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Functional analysis of a leucine aminopeptidase from *Solanum tuberosum* L.

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Abstract. A protein encoded by a potato cDNA homologous to a leucine aminopeptidase (LAP) from bovine lens (Hildmann et al. 1992) was expressed in *Escherichia coli* cells and biochemically characterized by hydrolysis of leucine *p*-nitroanilide. Activity was highest under alkaline conditions with an optimum at about pH 10. Maximal activities were measured at 65° C. Apart from leucine *p*-nitroanilide the enzyme could also efficiently hydrolyze the *p*-nitroanilides of arginine and methionine. Complete inhibition of the enzyme was achieved by incubating bacterial extracts with bestatin and EDTA, which classifies the enzyme as a metalloprotease belonging to the same group as the homohexameric LAPs from mammals. Protein blots showed low constitutive expression of the LAP in all organs of potato plants: buds, flowers, tubers, roots and leaves. An increase in steady-state protein that was parallelled by an increase in total LAP activity was observed in leaf extracts after supplying jasmonic acid via the petioles. Plants containing the cDNA in antisense orientation behind the constitutive Cauliflower Mosaic Virus 35S promoter showed nearly complete reduction of the corresponding mRNA in leaves. However, in these plants LAP activities were only decreased by about 20% as compared to non-transgenic potato plants, while after feeding with jasmonic acid the activity of transgenic plants was reduced to about 5% of that of non-transgenic plants also induced by jasmonic acid. There was no phenotypic difference between wild-type and LAP antisense plants.

Key words: Antisense inhibition – Jasmonic acid – Leucine aminopeptidase – *Solanum*

Introduction

Peptidases are involved in many different physiological processes in any living cell. Their functions include the general turnover of proteins, removal of damaged and abnormally folded proteins, and mobilization of storage proteins. More specific functions attributed to peptidases are processing events during targeting of proteins into organelles, the activation of zymogens and the production of specific peptide hormone signals. In general, it is thought that proteins destined to be degraded are first hydrolysed by endopeptidases into smaller peptides. These are then attacked by exopeptidases – further classified as amino- or carboxypeptidases depending on their site of action on the peptide – resulting in the liberation of free amino acids.

Biochemical characterization of plant aminopeptidases has identified two classes of enzymes. The first group comprises thermolabile aminopeptidases of molecular weight approx. 60–90 kDa and a neutral pH optimum. They are strongly inhibited by heavy metals and SH-reagents. Most of the aminopeptidases characterized so far, i.e. from pea, barley, maize and wheat belong to this group (Waters and Dalling 1984, and references therein). However, one enzyme purified from barley (Sopanen and Mikola 1975) has been described as being similar to the leucine aminopeptidases (LAPs) from animals (Carpenter et al. 1973), *E. coli* (Vogt 1970) and *Salmonella typhimurium* (Miller and Mackinnon 1974). These are large (250–330 kDa) homohexameric metalloproteases that are inhibited by EDTA and bestatin. They are further characterized to be very heat-stable and to possess an alkaline pH optimum.

So far, very little is known about the molecular biology of plant aminopeptidases. The first aminopeptidase cloned from a plant was obtained by immunoscreening with antibodies prepared against plasma-membrane proteins of *Arabidopsis thaliana* (Bartling and Weiler 1992). The amino-acid sequence derived from the isolated cDNA showed homology to the LAP purified from bovine lens (Cuypers et al. 1982) and to the *xer*B gene

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Abbreviations: CaMV = cauliflower mosaic virus; CathInh = cathepsin D inhibitor; JA = jasmonic acid; LAP = leucine aminopeptidase; Leu-NA = leucine *p*-nitroanilide

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from *E. coli* (Stirling et al. 1989). The *xer*B gene product was first identified to be essential for stability of the ColE1 plasmid and was shown to possess LAP activity. It is probably identical to the aminopeptidase I from *E. coli* characterized by Vogt (1970). Bartling and Weiler (1992) have partially characterized the aminopeptidase from *A. thaliana* by its overexpression in *E. coli* and have proven the functional similarity to the aminopeptidases from bovine lens and *E. coli*.

By screening for genes the RNA levels of which were induced by wounding, jasmonic acid (JA) and abscisic acid, Hildmann et al. (1992) isolated four cDNAs from potato plants. The amino-acid sequence derived from one of these cDNAs was homologous to the bovine lens LAP and the *E. coli xer*B gene product. In order to obtain further insight into the possible biological function of this aminopeptidase, we analyzed the kinetic parameters of the enzyme by means of overexpressing it in *E. coli*. Furthermore, its pattern of expression was investigated in plants at the levels of RNA, protein and activity. Finally, by creating transgenic potato plants with a largely reduced aminopeptidase activity, we attempted to elucidate its physiological function.

Materials and methods

Recombinant-DNA techniques and bacterial strains. Standard procedures were used for recombinant-DNA work (Sambrook et al. 1989). The DNA sequences were determined by the dideoxy method (Sanger et al. 1977). Plasmid sequencing was performed with T7 DNA polymerase (Pharmacia, Freiburg, Germany). Specific oligonucleotides for sequencing and for first-strand cDNA synthesis were made on a DNA synthesizer 380A (Applied Biosystems, Weiterstadt, Germany). *Escherichia coli* strain DH5α (ITC Biotechnology, Heidelberg, Germany) was cultivated and transformed using standard techniques (Sambrook et al. 1989). *Agrobacterium tumefaciens* strain C58C1 containing plasmid pGV2260 (Deblaere et al. 1985) was cultivated in YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$) and transformed as described by Höfgen and Willmitzer (1988). In order to express the LAP protein in *E. coli* the full-length LAP cDNA was subcloned into the *Bam*HI site of vector pET3a (Rosenberg et al. 1987) and the construct was transformed into strain B121DE3 (Studier and Moffatt 1986). For construction of the LAP antisense gene, a 1.5-kb *Bam*HI/*Hinc*II fragment from the cDNA encoding LAP (Hildmann et al. 1992) was cloned in inverted orientation behind the Cauliflower mosaic virus (CaMV) 35S promoter of plasmid pBinAR (Höfgen and Willmitzer 1990) between the *Sma*I and *Bam*HI restriction sites.

Isolating the 5′ end of the cDNA encoding LAP. An oligonucleotide hybridizing specifically 200 base pairs (bp) downstream of the 5′ end of the published sequence (Hildmann et al. 1992) was end-labelled by T4 polynucleotide kinase (Sambrook et al. 1989). Synthesis of cDNA was performed using the "You Prime" Kit (Pharmacia) according to the manufacturer's instructions. Oligonucleotides were separated from the cDNA by running a 5% denaturing polyacrylamide gel. The radioactively labelled cDNA was visualized by autoradiography for 20 min and the corresponding area was cut out from the gel. The cDNA was eluted by the "Crush and Soak" method (Sambrook et al. 1989), treated with DNA-polymerase Klenow fragment and cloned into the *Sma*I restriction site of the vector pGEM4 (Serva, Heidelberg, Germany). After transformation into *E. coli*, positive clones were identified by colony hybridization using the cDNA encoding the LAP as probe.

