Cloning, Expression, and Characterization of Tomato (Lycopersicon esculentum) Aminopeptidase P*

Received for publication, April 10, 2001, and in revised form, June 12, 2001
Published, JBC Papers in Press, June 22, 2001, DOI 10.1074/jbc.M103179200

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A cDNA (LeAPP2) was cloned from tomato coding for a 654 amino acid protein of 72.7 kDa. The deduced amino acid sequence was >40% identical with that of mammalian aminopeptidase P, a metalloenzyme. All amino acids reported to be important for binding of the active site metals and catalytic activity, respectively, were conserved between LeAPP2 and its mammalian homologues. LeAPP2 was expressed in Escherichia coli in N-terminal fusion with glutathione S-transferase and was purified from bacterial extracts. LeAPP2 was verified as an aminopeptidase P, hydrolyzing the aminoterminal-Xaa-Pro bonds of bradykinin and substance P. LeAPP2 also exhibited endoproteolytic activity cleaving, albeit at a reduced rate, the internal -Phe-Gly bond of substance P. Apparent \( K_m \) (15.2 ± 2.4 μM) and \( k_{cat} \) (0.94 ± 0.11 m/s) values were obtained for β-Lys(Alb)-Pro-Pro-pNA as the substrate. LeAPP2 activity was maximally stimulated by addition of 4 mM MnCl₂ and to some extent also by Mg²⁺, Ca²⁺, and Co²⁺, whereas other divalent metal ions (Cu²⁺, Zn²⁺) were inhibitory. Chelating agents and thiol-modifying reagents inhibited the enzyme. The data are consistent with LeAPP2 being a Mn(II)-dependent metalloprotease. This is the first characterization of a plant aminopeptidase P.

Proline is unique among the proteinogenic amino acids in that its side chain is bonded to both the α-carbon and the amino group. The resulting cyclic structure imposes conformational restraints on proline-containing peptides relevant for structure and function of many physiologically important biomolecules. A key role for proline residues is the protection against nonspecific proteolytic degradation. Hence proline is frequently found and conserved in peptide hormones, neuropeptides, and growth factors (1–3). Many bioactive polypeptides share a Xaa-Pro motif at their N termini shielding them against nonspecific N-terminal degradation. The degradation of these peptides requires proteases with specificity for the Xaa-Pro motif including proline-selective dipeptidases (dipeptidyl peptidases II and IV, cleaving the post-Pro bond) and aminopeptidase P (Xaa-Pro aminopeptidase, cleaving the pre-Pro bond). Cleavage of the Xaa-Pro motif by either one of these peptidases may initiate the proteolytic degradation/inactivation of the peptide or may result in an altered bioactivity (2–4).

Aminopeptidase P (APP, EC 3.4.11.9) was first isolated from Escherichia coli (5) and has subsequently been characterized from many microbial and mammalian sources (reviewed in Ref. 4). Mammalian APPs are now known to comprise at least two distinct forms, a cytosolic form and a membrane-bound form attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (6–14). APPs hydrolyze the peptide bond between any amino acid and a penultimate proline residue at the N termini of oligopeptide and protein substrates. A free amino group is required at the N terminus and the scissile bond must be in the trans configuration (15). The hydrolysis of dipeptides is very slow compared with the hydrolysis of longer chains, indicating the existence of a third subsite for substrate binding, which was confirmed for E. coli and mammalian APPs (10, 15). Likely physiological substrates of APP include bradykinin, substance P, and peptide-YY (15–18), and APP has been implicated in the regulation of cardiovascular and pulmonary functions in vivo (19–21).

In higher plants, only very few peptides with hormone-like functions are presently known (22, 23), but a more general role for peptides as signal molecules in the regulation of plant defense, growth, and development is anticipated (24). Likewise, the proteases involved in the maturation and degradation of plant peptide hormones are still elusive. In the present work, we used a partial cDNA as a probe to isolate the cDNAs of two APPs from tomato. One of the enzymes (LeAPP2) was functionally expressed in E. coli, purified from bacterial extracts, and characterized biochemically. This is the first characterization of an APP from any plant source.

