   Y   V   D   A  420
                                  Fragment 5
1261 TTC GCC GGC AAC GCG CAG GGC ATC CTG TCC TAC GAC GCG GCG AGC AAG GCC GGC AGC CTG 1320
421  F   A   G   N   A   Q   G   I   L   S   Y   D   A   A   S   K   A   G   S   L  440

1321 GCA GTC GAC TTC AGC GGG GAC CGC CAT GCC GAT TTC GCG ATC AAT CTG ATC GGC CAG GCG 1380
441  A   V   D   F   S   G   D   R   H   A   D   F   A   I   N   L   I   G   Q   A  460

1381 ACC CAG GCC GAC ATC GTG CTC TGA CGCGCTGAAGCGCTGACGTTCGGTGCCGGTACGGACATGCTCCGTCG 1451
461  T   Q   A   D   I   V   L  ***

1452 CGGCCGGGCACTGTTCGAATTGATAGGTGTCTTCCACAGGAGCGAAGTTGTGAACTGTGGCGCGCCGTCGGCCGGCGGC 1530

1531 CTGGGCAGCAAGCAGGGCCCCGGCGCCTGGCGCGTCTGCGGTTCGAAGCGAGGATGGCGGCGGGCTTCGCGTGTTATCC 1609

1610 GATGTTCCAGGCTTGAAGGAGATCTCTCGATGTCGGCAAGCGCGAAGCTCTCCAGGATGGTTTGCTTGCTCTGCGGTTT 1688

1689 TTTCAGCACAGGTATCAGTATGGCCAGCAGTCTGATTCTTCTCAGCGCTTCCGATCTCGCGGGCAATGGACCCTGCAGC 1767

1768 AGGACGAGGTCAGCCCGCGATCTGCCACCTGGAGCTGGCCGACAGCGAAGTGGCGGAACGCAGTGGCTACGACCTGGCG 1846

1847 CGATACCGCCTGCCTCGCGACCTGCCCAGGCAGCCGCGCCGCTGGAGCCTACCCGGCCGGGATCGCCTGCTCGAACGCG 1925

1926 GCGGCCTGACCCTGATGCTCCTCGGTCCAGGGCGAGGGCGACTAACCGGGTGCAGCGAGAGCGGCGGGCAGTTGGTGCT 2004

2005 GCGCGGCGACGCCCTAGCTCGCTGCGGGTCAGACCACGAAGCGACCGATCATGCCCTTGAGGTCGTTGGCCAGGCGCGA 2083

2084 CAGCTCGTGGGNGTGCGGGTCTCGTCGGCGCCCAGGGTGGTCTGCACGGCAACGTCGCGGATGTTCACCAGGTTGCGAT 2102

2103 CGACTCGCGGGCGGGGGATCCTCTAGAG 2130
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FIG. 1—*Continued.*

strands. Both the N-terminal side amino acid sequence of the purified AP (Gly-1 to Gly-20) and the four internal lysyl peptides, fragment 1 (Ala-49 to Thr-71), fragment 2 (Tyr-75 to Gly-83), fragment 3 (Val-112 to Leu-122), and fragment 4 (Asp-232 to Gly-240), derived from AP corresponded completely to the deduced amino acid sequences. The G+C content of this mature AP gene was 64.0%.

ATG, the initiation methionine codon, is located 25 to 27 bp upstream of the mature protein coding sequence. A Shine-Dalgarno-like sequence was also observed upstream from this ATG codon. However, another possibility is that Leu is the initial amino acid of the mature AP protein (see below), since the termination codon TGA was observed at nucleotide positions 1402 to 1404 bp. In addition, the M_r determined by Inoue et al. (10) supports these results. We were able to observe a palindromelike structure or a putative transcription terminator (1425 bp to 1433 bp), which also supported the existence of termination codon at this site.

Comparison of amino acid composition. In a previous study, Morihara et al. (29) reported the amino acid composition of *Pseudomonas* AP by using a purified protein sample. A comparison of the amino acid composition of Morihara et al. with ours is shown in Table 1. The amino acid composition deduced from our DNA sequence was similar to that determined from purified AP.

Hydropathy profile. Hydrophobic amino acids were observed in the initial sequence (amino acid 1 to 325). The amino acid sequence of the C-terminal region (amino acid 326 to 467) was strongly hydrophilic.

Amino acid sequence comparison. The amino acid sequence of *Pseudomonas* AP was compared with that of *Serratia* protease. The *Serratia* protease is a Zn-metalloproteinase (19), and its substrate specificity is very similar to that of *Pseudomonas* AP (18, 23). In addition, the amino acid sequence of *Serratia* protease has also been elucidated (30). The frequency of identity appears to be most evident in the central position of the molecule (Fig. 2).

