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Prolyl Aminopeptidase from *Serratia marcescens*: Cloning of the Enzyme Gene and Crystallization of the Expressed Enzyme[1]

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We cloned and sequenced the *Serratia marcescens* prolyl aminopeptidase (SPAP) gene. Nucleotide sequence analysis revealed an open reading frame of 951 bp, encoding a protein of 317 amino acids with a predicted molecular weight of 36,083. The expressed enzyme was purified about 90-fold on columns of Toyopearl HW65C and DEAE-Toyopearl, with an activity recovery of 30%. The apparent molecular weight of the purified enzyme was 36,000 and 38,000 as estimated by SDS-PAGE and gel filtration, respectively. The enzyme was not inhibited by diisopropyl phosphofluoridate (DFP) or phenylmethylsulfonyl fluoride (PMSF), but was markedly inhibited by 3,4-dichloroisocoumarin (DCIC). Crystals of the enzyme were grown by the hanging drop vapor diffusion method using PEG6000 as a precipitant at pH 6.5. The crystals are tetragonal with cell dimensions $a=b=65.6$ Å, and $c=169.8$ Å, a space group $P4_12_12$ or $P4_32_12$, and probably contain one monomer in the asymmetric unit. They diffract to at least 2.22 Å resolution.

Key words: α/β hydrolase fold family, crystallization, nucleotide sequence, prolyl aminopeptidase, *Serratia marcescens*.

Prolyl aminopeptidase (PAP, proline iminopeptidase; EC 3.4.11.5) catalyzes the removal of N-terminal proline from peptides, and has been the object of many studies since the first report of the *Escherichia coli* enzyme by Sarid *et al.* (*1*). PAP has been identified mainly in bacteria, and in some plants. Several investigators have purified PAP from these sources (*2-6*). In these reports, it was assumed that PAP is a sulfhydryl peptidase, since it is inhibited by *p*-chloromercuribenzoate (PCMB) and heavy metals, and because other reagents such as diisopropyl phosphofluoridate (DFP) have little or no influence. However, we demonstrated by site-directed mutagenesis, that serine and not cysteine residues are essential for the activities of the *Aeromonas sobria* and *Bacillus coagulans* PAPs (*7*). Several *pap* genes have been cloned and sequenced from *A. sobria, B. coagulans, Flavobacterium meningosepticum, Hafnia alvei, Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Neisseria gonorrhoeae* (*8-13*). Alignment of the sequences and a consideration of the enzymatic properties, showed that the PAP enzymes could be classified in two groups (*8*). One consists of monomers of approximately 30 kDa. They are rather small enzymes with a strict specificity for proline terminals and they cannot act on large peptides (*Bacillus, Flavobacterium, Neisseria*, and *Lactobacillus* PAPs). The second group comprises multimeric large enzymes of around 200 kDa, with a broader specificity extending to hydroxyproline terminals. These enzymes also act on longer peptide substrates (*Aeromonas* and *Hafnia* PAPs).

The analysis of the PAP sequences and homology searches of several databases suggested that these enzymes are related to a wide variety of hydrolases sharing identities of around 20%. These hydrolases belong to the α/β hydrolase fold family, according to three-dimensional analyses (*10*). On the other hand, the proline-recognizing mechanism of the proline-specific peptidases remains unknown, since the PAP sequences show no significant similarity to any other peptidases, or to any of the numerous known proline-specific peptidases.

To provide the basis for a detailed study of the structural relationships of PAPs with enzymes of the α/β hydrolase fold family and the other proline-specific peptidases, we obtained crystals suitable for X-ray analysis. We confirmed the presence of the activity in *Serratia marcescens*, and isolated the gene encoding the enzyme. In this paper, we report the nucleotide sequence of the *S. marcescens* PAP gene, as well as the crystallization and preliminary X-ray analysis of the expressed enzyme.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media—The hosts for cloning were *E. coli* DH1, DH5α, and JM105. Plasmids pBR322, pUC18, pUC19, and pBluescript SK(+) were used as vectors. Bacteria were grown in Luria-Bertani broth (LB-broth).

