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Purification and characterization of an aminopeptidase from the chloroplast stroma of barley leaves by chromatographic and electrophoretic methods

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Abstract

Aminopeptidases catalyze the cleavage of amino acids from the amino terminus of protein or peptide substrates. Although some aminopeptidase activities have been found in plant chloroplasts, the identity of these proteins remains unclear. In this work, we report the purification to apparent homogeneity of a soluble aminopeptidase from isolated barley chloroplasts which preferentially degraded alanyl-*p*-nitroanilide (Ala-pNA). After organelle isolation in a density gradient and precipitation of soluble proteins with ammonium sulfate, the proteins were purified in three consecutive steps including hydrophobic interaction, gel permeation and ion-exchange chromatographies. The purified enzyme appeared as a single band with a M_r of ~84 000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. The M_r of the native enzyme was estimated to be ~93 000 by gel permeation chromatography, suggesting that the protein is a monomer. Mass spectrometry analysis of tryptic digests indicates that the primary structure of the protein has not been reported previously. The enzyme was characterized as a metalloprotease as it could be totally inhibited by 1,10-phenanthroline. Strong inhibition could also be observed using the specific aminopeptidase inhibitors amastatin and bestatin. Besides Ala-pNA, the purified protein could also cleave with decreasing activity glycyl-pNA, leucyl-pNA, lysyl-pNA, methionyl-pNA and arginyl-pNA. The possible physiological role of this enzyme in the chloroplast stroma is discussed.

Keywords: Purification; Chloroplast stroma; Enzymes; Aminopeptidase

1. Introduction

Aminopeptidases catalyze the cleavage of amino acids from the amino terminus of protein or peptide substrates. They play an essential role in intracellular maturation and turnover of proteins and in the regulation of other fundamental biological processes. There is substantial information about structure, mechanism of action, biological function and regulation of gene expression of several aminopeptidases in prokaryotes and mammals (for a review, see [1]). In contrast, little is known about aminopeptidases in plants and particularly in the chloroplast.

Several reports in the 1970s and 1980s demonstrated the occurrence of aminopeptidases in plant tissues [2–6]. A number of proteins with amino-

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peptidase activity were also purified to homogeneity from whole extracts and partially characterized [7–9]. In addition, cDNAs homologous to leucyl-aminopeptidases of bacteria and animals were isolated from cDNA libraries of *Arabidopsis taliana* [10] and potato [11]. However, the subcellular localization of these proteins remains unclear.

Using isolated plastids as starting material, some aminopeptidase activities have also been detected and partially purified [12,13]. Nevertheless, the purification protocols used in these works yielded non-homogeneous preparations which did not permit the direct identification of the protein involved in substrate hydrolysis. As an exception, El Amrani et al. [14] purified an aminopeptidase from etioplasts of sugar beet cotyledons using a five-step protocol and obtained a homogeneous protein preparation as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

In this study, an aminopeptidase was detected in chloroplasts of barley leaves and purified to homogeneity using a protocol with three chromatographic steps. The aminopeptidase was identified as a novel monomeric protein and characterized concerning to structural and enzymic properties.

2. Materials and methods

2.1. Plant material, chloroplast isolation and stroma extract preparation

Barley (*Hordeum vulgare* L. cv Angora) seedlings sown in vermiculite, were grown for 8 days under a 12 h photoperiod at a light intensity of 100 W m^{-2}, at 24°C. The primary leaves were cut, placed at 4°C for 1 h and then ground with a blender in a medium consisting of 50 m*M* Tris–HCl pH 8.0, 330 m*M* sorbitol, 10 m*M* $MgCl_2$, 1 m*M* 1,4-dithioerytrit (DTE), 1 m*M* EDTA and 1% bovine serum albumin (BSA). The resulting homogenate was filtered through Miracloth (70 μm pores) and then centrifuged for 1 min at 2000 *g*. The pellets were washed with the same buffer without BSA and centrifuged again as described above. Intact chloroplasts were isolated by centrifugation on a density gradient as previously described [15]. The washed chloroplast pellets were subjected to hypo-osmotic shock by incubation in aqua dest. on ice for 10 min. Afterwards, the medium was adjusted to a final concentration of 50 m*M* Tris–HCl pH 8.0, 10 m*M* $MgCl_2$ and 1 m*M* ATP. The lysate was then centrifuged for 10 min at 48 226 *g* to yield a crude stromal extract in the supernatant.