TABLE 1. Amino acid composition of AP

Amino acid	No. of residues	
	Determined from purified enzyme[a]	Predicted from DNA sequence
Asp	67.2 (Asp + Asn)	41
Asn		28
Thr	24.5	26
Ser	42.8	46
Glu	36.1 (Glu + Gln)	11
Gln		25
Pro	10.8	14
Gly	66.3	52
Ala	58.9	54
Val	25.0	27
Met	0	1
Ile	17.1	17
Leu	38.1	40
Tyr	20.9	23
Phe	19.9	23
Lys	15.8	15
His	6.0	7
Arg	7.2	10
Cys	0	0
Trp	6.2	7

[a] Calculated assuming a molecular weight of 49,507 and by using the data of Morihara et al. (21, 24).

DISCUSSION

Recent studies into the molecular genetics of *P. aeruginosa* have revealed the amino acid sequences of exotoxin A (13, 14, 33, 39), hemolysin (24), carboxypeptidase G2 (17), pilin (31, 36), and elastase (2, 4, 35, 42), as well as molecular size of proteins (3, 10) or *P. aeruginosa* gene expression (3, 5, 6). AP is also thought to be one of the important pathogenic factors in *Pseudomonas* infection, and a vaccine involving this protein mixture has been shown to be effective (9). Molecular genetic approaches are therefore expected to yield important information in this area of research.

We have cloned the *Pseudomonas* AP gene and determined the entire DNA and amino acid sequence. The details of this gene cloning have been reported previously (1). The translated gene product of the pAPDS2 clone displayed both the AP activity and almost the same M_r as that of purified AP. In addition, the characteristics of the deduced amino acid sequence of AP were similar to those of purified *Pseudomonas* AP. These results strongly indicate that the gene we cloned is a true AP gene.

We tentatively decided that the initiating amino acid of the mature protein is Gly on the basis of only amino acid sequence data. If this is the case, some other proteolytic enzyme might break the signal peptide between Lys and Gly. Another possibility is that Leu-2 is the initiating amino acid, since Ala is a rather common signal sequence terminal amino acid (16) and Ser-Leu-Ala (−6 to −4) is the typical amino acids of the "(−3, −1)-rule" for a signal sequence cleavage

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AP    1  GRSDAYTQVDNYLHAYARGGDELVNGHPSYTVDQAAEQILRGQASWQ--KAPGDSVLTLS  57
SP    1  AATTGYDAVDDLLHYHERGNGIQINGKDSFSNEQAGLFITRENQIWNGYKVFGQPV-KLT  59

AP   58  YSFLTKPNDFFNTPWKYVSDIYSLGKFSAFSAQQQAQAKLSLQSWSDVTNIHFVDAGQGH 117
SP   60  FSF---PDYKFSST--NVAGDTGLSKF---SAEQQQQAKLSLQSWADVANITFTEVAAGQ 111

AP  118  QGDLTFGNFS------SSVGGAAFAFLPD---VPDALKGQSWYLINSSYSANVNPANANY 168
SP  112  KANITFGNYSQDRPGHTDYGTQATAFLPNTIWQGQDLGGQIWYNVNQS--NVKHPATEDY 169

AO  169  GRQTLTHEIGHTLGLSTP-DYNAGEGDPTYADATYAEDTRAYSVMSYWEEQNTGQDFKGA 227
SP  170  GRQTFTHEIGHALGLSHPGDYNAGEGNPTYRDVTYAEDTRQFSLMSYWSETNTGGDNGGH 229

AP  228  YSSAPLLDDIAAIQKLYGANLTTRTGDTVYGFNSDTERDFYSATSSSSKLVFSVWDAGGN 287
SP  230  YAAAPLLDDIAAIQHLYGANLSTRTGDTVYGFNSNTERDFLSTTSNSQKVIFAAWDAGGN 289

AP  288  DTLDFSGFSQNQKINLNEKALSDVGGLKGNVSIAAGVTVENAIGGSGSDLLYGNDVANVL 347
SP  290  DTFDFSGYTANQRINLNEKSFSDVGGLKGNVSIAAGVTIENAIGFRQR-LIVGNAANNVL 348

AP  348  KGGAGNDILYGGLGADQLWGGAGADTFVY-AISPSPPRAPDTLRDFVSGQDKIDLSGLDA 406
SP  349  KGGAGNDVLFGGGGADQLWGGAGLDIFVFSAASDSAPGASDWIRDFQKGIDKIDLSFFNL 409

AP  407  FVNGGLVLQYVDAFAGNAQGILSYDAS-KAGSLAVDFSGDRHADFAINLIGQATQADIVL 466
SP  409  EAQSSDFIHFVDHFSGAAGALLSYNASNNVIDLSVNIGGHQAPDFLVKIVGQVDVATDFIV 470
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FIG. 2. Comparison of the amino acid sequence of AP with that of *Serratia* protease. The amino acid sequence of AP is from Fig. 1, and that of *Serratia* protease is from Nakahara et al. (30). Gaps (indicated by dashes) were introduced to increase the similarity, and matching amino acids are indicated by asterisks. The probable zinc ligands are boxed.