Materials—Restriction endonucleases and various DNA modifying enzymes were purchased from Takara Shuzo and Toyobo. ⊿Tth DNA Polymerase Sequencing PRO was from

[1] The nucleotide sequence presented in this paper was entered into the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number D87897.

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Abbreviations: PAP, prolyl aminopeptidase; DFP, diisopropyl phosphofluoridate; DCIC, 3,4-dichloroisocoumarin; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; Pro-2NNap, proline-β-naphthylamide.

Toyobo and [α-^{32}P]dCTP was from Amersham. Agarose I was from Dojin Chemicals, and calf intestine alkaline phosphatase was from Boehringer-Mannheim.

Preparation of Gene Library and Screening of the Enzyme Gene—*S. marcescens* chromosomal DNA was prepared by the method of Saito and Miura (*14*), digested with *Bam*HI, and ligated into the dephosphorylated *Bam*HI site of pBR322. The ligated plasmid mixture was used to transform *E. coli* JM105. The transformants were screened for expression of the PAP gene by measuring activity with proline-β-naphthylamide (Pro-2NNap) as the substrate, in 96-well polystyrene assay plates.

Subcloning and Nucleotide Sequencing—Restriction endonuclease fragments of chromosomal DNA were subcloned into pUC18, pUC19, or pBluescript SK(+), following standard procedures (*15*). Nucleotides were sequenced by dideoxy chain termination.

Assay of Prolyl Aminopeptidase Activity—PAP activity was assayed using Pro-2NNap as the substrate, according to the method of Yoshimoto and Tsuru (*6*).

One unit of the enzyme activity was defined as the amount of enzyme required to release 1 μmol of β-naphthylamine per min. The protein concentration was determined by the method of Bradford (*16*) or by measuring the absorbance at 280 nm ($A_{280}^{1\%}=10.0$).

Purification of the Expressed Enzyme—*E. coli* DH1/pSPAP-HE (Fig. 1) was aerobically cultured in 12 liter N-broth containing ampicillin (50 mg/liter) at 37°C for 12 h, using a New Brunswick jar fermentor. Cells were harvested by centrifugation, washed, and resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA. The cells were disrupted with glass beads in a Dyno-Mill, the clear supernatant was treated with protamine sulfate (18 mg/g of wet cells), and the mixture was centrifuged at 8,000$\times g$ for 30 min. The supernatant was fractionated with ammonium sulfate from 25 to 55% saturation. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 25% saturation ammonium sulfate, and applied to a column of Toyopearl HW65C (6$\times$15 cm) equilibrated with the above buffer. The column was washed, and the adsorbed enzyme was eluted with a decreasing linear gradient of ammonium sulfate concentration from 25 to 0% saturation in 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA. The enzyme in the active fractions was precipitated with ammonium sulfate, and the precipitate was dissolved in a small volume of 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA. The resulting solution was desalted, applied to a DEAE-Toyopearl column (6$\times$15 cm) and eluted with a linear gradient of 0 to 0.5 M sodium chloride. Active fractions were combined and concentrated by ultrafiltration using an Amicon apparatus (PM-10) and dialyzed against 20 mM Tris-HCl buffer (pH 8.0).

Crystallization, Data Collection, and Processing—Crystals of PAP were grown at 20°C by the hanging drop vapor diffusion method. Drops of 10 μl were prepared by mixing 5 μl of the protein (28 mg/ml) with 5 μl of reservoir solution (0.1 M Na cacodylate pH 6.5, 0.2 M Na acetate, and 20% PEG 6000).

X-Ray data were collected by an oscillation method at 20°C using a Rigaku R-AXIS IIc area detector with Cu $K\alpha$ radiation, which was generated by a Rigaku RU200 rotating-anode generator, operating at 36 kV and 84 mA and focused using a Supper double-focusing mirror. Laue group and unit-cell parameters were determined by data-processing software (*PROCESS*) provided with the R-AXIS system. The best diffraction data from a crystal were collected up to 2.22 Å resolution. The diffraction data were analyzed with the program *PROCESS*.