2.2. Assay of peptidase activity

The standard assay mixture (0.2 ml) contained 100 m*M* Tris–HCl pH 7.6, 1 m*M* substrate and 20 μl of enzyme preparation. The mixture was incubated at 37°C for 30 min and the reaction stopped by addition of 800 μl 1% SDS solution. Activity was indicated by the release of free nitroanilide (pNA) or β-naphtylamide (*βNA*) which was measured photometrically (410 nm) or fluorometrically (excitation: 380 nm, emission 410 nm), respectively. The amount of pNA or *βNA* released was calculated using standard calibration curves for the pure compounds. Glycyl-pNA (Gly-pNA), leucyl-pNA (Leu-pNA), lysyl-pNA (Lys-pNA), metionyl-pNA (Met-pNA), arginyl-pNA (Arg-pNA), prolyl-pNA (Pro-pNA), alanyl-*βNA* (Ala-*βNA*), leucyl-*βNA* (Leu-*βNA*), phenylalanyl-*βNA* (Phe-*βNA*), tryptophanyl-*βNA* (Trp-*βNA*) and tyrosyl-*βNA* (Tyr-*βNA*) were provided by Sigma (Deissenhofen, Germany) and alanyl-pNA (Ala-pNA), phenylalanyl-pNA (Phe-pNA), valyl-pNA (Val-pNA) and cysteyl-pNA (Cys-pNA) by Bachem (Heidelberg, Germany).

2.3. Enzyme purification

Solid ammonium sulfate was added to the crude stromal extract to reach a saturation of 60%. After stirring for 30 min at 8°C, the preparation was centrifuged for 15 min at 48 226 *g*. The supernatant was discarded and the protein pellet was stored up to use at −24°C. Afterwards, the pellet was resuspended in buffer B consisting of 50 m*M* Tris–HCl pH 8.0 and 0.8 *M* ammonium sulfate and centrifuged for 15 min at 48 226 *g*. The resulting supernatant was loaded onto a column (2.6×20 cm) packed with Phenyl-Sepharose High Performance previously equilibrated with buffer B. Proteins were eluted with 200 ml of a linearly decreasing salt gradient (800–0

m*M* ammonium sulfate in 50 m*M* Tris–HCl pH 8.0). Using a flow rate of 2 ml/min, 8 ml fractions were collected and tested for aminopeptidase activity. The active fractions were pooled and concentrated with Centricon-30 concentrators (Amicon, Beverly, MA, USA) down to 2 ml. The protein sample was loaded onto a column (1.6×100 cm) filled with Superdex 75 and pre-equilibrated with a buffer containing 50 m*M* Tris–HCl pH 8.2 and 140 m*M* NaCl. The proteins were eluted with the same buffer at a flow rate of 0.3 ml/min, collected in 2 ml fractions and tested for peptidase activity. Active fractions were diluted with 50 m*M* Tris–HCl pH 8.2 to obtain a final salt concentration below 70 m*M* NaCl and loaded onto a prepacked MonoQ HR 5/5 column equilibrated with 50 m*M* Tris–HCl pH 8.2. Proteins were eluted with 40 ml of a linearly increasing salt gradient (0–150 m*M* NaCl in 50 m*M* Tris–HCl pH 8.2) and the column finally washed with 1 *M* NaCl. Fractions of 2 ml were collected at a flow rate of 1 ml/min and assayed for peptidase activity. All chromatographic separations were carried out at 4°C and performed on a Pharmacia LCC-500 liquid chromatography system (FPLC). All chromatography columns and media were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Aminopeptidase activity was measured using Ala-pNA as substrate under standard conditions.

