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Lysine aminopeptidase of *Aspergillus niger*

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Conserved regions within the M1 family of metallo-aminopeptidases have been used to clone a zinc aminopeptidase from the industrially used fungus *Aspergillus niger*. The derived amino acid sequence of ApsA is highly similar to two yeast zinc aminopeptidases, LAPI and AAPI (53·3 and 50·9% overall similarity, respectively), two members of the M1 family of metallo-aminopeptidases. The encoding gene was successfully overexpressed in *A. niger* and the overexpressed product was purified and characterized. Aminopeptidase A was found to be active towards a number of amino acid p-nitroanilide (*p*NA) substrates, viz. K-*p*NA, R-*p*NA, L-*p*NA, M-*p*NA, A-*p*NA and F-*p*NA. The most preferred N-terminal amino acid is lysine and not leucine, arginine or alanine, the N-terminal amino acids preferred by the yeast homologues. The K_m and K_{cat} for K-*p*NA and L-*p*NA were 0·17 mM and 0·49 μkat mg^{-1}, and 0·16 mM and 0·31 μkat mg^{-1}, respectively. The pH optimum of the enzyme is between 7·5 and 8, whereas the enzyme is stable between pH 5 and 8. The enzyme is inhibited by the metal chelators EGTA, EDTA and 1,10-phenanthrolin. Bestatin was also able to inhibit the activity.

Keyword: metallopeptidase

INTRODUCTION

Intracellular proteolytic degradation is important for the elimination of damaged proteins, for the modulation of protein levels and the maintenance of amino acid pools. The major cytoplasmic proteolytic process in eukaryotes is the ubiquitin proteasome pathway leaving the cell with short peptides, ranging from 3 to 22 residues in length (Tomkinson, 1999; Kisselev *et al.*, 1999). These peptides, and peptides resulting from the breakdown of protein substrates that are imported from the external milieu via peptide transporters, are further degraded to amino acids by tri-, di-, carboxy- and aminopeptidases.

The majority of aminopeptidases belong to the M1 family of peptidases; they are metalloenzymes (Van Wart, 1996) which require zinc for enzymic activity and share the zinc binding motif HEXXH (Jongeneel *et al.*, 1989; Hooper, 1994).

The evolutionary tree of the M1 family of metallo-aminopeptidases (Barret *et al.*, 1998) shows that this family can be divided into three main groups. Two groups, the aminopeptidase N group and the leukotriene A4 hydrolase group, have been fully characterized. The remaining aminopeptidases within the M1 family are grouped together, mainly because they share a high sequence similarity. However, peptidases from this group that have been biochemically characterized differ considerably in their characteristics.

Two yeast enzymes from the M1 group have been actively studied (Hirsch *et al.*, 1988; Garcia-Alvarez *et al.*, 1991; Caprioglio *et al.*, 1993). In the case of industrially used filamentous fungi, like *Aspergillus* spp., only aminopeptidase activities from *Aspergillus oryzae* have been described. *A. oryzae* produces at least seven aminopeptidase activities (Nakadai & Nasuno, 1977) of which four have been purified (Nakadai & Nasuno, 1977; Nakadai *et al.*, 1973a, b, c) and one has been cloned (Blinkovsky *et al.*, 2000).

Our aim is to characterize the pathways involved in protein catabolism in *Aspergillus niger*. So far seven endopeptidases (see van den Hombergh *et al.*, 1997, and references therein), one maturase (Jalving *et al.*, 2000) and two carboxypeptidases (van den Hombergh *et al.*, 1994; Dal Degan *et al.*, 1992; Svendsen & Dal Degan, 1998) of *A. niger* have been cloned and characterized. To date no aminopeptidases of *A. niger* have been cloned or characterized. Here we present the characterization of the *apsA* gene encoding an intracellular zinc amino-

Abbreviation: *p*NA, *p*-nitroanilide.

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peptidase of *A. niger* and the characterization of the purified enzyme.