RESULTS

Cloning of the Prolyl Aminopeptidase Gene—Among about 1,200 transformants screened, one with enzyme activity harbored a plasmid with a 6.0 kbp insert (pSPAP 1). The restriction map of the insert is shown in Fig. 1. Based on this map, we constructed various subclones and compared their enzymatic activities. *E. coli* harboring pSPAP-HE had an enzyme activity level 120-fold higher than that conferred by pSPAP 1, so this clone was used for the purification procedure.

The identity of the cloned gene as *pap* was further confirmed by Southern hybridization, using the 6.0 kbp fragment as a probe. Only one band of the *Bam*HI-digested *S. marcescens* chromosomal DNA hybridized with the labeled probe, and it was about the same size as that of the insert in pSPAP 1. This result not only confirmed the identity of the cloned gene, but also showed that PAP exists as a single gene in *S. marcescens* (data not shown).

Nucleotide Sequence—We sequenced the nucleotides of

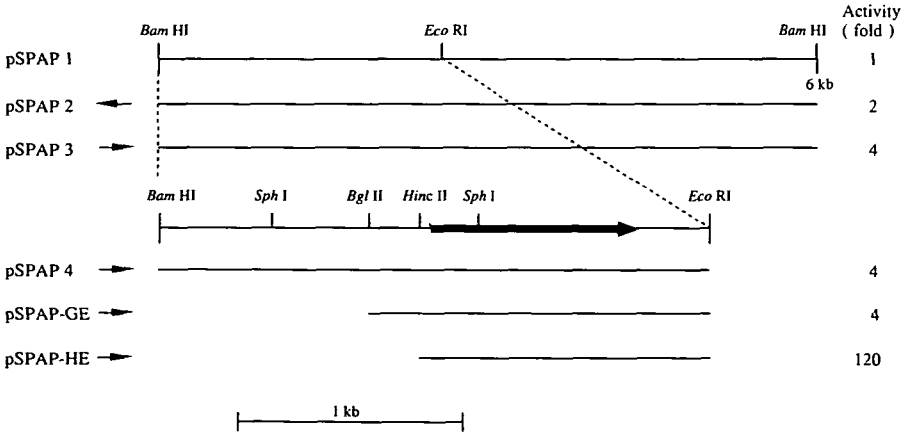

Fig. 1. **Restriction endonuclease map of the 6.0 kbp chromosomal DNA fragment.** The large arrow indicates the position and orientation of the enzyme gene, and the small arrows indicate the orientation of the promoter on the vector. For each plasmid, the vector was as follows: pSPAP 1/pBR322, pSPAP 2 to pSPAP HE/pUC18 or pUC19.

the insert in pSPAP 4. We found an open reading frame of 951 bp beginning with an ATG methionine codon (Fig. 2). The protein deduced from the nucleotide sequence was composed of 317 amino acid residues with a molecular weight of 36,083. Upstream from the initiation codon, there were putative −35 and −10 regions, and an inverted repeat downstream of the termination codon.