2.4. Protein determination and SDS–PAGE

Total protein content was determined by the method of Bradford [16] using BSA as standard. SDS–PAGE analysis of proteins was carried out in a Mighty Small II electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA), essentially as previously described [15]. A protein sample of 22–25 μl in electrophoresis buffer were loaded per lane of 8% polyacrylamide gel. The proteins were electrophoresed at 8°C for 90 min using 20 mA per gel at a maximal voltage of 200 V. Afterwards, proteins were fixed by incubation of gels in 10% (v/v) acetic acid and 40% (v/v) ethanol for 30 min and visualised by using a Silver Stain Plus Kit™ (Bio Rad, München, Germany) according to the manufacturer's instructions. Proteins to be used for tryptic digestion were alternatively stained with Coomassie Brilliant Blue R-250 (Roth, Karlsruhe, Germany).

2.5. Molecular size estimation

The size of the native protein was estimated by chromatographing the purified protein on a column (1.6×80 cm) filled with Superdex 200 (Amersham Pharmacia Biotech, Freiburg, Germany) using ferritin (M_r=440 000), catalase (M_r=232 000) aldolase (M_r=150 000), BSA (M_r=67 000) and chymotrypsin (M_r=25 000) as standard (Amersham Pharmacia Biotech, Freiburg, Germany). The column was pre-equilibrated and eluted with 50 m*M* Tris–HCl pH 8 and 150 m*M* NaCl.

2.6. Tryptic in-gel digestion and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI–TOF-MS)

After SDS–PAGE, 10 protein bands (~10 μg protein) from the Coomassie Blue-stained gel were excised and washed twice in 1 ml of 100 m*M* NH_4HCO_3. The solvent was discarded, and the gel pieces were washed in 500 μl of 50% (v/v) CH_3CN and 100 m*M* NH_4HCO_3 for 1 h, dehydrated in 100 μl CH_3CN for 10 min, dried and reswelled in 50 μl 100 m*M* NH_4HCO_3 containing 1 μg trypsin (Roche, Mannheim, Germany) for 10 min. After addition of 150 μl 100 m*M* NH_4HCO_3, the digestion was carried out at 37°C overnight. The supernatant was saved and the peptides were extracted from the gel slices twice with 200 μl of 50% (v/v) CH_3CN and 0.1% (v/v) trifluoroacetic acid (TFA) for 20 min. The supernatant and extracts were combined and concentrated under vacuum. The lyophilized digests were dissolved in 10% aqueous formic acid, loaded on a ZipTip™ (Millipore, Eschborn, Germany), washed with 0.1% (v/v) TFA and eluted in one step with 50% (v/v) CH_3CN. Typically, samples were prepared for MALDI–TOF-MS by mixing 0.5 μl sample and 0.5 μl matrix solution (10 mg 2,5-dihydroxybenzoic acid in 1 ml water) on the target. Post source decay (PSD) spectra were recorded on a home built reflectron mass spectrometer with delayed extraction technique using a final accelerating voltage of 20 kV and a 337 nm laser [17].

2.7. Characterization of enzymic activity

After ion-exchange chromatography, the purified enzyme was desalted on a Sephadex G-25M column (Amersham Pharmacia Biotech, Freiburg, Germany) using buffer 20 m*M* Tris–HCl pH 7.6. The pH dependence of the enzyme was established using buffer 100 m*M* Tris–HCl in a pH range between 6.8 and 9.0. For inhibition experiments, the enzyme was pre-incubated for 2 h with protease inhibitor at room temperature, and then assayed for aminopeptidase activity using Ala-pNA as substrate. Amastatin and bestatin were added together with the substrate, and the release of pNA was monitored continuously. Other effectors were mixed with the reaction medium, and the activity was measured under standard conditions. EDTA, 1,10-phenanthroline, phenylmethylsulfonylfluoride (PMSF), isovaleryl-valinyl-valinyl-statyl-alanyl-statine (Pepstatin A) and iodacetamide were provided by Sigma (Deissenhofen, Germany), Pefablock™ by Roche (Mannheim, Germany), and L-*trans*-epoxysuccinyl-leucyl-agmatine (E-64), [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-valinyl-valinyl-aspartate (amastatin) and [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine (bestatin) by Bachem (Heidelberg, Germany).

