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Prolyl Aminopeptidase Is Also Present in *Enterobacteriaceae*: Cloning and Sequencing of the *Hafnia alvei* Enzyme-Gene and Characterization of the Expressed Enzyme

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The *Hafnia alvei* prolyl aminopeptidase gene (*hpap*) was cloned and sequenced, the expressed enzyme (HPAP) was purified to homogeneity and thoroughly characterized. An open reading frame of 1,281 bp was found to code for the enzyme, resulting in a protein of 427 amino acids with a molecular weight of 48,577. HPAP resembles the *Aeromonas sobria* enzyme, having 45% identity and the same distinctive properties with respect to size and substrate specificities. Both enzymes show similar chromatographic behavior, and HPAP could be purified following the procedure previously described for the *Aeromonas* enzyme. HPAP was found to be resistant to diisopropylphosphofluoridate as are most of the prolyl aminopeptidases hitherto described. In spite of this similarity, no inhibition by 1 mM *p*-chloromercuribenzoate solution could be detected. Significant inhibition was, however, observed when the enzyme was incubated with 3,4-dichloroisocoumarin. This study confirms the presence of two types of prolyl aminopeptidases, of which the *Hafnia* and *Aeromonas* enzymes constitute one group and the *Bacillus*, *Neisseria*, and *Lactobacillus* enzymes the other, and describes the cloning of the first prolyl aminopeptidase gene from an *Enterobacteriaceae*.

Key words: aminopeptidase, *Hafnia alvei*, iminopeptidase, proline.

The studies reported to date have defined prolyl aminopeptidase (PAP, proline aminopeptidase, Pro-X aminopeptidase, proline iminopeptidase) [EC 3.4.11.5] as an activity cleaving amino-terminal proline residues with high specificity, acting almost exclusively on short peptides. From its inhibition by *p*-chloromercuribenzoate (PCMB) and heavy metals, and the fact that other modifying reagents such as diisopropylphosphofluoridate (DFP), have little or no influence, PAP had long been assumed to be a sulfhydryl peptidase (*1-13*). However, we were recently able to demonstrate by site-directed mutagenesis that serine and not cysteine residues are essential for the activities of the *B. coagulans* and *A. sobria* prolyl aminopeptidases (*14*).

Another important finding was that the PAP enzymes could be classified in two groups, on the basis of their size (molecular weight) and stringency of substrate specificity. One consists of monomers of approximately 30 kDa, as rather small enzymes, comparatively unstable, being inhibited by some thiol-blocking agents, with a strict specificity for proline terminals, and lacking the ability to act on large peptides (*Bacillus*, *Neisseria*, and *Lactobacillus* PAPs). The second group comprises stable, multimeric large enzymes of around 200 kDa, with a broader specificity extending to hydroxyproline terminals, a preference for longer peptide substrates, and a variable sensitivity to sulfhydryl enzyme inhibitors (*Aeromonas* PAP) (*12*).

The analysis of these PAP sequences and homology searches in different databases suggested that these enzymes could be related to a wide variety of hydrolases sharing identities of around 20%. No reliable explanation of their hydrolytic and proline-recognizing mechanism has been given to date, and since the PAP sequences show no significant similarity with any other peptidase nor with any of the numerous proline-specific peptidases reported to date, the nature of these mechanisms remained obscure (*10, 12*).

Interestingly, although the *Escherichia coli* PAP was the first one to be studied, being described in two papers by Sarid *et al.*, it has not been reported since *E. coli* PAP was reported to be Mn^{2+}-dependent and to have the ability to cleave poly-L-proline (*1, 2*). In this regard, Fanghanel *et al.*, in an extensive study, found that the PAP activity was uniformly present in *Serratia* and *Hafnia* strains, but no activity hydrolyzing L-proline-4-nitroanilide was detected in 74 different *E. coli* serotypes (*6*). In our studies, no PAP activity could be detected in the *E. coli* strains used for expression of the recombinant enzymes, using proline β-naphthylamide or peptide substrates. Then, the cloning of the prolyl aminopeptidase gene from *H. alvei* would allow us verify the presence of the activity in an *Enterobacte-*

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Abbreviations: APAP, *Aeromonas sobria* prolyl aminopeptidase; BPAP, *Bacillus coagulans* prolyl aminopeptidase; DEP, diethylpyrocarbonate; DFP, diisopropylphosphofluoridate; DCIC, 3,4-dichloroisocoumarin; HPAP, *Hafnia alvei* prolyl aminopeptidase; IAA, iodoacetic acid; NEM, *N*-ethylmaleimide; LAP, leucyl aminopeptidase; LPAP, *Lyophyllum cinerascens* prolyl aminopeptidase; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethyl sulfonylfluoride; Pro-βNA, proline β-naphthylamide.

riaceae and could be the base for future studies of the now hypothetical *E. coli* enzyme. On the other hand, since the characterization of PAP enzymes from sources as diverse as possible is a good means to identify their conserved characteristics, we attempted the cloning, sequencing and studies of the prolyl aminopeptidase from *H. alvei* and report our results and conclusions in this work.