The identity between this and the reported sequences of

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      -35                  -10
   1 GTTGACCTGCCGGGCGGCTTCCGCTTTGATGAAAGCATGACTCACAGAGAGACGGAAAGCGCCATGGAACAATTACGAGGTTTGTACCCG
                                                                  M  E  Q  L  R  G  L  Y  P
  91 CCGTTAGCGGCATACGACAGCGGTTGGCTGGATACTGGGGATGGCCACCGGATCTACTGGGAGCTGAGCGGTAACCCGAACGGTAAACCG
     P  L  A  A  Y  D  S  G  W  L  D  T  G  D  G  H  R  I  Y  W  E  L  S  G  N  P  N  G  K  P
 181 GCGGTATTCATTCACGGCGGGCCGGGCGGCGGCATCTCCCCGCATCATCGTCAGCTGTTCGATCCCGAACGCTACAAGGTGCTGCTGTTC
     A  V  F  I  H  G  G  P  G  G  G  I  S  P  H  H  R  Q  L  F  D  P  E  R  Y  K  V  L  L  F
 271 GATCAGCGCGGTTGCGGCCGCTCCCGTCCGCATGCCAGCCTGGACAACAACACCACCTGGCATTTGGTGGCCGATATCGAGCGGCTGCGC
     D  Q  R  G  C  G  R  S  R  P  H  A  S  L  D  N  N  T  T  W  H  L  V  A  D  I  E  R  L  R
 361 GAAATGGCCGGCGTCGAGCAGTGGCTGGTGTTCGGCGGTTCCTGGGGATCGACGCTGGCGCTCGCCTATGCGCAGACCCACCCGGAGCGC
     E  M  A  G  V  E  Q  W  L  V  F  G  G  S  W  G  S  T  L  A  L  A  Y  A  Q  T  H  P  E  R
 451 GTCAGTGAAATGGTGCTGCGCGGCATCTTCACCCTGCGCAAGCAGagGCTGCATTGGTATTACCAGGACGGCGCTTCGCGCTTCTTCCCG
     V  S  E  M  V  L  R  G  I  F  T  L  R  K  Q  R  L  H  W  Y  Y  Q  D  G  A  S  R  F  F  P
 541 GAAAAATGGGAGCGCGTGCTGTCCATCCTCTCGGATGACGAGCGTAAAGACGTGATCGCCGCCTACCGGCAGCGGCTGACCTCGGCCGAT
     E  K  W  E  R  V  L  S  I  L  S  D  D  E  R  K  D  V  I  A  A  Y  R  Q  R  L  T  S  A  D
 631 CCGCAGGTGCAGCTTGAAGCGGCCAAACTGTGGAGCGTGTGGGAAGGGGAGACGGTCACGCTGCTGCCGAGCCGCGAATCCGCTTCGTTC
     P  Q  V  Q  L  E  A  A  K  L  W  S  V  W  E  G  E  T  V  T  L  L  P  S  R  E  S  A  S  F
 721 GGCGAGGATGATTTCGCGCTGGCGTTCGCCCGCATTGAAAACCACTACTTCACCCATCTGGGCTTCCTGGAAAGCGACGACCAACTGCTG
     G  E  D  D  F  A  L  A  F  A  R  I  E  N  H  Y  F  T  H  L  G  F  L  E  S  D  D  Q  L  L
 811 CGCAACGTGCCGCTGATCCGCCATATTCCGGCGGTGATCGTCCACGGCCGCTATGACATGGCCTGCCAGGTGCAGAACGCCTGGGACCTG
     R  N  V  P  L  I  R  H  I  P  A  V  I  V  H  G  R  Y  D  M  A  C  Q  V  Q  N  A  W  D  L
 901 GCCAAGGCCTGGCCGGAGGCGGAGCTGCATATCGTGGAAGGGGCGGGGCATTCGTATGATGAGCCGGGCATTTTGCACCAGCTGATGATC
     A  K  A  W  P  E  A  E  L  H  I  V  E  G  A  G  H  S  Y  D  E  P  G  I  L  H  Q  L  M  I
 991 GCCACCGACCGGTTTGCCGGCAAATGACGCAAAAAAACCCGCCGTAACGGCGGGTTTTTCTATTTAGCCTTCGGCTCGTACGGCAGGCGC
     A  T  D  R  F  A  G  K  *
1081 GAAAACTTGTGGGATTCCGCGCGGTAGTGCGCCACGCGCTCGCGAAAATAGTCGCGCAGCGCTTCCGGTTGCTCGCGCTCCACACTTCC
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Fig. 2. **Nucleotide sequence of the *S. marcescens* prolyl aminopeptidase gene and its deduced amino acid sequence.** Putative −35 and −10 sequences are underlined. Double underline indicates the putative transcription terminator.

