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Purification and properties of an intracellular leucine aminopeptidase from the fungus, *Penicillium citrinum* strain IFO 6352

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An intracellular leucine aminopeptidase (LAP) from *Penicillium citrinum* (IFO 6352) was purified to homogeneity using three successive purification steps. The enzyme has a native molecular mass of 63 kDa using HPLC gel filtration analysis and a molecular mass of 65 kDa when using SDS-polyacrylamide gel electrophoresis. This monomeric aminopeptidase showed maximum enzyme activity at pH 8.5. An optimum temperature was 45–50°C when L-Leu-*p*-nitroanilide (*p*NA) was the substrate, and enzyme activity drastically decreased above 60°C. The Michaelis–Menten constants for L-Leu-*p*NA and L-Met-*p*NA were 2.7 mM and 1.8 mM, respectively. When the enzyme reacted with biosynthetic methionyl human growth hormone, it showed high specificity for N-terminal methionine residue and recognized a stop sequence (Xaa-Pro). The aminopeptidase was inactivated by EDTA or 1,10-phenanthroline, indicating that it is a metallo-exoprotease. Enzyme activity was restored to 90% of maximal activity by addition of Co^{2+} ions. The activity of EDTA-treated enzyme was restored by addition of Zn^{2+}, but reconstitution with Ca^{2+}, Mg^{2+} or Mn^{2+} restored some enzyme activity. It is likely that Co^{2+} ions play an important role in the catalysis or stability of the *Penicillium citrinum* aminopeptidase, as zinc plays a similar function in other leucine aminopeptidases.

Keywords: leucine aminopeptidase; *Penicillium citrinum*; methionine removal

Introduction

Leucine aminopeptidase (LAP), a class of cellular exoproteases widely distributed in nature, catalyzes hydrolysis of the peptide bond which is adjacent to free α-hydrophobic amino acids such as Leu. It is generally accepted that for both animals and bacteria, aminopeptidases are involved during key metabolic events occurring in the process of recovering amino acids from abnormal polypeptides or during the process of regular turnover of cytosolic proteins. A variety of LAPs from many tissues and organs has been isolated and characterized [22,23]. The enzymes were obtained from *Escherichia coli* [24], *Aeromonas proteolytica* [19], marine pseudomonads [16] and *Arabidopsis thaliana* [2]. Several genes encoding for LAP have been also isolated [2,11,21] and amino acid sequences determined [10,11]. Despite the availability of a substantial amount of knowledge about bacterial and mammalian aminopeptidases, the entire function of aminopeptidases remains to be elucidated.

Leucine aminopeptidases are exopeptidases belonging to a class of zinc-requiring metalloproteases. A few are exceptional enzymes that require cobalt ion for full enzyme activity. Leucine aminopeptidases show a variety of physico-chemical properties which depend on enzyme source [22]. Bovine lens leucine aminopeptidase, a mammalian aminopeptidase which has been extensively studied, is a hexamer of identical chains having a total molecular mass of 324 kDa. Each subunit of the hexamer has a metal-binding site for two zinc atoms [1]. A leucine aminopeptidase from *Aeromonas proteolytica* is a monomer with a molecular mass of 30 kDa and two zinc atoms also bind to this enzyme [11]. Similar metal-binding features have also been found in *Streptomyces griseus* leucine aminopeptidase, which was a well-characterized bacterial aminopeptidase [20]. In spite of variable enzyme sources and physicochemical properties, a common feature found in aminopeptidases is the binding of one or two zinc atoms per monomer or subunit. This suggests that aminopeptidases are highly similar in their structures and that LAPs constitute an enzyme superfamily, having evolutionarily-conserved regions.

A substantial body of primary sequences for bacterial or mammalian aminopeptidases is available [8]. In addition, the three-dimensional structure complexed with bestatin [6] and metal-substituted enzyme kinetics [1,3,18,23] are also well defined. However, leucine aminopeptidases from the fungi have still not been well characterized. An EDTA-insensitive prolyl aminopeptidase from *Penicilium camemberti* and a monomeric fungal alanine aminopeptidase from the fungus, *Saccobolus platensis* were purified and characterized [9,17]. Recently, *Saccharomyces cerevisiae* methionine aminopeptidase has also been reported [7].

In recent years, researchers in biotechnology have been interested in the unique features of leucine aminopeptidase, which is a high substrate specificity being able to recognize the Xaa-Pro sequence (nonpolar amino acids are preferred in the position of Xaa) as a stop signal [3,4]. This characteristic of the enzymes can be used, with high efficiency, in maturation steps of several protein drugs which hold an extra methionyl residue at the N-terminus when they are produced intracellularly in genetically engineered microorganisms.