**EXPERIMENTAL PROCEDURES**

**Cloning of LeAPP2**—All basic molecular techniques were adapted from published protocols (25, 26). A cDNA library from tomato shoot tissue in JZAP (Stratagene, La Jolla, CA) was used (27) and 9.5 × 10⁶ plaque-forming units were screened on nitrocellulose membranes. A partial tomato cDNA (LeAPP1) with sequence similarity to human aminopeptidase P was used as a probe. Hybridization with the radiolabeled cDNA (Prime-It II system, Stratagene) was performed at 42 °C in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 2× Denhardt’s solution (1× Denhardt’s solution: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 50 mM potassium phosphate buffer (pH 7.0), and 200 μg/ml of denatured salmon sperm DNA. Filters were washed in 0.5× SSC, 0.5% SDS at 60 °C and were subsequently exposed to x-ray film (Kodak X-Omat AR) using an intensifying screen. Individual positive clones were identified in two consecutive rounds of screening. Recombinant pBluescript cDNA phagemids were excised in vivo using the

* This work was supported by Grant No. 31.5685.99 of the Swiss National Science Foundation (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) AJ308541 and AJ310676.

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1 The abbreviations used are: APP, aminopeptidase P; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time of flight/mass spectrometry; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

J. Strassner and A. Schaller, unpublished observations.
Characterization of Tomato Aminopeptidase P

ExAssist helper phage according to the recommended procedure (Stratagene). RACE-PCR was performed to obtain full-length cDNAs using the SMART RACE cDNA amplification kit (CLONTECH, Palo Alto, CA) according to the manufacturer's instructions. In a first step, single-stranded cDNA was synthesized from total RNA of tomato leaves using M-MLV reverse transcriptase (Promega, Madison, WI) and oligo(dT) as the primer. Subsequently, the full-length LeAPP1 and LeAPP2 cDNAs were amplified using gene-specific primers (5'-CTGGGAAAGCTCTGAAGAACCTTGTT-3' and 5'-AATGAGGACTTGCAAGCGTCG-3' (Microsynth, Balgach, Switzerland) and the universal primer provided with the kit. The PCR products were gel-purified and cloned into pCR-Script (Stratagene). The identity of all PCR-generated clones was confirmed by sequence analysis of at least three independent PCR products using fluorescent dideoxy chain terminators in the cycle sequencing reaction (PerkinElmer Life Sciences) and the Applied Biosystems model 373A DNA sequencer.

Northern and Southern Blot Analysis—RNA was isolated from different tissues of tomato plants using a phenol-based extraction procedure (26). Total RNA (5 μg) was separated on formaldehyde/agarose gels and transferred to nitrocellulose membranes according to standard protocols (26). For Southern blot analysis, genomic DNA was extracted from tomato leaf tissue using the Nucleon Phytopure Biotech. Ten μg of DNA was restricted using the enzymes indicated in the legend to Fig. 2 and were subjected to DNA electrophoresis using the radiolabeled 3´-untranslated region of the LeAPP cDNAs as probes. Hybridization, washing, and evaluation of blots were done as described (28).

Expression of LeAPP2—The open reading frame of LeAPP2 was amplified by PCR using Ffu Turbo DNA polymerase (Stratagene) and synthetic oligonucleotide primers (forward primer: 5´-ATGGCCGATA-CACCTGGAAC-3´; reverse primer: 5´-GGGTGACTTAAAGACCATC-GAACATCTGA-3´ (Microsynth, Balgach Switzerland)). The PCR product was cloned into the StuI/KpnI sites of pGEX-G (29) a derivative of pGEX-3x (Amersham Pharmacia Biotech), to yield pGEX-APP2. This construct allows expression of LeAPP2 in NT-terminal fusion with glutathione S-transferase (GST) in E. coli under control of the IPTG-inducible lac promoter. The expression construct was verified by sequence analysis and was transformed into E. coli BL21 codon plus (DE3)-RIL (Stratagene). Cultures (500 ml) were grown at 37 °C to an A600 of 1.0; IPTG was added to a final concentration of 1 mM, and cultures were grown for another 2 h at room temperature. The cells were harvested by centrifugation (2500 × g, 15 min, 4 °C) and stored at −80 °C.