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PEPN   1:M--------------------------KTGTKIITLDNGYHLWTNTQGEGDIHLLALHGGPGGNHEYWEDTAEQLKKQGLNVQVTMYDQLGSLYSDQPDYSDP-------
BPAP   1:M--------------------------YTEGFIDVTGGRVSFQKFDENGGGTPVIVLHGGPG----SSCYSLLGLKALAKDRPVILYDQLGCGKSDRP------------
FPAP   1:M--------------------------IPITTPVGNFKVWTKRFGTNPKIKVLLLHGGPA--MTHEYMECFETFFQREGFEFYEYDQLGSYYSDQP------------
LPAP   1:M------------------------MQITEKYLPFGNWQTYCRIVGEATDRAPLLLLHGGPG--SSHNYFEVLDQVAEKSGRQVIMYDQLGCGNSSIP----------DD
SPAP   1:M-----THRETESAMEQLRGLYPPLAAYDSGWLDTGDGHRIYWELSGNPNGKPAVFIHGGPG----GGISPHHRQLFDPERYKVLLFDQRGCGRSRP-HASL--------
APAP   1:MSSPLHYVLDGIHCEPHFFTVPLDHQQPDDEETITLFGRTLCRKDRLDDELPWLLYLQGGPGFGAPRPSANGGWIKRALQEFRVLLLDQRGTGHSTPIHAELLAHLNPRQ
HPAP   1:M-IEREYLLAGMWVREHRLSVPLNWFDENDPRRITVFARELSEGGKAAEDLPCLLFLQGGPGGKSPRPTSKGGWLGEALKSFRVILLDQRGTGQSSRIESSVIRNMDVDQ

PEPN  78:EIAKKYLTYEYFLDEVDEVREKLGLD--NFYLIGQSWGGLLVQEY---------AVKYGQHLKGAIISS----------MVDEIDEYVASVNR
BPAP  69:MDTTLWRLD-RFVEELAQIRQALNLD--EVHILGHSWGTTLAAAY---------CLTKPSGVKSVIFSS----------PCLSAPL-WEQDQK
FPAP  69:TDEKLWNID-RFVDEVEQVRKAIHADKENFYVLGNSWGGILAMEY---------ALKYQQNLKGLIVAN----------MMASAPE-YVKYAE
LPAP  75:QAETAYTA--QTWVKELENVREQLGLD-QIHLLGQSWGGMLALIYL--------CDYQPEGVKSLILSS----------TLASAKL-WSQELH
SPAP  93:--DNNTTW--HLVADIERL-REMAGVE-QWLVFGGSWGSTLALAY---------AQTHPERVSEMVLRG----------IFTLRKQRLHWYYQDG
APAP 111:QADYLSHFRADSIVRDAELIREQLSPDHPWSLLGQSFGGFCSLTYLSLFPDSLHEVYLTGGVAPIGRSADEVYRATYQRVADKNRAFFARFPHAQAIANRLATHLQRHDV
HPAP 110:PADYLSYFRADSIVADAEHLRKTQFGGRKWSTLGQSFGGFITLTYLSQAPEGLAACYITGGLPSIKPDAEQLYQRTYQKLKEKNQIFFSRYPHLQQQINRIADVLNEQDV