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In this study, we report on the purification and partial characterization of a 65-kDa intracellular leucine aminopeptidase from the fungus, *Penicillium citrinum* strain IFO 6352. This study also demonstrates that this fungal aminopeptidase can be used efficiently for the maturation of a recombinant protein through *in vitro* processing.

Materials and methods

Materials

All of the synthetic chromogenic substrates, L-Leu-*p*NA, L-Met-*p*NA, L-Ala-*p*NA, Gly-Phe-*p*NA, L-Lys-*p*NA, Gly-*p*NA, Gly-Pro-*p*NA, L-Phe-*p*NA, L-Pro-*p*NA, were obtained from the Sigma Chemical Co (St Louis, MO, USA). The protease inhibitors used in this study were purchased from Sigma Chemical Co or Boehringer Mannheim Biochemica (Mannheim, Germany). Distilled and deionized water were used to study the effect of metal ions on enzyme activity (Millipore, Bedford, MA, USA). The standard molecular mass markers for SDS-polyacrylamide gel electrophoresis analysis were obtained from Bio-Rad (Herculis, CA, USA). Two chromatographic resins, S-Sepharose FF and Sephacryl S-200 HR were obtained from Pharmacia LKB Biotechnology Inc (Uppsala, Sweden). Thin layer polyacrylamide gel for protein pI determination was purchased from Serva Co (Whichita Falls, TX, USA) and pI standards came from Pharmacia. All other reagents were of analytical grade.

Microorganism and culture conditions

Penicillium citrinum (IFO 6352) was used as a source of the enzyme. The following medium was used for culturing cells: 1% glucose, 1% bacto-peptone, 0.5% yeast extract, 0.2% potassium dihydrogen phosphate. The pH was adjusted to 7.0 with 1 N sodium hydroxide before the medium was autoclaved. For the primary culture, mycelium tips grown on a 1.5% agar plate were inoculated into a 500-ml culture flask containing 200 ml of medium. The culture was then incubated for about 72 h at 30°C at 150 rpm on a gyratory shaker (Lab-Line Instruments, Inc, Melrose Park, IL, USA). Thereafter, a total cell suspension of the primary culture was transferred to a 5-L culture vessel (Korea Fermentor Co, Korea) containing 3 L of medium. Cells were grown aerobically for 2 days with an air flow of 3 L per minute and an agitator speed of 450 rpm at 30°C.

Aminopeptidase assay

Aminopeptidase activity was measured spectrophotometrically at 405 nm using a synthetic substrate, L-Leu-*p*NA. In brief, the 0.1-ml enzyme solution was mixed with 1 mM L-Leu-*p*NA in 0.9 ml of 100 mM Tris buffer (pH 8.0) and incubated at 37°C for a given time; 0.1 ml of 70% acetic acid was added to terminate the reaction. One unit of aminopeptidase is defined as the amount of enzyme per milliliter required for producing 100 micromoles of *p*NA per minute under the described conditions. Reaction mixture without substrate was used as a blank in each measurement. All of the assays in the study were performed in duplicate.

Protein assay and polyacrylamide gel electrphoresis

The amount of protein was determined as described previously using bovine serum albumin as the standard [5]. SDS-polyacrylamide gel electrophoresis was performed in slab gels containing 15% acrylamide [14]. The amount of protein was estimated using the reducing silver-staining method [12]. Isoelectrofocusing was carried out as recommended by the manufacturer, using ready-made pH 3–10 horizontal gels (Serva).

Purification of an intracellular aminopeptidase

The following purification steps were carried out at 4°C. Aggregated fungal cells were harvested by gravity filtration through two layers of small porous nylon cloth. The mycelium was washed twice with water purified by reverse osmosis to remove the medium components from harvested cells and frozen at −70°C until used. About 200 g of cells was suspended in 1.5 L of 50 mM sodium phosphate (pH 6.8) containing 1 mM phenylmethylsulphonylfluoride (PMSF) and homogenized in a Waring blender until the clustered cells were disaggregated. Thereafter, the cells were completely disrupted with a bead-beater (Biospec Products, Bartlesville, OK, USA) for three cycles of 2 min (sample volume *vs* bead volume = 1 : 2) at 5-min intervals on ice bath. After centrifugation at $12000 \times g$ for 30 min at 10°C, the supernatant phase was treated with ammonium sulphate. The insoluble residue obtained at 30% ammonium sulphate saturation was discarded. The resulting 700 ml of supernatant fluid was concentrated to 200 ml in an ultrafiltration apparatus (YM10 membrane, Amicon, USA).