3. Results

3.1. Enzyme purification

An enzyme hydrolyzing Ala-pNA was detected in the soluble fraction of isolated chloroplasts from barley leaves and purified to homogeneity using a four-step purification protocol. After ammonium sulfate precipitation, proteins were separated by hydrophobic interaction chromatography on a phenyl-sepharose column (Fig. 1A). The aminopeptidase activity eluted typically at $(NH_4)_2SO_4$ concentrations below 0.25 *M*. When this purification step was carried out at pH 7.5 or 8.5 or using NaCl instead of $(NH_4)_2SO_4$ no significant changes in specific activity were obtained (data not shown). Afterwards, proteins were concentrated in Centricon-30 devices without loss of activity and separated by gel permeation chromatography in a Superdex 75 column (Fig. 1B). The activity was eluted from the column as a single peak. This purification step not only yielded an important increase in specific activity (Table 1), but also served to change the medium (50 m*M* Tris–HCl pH 8.2 and 140 m*M* NaCl) for ion-exchange chromatography. The fractions containing aminopeptidase activity were pooled and NaCl concentration was adjusted to 70 m*M* before application onto a Mono-Q column. Proteins bound to the Mono-Q column were eluted with a NaCl gradient (Fig. 1C). The desorption of the aminopeptidase occurred at 125 m*M* NaCl. Using this procedure, more than one third of the initial activity was typically recovered (Table 1).

To identify the protein responsible for enzymic activity, each fraction obtained after chromatography was analyzed by SDS–PAGE. A band with a Mr of ~84 000 showed the same elution profile when compared with the aminopeptidase activity (data not shown). As shown in Fig. 2, this protein was specifically enriched during purification and appeared in the most purified fraction (lane 4) as a single band. Another fainter band at a M_r of ~70 000 was observed in some experiments after purification on Mono-Q column, but could be removed when a second ion-exchange chromatography was included at the end of the purification procedure (data not shown).

3.2. Structural properties

The M_r of the native protein was estimated to be 93 000 by gel permeation chromatography on Superdex 200. Since this mass agrees very well with that determined in SDS–PAGE analysis, it was concluded that the protein has a monomeric structure.

In a first approach to obtain the amino-terminal sequence, the purified protein was transferred onto polyvinylidenfruoride (PVDF) membranes and subjected to Edman degradation. However, no sequence was detected, probably because of N-terminal blockage. Therefore, the denatured protein was digested in-gel with trypsin and the masses of the resulting peptides analysed by MALDI–TOF-MS. After subtraction of peaks from self-digested trypsin, the masses of 39 peptides were entered in the PeptideSearch programm of EMBL (see www.embl-heidelberg.de / services / PeptideSearch