Purification of Recombinant LeAPP2—E. coli cells were resuspended in 20 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 1 mM EDTA containing 0.1 mg/ml DNase I and 1 mg/ml lysozyme. After 20 min at room temperature, cells were lysed by sonication. The cell debris was removed by centrifugation (35000 × g, 15 min, 4 °C), and the supernatant was subjected to affinity chromatography on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech; 1-ml bed volume) equilibrated in buffer A. After extensive washing with buffer A and buffer B (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl), the fusion protein (GST-LeAPP2) was eluted with buffer B containing 10 mM reduced glutathione. The progress of protein purification was monitored by SDS-polyacrylamide gel electrophoresis performed on 10% polyacrylamide gels using the buffer system described by Laemmli (30). Gels were stained for protein detection using Coomassie Brilliant Blue R250.

Steady State Kinetic Analyses—For continuous assay of LeAPP2 activity, H-Lys(Abz)-Pro-Pro-[2H3]H-Leu (Amer sham) was used as a substrate (31). A 50 μl reaction mixture containing 1 μg of LeAPP2, 25 μl of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA), 0.1 mM Z-Fmoc-Phe-OMe and 50 μM H-Leu was incubated at 37 °C for 10 min. The hydrolysis products were separated by high performance liquid chromatography on a C18 reverse phase column and quantified by mass spectrometry.

RESULTS
Molecular Cloning of LeAPP1 and —A partial cDNA with sequence similarity to human APP was serendipitously isolated3 in a genetic screen for the identification of proteases involved in either the maturation or the degradation/inactivation of systemin an oligopeptide signal molecule involved in wound signal transduction (32). The screen had been devised for the identification of endopeptidases with known specificity (33). Hence, the isolated cDNA, which encodes an APP, i.e. an exopeptidase, likely represents a false positive. The sequence was completed at its 5´-end by RACE-PCR and termed LeAPP1 (GenBank™ accession number AF308541). Using the LeAPP1 cDNA as a probe, a tomato cDNA library was screened at moderate stringency resulting in the isolation of seven clones, four of which corresponded to LeAPP1 although three appeared to encode a second aminopeptidase, i.e. LeAPP2. As indicated by a sequence comparison with the LeAPP1 cDNA, all three clones were incomplete at their 5´-ends, and RACE-PCR was performed to obtain the full-length sequence (GenBank™ accession number AJ310676). The LeAPP2 cDNA encompasses an open reading frame of 1962 base pairs coding for a protein of 654 amino acids with a calculated Mr, 72,682. The deduced amino acid sequence was compared with those of LeAPP1 and the cytosolic and membrane-bound forms of human aminopeptidase P (82, 46, and 41% sequence identity, respectively; Fig. 1). All amino acid residues implicated in the binding of active site metal (Asp-451, Asp-462, His-525, Glu-561, Glu-574; Ref. 34) and two further histidines (His-432 and His-535, numbers refer to the LeAPP2 sequence) proposed to play a role during catalysis in proton shuttling from the dinuclear metal center to the solvent (35) are conserved between the four sequences. There are no obvious sequence elements for targeting to subcellular compartments, and therefore, LeAPP2 is likely to be a cytosolic enzyme. This conclusion is in agreement with the higher overall similarity of LeAPP2 with the cytosolic form of human APP as compared with the membrane-bound form, which contains N- and C-terminal signal sequences (12). Whereas the sequence similarity between the tomato and human enzymes is high in the N- and C-terminal regions, a stretch of low similarity extends from amino acids 237 to 393 of LeAPP2 where there are two insertions in the plant sequences (amino acids 237–252 and 373–392 of LeAPP2) lacking a counterpart in the human proteins.