METHODS

Strains, DNA and RNA techniques. *Escherichia coli* DH5α and LE392 were used as hosts for recombinant plasmids and λ DNA. *Aspergillus niger* strains N402 (*cspA1*) and NW219 (*cspA1 pyrA6 leuA1 nicA1*) are derived from *A. niger* N400 (CBS 120.49). Transformation and DNA digestion with restriction enzymes, ligation and agarose electrophoresis were standard procedures as described by Sambrook *et al.* (1989). Cloned fragments were sequenced using the Thermo Sequenase Fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP from Amersham Pharmacia Biotech and an ALF automated sequencer (Amersham Pharmacia Biotech). Hybridizations were done in SHB [6 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M sodium citrate), 5 × Denhardts' solution (Sambrook *et al.*, 1989), 0·5 % (w/v) SDS and 100 μg denatured herring sperm DNA ml^{-1}] at 65 °C; washing was performed at 65 °C using a final wash step of 1 × SSC plus 0·1 % SDS. For Northern analysis, strains were either grown for 17 h in minimal medium (Pontecorvo *et al.*, 1953) supplemented with 1 % (w/v) glucose and then harvested or grown for 17 h in minimal medium supplemented with 1 % (w/v) fructose and then transferred to minimal medium using various other carbon or nitrogen sources as supplements. Glucose, fructose or galactose (all 1 %, w/v) were used as carbon sources; 75 mM NH_4Cl or 70 mM $NaNO_3$ were used as nitrogen sources. In addition we used 0·5 % (w/v) BSA (pH 3 and 8), 0·5 % (w/v) casein (pH 3 and 8), 0·5 % (w/v) collagen (pH 3 and 8) or 0·5 % (w/v) elastin (pH 3 and 8) as sole carbon and nitrogen sources. The cultures were grown as 50 ml cultures in 250 ml Erlenmeyer flasks in an Innova incubator shaker (New Brunswick Scientific) at 250 r.p.m. at 30 °C. Mycelium was collected by filtration over a nylon gauze, frozen in liquid nitrogen and then ground in a Braun II dismembrator. Total RNA was isolated using Trizol Reagent (Life Technologies). RNA concentrations were determined spectrophotometrically and equal amounts of RNA were denatured with glyoxal by standard techniques (Sambrook *et al.*, 1989) and separated on a 1·6 % (w/v) agarose gel. RNA blots were hybridized at 42 °C in SHB to which 10 % (w/v) dextran sulphate and 50 % (v/v) formamide were added. Washing was performed at 65 °C to a final stringency of 1 × SSC plus 0·1 % SDS. The blots were hybridized with the 18S rDNA gene of *A. niger* as a loading control.

Cloning of *apsA*. An *apsA* PCR product was generated using degenerate primers based on regions conserved between the metallo-aminopeptidase amino acid sequence of *Saccharomyces cerevisiae* (accession nos P37898, P32454), *Mus musculus* (AAC52409), *Homo sapiens* (P15144), *Oryctolagus cuniculus* (S07099), *Haemonchus contortus* (CAA63897) and *Rattus norvegicus* (AAB38021).

The conserved peptide sequences GAMENWG and HELAHQW were used to design a forward primer, 5′-GGIGCNATGGARAAYTGGGG-3′, and a reverse primer, 5′-AAICCRAACCAYTGRTGNGC-3′, respectively (standard IUB-IUPAC symbols are used to indicate the nucleotide mixtures, I denotes inosine). A standard PCR was performed on genomic DNA of *A. niger* N402 using an equal amount of both primer mixtures and an annealing temperature of 50 °C. The amplified product was cloned in pGEM-T (Promega) and sequence analysis followed. The PCR product was used as a probe in the screening of a λ EMBL4 genomic library of *A. niger* N400 by standard methods to obtain the *apsA* gene. Three phages were isolated and from one positive phage a 1·7 kb *Eco*RI–*Bam*HI fragment and a partially overlapping 2·7 kb *Sal*I fragment were subcloned in pUC19 and sequenced over both strands. cDNA of *apsA* was generated by RT-PCR, using the enhanced avian RT-PCR kit of Sigma, according to the supplier's instructions.

