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 (pNA) substrates, viz. K-pNA, R-pNA, L-pNA, M-pNA, A-pNA and F-pNA. 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-pNA and L-pNA 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-phenanthroline. Bestatin was also able to inhibit the activity.

**Keyword:** metallopeptidase

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**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 or 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 aminopeptidase...
peptidase of A. niger and the characterisation 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 × Denhardt’s’ solution (Sambrook et al., 1989), 0.5% (v/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 NH₄Cl or 70 mM NaNO₃ 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 16% (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* (AA838021).

The conserved peptide sequences GAMENWG and conserved peptide sequences 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 EcoRI–BamHI fragment and a partially overlapping 2.7 kb SaI 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.

Protein and nucleotide sequence analyses were done with the program DNASAR (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 EcoRI–BamHI fragment and the partially overlapping 2.7 kb SaI fragment were merged, resulting in pLM4102 (Fig. 1). Plasmids pLM4102 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 (10 000 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 Ts7 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 (NH₄)₂SO₄ was added to 30% saturation. After centrifugation (15 min, 10 000 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 (NH₄)₂SO₄. Bound protein was eluted using a 200 ml linear gradient from 1 to 0 M (NH₄)₂SO₄ in
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 (pNA) 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 pNA min⁻¹ L-pNA, P-pNA, R-pNA, F-pNA, A-pNA, M-pNA and K-pNA were obtained from Sigma and V-pNA, G-pNA, I-pNA and E-pNA 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 result of the protease inhibitors bestatin, 1,10-phenanthroline, EDTA, EGTA, PMSF, tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl lysyl chloromethyl ketone (TLCK), leupeptin 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-phenanthroline 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 EcoRI–BamHI fragment and a partially overlapping 2.7 kb SalI 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 pLM4102. 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 pNA substrates: K-pNA, R-pNA and L-pNA, substrates that are preferred by the two homologous yeast enzymes, and F-pNA, 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-pNA was used as substrate. Furthermore a 19- (Tr7) and 12-fold (Tr8) increase in activity towards R-pNA, and a 9- (Tr7) and 6-fold (Tr8) increase in activity towards L-pNA were found. A cell extract of A. niger hydrolyses F-pNA, 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-pNA, R-pNA and L-pNA.
Fig. 2. Molecular characterization of apsA multicopy strains. (a) Southern analysis of apsA transformants. HindIII-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.

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 \(A.\) \textit{oryzae} isolated from culture broth differ considerably in their molecular mass from ApsA (Nakadai & Nasuno, 1977; Nakadai \textit{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 \(K_{\text{in}}\) and \(K_{\text{cat}}\) for K-\(p\)NA and L-\(p\)NA were 0·17 mM and 0·49 \(\mu\)kat mg\(^{-1}\), and 0·16 mM and 0·31 \(\mu\)kat 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 \(A.\) \textit{niger} Tr7

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

* One unit of enzyme activity (U) is defined as the amount of enzyme that produces 1 \(\mu\)M \(p\)NA min\(^{-1}\). Activity was determined with 2 mM K-\(p\)NA as a substrate.
The enzyme is active between pH 7.5 and 8.0 which is close to the optimal 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.

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-Phenanthroline 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. ZnCl₂ 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 ZnCl₂.

In this study we cloned and characterized the first aminopeptidase gene of the M1 family of aminopeptidase.
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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REFERENCES


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