MATERIALS AND METHODS

Materials—Restriction endonucleases, Klenow enzyme, T4 DNA ligase, other DNA-modifying enzymes, deletion, and M13 sequencing kits and primers for sequencing were purchased from Takara Shuzo or Toyobo. [^{35}S]dCTP and [^{32}P]dCTP were from Amersham. Sequenase and T7-Gen *in vitro* mutagenesis kit were obtained from U.S. Biochemical, and Agarose I was from Dojin Chemicals. Proline β-naphthylamide (Pro-βNA), Fast Garnet GBC salt, lysozyme, and RNase A were from Sigma. Calf intestine alkaline phosphatase was obtained from Boehringer-Mannheim. Oligonucleotides were either synthesized by the phosphoramidite method using a Pharmacia LKB DNA synthesizer, or obtained from Nippon Gene. *Pseudomonas fragi* endoproteinase Asp-N was from Boehringer-Mannheim. Peptide substrates were from Bachem Feinchemikalien AG or Peptide Institute. All other chemicals and solvents were of the highest grade available from Nacalai Tesque or Wako Pure Chemical Industries.

Bacterial Strains, Plasmids, and Media—Several *Hafnia* strains were obtained from the Institute for Fermentation of Osaka (IFO), and after determining the activities of many type cultures, the strain *Hafnia alvei* IFO 3631, which showed the highest activity, was selected. *E. coli* HB101 (F$^-$, *hsd*S20 (*r*B$^-$, *m*B$^-$), *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (Smr), *xyl*-5, *mtl*-1, *sup*E44m, l-), XL1-blue [*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*, *rel*A1, *lac*$^-$, F′(Tn10, *pro*AB, *lac*I^q *Z* M15)], DH1 [F$^-$, *rec*A1, *gyr*A96, *thi*-1, *hsd*R17 (*rk*$^-$, *mk*$^-$), *sup*E44, *rel*A1, *l*$^-$], and DH5α [*sup*E44, Δ*lac*U169 (ϕ80*lacZ*ΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1] were used as hosts. Plasmids pBR322, pUC18, and pUC19 were used for cloning and sequencing. Phagemids pBluescript SK (+) and (−), and M13 phages were used for routine cloning procedures, the construction of deletion mutants, and for nucleotide sequencing with single-strand templates. Bacteria were grown in Luria-Bertani broth (LB-broth, 1% tryptone, 1% NaCl, and 0.5% yeast extract).

Construction of Genomic Library, and Screening—*Aeromonas sobria* was grown at 30°C in LB broth supplemented with salts (0.05% magnesium sulfate, 0.001% ferrous sulfate, 0.001% zinc sulfate, 0.0001% cupric sulfate, 0.0001% manganese sulfate, 0.0001% calcium chloride, and 0.1% potassium phosphate dibasic), with vigorous agitation. Chromosomal DNA was prepared following the method of Saito and Miura (*15*); plasmid pBR322 was used as the vector and *E. coli* DH1 as the host. For selection, either ampicillin (50 μg/ml) or tetracycline (25 μg/ml) was added to the medium. Individual colonies of DH1 cells transformed with the pBR322 plasmids from the genomic library were picked from plates and transferred into the wells of microtiter plates containing 180 μl of broth. After overnight incubation, 50 μl of the culture was transferred to another plate and mixed with 50 μl of 2 mM Pro-βNA. The plate was incubated overnight at 30°C and the reaction was stopped by adding 50 μl of the Fast Garnet GBC salt solution (1 mg/ml in 1 M acetate buffer, pH 4.0, containing 10% Triton X-100). Rapid red color development indicated the presence of high enzymatic activity.

DNA Manipulation, and Subcloning—Plasmid DNA was isolated by the alkaline extraction procedure (*16*) or by CsCl-ethidium bromide equilibrium density gradient centrifugation. Competent cells for transformation were prepared by rubidium chloride treatment. All other procedures were done following the standard procedures (*17*). The fragment containing the *pap* gene was subcloned based on the restriction enzyme map constructed, and deletion analysis of these subclones indicated the location of the gene and its transcription direction.

Nucleotide Sequencing—Sequence data were generated from single-stranded M13 or Bluescript subclones, containing inserts in both orientations, and deleted plasmids obtained by exonuclease III digestion (*18*), by the dideoxynucleotide chain-termination method using Sequenase (United States Biochemical). To overcome compression, dGTP was replaced by dITP, 7-deaza-dGTP (United States Biochemical), or 7-deaza-dGTP and dATP mixes (Pharmacia P-L Biochemicals) in some reaction mixtures.

Routinely, 5% polyacrylamide gels containing 20% v/v formamide were used for running sequencing reaction mixtures. Sequence data were analyzed using Genetyx-Mac software (Software Development).