Bacterial extracts for measuring LAP activities. B121(DE3) cells transformed with the LAP cDNA in vector pET3a were grown in 250 ml YT medium (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 100 $\mu g \cdot ml^{-1}$ carbenicillin at 28° C. At an OD_{600} of 0.2–0.5, 1 mM *iso*propyl β-D-thiogalactopyranoside was added and the culture maintained under these conditions for 3–4 h. Cells were collected by centrifugation, the pellet resuspended in 10 ml lysis-buffer [50 mM Tris-HCl, 10% glycerol, 1 mM phenylmethyl-sulfonyl fluoride (PMSF); pH 7.5] and lysed using a French-Pressure Cell Press (SLM Aminco, Colora Messtechnik, Lorch, Germany). Homogenates were cleared by centrifugation at $12000 \cdot g$ for 15 min at 4° C and the supernatant was taken for activity measurements.

Plant extracts for measuring LAP activities. Frozen plant material was homogenized in extraction buffer (50 mM Tris-HCl, pH 7.5; 1 mM PMSF; 10% glycerol; 0.5 mM EDTA; 0.5 mM EGTA; 0.1% Triton X-100; 5 mM $MgCl_2$; 5 mM $MnCl_2$; 1 mM β-mercaptoethanol). The extract was centrifuged for 5 min at $15000 \cdot g$ at 4° C and the supernatant taken for measuring aminopeptidase activity and determining the protein concentration according to Bradford (1976).

Assay of aminopeptidase activity. Aminopeptidase activity was measured spectrophotometrically at 405 nm by the release of *p*-nitroaniline from the hydrolysis of L-amino acid *p*-nitroanilides ($\varepsilon_{405} = 9450\ M^{-1} \cdot cm^{-1}$). Unless otherwise stated, 20 µl of bacterial or plant extract was incubated with 50 mM Tris-HCl, (pH 8.5), 1 mM $MnCl_2$ in a final volume of 1 ml at 30° C. The reaction was started by adding 1 mM substrate (final concentration). L-leucine *p*-nitroanilide (Leu-NA) was obtained from Merck (Darmstadt, Germany), all other *p*-nitroanilides from Sigma (Deisenhofen, Germany).

Plant material. Solanum tuberosum L. cv Désirée was obtained from Saatzucht Fritz Lange KG (Bad Schwartau, Germany). Plants in tissue culture were maintained at 22° C under a 16 h light–8 h dark regime (irradiance 450 µmol photons $\cdot m^{-2} \cdot s^{-1}$), 50% relative humidity, on MS medium (Murashige and Skoog 1962) containing 2% sucrose. Plants in the greenhouse were cultivated in soil under a light-dark regime of 16 h light (22° C) and 8 h (15° C) dark (irradiance 300 µmol photons $\cdot m^{-2} \cdot s^{-1}$) at 60% relative humidity.

Potato transformation. Solanum tuberosum L. cv. Désirée was transformed by Agrobacterium-mediated gene transfer using *A. tumefaciens* strain C58C1:pGV2260 as described by Rocha-Sosa et al. (1989).

Treatment with jasmonic acid (JA). Petiole feeding experiments were performed as described (Hildmann et al. 1992). Plants used were of the eight-leaf stage.

Extraction of RNA and Northern blot experiments. Total RNA was extracted from frozen plant material (Logemann et al. 1987). Polyadenylated RNA was isolated using oligo(dT) cellulose type 7 (Pharmacia) according to Sambrook et al. (1989). Ribonucleic acid was denatured in 40% formamide, subjected to agarose-gel electrophoresis (1.5% agarose, 15% formaldehyde) and blotted onto nylon membranes (Hybond N; Amersham Buchler, Braunschweig, Germany). Membranes were hybridized in a buffer containing polyethylene glycol and 40% formamide (Amasino 1986). Radioactive labelling of DNA probes was performed using a multiprime labelling kit (Amersham). Filters were washed three times for 10 min at 60° C with 0.5 × SSC/0.1% SDS (1 × SSC = 0.15 M NaCl, 0.015 M Na-citrate). *Bam*HI inserts of plasmids No. 4 and No. 17, encoding cathepsin D inhibitor (CathInh) and LAP from potato (Hildmann et al. 1992), were used for labelling reactions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Proteins were extracted from frozen tissue in Laemmli buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 10% β-mercapto-

ethanol, pH 6.8) and separated on 10% SDS-polyacrylamide gels (Laemmli 1970). For Western-blot analysis, proteins were transferred onto nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany; cellulose nitrate filter BA85, 0.45 µm) using a semi-dry electroblotting apparatus (Multiphor II; LKB-Pharmacia, Bromma, Sweden). Immunodetection was performed using a commercial biotin-streptavidin-alkaline phosphatase system (Amersham) according to the manufacturer's instructions.

Production of polyclonal antisera against the LAP protein. For high-level expression, B121(DE3) cells transformed with pET3a containing the LAP cDNA were grown at 37° C and induced to express the protein by treatment with 2 mM *iso*propyl β-D-thiogalactopyranoside for 5 h. Under these conditions the LAP accumulated in inclusion bodies which were centrifuged down at 12000·*g*, after having lysed the cells as described above. The inclusion bodies were solubilized at 65° C in Laemmli buffer (see above) and run on a preparative 10% polyacrylamide gel. After electrophoretical separation of the proteins the gel was incubated in 250 mM KCl for 10 min. Protein bands appeared opaque, and the LAP band was cut out. The protein was electroeluted from the gel using an electroeluting chamber (Bio-Rad, Munich, Germany) and concentrated using Centricon 30 filtration units (Amicon, Denver, Colo., USA). Rabbits were immunised and boosted several times subcutaneously with 50–150 µg protein each time. Leucine aminopeptidase protein was coupled to CNBr-activated Sepharose (Pharmacia) and the antiserum affinity-purified according to the manufacturer's instructions. Antiserum was diluted 1:200 in TBST (20 mM Tris, 500 mM NaCl, pH 8.0, 0.1% Tween-20, 1% bovine serum albumin).

Results

Isolating the complete cDNA for LAP. As the cDNA encoding the putative LAP was not completely full-length (Hildmann et al. 1992) the 5′ end to this specific transcript was isolated from a selective cDNA library specifically designed to match this end (see *Materials and methods*). The longest clone (272 bp) was sequenced and, within the 200 bp overlap, showed identity between the newly isolated and the published cDNAs. The two cDNAs were fused, resulting in a cDNA of 1928 bp. This cDNA contains an open reading frame with a codon for methionine at nucleotide position 17 and a termination codon at position 1736. This reading frame would code for a protein with 573 amino acids and a theoretical molecular weight of 60.5 kDa. The ATG codon at nucleotide position 17 and the sequence elements surrounding it are in good agreement with Kozak's rule (Kozak 1984) for translational start sites.