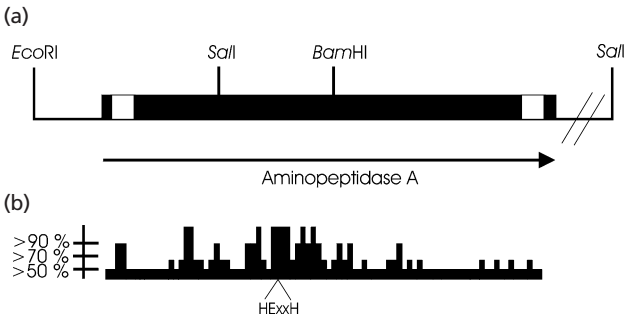

Fig. 1. Sequence characteristics of the *A. niger apsA* gene. (a) Partial restriction map of the *apsA* genomic region. The positions of the restriction enzymes used in the cloning strategy are indicated. The ORF is indicated with a filled box; an arrow indicates the direction of transcription. The position of the two introns encountered in the ORF are indicated by open boxes. (b) Identity between *A. niger* ApsA, and LAPI and AAPI of yeast. The multiple alignment was created with the program CLUSTAL X. The ApsA ORF is presented as a solid line and regions with more than 50 % identity are indicated. The percentage identity was calculated using a window size of 10. The zinc-binding motif (HExxH) is indicated.

Protein and nucleotide sequence analyses were done with the program DNASTAR (Lasergene). The BLAST algorithm (Altschul *et al.*, 1997) was used to search the public databases. Multiple alignments were made with CLUSTAL X (Jeanmougin *et al.*, 1998).

Plasmid construction and overexpression of ApsA. The 1·7 kb *Eco*RI–*Bam*HI fragment and the partially overlapping 2·7 kb *Sal*I fragment were merged, resulting in pIM4102 (Fig. 1). Plasmids pIM4102 and pGW635, which contain the *A. niger pyrA* gene, were used to co-transform *A. niger* NW219 according to Kusters-van Someren *et al.* (1991). $PyrA^+$ transformants were screened for enhanced aminopeptidase activity in cell extracts. For this, ground mycelium was extracted with 100 mM sodium/potassium phosphate buffer at pH 7·2 and clarified by centrifugation (10000 ***g*** for 15 min at 4 °C). Aminopeptidase activity in these cell extracts was determined as described below and protein concentrations were determined by the bicinchoninic acid method as described by the supplier (Sigma).

Purification of *A. niger* ApsA. Strain Tr7 was grown for 17 h in minimal medium (Pontecorvo *et al.*, 1953) supplemented with 2 % glucose, 0·2 % (w/v) meat peptone, 0·1 % (w/v) yeast extract, 0·1 % (w/v) peptone 140 and 0·03 % (w/v) yeast ribonucleic acids. The mycelium was harvested and ground. The ground mycelium was suspended in 100 mM sodium/potassium phosphate buffer, pH 7·2, and stirred for 15 min at 4 °C. Cellular debris was removed by centrifugation. Then $(NH_4)_2SO_4$ was added to 30 % saturation. After centrifugation (15 min, 10000 ***g***) the supernatant was loaded onto a 20 ml Phenyl Sepharose high performance column (Pharmacia Biotech), pre-equilibrated with 50 mM triethanolamine (TEA), pH 7·0, and 1 M $(NH_4)_2SO_4$. Bound protein was eluted using a 200 ml linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in

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20 mM TEA, pH 7·0. The fraction size was 5 ml. The active fractions were pooled, dialysed against 20 mM TEA, pH 7·0, and loaded onto a 1 ml Resource Q column (Pharmacia Biotech) pre-equilibrated with 20 mM TEA, pH 7·0. The bound protein was then eluted using a 15 ml linear gradient from 0 to 0·4 M NaCl in TEA, pH 7·0. The fraction size was 1 ml.