The 3´-untranslated regions of the LeAPP1 and 2 cDNAs were used as probes on gel blots of tomato genomic DNA (Fig. 2, A and B). The hybridization pattern obtained with the probe derived from the LeAPP1 cDNA may indicate the existence of a third, closely related gene in the haploid tomato genome (Fig. 2A). The LeAPP2 probe, on the other hand, was found to be gene-specific and was thus suitable for the analysis of LeAPP2 expression on RNA gel blots (Fig. 2C). A single class of LeAPP2 transcripts was detected in all tissues analyzed, being more prevalent in tomato roots and cultured cells than in flowers, leaves, and cotyledons (Fig. 2C).

Expression, Purification, and Catalytic Activity of LeAPP2—The open reading frame of the LeAPP2 cDNA was cloned into the expression vector pGEX-G and expressed in E. coli in N-terminal fusion with GST. From 1 liter of E. coli culture, 4 mg of soluble GST-LeAPP2 were purified by affinity chroma-
tography (Fig. 3). The apparent molecular mass of the purified protein of 105 kDa is consistent with the mass expected for the GST (26 kDa)-Le APP2 (72.7 kDa) fusion protein. A few minor contaminants with estimated masses of 70, 43, and 35 kDa, respectively, co-purified during affinity chromatography and likely represent degradation products of GST/H18528 Le APP2 as indicated by N-terminal amino acid sequence analysis (data not shown). Efficient cleavage of the GST moiety of the fusion protein by factor Xa treatment proved to be impossible. Hence, the GST/H18528 Le APP2 fusion protein was used in all further studies. GST/H18528 Le APP2 could be stored at -20 °C or, in concentrated solution (0.5 mg/ml), at 4 °C for several weeks without a significant loss of activity.

The proteolytic activity of GST-LeAPP2 was assayed using an internally quenched, fluorogenic substrate (H-Lys(Azb)-Pro-Pro-pNA, (31)). The release of the N-terminal Lys(Azb) by aminopeptidase P activity was followed spectrofluorometrically. The reaction rate was a linear function of the protein concentration over the investigated range of 0–2 mg/ml. LeAPP2 activity was affected by addition of divalent cations (Fig. 4A). Increasing concentrations of MnCl2 and MgCl2 stimulated LeAPP2 activity with MnCl2 being most effective at a concentration of 4 mM. CaCl2 and CoCl2 stimulated LeAPP2 activity at 0.4 mM but were inhibitory at 4 mM. Complete inhibition of LeAPP2 activity was observed after addition of 4 mM ZnCl2 or CuSO4 (Fig. 4A). LeAPP2 activity was also inhibited by metal-chelating agents with 1,10-phenanthroline being much more effective than EDTA (half-maximal inhibition at 36 and 340 μM, respectively). Furthermore, treatment with the
Catalytic properties of GST-LeAPP2. Unless otherwise indicated, assays of LeAPP2 activity were performed in 0.1 M Tris-HCl, pH 7.5 in the presence of 4 mM MgCl2 using H-Lys(Azb)-Pro-Pro-pNA (50 μM) as the substrate. A, divalent metal ion dependence of GST-LeAPP2 activity. The activity of GST-LeAPP2 in the presence of increasing concentrations of MnCl2, MgCl2, CaCl2, CoCl2, ZnCl2, or CuSO4 was analyzed and is expressed as percent of the activity observed in the absence of added metal ions (100% = 26 pkat/ml). The relative error of individual data points was between 0.01 and 0.2%. B, GST-LeAPP2 activity as a function of pH. GST-LeAPP2 activity was assayed in 0.1 M Tris-HCl buffer in presence of 4 mM MnCl2. It is expressed in percent of the maximum activity observed at pH 7.5 (100% = 50 pkat/ml). C, substrate dependence of GST-LeAPP2 activity. GST-LeAPP2 (250 ng/ml) activity was assayed with increasing concentrations of H-Lys(Azb)-Pro-Pro-pNA, and apparent catalytic constants were derived from a double-reciprocal (Lineweaver-Burk) plot of the data. The values are the means ± S.D. of eight independent experiments.