Enzyme Activity Assay—The PAP activity was assayed by measuring the amount of β-naphthylamine liberated from Pro-βNA, as described previously (*5*). One unit of the enzyme activity was defined as the amount of enzyme releasing 1 μmol of β-naphthylamine per min. The protein concentration was determined by the method of Bradford (*19*) or by measuring the absorbance at 280 nm. For substrate specificity studies using peptide substrates, the liberated proline was determined using ninhydrin (*20*) or by HPLC.

Culture of E. coli Transformants and Purification of Prolyl Aminopeptidase—*E. coli* harboring plasmid pHAP-2 was grown in 12 liters of N-broth [1% meat extract, 1% polypepton, and 0.5% NaCl, pH 7.0, containing ampicillin (50 μg/ml)] in a fermentor at 30°C. Growth was followed by measuring absorbance at 650 nm. All the purification procedures were done at 4°C. The washed cells (50 g wet weight) were suspended in 800 ml of 20 mM Tris-HCl buffer, pH 8.0 (Tris buffer), and disrupted for 10 min with glass beads in a Dyno-Mill. The glass beads were removed by decantation and the disrupted cell suspension was centrifuged at 8,000 × *g* for 15 min. The supernatant was treated with protamine sulfate solution (final concentration, 18 mg per gram of wet cells), then the mixture was kept for 30 min and centrifuged. Ammonium sulfate was added to the supernatant to make it 40% saturated, the solution was allowed to stand for 1 h, and the precipitated proteins were recovered by centrifugation. The pellet obtained was dissolved in Tris buffer containing 20% saturation ammonium sulfate, and the clear solution was applied to a column (6 × 15 cm) of Toyopearl HW-65C equilibrated with the above buffer. The enzyme was not adsorbed, but passed through the column leaving a large fraction of the unwanted proteins behind. The active fractions were combined, and the enzyme was precipitated

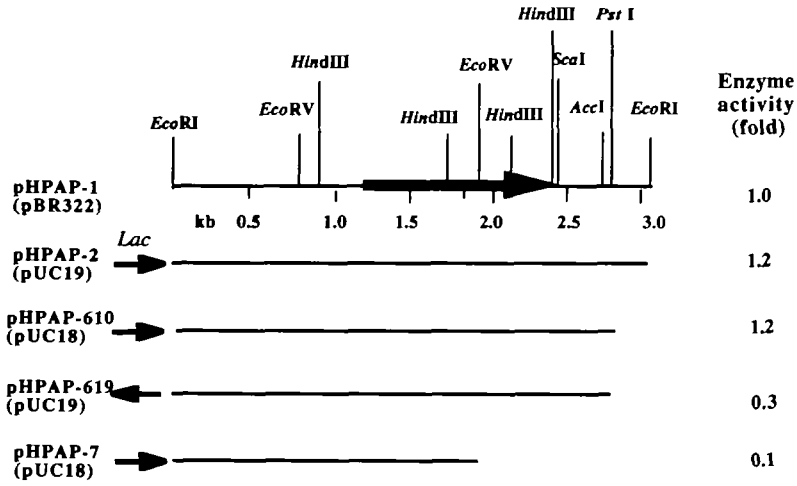

Fig. 1. **Restriction map of the region containing the *H. alvei* pap gene.** The enzymatic activity of each clone is compared to that of the clone bearing plasmid pHPAP-1. The arrows indicate the transcription orientation of the gene in each clone, with reference to that guided by the promoter on the vector.