Amino acid sequence analysis of the potato LAP. We compared the deduced amino acid sequences of the LAPs from *Arabidopsis thaliana* and potato (Fig. 1). A high similarity exists from the central part (beginning with amino acid 290) of the protein and extending to the C-terminus: 76% of the amino acids of the two sequences are identical. The N-terminus is more variable: only about 60% of the amino acids are shared. The

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S.t. MATLRVSSLLASSPSSLHCNPSVPTLCESSPRWAPSPSVTPLCSRRSKRIVHCIAGDTLGLTRPNES   67
A.                                                              MAH-----Q--ST   13

S.t. DAPKISIGAKDTDVVQWQGDLLAIGATENDLARDDNSKFKNPLLQRLDSKLNGLLSAASSEEDFSGK  134
A.   EPH---FT--EI--IE-K--I-VV-V--K---K-G----E--I-SKV-SH-S---AQV------T--   80
b.l.                       TDGLVLGIYSKEKEEDEPQFTSAGENFNKLVS-K-REILNISGPPL-   47

S.t. SGQSINLR.LPG.G..RITLVGLG.SSASSPTSYHSL.GEAAAAAAKSAQARNIAVSLASTDGLSAE  195
A.   P---TV--.---L-SK--A-I---.Q-V---VAF---.---V-TVS-AS-STSA-IV---..SV-D-  142
b.l. A-KTRTFYG-HEDFPSV.VV----KKT-GIDEQENWHE-KENIR--VA-GCRQ-QDLEIPSVEVDPC  113

S.t. SKINSASAIATGVMLGIFEDNRFRSESKTPALESLDILGLGTGPEI...ESKIKYAEHVCAGVILGR  259
A.   --LS-V--L-S-IV--L---G-YK----K-S-KAV--I-F---A-V...-K-L----D-SY---F--  206
b.l. GDAQA-AEG-VLGLYE.YD-LK.........QKR.KVVVSAKLHGSEDQEAWQRGVLFAS-QN-A-  166

S.t. ELVNAPANIVTPGALAEEAKKIASTYSDVITVNILDAEQCKELKMGAYLGVAAAATENPPYFIHLCF  326
A.   --I-S---VL--AV-----A-V-------F-A---NE---------S--A----SA.---H----VY  272
b.l. R-MET---EM--TKF--IVEENLKSA-IKTD-F-RPKSWIE-QE--SF-S-.-KGS-E--V-LEIHY  234

                                  *           *  *
S.t. KT.NSRERKTKIALVGKGLTFDSGGYNLKTGAGSKIELMKNDMGGAAAVLGAAKALGEIKPRGVEVH  392
A.   -P.PNGSV---L---------------I---P-CS-----F----S---------I-----P-----  338
b.l. -GSP-AS-.PPLVF----I------ISI-AA-N..MD--RA------TICS-IVSAAKLDLP.INIV  297

                                        *  *                          *  *
S.t. FIVAACENMISGAGMRPGDIVTASNGKTIEVNNTDAEGRLTLADALIYACNQVVDKIIDLATLTGAI  459
A.   ------------T------VI-----------------------V-----G-D--V--------C  405
b.l. GLAPL----P--KANK---V-R-R-----Q-D--------I-----C--HTFNPKV--NA------M  364

                                                          *
S.t. VTALGPSVAGAFTPSDGLAREVVVAAEASGEKLWRMPMEESYWES.MKSGVADMINTGP.RDGGAIT  524
A.   -I---T-M--IY----E--K--IA-S-R---------L------M.--------V---G.-A--S--  470
b.l. DI---SGAT-V--N-.SWMNKLFE-SIET-DRV----L--H-TRQVIDCQL--VN-I-KY-SA--C-  431

                              *  *
S.t. GALFLKQFVDEKVQWLHLDIAGPVW.SDEKK.NATGY.GVSTLVEWVLRNSL                  573
A.   A--------S-----M-I-M-----N..---KSG--F.--A------QK--S                  519
b.l. A-A---E--.THPK-A------VMTNK--VPYLRK-MA-RP-RFS..QD.-A                  478
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Fig. 1. Amino acid sequence derived from the complete LAP cDNA of potato (denoted *S.t.*) aligned with the LAP sequence of *Arabidopsis* (denoted *A.*) derived from cDNA PM25 (Bartling and Weiler 1992) and with the amino acid sequence of the bovine lens protein (denoted b.l.; Cuypers et al. 1982). Amino acids of the enzymes from *Arabidopsis* and bovine lens identical to the potato enzyme are indicated by *horizontal lines*. Gaps marked by *dots* were introduced for optimal alignment. Amino acids that bind bestatin in the bovine lens protein (Burley et al. 1991) are shown by *asterisks*

same tendency for both LAP sequences was observed when they were independently compared to the *Xer*B and bovine lens aminopeptidase sequences (Bartling and Weiler 1992; Hildmann et al. 1992). The potato sequence exceeds that of *A. thaliana* by 54 amino acids at the N-terminus. This sequence displays the main features of transit peptides of proteins destined for the mitochondrial matrix and the chloroplastic stroma (Keegstra et al. 1989; von Heijne et al. 1989). The extension contains a high percentage of serine residues (24%), with only 9.8% serine content in the mature protein. There is only one glutamate and no aspartate present as negatively charged amino acids. The sequence starts with the dipeptide methionine-alanine, which has been found in nearly all chloroplastic transit peptides analyzed so far. Further features reminiscent of transit peptides are the absence of any tyrosine residue, the presence of only a few proline, glycine and charged residues within the first ten amino acids and an overall positive charge of the putative transit peptide.

Expression of LAP activity in E. coli. In order to prove aminopeptidase activity for the protein encoded by the LAP cDNA in bacterial cells, the full-length LAP cDNA was cloned into the expression vector pET3a and transformed into *E. coli* strain B121(DE3). Under moderately inducing conditions, which did not allow the formation of inclusion bodies (see *Materials and methods*), a strong LAP activity could be measured in the bacterial extracts when Leu-NA was used as substrate. Unless otherwise stated, measurements were performed in 50 mM Tris-HCl, 1 mM $MnCl_2$, in a final volume of 1 ml at 30° C in a thermostatted cuvette. All experiments were done in triplicate and repeated with three independent extracts. As the absolute values for activities (but not the relative values) are dependent on the induction status of the different extracts, representative results are presented without giving standard deviations. All data presented on the activity of LAP have been corrected for the activity values obtained with the control extracts from B121(DE3) containing the vector pET3a without an insert. Bacterial extracts which had been boiled for 10 min did not evolve measurable *p*-nitroaniline in any experiment.

The dependence of the activity on different concentrations of Leu-NA was determined (Fig. 2a). The K_m value for Leu-NA was found to be about 0.38 mM under the conditions stated above. As at 1 mM the substrate concentration was saturating, this concentration was used in all further experiments. The hydrolysis rates for Leu-NA were determined under different pH conditions in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) buffer considering the molar extinction coefficients of the *p*-nitroanilide at the different pH values. Measurement of Leu-NA hydrolysis between pH 8.0 and pH 11.5 (final pH in assay) resulted in a bell-shaped curve with maximal activity of the protein at about pH 10.3 (Fig. 2b). The influence of temperature on LAP activity was also examined. After having checked that high temperatures did not result in activity loss, all extracts were incubated at 75° C for 10 min in order to