A

B

C

**DISCUSSION**

APP, like X-Pro dipeptidase (prolidase) and methionyl aminopeptidases of types I and II, belong to the M24 family in the clan MG of metalloproteases (36, 37). Whereas the overall sequence similarity between these enzymes is rather low, their C-terminal catalytic domains share a common structural feature called the pita-bread-fold (38). The structures of *E. coli* methionyl aminopeptidase and APP have been solved and two metal ions were found to be “sandwiched” in the pita-bread domain. The metal ions are liganded by two Asp, one His, and two Glu residues, respectively, which are strictly conserved in this family of proteases (35, 39, 40). The requirement of these residues for the catalytic activity of porcine APP has been demonstrated by site-directed mutagenesis (34). We report here the cloning and characterization of a related enzyme from tomato called LeAPP2. This is the first characterization of an aminopeptidase P from any plant. LeAPP2 shares considerable sequence similarity with both *E. coli* and mammalian APPs in both the C-terminal pita-bread- as well as in the N-terminal domains. All the amino acid residues involved in metal binding (34, 35) as well as two histidine residues implicated in proton shuffling between the solvent and the dinuclear metal center (35) are conserved in LeAPP2 (Fig. 1).

In addition to the structural similarity, LeAPP2 shares functional characteristics with known APPs. LeAPP2 expressed and purified from *E. coli* as a GST fusion protein exhibited APP activity, releasing the N-terminal amino acid from peptides with a penultimate proline residue. It was found to process typical substrates of mammalian APPs, i.e. bradykinin and substance P. The catalytic properties as well as structural similarity indicate a closer relationship with the cysteine as compared with the membrane-bound forms of mammalian APPs. The pH optimum of 7.5 for LeAPP2 activity (Fig. 4B) is consistent with its localization in the cytoplasm. Similar to the cysteine APP from rat brain (7), LeAPP2 clearly preferred Arg-Pro-Pro- (bradykinin) over Arg-Pro-Lys- (substance P), which indicates an extended binding site for recognition of the P1 residue of the substrate as it was reported for *E. coli* and mammalian APPs (10, 15). Like the cysteine APPs from *E. coli*, *Rattus norvegicus*, and *Homo sapiens*, but unlike the membrane-bound enzymes from *R. norvegicus* and *Bos taurus*, the tomato enzyme tolerates a Lys residue in the P2 position.
LeAPP2 also hydrolyzed the N-terminal Pro-Pro-bond of processed bradykinin, albeit at a slower rate. Cleavage of the Pro-Pro-bond at the N terminus of oligopeptide substrates has also been reported for the cytosolic rat and E. coli APPs (7, 41). The rat cytosolic APP functionally expressed in E. coli, however, was found to be unable to hydrolyze the N-terminal Pro-Pro-bond of a synthetic oligopeptide substrate (13). Endopeptidase activity, i.e. the cleavage of the -Phe-Gly-bond in substance P, appears to be a unique feature of LeAPP2. The fact that protein preparations from E. coli cultures carrying the LeAPP2 activity are essentially the same as those observed for hcAPP (Fig. 4A) and, therefore, LeAPP2 is also likely to be a single Mn(II)-dependent enzyme.

There are conflicting reports in the literature with respect to the metal requirement of APPs. Until recently, supported by the crystal structures of E. coli methionyl aminopeptidase and APP, which revealed the presence of dinuclear metal centers in both enzymes (35, 39), two manganese (Mn(II)) or zink (Zn(II)) ions per subunit were considered necessary for maximum catalytic activity in cytosolic and membrane-bound APPs, respectively (Ref. 14, and references therein). For methionyl aminopeptidase, on the other hand, two equivalents of cobalt (Co(II)) were proposed to be required based on the reproducible observation of highest activity in vitro in the presence of Co(II). Both the nature and the amount of metal required in vivo have recently been questioned, however.