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                                                                    CGGCAAC
CTCTCCGCTGCAATATATCAAATCCTTCCGCTTGCATAAGGCGCGAATGATGATGTTGCATGACGGGCTAAAAGCCAGCA
CTGCGGCCATTCGCGTGGGTTATGAGAGCGCTTCGCAGTTTAGCCGTGAATATAAACGTTTCTTCGGTAATACGCCGAGT
GAGCAAATGACGCGCTACCGCGAAGGTGGCTTGAGCATCGTTGAGCGATAAAAACGAATTTTACCGCAGAGTGGTCTGCC
        10        20        30        40        50        60        70        80
ATGATTGAAAGGGAATATCTGTTAGCCGGTATGTGGGTGCGAGAGCATCGTCTATCGGTGCCGCTAAACTGGTTTGATGA
M  I  E  R  E  Y  L  L  A  G  M  W  V  R  E  H  R  L  S  V  P  L  N  W  F  D  E
        90       100       110       120       130       140       150       160
AAATGATCCGCGCCGTATTACGGTTTTTGCACGTGAGTTATCAGAGGGCGGCAAAGCGGCAGAAGATTTACCATGTTTGC
 N  D  P  R  R  I  T  V  F  A  R  E  L  S  E  G  G  K  A  A  E  D  L  P  C  L  L
       170       180       190       200       210       220       230       240
TGTTCTTACAAGGCGGCCCTGGCGGGAAATCGCCGCGACCAACCAGTAAAGGCGGATGGTTAGGCGAGGCGCTGAAAAGC
  F  L  Q  G  G  P  G  G  K  S  P  R  P  T  S  K  G  G  W  L  G  E  A  L  K  S
       250       260       270       280       290       300       310       320
TTTCGCGTGATCTTGCTTGATCAACGCGGCACGGGGCAGAGCAGCAGGATCGAATCGAGCGTGATCCGCAATATGGACGT
F  R  V  I  L  L  D  Q  R  G  T  G  Q  S  S  R  I  E  S  S  V  I  R  N  M  D  V
       330       340       350       360       370       380       390       400
GGATCAACCCGCTGACTATCTCAGCTATTTCCGCGCTGATTCTATTGTGGCAGATGCTGAGCATCTGAGAAAAACGCAGT
 D  Q  P  A  D  Y  L  S  Y  F  R  A  D  S  I  V  A  D  A  E  H  L  R  K  T  Q  F
       410       420       430       440       450       460       470       480
TTGGTGGCCGTAAATGGAGCACCTTAGGGCAGAGTTTTGGTGGCTTTATCACCCTGACTTATTTATCTCAGGCCCCAGAA
  G  G  R  K  W  S  T  L  G  Q  S  F  G  G  F  I  T  L  T  Y  L  S  Q  A  P  E
       490       500       510       520       530       540       550       560
GGGCTTGCAGCCTGCTATATCACCGGCGGTTTACCATCTATCAAACCCGATGCCGAGCAGCTGTATCAGCGCACCTATCA
G  L  A  A  C  Y  I  T  G  G  L  P  S  I  K  P  D  A  E  Q  L  Y  Q  R  T  Y  Q
       570       580       590       600       610       620       630       640
GAAGCTGAAAGAAAAGAATCAGATTTTCTTCTCGCGCTATCCCCACTTGCAGCAGCAAATCAACCGTATTGCAGACGTGC
 K  L  K  E  K  N  Q  I  F  F  S  R  Y  P  H  L  Q  Q  Q  I  N  R  I  A  D  V  L
       650       660       670       680       690       700       710       720
TAAACGAGCAGGATGTGTACCTGCCCGATGGCGATATCCTCACCGTACAGCGTTTGCAAACGCTGGGTATTCAGTTAGGT
  N  E  Q  D  V  Y  L  P  D  G  D  I  L  T  V  Q  R  L  Q  T  L  G  I  Q  L  G
       730       740       750       760       770       780       790       800
ATGAGTGAAGGTTATGAGAGTTTGCTGTGGTTGTTTGATGAAGCTTTTAACCATGAGGGAGAGCTGAGCGATACGTTTTT
M  S  E  G  Y  E  S  L  L  W  L  F  D  E  A  F  N  H  E  G  E  L  S  D  T  F  L
       810       820       830       840       850       860       870       880
ATCGCAGGTGATGCATCTCACCGGTTTCACCGAGCATCCTCTGTACGCTGTTCTGCACGAAAGTATTTATGCTGATAACC
 S  Q  V  M  H  L  T  G  F  T  E  H  P  L  Y  A  V  L  H  E  S  I  Y  A  D  N  R
       890       900       910       920       930       940       950       960
GTAGTGGGGCAACTGACTGGGCAGCGCAGCGCGTGCATGACACGTTACCTGAGTTTCAAACCGACTGCCGCCCGCTGTTG
  S  G  A  T  D  W  A  A  Q  R  V  H  D  T  L  P  E  F  Q  T  D  C  R  P  L  L
       970       980       990      1000      1010      1020      1030      1040
TTAACGGGTGAGATGATCTATCCGTGGATGTTTGATGAAATGCAGTGTTTACGACCGTTCCGCGATGCAGTTCACCAGTT
L  T  G  E  M  I  Y  P  W  M  F  D  E  M  Q  C  L  R  P  F  R  D  A  V  H  Q  L
      1050      1060      1070      1080      1090      1100      1110      1120
GGCGCAGCGTTCAGAATGGCAACCGTTGTATGACGCGGAGCGCTTGGCTGCCAATACGGTGCCAGTGGTTGCGGCGGTTT
 A  Q  R  S  E  W  Q  P  L  Y  D  A  E  R  L  A  A  N  T  V  P  V  V  A  A  V  Y
      1130      1140      1150      1160      1170      1180      1190      1200
ATTACAATGACATGTATGTAGATATTGGTTTATCGCTGGAAACGGCCAAGCATATTGGCAACGTGGAAACGTGGATCACC
  Y  N  D  M  Y  V  D  I  G  L  S  L  E  T  A  K  H  I  G  N  V  E  T  W  I  T
      1210      1220      1230      1240      1250      1260      1270      1280