PEPN 150:RRQEVLPQTEIDF---MHEC------EKNND----------YDNQRYQ----DDVQILNINFVDRKQPSKLYHLKDIGGSA---VYHAFQGDNEFVITGK--
BPAP 139:RNLKKLPLDVQET---INRC------EENGT----------TDSEEFA----AAIEVFGKHFVNRLEKQPEWLEQKPSGYRNADIYNIMWGPSEFTVLGN--
FPAP 141:VLSKQMKPEVLAE---VRAI------EAKKD----------YANPRYT----ELLFPNYYAQHICRLKEWPDALNRSLKHVNSTVYTLM-QGPSELGMSSDA
LPAP 146:RLIKYLPKGEQAA---IK--------EAETTGN----------YDSLAYQ----AANAHFMDQHAIKLTPDLPEPVLRKKKGG---SLAYLTG-WGPNEYTPIG
SPAP 163:ASRFFPEKWERVL---SI-L-----SDDE----------RKDVIAAYRQRLTSADPQVQLEAAKLWSVWEGETVTLLPSRESASFGEDDF-ALAFAR-IENHYFTHLGF
APAP 221:RLPNGQRLTVEQLQQQGLDLGASGAFEELYYLLEDAF--IGEKLNPAFLYQVQAMQPFNTNPVFAILHELIYCE--GAASHWAAERVRGEFPALAWAQGKDFAFTGEMIF
HPAP 220:YLPDGDILTVQRLQTLGIQLGMSEGYESLLWLFDEAFNHEGELSDTFLSQVMHLT-GFTEHPLYAVLHESIYADNRSGATDWAAQRVHDTLPEFQTDCRPLL-LTGEMIY

PEPN 224:-LKDWHFRDQLKNI---KVPTLLTFG-ENETM-------PISTAKIMQKEIPNSRLVTTP----------DGGEHHMVDNPDVYYKHLADFIREVENGTFKGE
BPAP 216:-LKNFDCTTQLKEI----TCPSLYTCG-RFDEA-------TPETTEYYSSLTPKSKFHVFE----------KSAEMPYIEEPEEYLAVIGDFLNSI
FPAP 219:RLAKWDIKNRLHEI---ATPTLMI--GARYDTMD-------PKAM----EEQSKLVQKG---------RYLYCPNGS-ELAMWDDQKVFMDGVIKFIKDVDTKSFN
LPAP 221:NLHGYEYTDRLKDL---HLPALIT-SG-TDDL-C------TPLVAKSMYDNLP----------NARWELFAGCGEMPFVQENAKYQELLSDWLISQD
SPAP 251:LESDDQL--LRNVPLIRHIPAVIV-HG-RYDMACQVQNAWDLAKAWPEAELHIVE----------GAGESYDEPGILHQLMIATDRFAGK
APAP 327:PWMFEQFRELIPLKEAAHLLAEKADWGPLYDP-VQLARNKVPVACAVYAEDMYVEFDYSRETLKGLSNSRAWITNEYEENGLRVDGEQ-ILDRLIRLNRDC
HPAP 328:PWMFDEMQCLRPFRDAVHQLAQRSEWQPLYDA-ERLAANTVPVVAAVYYNDMYVDIGLSLETAKHIGNVETWITSEFEENGLRVGNVFAHLRQMMALRGVN
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Fig. 3. **Alignment of the amino acid sequences of the prolinase and prolyl aminopeptidases.** The amino acid residues that seem to be involved in the catalytic triads are shadowed, and others that were highly conserved are shown in bold. PEPN, *L. helveticus* prolinase; BPAP, *B. coagulans* PAP; FPAP, *F. meningosepticum* PAP; LPAP, *L. delbrueckii* PAP; SPAP, *S. marcescens* PAP; APAP, *A. sobria* PAP; HPAP, *H. alvei* PAP.

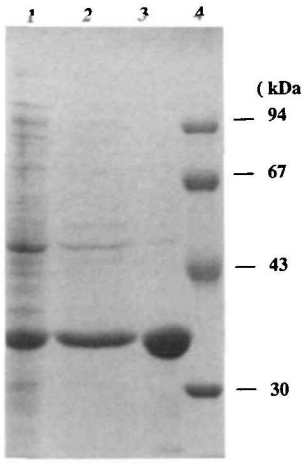

Fig. 4. **SDS-PAGE of the preparations obtained through the purification procedure.** SDS-PAGE was run on 10% polyacrylamide gels, which were subsequently stained with Coomassie Brilliant Blue R-250. Lane 1, cell-free extract; lane 2, pool after Toyopearl HW 65C; lane 3, pool after DEAE-Toyopearl chromatography; lane 4, protein size markers.