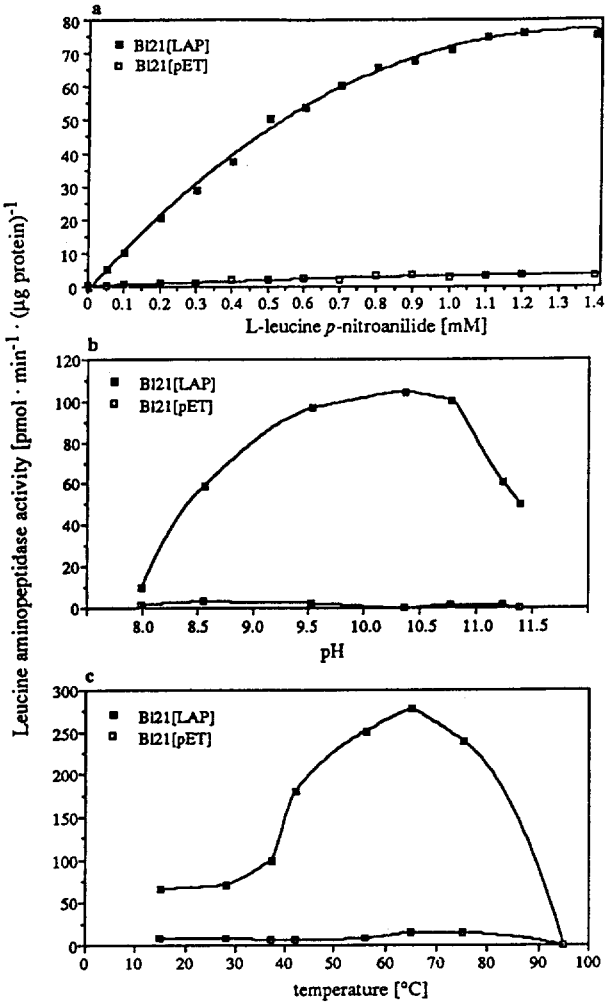

Fig. 2a–c. Properties of LAP from potato expressed in *E. coli*. **a** Dependence of LAP activity on the concentration of the substrate, L-leucine *p*-nitroanilide. Final substrate concentrations given: 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2 and 1.4 mM. Measurements were performed in 50 mM Tris-HCl (pH 8.5), 1 mM $MnCl_2$ at 30° C. **b** Dependence of LAP activity on pH. Measurements were performed in 50 mM Caps buffer at 30° C. **c** Temperature dependence of LAP activity. Measurements were performed in 50 mM Tris-HCl (pH 8.5), 1 mM $MnCl_2$ at the temperature indicated. B121[pET], activity of *E. coli* strain B121 (DE3) harboring plasmid pET3a. B121[LAP], activity of B121(DE3) transformed with plasmid pET3a that contains the LAP cDNA, corrected for the reference values of B121(DE3)

inactivate potential temperature-dependent effectors. Thereafter, bacterial extracts were pre-incubated for 10 min at the respective reaction temperature and the reaction was started by adding substrate. As shown in Fig. 2c the enzyme was already active at 15° C but activity increased as the temperature was raised. Optimal hydrolysis was measured at about 65° C where activity was 4-fold that at 28° C.

Various putative effectors of the LAP protein were investigated (Table 1). The extracts were pre-incubated with the respective effector at room temperature for 30 min. Activities were compared with those of extracts also kept at room temperature but pre-incubated in 50 mM Tris-HCl (pH 8.5) without additives. The basal

LAP activity could be enhanced about 1.8-fold by the addition of 1 mM $MnCl_2$; $MgCl_2$ was also slightly stimulatory whereas 1 mM Zn^{2+} inhibited the enzyme nearly completely. The serine-protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), at a concentration of 1 mM, resulted in a decreased LAP activity (0.74-fold). This might be explained by the rather high percentage of serine residues (9.8%) of the LAP, which after covalent modification could affect the overall structure and thereby the function of the protein. Pepstatin (0.1 mM), an aspartate-protease inhibitor, had no effect on enzymatic activity while 1 mM EDTA as an inhibitor of metalloproteases inhibited the activity completely. Bestatin, [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine hydroxide, which specifically binds to metalloproteases of the LAP type from bovine lens (Burley et al. 1991) also abolished the activity of this LAP. No significant changes of activity were observed after incubating extracts with 1 mM β-mercaptoethanol, 0.1% Triton X-100 or 0.1% Tween-20 (data not shown).

The substrate specificity of the LAP was studied using commercially available amino-acid *p*-nitroanilides other than Leu-NA, each at 1 mM final concentration, and hydrolysis was compared with that of Leu-NA (Table 2). Both Arg-NA and Met-NA were more efficiently hydrolyzed than Leu-NA (about 4.6-fold and 1.2-fold, respectively) while Glu-NA and Ala-NA were not accepted as substrate. Very low specific activities were measured with Val-NA, Pro-NA, Lys-NA and Phe-NA.

Table 1. Influence of various effectors on LAP activity. The LAP activity was measured after pre-incubating bacterial extracts for 30 min at room temperature with or without (controls) the effectors. Activities (±SD) are expressed as a percentage of that of control extracts

Treatment	Activity (%)
none	100
0.1 mM bestatin	0
1 mM EDTA	0
1 mM PMSF	74±4.5
0.1 mM pepstatin	100±2.0
1 mM $ZnCl_2$	1.8±1.3
1 mM $MnCl_2$	180±53
1 mM $MgCl_2$	115±5.5

Table 2. Substrate specificity of LAP from bacterial extracts. The hydrolysis of different amino-acid *p*-nitroanilides (1 mM final concentration) was measured and compared with that of leucine *p*-nitroanilide (taken as 100%)

Substrate	Activity (%)
Leu-NA	100
Arg-NA	463.6±160
Met-NA	122.8±5.3
Val-NA	6.5±0.9
Pro-NA	4.0±0.7
Lys-NA	4.0±1.4
Phe-NA	1.0±0.9
Ala-NA	0
Glu-NA	0

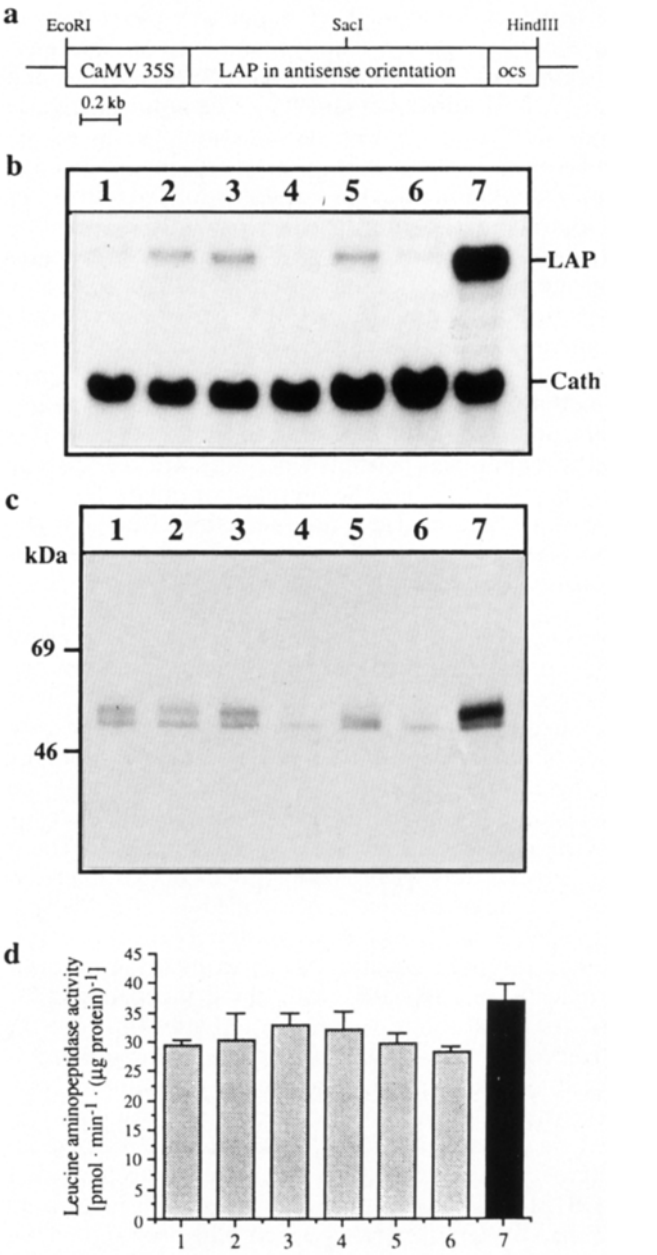

Fig. 3a–d. Structure of the chimeric antisense gene and analysis of transgenic potato plants with reduced expression of LAP. Extracts from leaf discs of the different transgenic plants No. 7 (*1*), No. 11 (*2*), No. 35 (*3*), No. 40 (*4*), No. 44 (*5*), No. 46 (*6*) and of control plants (*7*) were analyzed after floating on JA. **a** Structure of the chimeric LAP antisense gene used for transformation of potato plants. **b** Analysis of LAP mRNA expression in leaf discs of transgenic and control plants after incubation in 50 µM JA for 25 h. Total RNA (15 µg per lane) was hybridized to multiprime-labelled LAP- and CathInh-specific probes. **c** Immunoblot analysis of LAP protein expression in leaves of transgenic and control plants; 4 µg of total protein was loaded per lane. Each extract is a mixture prepared from five transgenic plants of each particular genotype, or from eight control plants. **d** Analysis of LAP activity in leaves of transgenic and control plants. The same plants as analyzed by immunoblot in **c** were taken for activity measurements. The activity values are the calculated means±SD of eight control plants or five transgenic plants of each genotype