Biochemical characterization of ApsA. Aminopeptidase activity was determined as described by Atlan *et al.* (1994). A range of amino acids coupled to *p*-nitroanilide (*p*NA) were used as substrate. Standard conditions were 2 mM substrate in 67 mM sodium/potassium phosphate buffer at pH 7·2 and 30 °C. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μM *p*NA min^{-1} L-*p*NA, P-*p*NA, R-*p*NA, F-*p*NA, A-*p*NA, M-*p*NA and K-*p*NA were obtained from Sigma and V-*p*NA, G-*p*NA, I-*p*NA and E-*p*NA were obtained from Bachem. The optimal pH for enzymic activity was determined using McIlvaine buffers at pH values ranging from 3 to 8, 200 mM HEPES plus 300 mM NaCl at pH values from 7·2 to 8·5 and 50 mM Tris/glycine buffer at pH values from 8 to 10. The pH stability of ApsA was tested by preincubation of the purified enzyme in McIlvaine buffer of different pH values ranging from 2·2 to 8 at 30 °C for 90 min followed by the standard enzyme reaction. The temperature stability of ApsA was tested by preincubation of the purified enzyme at 0, 30, 40, 50 and 60 °C for 60 min in sodium/potassium phosphate buffer, pH 7·2, followed by the standard enzyme reaction. Here the 0 °C preincubated sample was used as a reference to calculate the residual activity.

The effect of the protease inhibitors bestatin, 1,10-phenanthrolin, EDTA, EGTA, PMSF, tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl lysyl chloromethyl ketone (TLCK), leupepstatin and iodoacetamide on enzymic activity was measured in sodium/potassium phosphate buffer at pH 7·2. The purified enzyme was preincubated with the respective compound for 30 min at 30 °C. After the preincubation period substrate was added, followed by the standard enzyme assay. The metal ion requirement of ApsA for activity was tested by preincubating the enzyme with EDTA, EGTA or 1,10-phenanthrolin for 15 min at 37 °C or for 17 h at 4 °C.

RESULTS AND DISCUSSION

Cloning of the *A. niger* ApsA and analysis of the gene

The amino acid sequences GAMENWG and HELAHQW, which are conserved between zinc aminopeptidases of nearly all subgroups of aminopeptidases within the M1 family, were used to design primers. These primers were used in a PCR reaction on genomic DNA of *A. niger* and resulted in a 130 bp product. A 1·7 kb *Eco*RI–*Bam*HI fragment and a partially overlapping 2·7 kb *Sal*I fragment which hybridized to this PCR product (Fig. 1) were subcloned and sequenced over both strands. Sequence analysis showed that together these fragments harbour the complete putative aminopeptidase A gene (*apsA*) and up- and downstream sequences. RT-PCR techniques were used to generate *apsA* cDNA. The cDNA and the genomic DNA sequences were identical except for two intervening sequences in the ORF, one 152 bp downstream of the start codon and one 48 bp upstream of the stop codon (Fig. 1).

The encoded protein is similar to the M1 family of metallo-aminopeptidases (Barret *et al.*, 1998). ApsA has highest similarity with LAPI (encoded by *APE2*), (Garcia-Alvarez *et al.*, 1991) and AAPI (encoded by *AAPI*), (Caprioglio *et al.*, 1993), both from *S. cerevisiae* (53·3 and 50·9 % overall identity, respectively; Fig. 1). The region of highest identity is found at the N-terminal part of the enzyme. The encoded protein contains the signature sequence of the M1 family of zinc peptidases (HEXXH) (Jongeneel *et al.*, 1989; Hooper, 1994) and this sequence was found in a region that is most conserved between ApsA, AAPI and LAPI (Fig. 1).

Northern analysis of *A. niger* N402 grown on several carbon and nitrogen sources showed that the *apsA* messenger levels were independent of the carbon or nitrogen source used (results not shown). The yeast aminopeptidase genes *APE2* and *AAPI* are also constitutively expressed, although yeast *AAPI* mRNA, which is present during all phases of growth, is reported to be more abundantly expressed in exponentially growing yeast cells (Caprioglio *et al.*, 1993).

Since ApsA does not contain a known secretion signal nor a known organellar targeting signal and since *apsA* transcript levels are apparently not influenced by the carbon or nitrogen sources tested, we conclude that ApsA is, like LAPI and AAPI, located in the cytosol.