AGTGAATTTGAACACAATGGTTTGCGGGTGGGGAACGTGTTTGCTCACCTACGCCAGATGATGGCGCTGCGCGGCGTGAA
S  E  F  E  H  N  G  L  R  V  G  N  V  F  A  H  L  R  Q  M  M  A  L  R  G  V  N
      1290
TTAAAGCCCCTTTATTTTCAAACATAAAATTCAAACGTAAAAAAACCCGCCATTGGCGGGTTTTTTATGACTCATACTTA
 *
TCAGACATTCTCTAAGCATAAAGCTT
```

Fig. 2. **Primary structure of the *H. alvei* prolyl aminopeptidase.** The underlined regions indicate the positions for the putative promoters and ribosome-binding sites. The dotted sequences are those found by amino acid sequence analysis of peptides obtained from endoproteinase Asp-N digestion of the purified enzyme.

by adding ammonium sulfate to 80% saturation. The recovered precipitate was dissolved in Tris buffer and the resulting solution was desalted (Sephadex G-25) and applied to a DEAE-Toyopearl column, which was developed with a linear gradient of 0 to 0.5 M sodium chloride.

Nucleotide Sequence Accession Number—The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D61383 (*Hafnia alvei* prolyl aminopeptidase).

RESULTS

Cloning and Nucleotide Sequencing—After assaying 3,960 colonies from the different genomic libraries, one clone bearing a plasmid with a 3.2-kb insert at the *Eco*RI site was found to express the activity. The respective restriction enzyme map was estimated and is shown in Fig. 1. Based on this map, various subclones were constructed and their enzymatic activities were compared. The vector was changed to pUC19 (pHPAP-2) resulting in 1.2-fold increase in activity, and this clone was used for the purification procedure.

The correspondence of the cloned gene with *hpap* was further confirmed by Southern hybridization, using the fragment in plasmid pHPAP-2 as a probe. Only one band of the *Eco*RI digested *H. alvei* chromosomal DNA hybridized with the labeled probe, and its size was approximately the same as the insert obtained in pHPAP-1. This result not only confirms the identity of the cloned gene but also shows that *hpap* might exist as a single gene in *H. alvei* (data not shown).

Subclones and deletion mutants were constructed and sequenced, resulting in the determination of the sequence of a 1,633-bp fragment harboring the whole coding sequence (Fig. 2). The open reading frame was found to have a length of 1,281 bp, encoding a protein of 427 amino acids and a molecular mass of 48,577 Da. The correspondence of this gene to the HPAP protein was confirmed by amino acid sequence analysis of the expressed enzyme. The underlined amino acids in the figure are those determined for peptides obtained after endoproteinase Asp-N digestion of the purified enzyme. The amino terminal could not be detected, suggesting the possibility that it is blocked, and the assignation of the initiation methionine is tentative.

Comparison of the Primary Structures of the Enzymes—Variable identity percentages were found between this sequence and the reported sequences of *B. coagulans*, *N. gonorrhoeae*, *L. delbrueckii*, and *A. sobria* prolyl aminopeptidases and *Lactobacillus helveticus* prolinase (*pepN*).

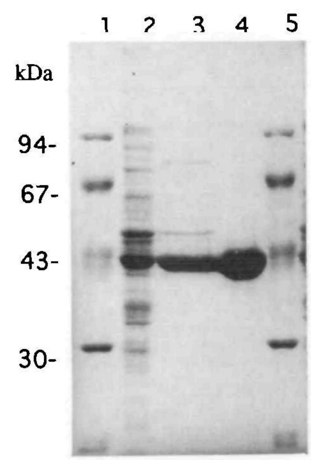

Fig. 4. **SDS-PAGE of preparations obtained through the purification procedure.** Lane 1, protein size markers; lane 2, cell-free extract; lane 3, pool after Toyopearl HW-65C; lane 4, pool after DEAE-Toyopearl chromatography; lane 5, protein size markers.

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HPAP   1 MIEREYLLAGMWVRE-HRLSVPLNWFDENDPRRITVFARELSEGGKAAEDLPCLLFLQGGPGGKSPRPTSKGGWLGEALKSFRVILLDQRG
APAP   1 MSSPLHYVLDGIHCEPHFFTVPLDHQQPDDEETITLFGRTLCRKDRLDDELPWLLYLQGGPGFGAPRPSANGGWIKRALQEFRVLLLDQRG
BPAP   1 M--YTEGFI------------DVTGGRV------------SFQKFDENGGGTPVIVLHGGPGSSCYS---LLGLKALAK-DRPVILYDQLG
NPAP   1 MYEIKQPFHSGY---------LQVSEIH-----------QIYWEESGNPDGVPVIFLHGGPGAGASPE--CRGFFNPDV-FRIVII-DQRG
LPAP   1 M--M-QITEKYL---------PFGNWQ------------TYCRIVGEATDRAPLLLLHGGPGSSHNYFE-VLDQVAEKS-GRQVIMYDQLG
PEPN   1 MKTGTKI--------------ITLDNG------------YHLWTNTQGEGDIHLLALHGGPGGNHEYWEDTAEQLKKQGLNVQVTMYDQLG

                                                       #