Construction of a chimeric "antisense" LAP gene. In order to obtain further insight into the physiological role of the LAP we decided to down-regulate LAP activity in transgenic potato plants via an antisense-RNA approach. To this end a 1.5-kb cDNA coding for the potato LAP was inserted in reverse orientation between the CaMV 35S promoter and the terminator of the octopine-synthase gene in pBinAR (Höfgen and Willmitzer 1990). This construct (Fig. 3a) was used to transform potato plants via *Agrobacterium tumefaciens* (Rocha-Sosa et al. 1989).

Screening for transgenic potato plants having reduced amounts of LAP RNA in their leaf tissue. In plant leaf tissue which has neither been wounded nor treated with abscisic acid or JA LAP mRNA can hardly be detected when 30 µg total RNA is analyzed in Northern blots (Hildmann et al. 1992). For this reason, plants were screened for reduced accumulation of LAP mRNA after treatment with JA. Leaf discs of plants randomly selected from tissue culture were floated on 50 µM JA for 25 h in the dark; total RNA was isolated from this tissue and analyzed in RNA blots. To exclude the possibility that the explant did not respond to the JA treatment in general, the blots were simultaneously probed with a CathInh cDNA. The CathInh mRNA, like LAP mRNA, only accumulates in leaf tissue after treatment with abscisic acid or JA or wounding (Hildmann et al. 1992). From 50 transgenic plants analyzed in this way, four plants (Nos. 7, 11, 35, 44) had significantly reduced amounts of LAP mRNA and, in the case of two plants (Nos. 40 and 46) no LAP mRNA could be detected at all (Fig. 3b). In all plants, CathInh mRNA was present, thus proving that the JA treatment had stimulated expression of other genes not down-regulated by the LAP antisense gene (Fig. 3b). The transgenic plants showing reduced accumulation of LAP mRNA after treatment with JA were transferred to soil and grown under standardized greenhouse conditions. There were no visible differences between transgenic plants and non-transgenic control plants.

Analysis of the expression of LAP protein in leaf extracts of potato plants containing reduced amounts of LAP mRNA. In order to determine whether or not the reduction in LAP mRNA was reflected at the protein level, an affinity-purified rabbit polyclonal antiserum raised against the isolated LAP overexpressed in *E. coli* was used in protein-blot experiments. Because of the very low levels of LAP mRNA in leaves of non-induced potato plants we were surprised to find an appreciable amount of immunodetectable protein in leaf extracts when only 4 µg total protein was loaded (Fig. 3c, lane 7). The size of the immunoreacting protein was about 54 kDa. In most immunoblots the antibodies identified two polypeptides in leaf extracts. As the separation into the two immunoreactive bands was reproducible in most, but not all, gels we cannot be completely sure that the recognized polypeptides represent isoforms of the LAP protein. Leaf samples were taken from the transgenic (Nos. 7, 11, 35, 40, 44, 46) and non-transformed plants grown under greenhouse conditions and subjected to Western blot analysis. Levels of immunologically detectable LAP protein were lower in the extracts of all transformants than in leaf extracts from non-transgenic plants (Fig. 3c). However, a strong reduction was only observed for the two transgenic plants (Nos. 40, 46) which were devoid of LAP mRNA (Fig. 3b). In these plants the two immunoreactive bands were affected to different degrees, which favours the idea of two closely related LAP isoforms.

Analysis of total LAP activity in potato plants containing reduced amounts of LAP protein in leaves. The total LAP activity of non-transgenic plants and of those transgenic plants that expressed less immunoreactive LAP protein than wild-type plants was determined by measuring the hydrolysis of Leu-NA caused by potato leaf extracts. Specific LAP activities of the transgenic plants were between 28.4 ± 0.9 and 33.0 ± 2.0 pmol·min^{-1}·(µg protein)$^{-1}$ while those of control plants were 37.0 ± 2.8 pmol·min^{-1}·(µg protein)$^{-1}$ (Fig. 3d; Table 3, first column). This means that the total LAP activity in the transgenic plants was reduced to only a minor degree, with residual activities ranging between 77% and 89% of wild-type activity (Table 3, second column). Comparing the hydrolysis rates of Leu-NA (Fig. 3d) with the signal strength of the LAP protein (Fig. 3c) in the respective transgenic plants gives the impression that enzymatic activities are largely independent of the amount of LAP protein present in the extract.

Leucine aminopeptidase mRNA and LAP activity increase in wild-type potato plants after treatment with JA for 72 h and are efficiently suppressed by the LAP antisense gene in transgenic plants during this period. A massive accumulation of LAP mRNA is observed in leaf extracts after treating potato plants with JA for 24 h either by spraying, floating or by providing JA through petioles (Hildmann et al. 1992), which should result in an increase in LAP activity. To test this assumption, detached leaves of non-transgenic and transgenic potato plants were fed 50 µM JA via their petioles under greenhouse

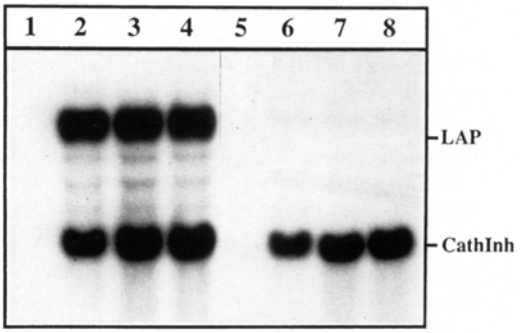

Fig. 4. Analysis of LAP mRNA expression in leaf extracts from transgenic potato plant No. 40 (*lanes 5–8*) and control plants (*lanes 1–4*) before (*1, 5*) and after feeding 50 µM JA via the petiole for 25 h (*2, 6*), 48 h (*3, 7*) and 72 h (*4, 8*). Total RNA (15 µg per lane) was hybridized to multiprime-labelled LAP- and CathInh-specific probes. The LAP transcript size was approx. 2.0 kb, the CathInh transcript 0.9 kb

Table 3. Leucine aminopeptidase activities in extracts of leaves from control and transgenic potato plants prior to and after feeding with 50 μM JA via their petioles for 25, 48 and 72 h under greenhouse conditions. Values are means of eight control plants or five transgenic plants of each genotype. Absolute activities (±SD) are expressed in pmol·min^{-1}·(μg protein)$^{-1}$. Relative activities of the transgenic plants are referred to control plants taken as 100%

Plant	Incubation in 50 μM JA for							
	0 h		25 h		48 h		72 h	
	LAP activity		LAP activity		LAP activity		LAP activity	
	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
Control	37.0±2.8	100%	58.0±12	100%	84.0±22	100%	188±42	100%
7	29.4±0.9	79%	21.8±3.7	38%	15.2±1.0	18%	15.0±1.7	8%
11	30.3±4.7	82%	28.6±2.1	49%	20.3±3.8	24%	20.5±6.5	11%
35	33.0±2.0	89%	35.0±7.2	62%	29.0±1.6	34%	39.0±3.1	21%
40	32.0±3.3	87%	21.0±3.4	36%	11.0±2.6	13%	9.2±1.4	5%
44	29.8±1.6	82%	28.6±4.4	49%	18.6±2.3	22%	22.9±7.3	12%
46	28.4±0.8	77%	18.6±1.9	32%	16.1±2.5	19%	10.6±2.4	6%

conditions. Samples were taken from the leaf blades before hormonal treatment and after 25, 48 and 72 h incubation in JA.