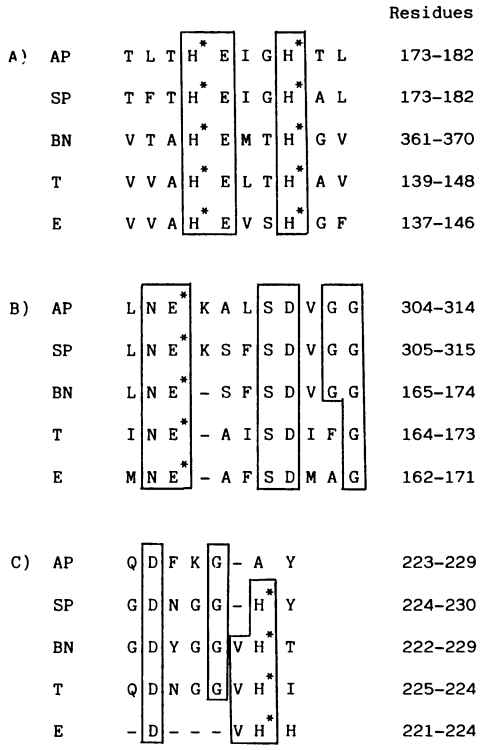

FIG. 3. Comparison of the regions containing Zn ligands and active sites of AP, *Serratia* protease, thermolysin, *B. subtilis* neutral protease, and *Pseudomonas* elastase. Abbreviations: SP, *Serratia* protease; T, thermolysin; BN, *B. subtilis* neutral protease; E, *Pseudomonas* elastase. The proposed Zn ligands and the active sites are indicated by single asterisks.

site (40). However, more thorough analysis is necessary to confirm this.

Although it has not yet been determined what metal is essential for the activity of *Pseudomonas* AP, the substrate specifity is very similar to that of the *Serratia* protease but unlike that of typical neutral Zn-metalloproteinases such as thermolysin and *Pseudomonas* elastase, etc. Both thermolysin and *Pseudomonas* elastase exhibit their specificity against bulky or hydrophobic amino acid residues at the imino-side of the splitting point (23). In contrast, *Pseudomonas* AP and the *Serratia* protease do not show any similarity to them in their specificity against oxidized insulin B chain and synthetic peptides. However, both show very similar specificity against oxidized insulin B chain (23). Therefore, we compared the amino acid sequence of AP with that of *Serratia* protease (30) by empirically aligning the sequences of these two enzymes to maximize homology. The homology of total protein is about 55% (Fig. 2). The frequency of homology is greatest in the central position of the molecule (Fig. 2; AP numbers 36 through 403). In contrast, for the sequences of the N-terminal region (AP numbers 1 through 35) and the C-terminal region (AP numbers 404 through 467), homologies are relatively low.

The pattern of homology is particularly evident in the regions that include structurally and functionally important residues which have been assumed to be regions of Zn ligands in the *Serratia* protease (Fig. 3), where a comparison is also made with thermolysin (37), *B. subtilis* neutral protease (38, 43), and *Pseudomonas* elastase (4, 42). In thermolysin, the Zn ligands are His-142, His-146, and Glu-166, and the active site is His-231 (14, 43). Glu-143 and Asp-226 are also thought to participate in the catalysis (15). We have previously compared the amino acid sequence of *Pseudomonas* elastase with the active site sequence of thermolysin (4) and showed that a region containing two histidine residues (at positions 140 and 144) in the elastase was similar to the Zn binding region in thermolysin. The amino acid sequence of AP has also been shown to be similar to that of thermolysin (Fig. 3): three Zn ligands present in thermolysin are present in AP (His-176, His-180, and Glu-314). The Zn ligands and the active site of AP were searched for by comparing the structure of the enzyme with the structure of *Serratia* protease (30), *B. subtilis* neutral protease (38, 43), *Serratia* thermolysin (37), and *Pseudomonas* elastase (4, 42) (Fig. 3). The region from residue 224 to 230 in *Serratia* protease is similar to the active-site region in thermolysin (from residue 225 to 232) and to the region from residue 222 to 229 in *B. subtilis* protease. However, we were not able to observe this active-site histidine in AP.

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