Overexpression of the gene encoding ApsA in *A. niger*

A. niger strain NW219 was transformed with plasmid pIM4102. Nine transformed strains were further analysed for the occurrence of multiple integrations of the plasmid in the genome. Southern analysis showed that transformants 7 (Tr7) and 8 (Tr8) have the highest copy numbers of the integrated plasmid (Fig. 2). Messenger levels of *apsA* of Tr7 and Tr8 were compared to the messenger level of the wild-type strain by Northern analysis. Scanning of Northern blots revealed that compared to the wild-type strain both transformants have at least a tenfold increased messenger level of the correct size compared to the wild-type (Fig. 2).

Tr7 and Tr8 were analysed for increased aminopeptidase activity on four *p*NA substrates: K-*p*NA, R-*p*NA and L-*p*NA, substrates that are preferred by the two homologous yeast enzymes, and F-*p*NA, a substrate which is not hydrolysed by these yeast enzymes. Compared to the wild-type activity, cell extracts of Tr7 and Tr8 had a 36- and 24-fold increase in activity, respectively, when K-*p*NA was used as substrate. Furthermore a 19- (Tr7) and 12-fold (Tr8) increase in activity towards R-*p*NA, and a 9- (Tr7) and 6-fold (Tr8) increase in activity towards L-*p*NA were found. A cell extract of *A. niger* hydrolyses F-*p*NA, but the transformants did not display an increased hydrolytic activity on this substrate, thus both transformed strains overexpress a specific aminopeptidase with activity towards K-*p*NA, R-*p*NA and L-*p*NA.

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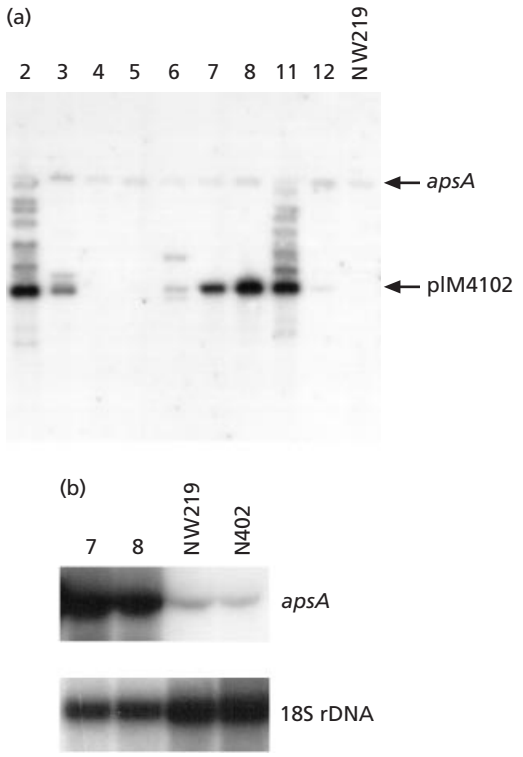

Fig. 2. Molecular characterization of *apsA* multicopy strains. (a) Southern analysis of *apsA* transformants. *Hin*dIII-digested genomic DNA of the parental strain (*A. niger* NW219) and nine transformed strains was analysed. The two hybridizing restriction fragments indicated originate from the endogenous *apsA* gene and from (multiple) integrations of pIM4102 in the genome. The other bands probably result from (partial) integrations of the plasmid at other loci. Transformed strain numbers are indicated above the lanes. (b) Northern analysis of *apsA* expression in the wild-type strain (*A. niger* N402), the parental strain (*A. niger* NW219) and transformed strains Tr7 and Tr8.