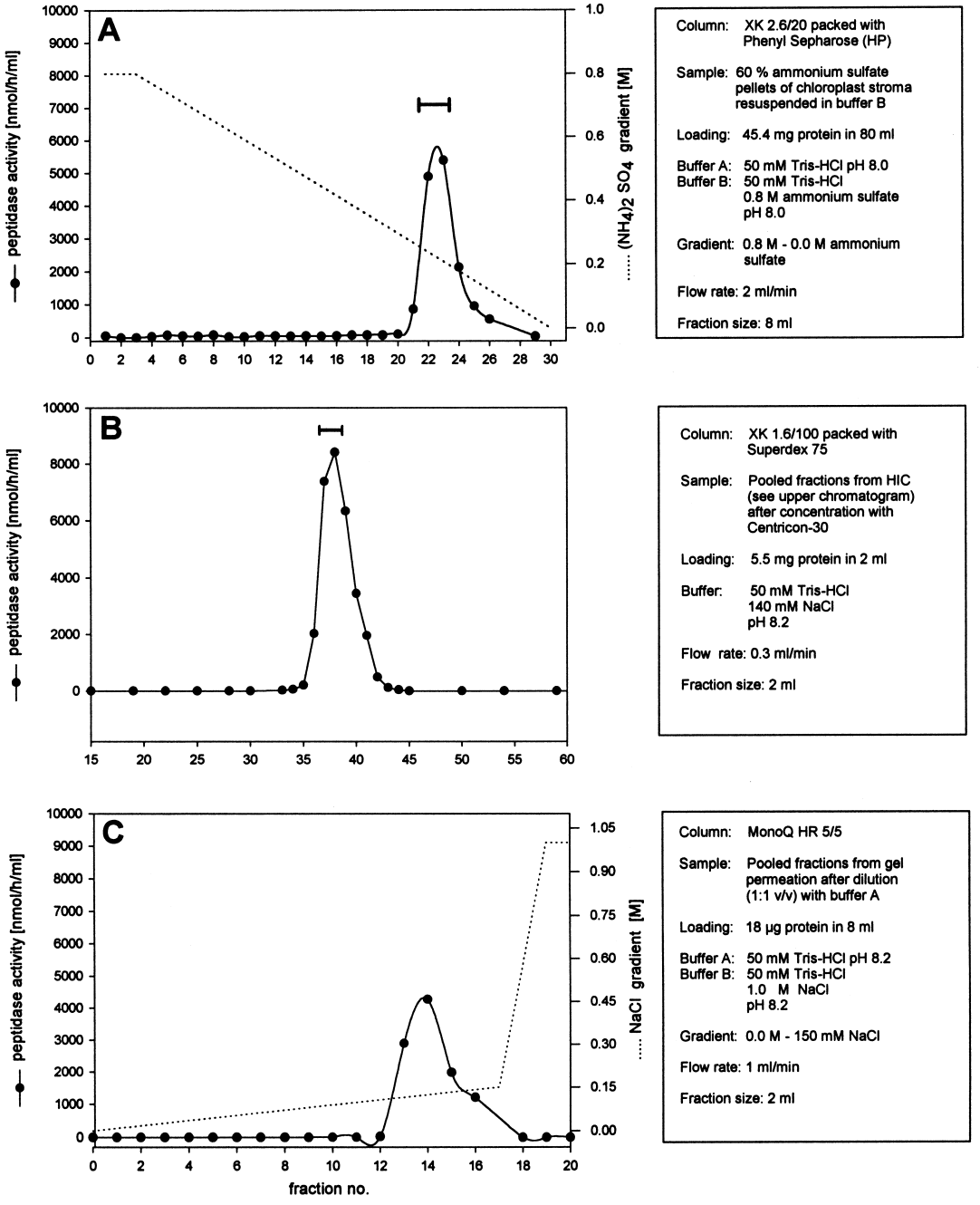

Fig. 1. Elution profile of aminopeptidase activity from hydrophobic interaction chromatography (A), Gel permeation chromatography (B) and ion-exchange chromatography (C). The pooled fractions are indicated with bars.

/FR-PeptideSearchForm.html). Searching the database a number of matches were obtained. However, the best of these matches involved the recognition of only 7 peptides, which represent a very low covering of the total sequence. Therefore, these matches were considered as not significant. In addition, the amino

Table 1
Purification of peptidase activity against Ala-pNA

Purification step	Activity[a] (nmol/h/ml)	Volume (ml)	Total activity (nmol/h)	Total protein[b] (mg)	Specific activity (nmol/h/μg prot.)	Recovery (%)	Purification (-fold)
Stroma	272.73	160	43636	57.5	0.759	100	1
AS precipitation	430.5	80	34440	45.4	0.758	78.9	1
HIC	2572.5	16	41160	5.55	7.416	94.32	9.77
Gel permeation	7898.7	3.8	30015.3	0.018	1667.5	72.4	236.7
Ion exchange	2490.6	6	14943.3	n.d.	–	34.2	–

[a] Peptidase activity was measured using Ala-pNA as substrate.
[b] Protein concentration was determined by the Bradford method. In the last purification step, the protein concentration was below the detection limit.

acid sequences of two selected peptides were obtained. Comparison of these two sequences (5 and 7 residues) with sequences registered in several databanks (including EMBL and SwissProt) did not give any significant matches. In combination, these results indicate that the primary structure of the aminopeptidase has not been reported previously.