Total RNA was extracted from control plants and transgenic plant No. 40, which shows the highest degree of inhibition of the LAP mRNA, and analyzed in Northern blots (Fig. 4). As expected, no LAP or CathInh mRNAs could be detected before treatment of plants with JA. In wild-type plants the levels of LAP mRNA had reached their maximum after 25 h and did not change with longer incubation times. Irrespective of the induction time the transgenic plant No. 40 did not accumulate LAP mRNA. Hybridization kinetics with the CathInh cDNA as probe showed no difference between transgenic and non-transgenic plants. This experiment proves that the inhibition by the LAP antisense gene is efficiently maintained at the RNA level irrespective of the induction status of the plant.

The specific activity of LAP in leaf extracts of wild-type potato plants increased in response to JA treatment, though the extent and time course did not reflect the corresponding mRNA accumulation. After 25 h (48 h, 72 h) of incubation the activity had risen by a factor of 1.6 (2.3, 5.1; see Table 3, upper row, for mean specific activities in wild-type plants). Measurements of LAP in the transgenic plants corroborated the results obtained from the Northern blot analyses, i.e. LAP activity was not enhanced in extracts of leaves which had been fed jasmonic acid via their petioles (Table 3), and relative to the wild-type plants there was a decline in activity at the different time points investigated (Table 3). The activity was maximally reduced to 5% of wild-type activity (transgenic plant No. 40). The same data were obtained when specific activities were related to leaf area instead of protein (data not shown) because, within the experimental period of 72 h, the protein content of the leaves was comparable between transgenic and non-transgenic plants. Except for plant No. 35, LAP activities in leaf extracts of the transgenic plants decreased with time of incubation with JA (Table 3). After 72 h, activities had declined to 50% (plant No. 7), 68% (No. 11), 29% (No. 40), 77% (No. 44) and 37% (No. 46) of their levels before treatment.

Kinetics of LAP protein expression and LAP activity in wild-type plants and in transgenic plant No. 40 during treatment with JA for 192 h. The petiolar-feeding experiment described above was pursued for a period of 192 h in order to investigate LAP activity in increasingly senescent leaves. Leaves of wild-type and transgenic plants fed with JA via their petioles started to turn yellow at the edges after 3 d of incubation. The yellow areas enlarged during the course of the experiment and leaves were completely yellow after 9 d. The phenotype of control leaves supplied with water for 9 d was comparable to that of leaves supplied with JA for 3 d. There was no phenotypic difference between non-transgenic and transgenic plants. Leaf samples taken for analyses originated from the greenest, i.e. innermost, parts of the leaf blade near the mid rib. Figure 5 shows time courses of LAP activity [pmol·min^{-1}·(cm^2 leaf area)$^{-1}$ in wild-type plants and transgenic plant No. 40 after incubation in water or JA, respectively, for 192 h. After 144 h treatment with JA, LAP activity in wild-type leaves reached a maximum, surmounting the initial level by a factor of 13 (activity in water-treated leaves: 2.6). Leaves of transgenic plant No. 40 maintained repressed levels of LAP activity irrespective of treatment with water or JA.

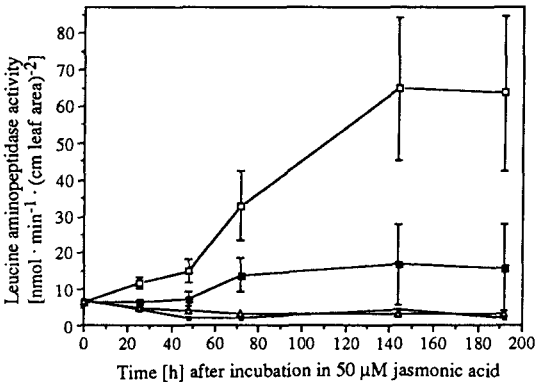

Fig. 5. Activity of LAP in leaf extracts from transgenic potato plant No. 40 and control plants fed 50 μM JA or H_2O via their petioles for a period of 192 h. Detached leaves and petioles from five independent transgenic and eight independent control plants were incubated under greenhouse conditions. Samples for LAP measurements were taken prior to and after incubation for periods of 25, 48, 72, 144 and 192 h. Mean specific activities (±SD) are expressed as pmol·min^{-1}·(cm^2 leaf area)$^{-1}$. *Curves* are labelled as follows: -□- control in JA, -■- plant No. 40 in JA, -▲- control in water, -△- plant No. 40 in water

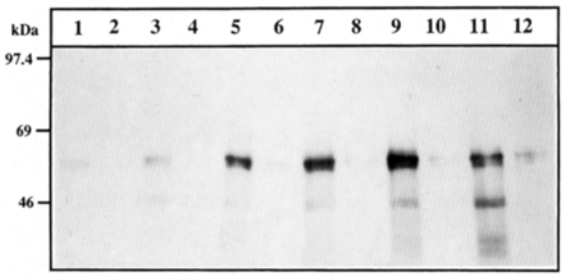

Fig. 6. Immunoblot analysis of LAP protein expression in leaves of transgenic potato plant No. 40 (*lanes 2, 4, 6, 8, 10, 12*) and control plants (*lanes 1, 3, 5, 7, 9, 11*) before (*1, 2*) and after feeding 50 µM JA via their petioles for 25 h (*3, 4*), 48 h (*5, 6*), 72 h (*7, 8*), 144 h (*9, 10*) and 192 h (*11, 12*). A 400-ng sample of total protein was loaded per lane

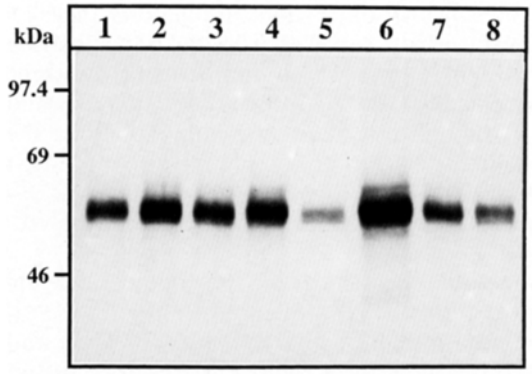

Fig. 7. Immunoblot analysis of LAP-protein expression in buds and flowers of potato plants during different stages of flower development. A 10-µg sample of total protein from the following extracts was loaded per lane: green buds (*1*), white buds (*2*), coloured buds (*3*), open flowers (*4*); sepals (*5*), petals (*6*), anthers (*7*) and ovaries (*8*) of open flowers

This could indicate that the enhanced level of Leu-NA hydrolysis by extracts of control leaves incubated in water is also caused by the LAP under study.