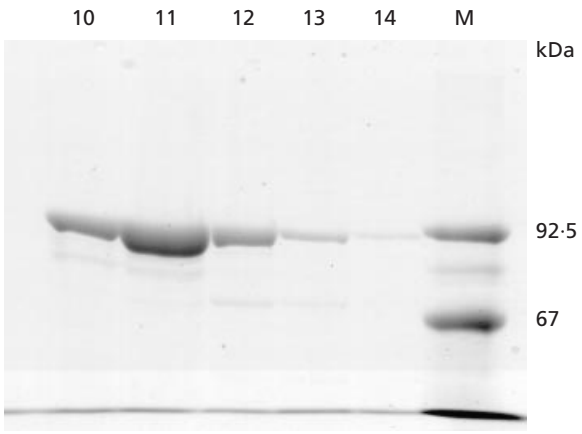

Fig. 3. Purification of ApsA from *A. niger*. A Coomassie-stained SDS-polyacrylamide gel (10 %) loaded with 20 µl of active fractions 10–14 eluted from the Resource Q column is shown. Fraction numbers are indicated above the lanes. Lane M, protein molecular mass markers.

Purification and biochemical properties of the enzyme

ApsA was purified from a cell extract of Tr7, resulting in an enzyme preparation with a specific activity of 12 U mg^{-1}. The final yield was 5 % (Table 1). The low yield after the first step is probably due to ammonium sulphate precipitation.

An analysis by 10 % SDS-PAGE of the active fractions revealed a protein band with an apparent molecular mass of 95 kDa, identical to the calculated molecular mass of the protein inferred from the amino acid sequence. The four aminopeptidases of *A. oryzae* isolated from culture broth differ considerably in their molecular mass from ApsA (Nakadai & Nasuno, 1977; Nakadai *et al.*, 1973a, b, c).

Fraction 11 contained the highest activity and was used for further characterization of the enzyme (Fig. 3). The enzyme efficiently hydrolyses K-*p*NA and R-*p*NA, suggesting that the enzyme prefers basic amino acids at the N-terminal end of the substrate. The K_m and K_{cat} for K-*p*NA and L-*p*NA were 0·17 mM and 0·49 µkat mg^{-1}, and 0·16 mM and 0·31 µkat mg^{-1}, respectively. The enzyme also hydrolyses M-*p*NA and has some activity towards A-*p*NA and F-*p*NA (Table 2).

The yeast homologues LAPI and AAPI have highest activity towards substrates that have at their N-terminal end respectively a leucine or a lysine and an alanine or an

Table 1. Purification of ApsA from *A. niger* Tr7

Data are means of three separate experiments in which differences did not exceed 10 %.

Step	Total activity (U)*	Total protein (mg)	Specific activity (U mg^{-1})*	Purification (-fold)	Yield (%)
Cell extract	280	390	0·72	1	100
Phenyl Sepharose	18·6	2·5	7·4	10	6·6
Resource Q	14·6	1·2	12·2	17	5·2

* One unit of enzyme activity (U) is defined as the amount of enzyme that produces 1 µM *p*NA min^{-1}. Activity was determined with 2 mM K-*p*NA as a substrate.

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Table 2. Substrate specificity of ApsA towards amino acid *p*NA substrates

Activity is relative to the activity towards K-*p*NA, which was 12 U mg^{-1}. The results are the means of three independent experiments in which differences did not exceed 10 %.

Substrate	Relative activity
K-*p*NA	1
R-*p*NA	0·6
M-*p*NA	0·4
A-*p*NA	0·1
F-*p*NA	0·1
V-*p*NA, G-*p*NA, E-*p*NA, I-*p*NA, P-*p*NA	<0·01

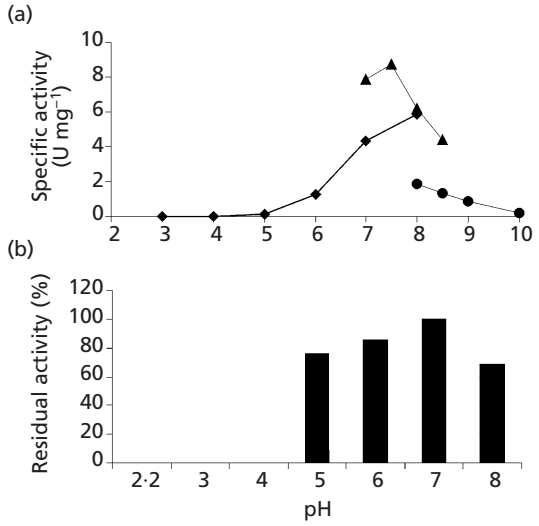

Fig. 4. Biochemical characterization of ApsA. (a) pH optimum of ApsA. The following buffers were used: McIlvaine (◆), HEPES (▲) and Tris/glycine (●). (b) pH stability of ApsA. Residual activity was calculated relative to a sample kept in 20 mM TEA, pH 7·0, at 30 °C.