HPAP  91 TGQSSRIESSVIRNMDVDQPADYLSYFRADSIVADAEHLRKTQFGGRKWSTLGQSFGGFITLTYLSQAPEGLAACYITGGLPSIKPDAEQL
APAP  92 TGHSTPIHAELLAHLNPRQQADYLSHFRADSIVRDAELIREQLSPDHPWSLLGQSFGGFCSLTYLSLFPDSLHEVYLTGGVAPIGRSADEV
BPAP  62 CGKSDRPMDTT--------------LWRLDRFVEELAQIRQALNLD-EVHILGHSWGTTLAAAY---------CLTKPSGVKSVIFS----
NPAP  68 CGRSHPYACAE--------------DNTTWDLVADIEKVREMLGIG-KWLVFGGSWGSTLSLAY---------AQTHPERVKGLVLR----
LPAP  66 CGNSSIPDDQA------------ETAYTAQTWVKELENVREQLGLD-QIHLLGQSWGGMLALIYL--------CDYQPEGVKSLILS----
PEPN  66 SLYSDQPDYSDPEI--------AKKYLTYEYFLDEVDEVREKLGLD-NFYLIGQSWGGLLVQEYA--------VKYGQH-LKGAIIS----

HPAP 182 YQRTYQKLKEKNQIFFSRYPHLQQQINRIADVLNEQDVYLPDGDILTVQRLQTLGIQLGMSEGYESLLWLFDEAFNHEGELSDTFLSQVMHL
APAP 183 YRATYQRVADKNRAFFARFPHAQAIANRLATHLQRHDVRLPNGQRLTVEQLQQQGLDLGASGAFEELYYLLEDAFIGE-KLNPAFLYQVQAM
BPAP 125 -------------------SPCLSAPLWEQDQKRNLKKLPLDVQETINR------------------------------CEENGTTDSEEF
NPAP 131 -----------------GIFLCRPSETAWLNEAGGVSRIYPEQWQKFVAP-----------------------------IAENRRNRLIEA
LPAP 132 -------------------STLASAKLWSQELHRLIKYLPKGEQAAIKE------------------------------AEKNGNYDSLAY
PEPN 135 ------------------SMVDEIDEYVASVNRRRQEVLPQTEIDFMHE------------------------------CEKNNDYDNQRY

                                                                                          *
HPAP 274 TGFTEHPLYAVLHESIYADNRSGATDWAAQRVHDTLPEFQTDCRPLLLTGEMIYPWMFDEMQCLRPFRDAVHQLAQRSEWQPLYDAERLAAN
APAP 274 QPFNTNPVFAILHELIYCEGAAS-HWAAERVRGEFPALAWAQGKDFAFTGEMIFPWMFEQFRELIPLKEAAHLLAEKADWGPLYDPVQLARN
BPAP 167 AAAIEVFGKHFVNRLEKQPEWLEQKPSGY-----------RNADIYNIMWGPSEFTVLGNLKNFDCTTQLKEITCPSLYTCGRFDEATPETT
NPAP 176 YHGLLFHQDEEVCLSAAKAWADWESYLIRFEPEGVDEDAYASLAIARLENHYFVNGGWLQGDKAILNNIGKIRHIPTVIVQGRYDLCTPMQS
LPAP 174 QAANAHFMDQHAIKLTPDLPEPVLRKKKG-----------GSLAYLTGWGPNEYTPIGNLHGYEYTDRLKDLHLPALITSGTDDLCTPLVA
PEPN 186 INFVDRKQP--SKLYHLKDIG-------------------GSAVYHAFQGDNEFVITGKLKDWHFRDQLKNIKVPTLLTFGENETMPISTA

                                              #
HPAP 366 TVPVVAAVYYNDMYVDIGLSLETAKHIGNVETWITSEFEHNGLRVGNVFAHLRQMMALRGVN
APAP 365 KVPVACAVYAEDMYVEFDYSRETLKGLSNSRAWITNEYEHNGLRVDGEQILDRLIRLNRDC
BPAP 248 EYYSSLTPK-------------------SKFHVFEKSAHMPYIEEPEEYLAVIGDFLNSI
NPAP 268 AWELSK--------------------AFPEAELRVVQAGHCAFDPPLADALVQAVEDILPRLL
LPAP 254 KSMYDNLPNA------------------RWELFAGCGHMPFVQENAKYQELLSDWLISQD
PEPN 256 KIMQK--------------------EIPNSRLVTTPDGGHHHMVDNPDVYYKHLADFIREVENGTFKGE
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Fig. 3. **Alignment of the amino acid sequences of the *H. alvei* (HPAP) (this work), *A. sobria* (APAP) (*12*), *B. coagulans* (BPAP) (*7*), *N. gonorrhoeae* (NPAP) (*9*), and *L. delbrueckii* (LPAP) (*10, 13*) prolyl aminopeptidases, and the *L. helveticus* prolinase (*21*).**

TABLE I. **Purification of the *H. alvei* prolyl aminopeptidase from *E. coli* DH1/pHPAP2.**

Step	Volume (ml)	Protein (g)	Total activity ($\times 10^{-4}$ U)	Activity recovery (%)	Specific activity (U/mg)	Purification (fold)
Cell-free extract	620	8.8	26.0	100	29.5	1.0
40% sat. ammonium sulfate fractionation	250	0.8	10.0	38	125	4.2
Toyopearl HW-65C	330	0.7	8.6	33	123	4.2
DEAE-Toyopearl	36	0.2	3.3	13	165	5.6

TABLE II. **Physicochemical properties of the prolyl aminopeptidases.**

	HPAP	APAP	BPAP
Optimum pH	7.0-7.5	8.5	8.0
pH stability[a]	5.5-10.5	4.5-8.3	5.5-8.2
Optimum temperature	55°C	55°C	40°C
Thermal stability[b]	55°C	57°C	38°C
Molecular weight			
by gel filtration	180,000	205,000	33,000
by SDS-PAGE	42,000	43,000	33,000
from nucleotide sequence	48,577	48,405	32,356
Form	Tetramer	Tetramer	Monomer
Isoelectric point	5.2	5.4	4.9

[a]70% of the enzymatic activity remained after incubation in each buffer at room temperature for 30 min. [b]50% of the enzymatic activity remained after incubation at each temperature and pH 8.0 for 15 min.

TABLE III. **Effect of chemicals on the prolyl aminopeptidase activities.** The enzymes were pre-incubated at 30°C for 10 min with each additive, and the remaining activities were assayed under the standard conditions.

Chemicals	mM	Remaining activity (%)		
		HPAP	APAP	BPAP
PCMB	0.1	121.9	3.9	0.7
	1.0	139.0	0	0
IAA	0.1	95.2	90.8	79.9
	2.0	71.5	69.1	2.2