To determine whether the augmented Leu-NA hydrolysis rates were due to elevated steady-state levels of LAP protein, leaf extracts of wild-type plants were analyzed before and after incubation in JA for 25 h, 48 h, 72 h, 144 h and 192 h. The immunoblot (Fig. 6) indicates a correlation between measurable LAP activity and amount of protein present. Extracts of transgenic plant No. 40, examined in parallel by immunoanalysis, contained hardly any detectable levels of LAP protein throughout the duration of the experiment.

Expression of the LAP protein in different organs of wild-type potato plants. Hildmann et al. (1992) detected that steady-state levels of LAP mRNA accumulated constitutively in green flower buds and to very low levels in tubers as well. No LAP mRNA was present in open flowers and roots. To study expression of LAP protein in the reproductive organ of potato plants we prepared protein extracts from buds of different developmental stages (green, white, coloured) as well as from open flowers that were additionally dissected into sepals, petals, anthers and ovaries. Figure 7 shows that roughly equal

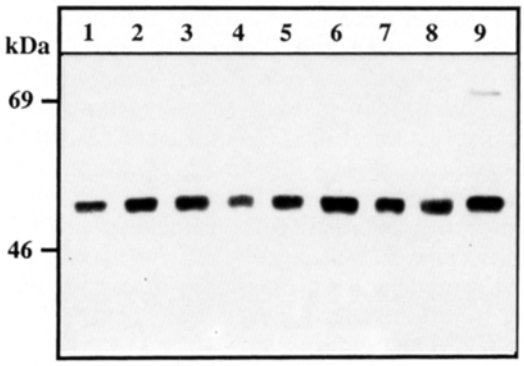

Fig. 8. Immunoblot analysis of LAP-protein expression in tubers during different stages of development, and in tuber-derived sprouts and roots. A 20-µg sample of the following protein extracts was loaded per lane: "Sink" tubers (*1–3*); tubers which had been stored at 4° C for six weeks (*4*), three months (*5*), five months (*6*) and six months (*7*); sprouts (*8*) and roots (*9*)

amounts of immunoreactive LAP protein were present during the different stages of flower development investigated. The highest relative amount of LAP protein had accumulated in petals of open flowers.

Protein extracts from sink tubers and tubers that had been stored for six weeks to 6 months (sprouting) at 4° C, and extracts from tuber-derived sprouts and roots contained approximately the same amount of LAP protein (Fig. 8). It seems that in tubers and roots the amount of LAP protein as a proportion of total protein is half as much as in buds and flowers: 20 µg of protein had to be loaded in order to get comparable signal strengths. Only one immunoreactive polypeptide could ever be detected in tuber extracts. When measuring LAP activity in tuber extracts no differences between sink, storage and source tubers were observed.

Discussion

Enzymatic characterization of the LAP cDNA from potato in E. coli

The LAP cDNA from potato was expressed in *E. coli* and biochemically characterized by its ability to hydrolyse amino-acid *p*-nitroanilides. Because of its sequence homology to the LAPs from *Arabidopsis thaliana* (Bartling and Weiler 1992) and bovine lens (Cuypers et al. 1982) our studies were primarily concentrated on the hydrolysis of Leu-NA. The enzyme from potato hydrolyzes Leu-NA efficiently upon expression in bacteria, the K_m value being 380 µM under the conditions used. This is comparable to the value of 500 µM obtained for the *Arabidopsis* enzyme (Bartling and Weiler 1992). The LAP from bovine lens has a lower affinity for Leu-NA; the K_m value was determined to be 2 mM (Hanson and Frohne 1976).

The LAP from potato is active over a broad alkaline pH range with an optimum at about pH 10.3. Such a high pH optimum has also been found for the aminopeptidase I from *E. coli* (Vogt 1970), which is probably

identical to the protein encoded by the *xer*B locus (Stirling et al. 1989). Bartling and Weiler (1992) obtained maximal activity for the enzyme from *Arabidopsis* at a pH of 7.5. Thus far, in plants, only the potato and barley aminopeptidases (Sopanen and Mikola 1975) have been found to display these highly alkaline pH optima of activity, which might function in regulating the degradation of peptides by compartmentation. Proteases with an acidic pH optimum of activity have only been isolated from vacuoles while those possessing neutral and alkaline optima have never been identified in this organelle (Vodkin and Scandalios 1979, Waters et al. 1982; and references therein).

Another outstanding feature of the LAP from potato is its heat-stability. Activity increases with rising temperature and reaches its maximum at about 65° C. The same thermal stability has been observed for the LAPs from *Arabidopsis* (Bartling and Weiler 1992) and bovine lens (Hanson and Frohne 1976). The proteases isolated from barley (Sopanen and Mikola 1975) and *E. coli* (Vogt 1970) were not analyzed for their heat-stability. It remains to be determined whether this characteristic of the enzyme is of physiological relevance to the plant.

The inhibition of the enzyme by EDTA and bestatin proved that the cloned LAP is a metalloprotease and functionally similar to the LAP from bovine lens (Cuypers et al. 1982; Burley et al. 1991). Upon incubation with $ZnCl_2$ the enzymatic activity was severly reduced while $MgCl_2$ and $MnCl_2$ enhanced its activity. The LAP from bovine lens is a homohexameric enzyme with the ability of each subunit to bind two Zn^{2+} ions. One Zn^{2+} ion is firmly co-ordinated while the second ion is easily exchangeable. It seems that one Zn^{2+} is essential for catalytic reaction (Hanson and Frohne 1976; Burley et al. 1991). When in excess, Zn^{2+} ions could occupy the "easily exchangeable" site, thereby displacing other metal ions important for the catalytic reaction. As Mg^{2+} and Mn^{2+} enhance hydrolysis rates in case of the potato as well as the *Arabidopsis* enzyme (Bartling and Weiler 1992), these ions are possible candidates to occupy this "easily exchangeable" site and might be involved in the reaction.

The LAP from potato had a higher specific activity for Arg-NA and Met-NA than for Leu-NA; Ala-NA and Glu-NA were not accepted as substrates while Val-NA, Pro-NA, Lys-NA and Phe-NA were poorly hydrolysed. These data cannot easily be explained as there is no obvious specificity based on the properties of the respective amino acids. A direct comparison with the other characterized aminopeptidases is not possible because different substrates were analyzed. Vogt (1970) and Sopanen and Mikola (1975) showed that a few di- and tripeptides from *E. coli* and barley, respectively, were best hydrolyzed if leucine or methionine were at the N-terminus. Amides, anilides and β-naphtylamides of leucine and other aliphatic amino acids of bovine lens were cleaved best by LAP in the order of Leu > Phe > Val > Ala > Gly (Hanson and Frohne 1976). Burley et al. (1991), after having resolved the three-dimensional structure of the bovine lens LAP with its inhibitor, bestatin, suggested a model for substrate binding and enzyme-catalyzed hydrolysis and concluded that only amino acids of the C-terminus of the enzyme are involved in catalysis. The C-terminal region is 47% homologous between the enzymes from bovine lens and potato (Hildmann et al. 1992). The amino acids which form hydrophobic pockets in the active site of bovine lens LAP, thereby stabilising the binding of the D-phenylalanine (Met-270, Thr-359, Ala-451 and Met-454) and L-leucyl (Asn-330, Ala-333 and Ile-421) side chains of bestatin, could correspond to Met-364, Thr-454, Ala-545, Val-548 and Asn-425, Ala-428 and Thr-515, respectively, in the potato enzyme (Fig. 1). Other amino acids of the bovine lens LAP forming hydrogen bonds to the bestatin backbone (Lys-262, Asp-273, Gly-362) seem to be completely conserved in the protein from potato (Lys-354, Asp-367 and Gly-457). All of the residues mentioned are identical in the enzymes from potato and *Arabidopsis* (Fig. 1). Assuming bestatin can serve as a model for substrate binding, the highly conserved amino acids of the three enzymes indicate that their substrate specificity should be similar. Although not likely, we cannot exclude the possibility that the bacterially expressed enzyme might develop artificial affinities for substrates due to additional amino acids at its N-terminus expressed from the vector.