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

	SPAP	BPAP	APAP	HPAP
Optimum pH	8.0	8.0	8.5	7.5
pH stability[a]	7.0-9.0	5.5-8.0	4.5-8.3	5.5-10.5
Optimum temperature	45°C	40°C	55°C	42°C
Thermal stability[b]	45°C	38°C	57°C	45°C
Molecular weight				
Gel filtration	38,000	33,000	205,000	180,000
SDS-PAGE	36,000	33,000	43,000	42,000
Form	Monomer	Monomer	Tetramer	Tetramer

[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. SPAP, BPAP, APAP, and HPAP: *S. marcescens, B. coagulans* (*9*), *A. sobria* (*8*), and *H. alvei* (*11*) prolyl aminopeptidases.

B. coagulans, F. meningosepticum, L. delbrueckii, A. sobria, and *H. alvei* prolyl aminopeptidases (*8-12*) and *Lactobacillus helveticus* prolinase (pepN) (*17*) varied. SPAP belongs to the *Bacillus, Flavobacterium, Lactobacillus* PAPs, and pepN group with 24-36% identity. Although the overall identity level is low among all these enzymes, all the sequences can be aligned with good conservation of the catalytic residues (Fig. 3).

Purification and Characterization of the Expressed Enzyme—The expressed enzyme was purified 90-fold to homogeneity, with an activity recovery of 30%. The final preparation migrated as a single band on SDS-PAGE, corresponding to a molecular weight of 36,000 (Fig. 4), which agreed well with that calculated from the deduced amino acid sequence.

Table I shows the physicochemical properties, and Table II the effects of inhibitors on the purified SPAP enzyme, as well as on the *Bacillus, Aeromonas*, and *Hafnia* enzymes (*8, 9, 11*). The molecular weight of the purified enzyme was estimated to be 36,000 and 38,000 by SDS-PAGE and gel filtration, respectively, suggesting that the enzyme is a monomer. As shown in Table II, *Serratia, Bacillus*, and

TABLE II. **Effect of inhibitors on prolyl aminopeptidase activities.**

Inhibitor	mM	Remaining activity (%)			
		SPAP	BPAP	APAP	HPAP
EDTA	1.0	88.9	123.6	99.2	102.1
	5.0	87.6	109.5	103.6	107.1
PMSF	0.1	102.0	108.4	97.1	96.5
	1.0	59.9	59.4	94.9	83.6
DFP	0.1	76.6	79.8	74.4	98.6
	1.0	9.3	4.1	14.3	91.1
DCIC	0.1	0.1	85.4	72.6	84.7
	1.0	0	24.9	15.5	41.7
PCMB	0.1	3.2	0.7	9.6	121.9
	1.0	0	0	0	139.1

SPAP, BPAP, APAP, and HPAP: *S. marcescens, B. coagulans* (*9*), *A. sobria* (*8*), and *H. alvei* (*11*) prolyl aminopeptidases.

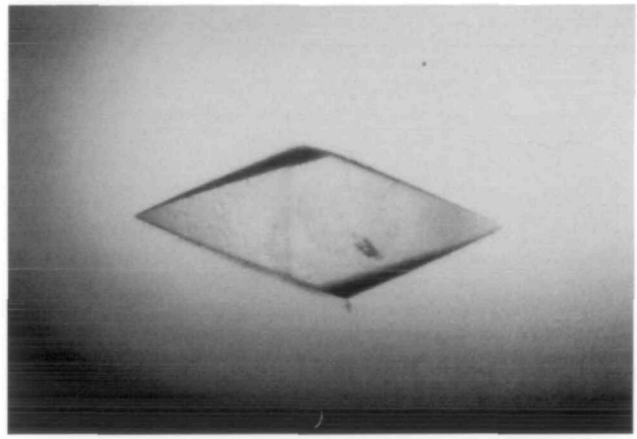

Fig. 5. **Photomicrograph of a crystal of *S. marcescens* prolyl aminopeptidase.** The crystal was obtained by the hanging drop method, by mixing 5 μl of protein (28 mg/ml) with 5 μl of reservoir buffer containing 20% PEG 6000 (pH 6.5).