arginine. Thus their substrate specificity differs from the *Aspergillus* enzyme. However, for AAPI Caprioglio *et al.* (1993) did not report on enzyme activities on substrates other than alanine-, arginine- and leucine-napthylamide.

In the cell extracts of Tr7 and Tr8, the highest increase in activity was found towards K-*p*NA and R-*p*NA, followed by L-*p*NA. However, no increase in activity towards F-*p*NA was found. The purified ApsA, however, shows some activity towards F-*p*NA. This suggests that other, more specific F-*p*NA hydrolysing activities present in the cell extract are dominant over a relatively small increase in the two transformants.

The enzyme is active between pH 5 and 9 (Fig. 4). The optimal pH is between 7·5 and 8·0 which is close to the pH of the *Aspergillus* cytosol of pH 7·8 (Hesse *et al.*, 2000). This slightly differs from the optimal pH of LAPI (pH 7·5) (Trumbly & Bradley, 1983). The optimal pH of AAPI has not been reported.

Table 3. Effects of chemicals on the activity of the purified protease

Activity is expressed as the percentage of residual activity after 30 min incubation of the enzyme with the inhibitor; 100 % is 2·1 U mg^{-1}. Results are the means of three experiments; differences did not exceed 10 %. TPCK: tosyl phenylalanyl chloromethyl ketone; TLCK tosyl lysyl chloromethyl ketone.

Inhibitor	Concentration (mM)	Residual activity (%)
None	0	100
PMSF	1	100
Leupepstatin	0·042	98
TPCK	0·020	95
EDTA	5	100
EGTA	5	100
1,10-Phenanthrolin	5	<1
Iodoacetamide	0·040	100
Bestatin	0·002	60
TLCK	0·020	94
$ZnCl_2$	0·5	<1

ApsA is stable in a pH range of 5–8 (Fig. 4) for 90 min with optimal stability at pH 7. Between pH 2·2 and 4 the enzyme is unstable. The enzyme is stable up to a temperature of 40 °C for 1 h at pH 7·0. After 1 h at 50 °C, 60 % of the activity remained. After 1 h at 60 °C, no enzyme activity was left.

Several possible inhibitors were tested (Table 3). The three serine protease inhibitors tested had no effect on the enzyme activity. Iodoacetamide also did not have any effect on the activity of ApsA, indicating that the enzyme is not a cysteine aminopeptidase. The aminopeptidase inhibitor bestatin was able to inhibit ApsA activity. 1,10-Phenanthrolin was also able to inhibit the activity of ApsA, probably by chelating the metal ion bound in the enzyme. Surprisingly, the metal chelators EDTA and EGTA were not able to reduce the activity after a pre-incubation of 30 min at 30 °C. A 17 h incubation at 4 °C with EDTA or EGTA was necessary to reduce the activity of ApsA completely. This suggests that the metal ion is bound strongly to the ApsA enzyme.

$ZnCl_2$ was also capable of reducing ApsA activity completely. This effect is probably caused by the oxidation of a cysteine residue (nine are present in ApsA) located near the active site of ApsA, since addition of 5 mM β-mercaptoethanol prevented the inhibitory effects of $ZnCl_2$.

In this study we cloned and characterized the first aminopeptidase gene of the M1 family of amino-

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peptidases from a filamentous fungus. We also determined the general biochemical characteristics of the encoded enzyme. The substrate specificity is different from that of the yeast enzymes; lysine and not leucine, arginine or alanine is preferred at the N-terminal position. This genetic and biochemical characterization will enable further studies for the understanding of the *in vivo* roles of the aminopeptidases in degradation of (imported) peptides.

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