The amino-acid sequence of the potato enzyme aligned with the only other sequence of an aminopeptidase cloned from a plant, i.e. *Arabidopsis* (Bartling and Weiler 1992), revealed 76% identity of residues within the central and C-terminal part and 60% identity within the first 290 amino acids (Fig. 1). This high degree of similarity is largely reflected by the enzymatic parameters of the two proteins, with the exception of dependence on pH. Whereas Bartling and Weiler (1992) did not identify typical signal or transit peptides in the polypeptide from *Arabidopsis*, an N-terminal sequence that resembles chloroplastid and mitochondrial transit peptides (Keegstra et al. 1989; von Heijne et al. 1989) is present in the LAP from potato. Remarkably, this sequence of 54 amino acids is exactly extending the polypeptide from *Arabidopsis*, arguing for different subcellular localizations of the proteins. This notion is further supported by the difference in the calculated (60.5 kDa) and experimentally determined (about 54 kDa) molecular weight of the potato enzyme. The observed differences in pH dependence of the LAPs from potato and *Arabidopsis* might play a physiological role due to the putative different compartmentation of the two enzymes.

Leucine aminopeptidase activity in leaf extracts of transgenic potato plants is hardly affected by introducing an antisense LAP gene. The introduction into potato plants of a chimeric gene containing the coding region of the potato LAP in a reversed orientation with respect to the CaMV 35S promoter resulted in a strong reduction of the expression of the corresponding sense RNA in leaf tissue after treatment with JA. However, even in those plants where the mRNA was reduced below the detection limit, immunodetectable LAP protein was still present. In the transgenic plants the measurable LAP

activity of leaf extracts was only reduced to about 80% of that of wild-type plants. The transgenic plants differed only slightly in their residual activities and these appeared to be independent of the respective LAP protein levels. These data point to the possibility that Leu-NA-hydrolysing activities other than that of the LAP under study contribute to the total measurable activity in potato leaves. Alternatively, the activity of the LAP protein might be up-regulated by some mechanism to guarantee a certain degree of LAP activity. The presence of several aminopeptidases in leaf extracts seems to be plausible as several have been identified by zymograms in extracts of seeds from pea (Elleman 1974) and barley (Kolehmainen and Mikola 1971; Sopanen and Mikola 1975), of embryos from maize (Vodkin and Scandalios 1980) and of primary leaves from wheat (Waters and Dalling 1984). After incubation in JA the total LAP activities decreased in the antisense plants and had declined by a factor of 1.3–2 after 72 h. The immunoblot shows that this decline cannot be accounted for by decreasing LAP protein levels but might be indicative of other (different) LAP activities present in the leaves. These other putative hydrolases could be subject to enhanced turnover rates and/or repressed expression after exposure of the plant to JA, thereby inducing an increased substitution for the LAP under study.

The transgenic plants were not phenotypically different from non-transgenic plants, either macroscopically or at the cellular level as shown by examination of semithin sections of chemically fixed potato leaves (data not shown). Also, transgenic potato plants overexpressing the LAP mRNA constitutively from the CaMV 35S promoter in leaf extracts did not differ in phenotype from non-transgenic plants (data not shown). With regard to wild-type plants, total LAP activity was increased by a factor of 1.2–1.5 in the different overexpressing plants (data not shown). It seems that the LAP activity is regulated in such a way that the introduced antisense and overexpressing genes do not disturb the total activity present in the cell.

A role for LAP in senescence? In potato plants grown under non-stressful optimal circumstances, LAP activity might play a subordinate role in metabolic processes. An argument supporting this hypothesis is the impressive potential of potato leaves to raise their total LAP activity by a factor of 13 when treated with jasmonic acid (JA) for 6 d. Immunoblots displaying increasing amounts of steady-state levels of the immunodetectable LAP protein suggest that the increasing amount of protein is responsible for the rise in activity. These petiole-feeding experiments might indicate a possible role of LAP during senescence. Petioles are confronted with a missing "sink" for photosynthetic assimilates and probably suffer from mineral deficiency. Both situations may cause senescence in plants (Thomas and Stoddart 1980, and references therein). We found that incubating petioles in water was sufficient to increase LAP activity by a factor of 2.6. Incubating petioles in JA results in considerably higher levels of activity, possibly because of higher endogenous concentrations of this hormone. The biochemical molecule(s) acting as inducer(s) of senescence is (are) not known. However, JA has been observed to induce senescence phenomena in a number of systems (Sembdner and Parthier 1993, and references therein). Thus, aminopeptidases might be involved in senescence induced by JA as the process of senescence is also accompanied by degradation of proteins.

Other putative functions of LAP? Apart from the LAP protein accumulating in leaves after treatment with JA, it was constitutively present in all of the analyzed organs of non-treated potato plants. The signals obtained in immunoblots using 4 μg total leaf protein were approximately comparable to 10 μg total protein from buds/flowers and 20 μg total protein from tubers, sprouts and roots. The LAP protein was expressed to the same degree early in flower development, i.e. in still-green buds, and at later stages, i.e. in white and coloured buds, and open flowers. Dissection into the different parts revealed the strongest expression in petals of open flowers. In tubers the LAP protein invariably accumulated during the "sink", storage and "source" stages of development, and there was no change in overall LAP activity. Thus, we can exclude the possibility that this aminopeptidase has a specialised function in releasing amino acids from storage proteins for development of the sprout.

The aminopeptidase could be involved in the activation or inactivation of peptide hormones like systemin that are important for the signal transduction pathway leading to the expression of defense-related proteins (Pearce et al. 1991). However, the levels and timely appearance of the likewise wound-inducible CathInh mRNA (Hildmann et al. 1992) was not altered in the LAP antisense or LAP-overproducing plants (data not shown). Since JA is also suggested as a mediator in the defence responses of plants against pathogens (Sembdner and Parthier 1993, and references therein) we investigated whether *Phytophtora infestans*, a fungal pathogen of potato plants, might induce the LAP and CathInh mRNAs. No such induction could be observed over a period of 72 h, neither in the compatible nor in the incompatible interaction of the plant with the fungal spores (applied at 2×10^6 spores per ml) (Caroline Hahn, Günter Schrittmatter, Max-Planck Institute für Züchtungsforschung, Cologne, Germany, data not shown). Furthermore, no LAP and CathInh mRNAs were expressed after leaves of potato plants were infiltrated with *Xanthomonas campestris* pv. *vesicatoria* (1×10^7/ml), a plant pathogenic bacterium that causes a non-host resistance response in potato (data not shown). These data show that the JA-inducible LAP is not involved in the defence of potato plants against at least some fungal and bacterial pathogens.

Thus, concerning the function of the LAP, its ubiquitous presence indicates a general role in protein turnover. The data obtained with the antisense plants indicate that the aminopeptidase may be easily substituted by other aminopeptidases present in the potato plant. During certain physiological processes, such as senescence, the LAP assumes a more prominent role as the strong rise in total LAP activity can be ascribed to LAP protein.

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