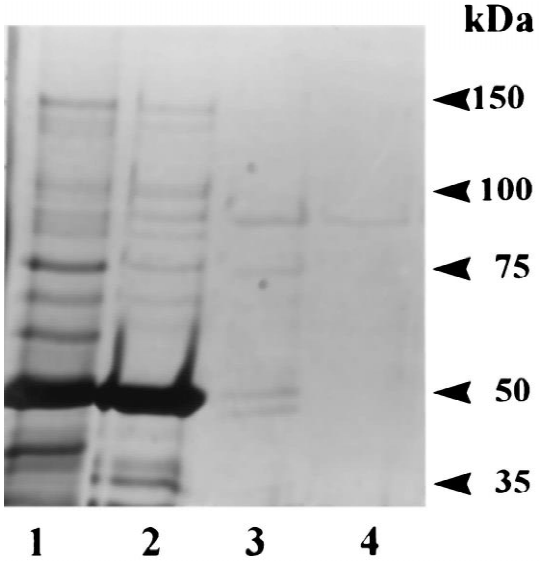

Fig. 2. SDS–PAGE analysis of purified aminopeptidase. Samples of resuspended pellets after ammonium sulfate precipitation (1), pooled fractions after hydrophobic interaction chromatography (2), pooled fractions after gel permeation chromatography (3) and the peak fraction after ion-exchange chromatography (4) were resolved in 8% polyacrylamide gels and proteins were stained with silver. M_r markers are indicated.

3.3. Enzymic properties

The enzymic properties of the isolated protein were preliminarily established using Ala-pNA as substrate. The enzyme was active in a broad pH range with an optimum at 7.6. When catalytic amounts of enzyme were incubated in 50 m*M* Tris–HCl pH 7.6 together with 1 m*M* Ala-pNA at 37°C, the concentration of free pNA increased linearly for several hours. The enzyme remained stable when incubated in the same buffer at 45°C for 15 min and became irreversibly inhibited over 55°C. In the presence of ammonium sulfate, the purified protein could be stored for several days at 0°C or −26°C without significant loss of activity. The addition of divalent (Mg^{+2}, Ca^{+2}) or monovalent (Na^{+}, K^{+}) cations to the incubation medium were not required for enzyme activity. Moreover, high or even moderate salt concentrations interfered with enzyme activity. For example, the enzyme was reversibly inhibited (50%) in the presence of 100 m*M* ammonium sulfate. Reduction agents (DTE or 2-mercaptoethanol) at a broad range of concentrations (0.01–100 m*M*) did not have any effect on the activity, indicating that no essential cysteines are involved in enzyme catalysis.

3.4. Substrate specificity

The purified protein was tested in its ability to hydrolyze several synthetic substrates under standard conditions. While amino acids with a free amino-terminus were cleaved with different effectiveness (Table 2), various amino-terminal blocked substrates

Table 2
Substrate specificity of purified peptidase activity

Substrate[a]	Relative activity[b] (% of control)
Ala-pNA	100
Gly-pNA	72
Leu-pNA	43
Lys-pNA	36
Met-pNA	20
Arg-pNA	18
Pro-pNA	5
Phe-pNA	3
Val-pNA	3
Cys-pNA	2
Ile-pNA	0.5
Ala-*βNA*	100
Leu-*βNA*	38.8
Phe-*βNA*	2.8
Trp-*βNA*	2.7
Tyr-*βNA*	0.7

[a] All substrates were used at a final concentration of 1 m*M*.
[b] Aminopeptidase activity after 1 h incubation was determined by measuring free pNA or β-NA. Hydrolysis of Ala-pNA and Ala-βNA were taken as reference values (100%).

were not hydrolyzed at all (data not shown). This suggests that the enzyme acts as an aminopeptidase. Amino acids having short aliphatic side chains (Ala-pNA or Ala-*βNA*, Gly-pNA) were preferred substrates. Leu-pNA, Lys-pNA, Met-pNA, Arg-pNA, Pro-pNA, Val-NA, Cys-pNA and Ile-pNA were also cleaved to a minor extent. Amino acids with acidic or aromatic side chains were hydrolysed not at all or very slowly.