Aeromonas PAPs were sensitive to PCMB, being completely inhibited at 0.1 mM, whereas neither DFP nor PMSF was significantly inhibitory at the same concentration.

Crystallization, Data Collection, and Processing—The enzyme was crystallized by the hanging drop vapor diffusion method using PEG 6000 as a precipitant. A bipyramidal crystal typically grew to $0.7 \times 0.3 \times 0.3$ mm within 4 days (Fig. 5).

The numbers of observed reflections and unique reflections were 50,926 and 16,306, respectively. Merging R factor based on intensity was 11.19% and completeness of data was 93.5%. X-Ray data indicated that the unit cell parameters are $a = b = 65.6$, $c = 169.8$ Å, and that the space group is $P4_12_12$ or $P4_32_12$. The assumption of one subunit per asymmetric unit leads to an empirically acceptable V_m value of 2.40 Å^3 Da^{-1}, corresponding to a solvent content of 48%.

DISCUSSION

We cloned the prolyl aminopeptidase gene from *S. marcescens*, then purified and crystallized the expressed enzyme. Fanghanel *et al.* firstly reported this activity and used it as a highly specific means of differentiating with high specificity *Serratia* and *Hafnia* strains from other *Enterobacte-*

riaceae, including *E. coli* (*18*). We cloned other *pap* gene including that from *Hafnia*, and categorized the PAPs into two groups based on size, substrate specificity, and sequence homology (*8*).

The open reading frame detected in the SPAP gene, encodes a protein consisting of 317 amino acid residues with a molecular weight of 36,083. The alignment of the sequences shown in Fig. 3 reveals the conservation of the active serine residues. Site-directed mutagenesis studies on *Bacillus* PAP have indicated that His267 is also essential for the enzymatic activity (*7*). This residue corresponds to His296 in *Serratia* PAP and it is also conserved in the other enzymes.

Cloning the PAP gene from *S. marcescens* allowed the expressed enzyme to be easily purified and its characteristics to be determined. SPAP was unaffected by chelators, but was potently inhibited by PCMB. These results are similar to those for the *Aeromonas* and *Bacillus* enzymes (*8, 9*). DFP was to some extent inhibitory, but the concentration required to inhibit the enzyme was more than ten times that of PCMB (Table III). *Serratia, Aeromonas, Bacillus*, and *Hafnia* PAPs considerably varied in their behavior with respect to almost all types of inhibitors, but a common inhibitory trend was evident with DCIC (*7, 11*).

The molecular weight of the purified enzyme was estimated to be 36,000 and 38,000, by SDS-PAGE and gel filtration, respectively, suggesting that the enzyme is a monomer, as are those of *Bacillus, Flavobacterium, Neisseria*, and *Lactobacillus*. Further, SPAP could not hydrolyze hydroxyproline β-naphthylamide (data not shown). Based on this evidence, *Serratia* PAP was included in the smaller and highly proline-specific group, which consists of *Bacillus, Neisseria, Lactobacillus*, and *Flavobacterium* PAPs (*8*).

The notion that all Pro-Y cleaving proline-specific peptidases share a common three-dimensional fold, namely that of the α/β-hydrolase fold family (*19*), has been proposed (*10, 20*). This notion would explain the failure to find a homologous primary structure in the many enzymes so far sequenced. There is no similarity between the members of the prolyl oligopeptidase family and the prolyl aminopeptidases, except for the region surrounding the active serine residue (*10, 20-22*). If the above notion is correct, then the determination of their three-dimensional structure should give a topology similar to the consensus of the α/β-hydrolase fold. Therefore, we performed crystallization studies.

SPAP was crystallized using hanging drop vapor diffusion. Preliminary X-ray analysis data were obtained from these crystals, but native crystals did not show any sign of decay during data collection up to 2.22 Å resolution. A search for heavy-atom derivatives suitable for phasing by multiple isomorphous replacement is under way.

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