E. coli methionyl aminopeptidase was shown to be maximally activated upon addition of only one Fe(II) ion and iron is likely to be the in vivo ligand. Whereas the first Fe(II) ion is bound with high affinity ($K_f = 0.3 \mu M$), the $K_f$ of the second metal binding site was reported to be 2.5 mM, and therefore, this site is likely to be unoccupied in vivo (42, 43). Likewise, the two metal binding sites in human cytosolic APP (hcAPP) appear to differ in affinity. Upon expression in E. coli, this enzyme was found to contain only one equivalent of Mn(II), and this was sufficient to support proteolytic activity (14). The hydrolysis of bradykinin and substance P by hcAPP was stimulated 2.7-fold upon further addition of Mn$^{2+}$, whereas Mg$^{2+}$, Cu$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$ were found to be inhibitory (in order of increasing inhibition, Ref. 14). The effects of divalent metal ions on LeAPP2 activity are essentially the same as those observed for hcAPP (Fig. 4A) and, therefore, LeAPP2 is also likely to be a single Mn(II)-dependent enzyme.

The function of the second metal ion binding site remains obscure. Roles in the regulation of proteolytic activity or in positioning the substrate by binding its N-terminal amine group have been proposed (34, 43). A competition of substrate and metal ion for the same binding site may explain the earlier observation that the inhibitory and stimulating effects of cations on APP activity can be substrate-dependent (44, 45).

The enzymatic properties of LeAPP2 were further characterized using H-Lys(Abz)-Pro-Pro-pNA as the substrate for which an apparent $K_m$ of 15.2 ± 2.4 $\mu M$ and a catalytic efficiency ($V_{max}/K_{app}$) of 0.94 ± 0.11 mM$^{-1} \cdot s^{-1}$ were derived from steady-state kinetic analyses (Fig. 4C). These values are within the range of catalytic constants reported for other APPs (2, 7, 9, 10, 14, 15, 19). Likewise, the inhibitor profile of LeAPP2 is typical for APPs. LeAPP2 was found to be inhibited by chelating agents with 1,10-phenanthroline being much more effective than EDTA. Consistent with the essential role of histidine residues in binding of the active site metal and in catalysis, LeAPP2 was inactivated by a histidine-modifying reagent (diethylpyrocarbonate). Inhibition by 2-mercaptoethanol and N-ethylmaleimide may indicate a functionally important cysteine residue. There is, however, no cysteine residue conserved between the two tomato and human enzymes (Fig. 1). Alternatively, thiol reagents may compete with the substrate as a ligand of the active site metal. The inhibition by both metal
chelators and thiol reagents has been reported widely for other APPs (7, 8, 10, 14, 19).

The function of LeAPP2 in planta remains obscure as long as the in vivo substrate(s) are elusive. They will include oligopeptides with an amino-terminal Xaa-Pro motif. Such peptides may arise during protein degradation implying a function for LeAPP2 in protein turnover. Physiological substrates may also include plant peptide hormones implying a function for LeAPP2 in the regulation of hormone stability/activity. Considering the role of mammalian APPs in the degradation of bradykinin and substance P, it is tempting to speculate on such a role for mammalian APPs in the degradation of bradykinin and substance P, it is tempting to speculate on such a role for LeAPP2. However, only very few peptide hormone-like signal molecules are known in plants (22), and none of them contains an N-terminal Xaa-Pro motif. They are therefore not likely to be substrates of LeAPP2. Yet peptides are anticipated to play a much broader role in plant signal transduction than presently appreciated (24), and they may require APPs for the regulation of activity.

Acknowledgments—We thank Dr. Peter Macheraux (ETH Zürich) for help with the MALDI-TOF/MS experiments and D. Frasson for excellent technical assistance.

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J. Biol. Chem. 2001, 276:31732-31737.
doi: 10.1074/jbc.M103179200 originally published online June 22, 2001

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