3.5. Inhibition profile

To characterize the catalytic mechanism for peptide hydrolysis, protease class-specific inhibitors were used (Table 3). After incubation with serine-, aspartyl-, or cysteine-protease inhibitors even at relatively high concentrations, the peptidase activity against Ala-pNA was not importantly affected. In contrast, the metal chelator 1,10-phenantroline abolished the activity completely at 1 m*M* and showed a significant inhibition at 10 μ*M*. This result strongly suggests that the enzyme belongs to the class of metalloproteases. For inhibition with EDTA, higher concentrations were required, probably due to a lower affinity of EDTA for an essential metal ion involved in catalysis. First experiments in order to restore enzyme activity after ion removal with 1,10-phenantroline were unsucessful.

The susceptibility to inhibition by the specific aminopeptidase transition-state analogs bestatin and amastatin was tested (Fig. 3). Both inhibitors showed a concentration-dependent effect on hydrolysis of Ala-pNA. Amastatin was very effective in a range of concentration of 1–10 μ*M*, whereas bestatin in-

Table 3
Effect of protease class-specific inhibitors on aminopeptidase activity

Inhibitor[a]	Specificity	Concentration (m*M*)	Residual activity[b] (% of control)
1,10-phenanthroline	metallo proteases	0.01	59.0
		0.1	17.2
		1.0	0.49
EDTA	metallo proteases	0.1	100
		1.0	89.8
		10	55.1
PMSF	serine proteases	1	76
Pefablock	serine proteases	1	100
Pepstatin A	aspartyl proteases	0.1	100
E-64	cysteine proteases	0.1	92
Iodacetamide	cysteine proteases	1	86

[a] Purified aminopeptidase was preincubated with protease inhibitors in buffer (50 m*M* Tris–HCl pH 7.6) at room temperature for 2 h.
[b] The remaining activity was measured using Ala-pNa as substrate under standard conditions. The values are relative to a control sample preincubated without inhibitors.

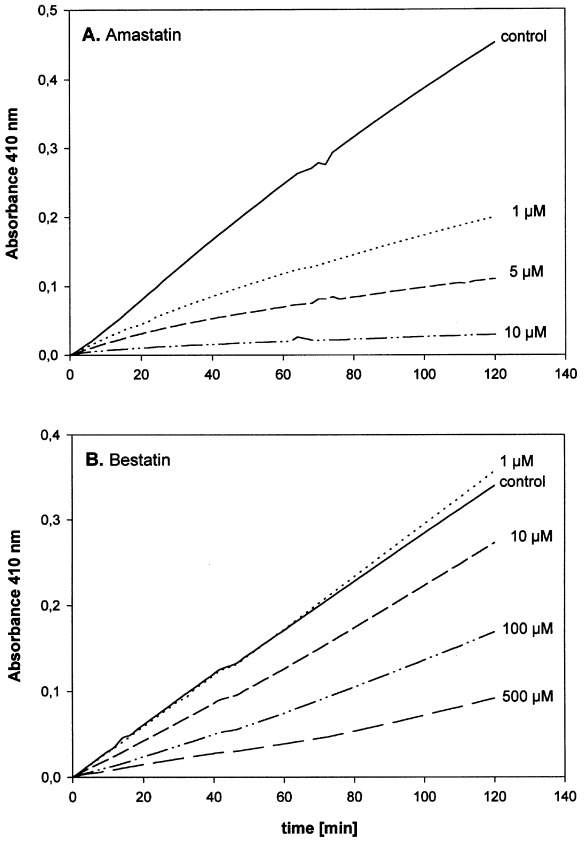

Fig. 3. Inhibition of aminopeptidase activity by amastatin (A) and bestatin (B). Aliquots of purified aminopeptidase were incubated with 1 m*M* Ala-pNA and different concentrations of inhibitors in 50 m*M* Tris–HCl pH 7.6. Product formation during the incubation time was measured by the increase of absorbance at 410 nm.

hibited the enzyme at relative high concentrations (87% at 100 µ*M*).