NEM	0.1	97.5	91.9	30.3
	2.0	100.3	62.1	0.6
DEP	0.1	25.7	11.6	91.0
	1.0	7.3	0	1.8
DCIC	0.1	84.7	72.6	85.4
	1.0	41.7	15.5	24.9
DFP	0.1	98.6	74.4	79.8
	1.0	91.4	17.3	4.7
PMSF	0.1	96.5	94.1	48.0
	1.0	83.6	91.4	18.3
Benzamidine	1.0	93.2	93.9	63.8
$ZnCl_2$	0.5	8.6	0.2	0
$MnCl_2$	0.5	79.0	78.0	0

Grouping the sequences according to their similarities put APAP and HPAP together with 45%, and BPAP, NPAP, LPAP, and pepN in another group with an identity percentage of 24-36%. Comparatively low values were obtained for mixed pairs like HPAP/BPAP and HPAP/NPAP with identities of 21%. In spite of this divergence, all the sequences could be aligned with a good conservation of the catalytic residues, as shown in Fig. 3.

Purification of the H. alvei Prolyl Aminopeptidase—Only two chromatographic steps were needed to obtain a homogenous preparation of the enzyme, as shown by the SDS-PAGE pattern in Fig. 4. Following this procedure, around 200 mg of pure HPAP could be obtained from a 12-liter culture, with a specific activity of 160 U/mg (Table I).

Characterization of the Expressed Enzyme—Table II shows the physicochemical properties, and Table III the results for the substrate specificity survey of the purified HPAP enzyme, together with those for the *Bacillus* and *Aeromonas* enzymes. Peptides containing proline at the amino terminal (*i.e.*, Pro-His-Lys, Pro-Gly-Gly, Pro-Val-Gly, Pro-βNA, Hyp-βNA) were hydrolyzed, but Ala-Pro-Ala and Ala-βNA and other peptides were unaffected by the enzyme (data not shown).

Effect of Chemicals on the Enzyme Activity—As shown in Table III, both APAP and BPAP were sensitive to PCMB, being completely inhibited at 0.1 mM, while no significant inhibition was observed with DFP or benzamidine at the same concentration. On the other hand, HPAP was not inhibited by PCMB but showed rather an increase in activity. Variable results were obtained for IAA, DEP, and NEM, but $ZnCl_2$ was manifestly inhibitory at 0.5 mM concentration toward the three enzymes. Variable effects were also shown by the serine enzyme inhibitors tested, but DCIC clearly inhibited the enzymes, although at a higher concentration (1 mM). DCIC inhibition was further studied by making parallel assays with well characterized enzymes. APAP and HPAP were both significantly resistant to denaturants such as urea and guanidine. Even at 5.4 M urea concentration, almost 90% of the APAP activity and 44% of that of HPAP remained after incubation, while BPAP was rapidly and effectively inactivated.

The three enzymes were unaffected by chelators as expected from previous reports. Dialysis of enzyme preparations for 24 h against 10 mM EDTA and 2 mM *o*-phenanthroline did not produce any significant change in the activities. Remarkably, when HPAP was dialyzed against 1 mM PCMB under the same conditions, 105.5% of the initial activity could be recovered.

DISCUSSION

With the results reported in this work, *Hafnia alvei* becomes the first *Enterobacteriaceae* in which the presence of a prolyl aminopeptidase activity has been definitively proved. This activity was, however, first by Fanghanel *et al.* who used it as a method to differentiate with high specificity *Hafnia* and *Serratia* strains from other *Enterobacteriaceae* including *E. coli*. The authors based their method on the finding that 74 different *E. coli* serotypes could not hydrolyze the substrate while high activities were easily detected in the *Hafnia* and *Serratia* strains (*6*), in clear disagreement with the reports by Sarid *et al.* regarding the presence of PAP activity in *E. coli*. In our study, the characterization of the expressed enzyme (physicochemical properties, substrate specificity, *etc.*) and the analysis of its amino acid sequence (alignment with other PAPs, homology search, *etc.*) conclusively identified the cloned gene as