4. Discussion

We have purified to homogeneity a chloroplastic protein that hydrolyses synthetic substrates for aminopeptidases. The protocol for protein purification described here has at least two important advantages. First, it is exclusively composed of widely known, standard chromatographic technics such as hydrophobic interaction, gel permeation and ion exchange. Second, it is a relative rapid procedure: starting from isolated chloroplasts the complete protocol can be carried out in about 12 h.

The purified enzyme was identified as a soluble monomeric protein. Like the majority of known aminopeptidases, it appears to have a metal ion in its active site since strong inhibition was observed after treatment with metal chelators (Table 3). Furthermore, it is inhibited by the naturally occurring compounds amastatin and bestatin. These two competitive inhibitors bind to metalloaminopeptidases in a tight and reversible manner and slowly form a transition state analog complex with the enzyme [18]. Whereas amastatin inhibited the purified enzyme at low concentrations, inhibition with bestatin occurred at more higher concentrations (Fig. 3). This differential response to amastatin and bestatin may be important for understanding the catalytic mechanism of the enzyme. One difference between these inhibitors is the structure of their N-terminal regions: while in amastatin it resembles a leucine residue, in bestatin resembles a phenylalanine residue. Interestingly, the purified enzyme hydrolysed with very much higher affinity N-terminal amino acids with aliphatic side chains, e.g. leucine, than amino acids with bulky hydrophobic side chains, e.g. phenylalanine (Table 2). This prompts us to speculate that the differences observed by inhibition with amastatin and bestatin reflect those given by substrate affinity.

The purified enzyme is present in the soluble fraction of chloroplasts and can release specific amino acids from the N-terminus of synthetic substrates (Table 2). It is important to note that the in vitro conditions used for maximal activation in the test mixture are compatible with physiological conditions in stroma. Whereas this aminopeptidase preferred amino acids with short aliphatic chains at the N-terminus, another aminopeptidase which was present in the same stroma preparations and is clearly different to the protein purified here, hydrolyses specifically amino acids with bulky hydrophobic side chains (unpublished observations). Thus, both stroma aminopeptidases may collaborate in unspecific degradation of oligopeptides. As expected for an aminopeptidase, the enzyme purified here could not hydrolyse succinyl-Ala-Ala-pNa, succinyl-Ala-Ala-Phe-pNa, Benzoyl-Arg-pNA and a variety of other short peptides with the N-terminus blocked (data not shown). However, these observations cannot exclude

that the enzyme acts also as an endopeptidase in vivo with physiological substrates. A novel enzyme (peptidase II_{mes}) recently purified from mesquite pollen [19], for example, presents a partial homology with the aminopeptidase N of *E. coli*, as well as some similarities with the enzyme described here, e.g., it hydrolyses typical aminopeptidase substrates, but not several commercial available endopeptidase substrates. Surprisingly, when this enzyme was incubated with some bioactive peptides that are involved in allergic responses (e.g. Angiotensin I), they became cleaved at internal positions. The direct degradation of larger substrates and proteins without participation of other proteases appears to be unlikely. In accordance with this idea, native or denatured ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [20] offered as an in vitro substrate was not degraded by the purified enzyme after several hours of incubation (unpublished observations). Alternatively, the aminopeptidase may play a separate, more specific function in the chloroplast. For example, it may regulate protein stability by protein N-terminal processing as has been demonstrated for other organisms [21,22].

To our knowledge, the protein isolated in this work represents the first direct identification of a protein with aminopeptidase activity in chloroplasts. The properties of this enzyme, however, are similar to some chloroplastic aminopeptidase activities characterized previously. In particular, those of AP2, which was purified from pea chloroplasts by Liu and Jaggendorf [12], agree very well with the enzyme studied here. Although the aminopeptidase purified by El Amrani et al. [14] from etioplasts of sugar beet seedlings also shows some similarity, a number of features are clearly distinct: it presents dimeric structure, different substrate specificity and maximal activity at pHs over 8.5. Other plant aminopeptidases with an uncertain subcellular localization might be also related [7,23]. The structural information of the aminopeptidase obtained in this work make now possible to determine the primary sequence of the protein. This should be of special significance to explore its function and regulation in the chloroplast.

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