hpap. It is highly probable that HPAP corresponds to the activity described by Fanghanel *et al.*, giving support to their conclusions, which are furthermore strengthened by the fact that no activity has been detected in the many *E. coli* strains we have used in our studies. In this way, it is likely that the *E. coli* activity described as prolyl aminopeptidase (proline iminopeptidase) by Sarid *et al.* was mistakenly characterized and had indeed a different specificity (*1, 2*). Further and more extensive studies are, however, necessary to solve this contradiction, and in this sense, the knowledge of the distribution of the enzyme in other *Enterobacteriaceae* is of great importance.

The cloning of the prolyl aminopeptidase gene from *Hafnia alvei* (*hpap*) allowed us purify very easily the expressed enzyme and determine its characteristics. On the other hand, the addition of its amino acid sequence to those already reported allowed us confirm the grouping of the prolyl aminopeptidases in two types on the basis of their size, substrate specificity stringency and sequence relatedness.

HPAP was found to be a tetramer of 48,577-Da subunits, encoded by an open reading frame of 1,281 bp (Fig. 2). The enzyme could be easily purified after two chromatographic steps and with a high yield, as much as 200 mg being obtained from a 12 liter culture with a 5.5-fold purification (Table I). The purification procedure was the same as that for the *Aeromonas* enzyme, to which HPAP showed not only the same chromatographical behavior but also a high sequence identity. Both enzymes were found to share 45% of identical amino acids, having the highest similarity among the prolyl aminopeptidases hitherto known (Fig. 3). HPAP could also hydrolyze hydroxyproline β-naphthylamide. On this evidence, the two enzymes can be placed in the same group, while the smaller, and highly proline specific *Bacillus, Neisseria*, and *Lactobacillus* enzymes (*12*) constitute another group.

The alignment of the sequences shown in Fig. 3 reveals the conservation of the active serine residue (*14*) and regions at the amino terminal. Recent site-directed mutagenesis studies on the *Bacillus* enzyme indicate that His267 is also essential for the enzymatic activity. This residue corresponds to His405 in HPAP and is analogously conserved in the other enzymes.

HPAP showed peculiar behavior when incubated with PCMB, the long-thought general inhibitor of the prolyl aminopeptidases (Table V). Though PCMB easily inhibited the other activities, HPAP was not affected even after dialysis of the enzyme preparation against 1 mM solutions. *Lyophyllum cinerascens* PAP (LPAP) was not affected by PCMB (*22*). However, the lack of sensitivity of LPAP towards typical serine enzyme inhibitors (DFP and PMSF), impeded the recognition of the significance of these results. APAP, HPAP, and BPAP showed great variability with respect to almost all types of inhibitors, but a common inhibitory trend could be observed with DCIC, a serine protease inhibitor that inactivates a wide variety of serine proteases but does not react with thiol, β-lactamase or metalloproteases (*23*). Although complete inhibition was not observed under the conditions described in the table, the significance of the inhibition level observed could be verified when assaying the behavior of well-characterized pyroglutamyl peptidase (*24*) and protease II (*25*), a sulfhydryl and serine peptidase, respectively.

In conclusion, the results reported in this work confirm the occurrence of at least two types of prolyl aminopeptidases, with similar enzymatic character but different size and specificity stringency. To the group containing only the *Aeromonas* enzyme it would be added the *Hafnia* enzyme, which is, however, peculiar in being resistant to PCMB. Although the distribution of the enzyme in Gram-negative and Gram-positive bacteria is more than clear from the studies reported to date, it is still unclear whether this activity is present in mammalian cells. This problem and that regarding the enzymatic mechanism are still unsolved, but, the knowledge of the properties of these two types of prolyl aminopeptidases, as well as the recognition of conserved sequences in both groups, could be the basis for future studies.

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