The protein was then dialyzed against 20 L of 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM PMSF for 20 h. The dialyzed protein was applied to a column of S-Sepharose FF (2.5 cm × 10 cm, Pharmacia) previously equilibrated with 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM PMSF. At a flow rate of 1 ml min^{-1}, the column was washed with five bed volumes of the equilibration buffer and eluted stepwise with 50 mM NaCl, 100 mM NaCl, and 200 mM NaCl, respectively, in equilibration buffer. The effluent was monitored at 280 nm and the aminopeptidase activity in each of the resulting fractions was measured. The fractions containing the enzyme activity were pooled and buffer-exchanged against gel filtration buffer containing 100 mM Tris (pH 8.0) and 6 M urea. After being concentrated to 0.5 ml by ultrafiltration using YM3 membrane (Amicon, USA), the protein solution was applied to a column of Sephacryl S-200 HR (1.5 cm × 50 cm, Pharmacia) connected to a FPLC LCC-500 controller at a flow rate of 0.15 ml min^{-1} using gel filtration buffer. The aminopeptidase activity was recovered within the range of between 33 and 40% of total column volume. Enzyme activity was measured and the fractions showing leucine aminopeptidase activity were analyzed using 15% SDS-polyacrylamide gel electrophoresis.

Determination of the optimal temperature and pH for the aminopeptidase

For optimum pH determination, 0.423 μg of the purified aminopeptidase was incubated in 50 mM Tris buffer at different pHs (7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10) for 20 min at 37°C. The enzyme activity was then measured as described

above at the pH at which the sample was incubated for 15 min. To determine optimum temperature, aliquots of the enzyme solution containing 2 μg of the enzyme were incubated in 100 mM Tris (pH 8.0) buffer at different temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65°C) for 20 min, and then the activities were measured at the temperature at which the sample had been incubated.

Determination of native molecular mass by HPLC gel filtration

The purified aminopeptidase was chromatographed by HPLC gel filtration on a Bio-Sil SEC-125 column (600 mm × 7.5 mm, Bio-Rad). Aprotinin (6.5 kDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa) were used as standards. The elution buffer for HPLC gel filtration chromatography was 50 mM sodium phosphate at pH 6.8. The partition coefficients of each protein and purified enzyme were determined.

Aminopeptidase reaction with biosynthetic methionyl human growth hormones

Recombinant methionyl human growth hormone (Met-hGH) from genetically engineered *Saccharomyces cerevisiae* was purified as previously described [25]. Ten units of purified aminopeptidase were incubated with 2 mg of biosynthetic Met-hGH in an appropriate tube containing 100 mM Tris buffer (pH 8.0), 1 mM PMSF, and 0.02% sodium azide at 37°C. After 15 h of incubation, the reaction mixture was buffer-exchanged with the same reaction buffer using MWCO 10000 Centricon (Amicon, USA) and further incubated for 5 h at 37°C. The hGH from the reaction mixture was easily isolated by passing through a Mono S (HR 5/5) column equilibrated with 20 mM sodium phosphate (pH 6.8). The flow-through fraction contained the aminopeptidase-free hGHs.

Protein sequencing

N-terminal amino acid sequencing of aminopeptidase-reacted hGHs was carried out according to the modified method reported by Applied Biosystems Inc. The sequences of a given protein were determined by Edman degradation using an Applied Biosystems model 471A Protein/Peptide Sequencer (Applied Biosystem Inc, CA, USA). The phenylthiohydantoin (PTH) derivatives of the amino acids were identified by reverse phase HPLC.

Effect of divalent metal ions on enzyme activity

Endogenous metals were removed by dialysis against 10 mM EDTA overnight at 4°C in order to examine the metal ion requirement of leucine aminopeptidase. EDTA-treated aminopeptidase was completely dialyzed by Centricon (MWCO 10000, Amicon, USA) against 100 mM Tris (pH 8.0). The purified enzyme (0.35 μg) was incubated with 1 mM of each metal ion for 15 min at 37°C, and restored enzyme activity was measured for 20 min at 37°C.

Results

Purification of the enzyme and molecular mass determination

An intracellular leucine aminopeptidase from the lysates of *Penicillium citrinum* was purified to homogeneity using a sequential combination of ammonium sulphate fractionation, cation exchange chromatography and gel filtration. The purification process is summarized in Table 1. During the aminopeptidase-hGH reaction process, we did not detect any hGH fragments, suggesting that the purified enzyme is free from endoprotease contamination. SDS-polyacrylamide gel electrophoresis of the purified aminopeptidase showed a single protein band with an estimated size of 65 kDa (Figure 1). The result of isoelectrofocusing indicates that *Penicillium citrinum* leucine aminopeptidase is an acidic protein with a pI value of 5.8 (Figure 2). The monomeric nature of the enzyme was shown by HPLC gel filtration. The homogeneous enzyme had an apparent molecular mass of 63 kDa under non-denaturing condition (data not shown).

Table 1 Purification of aminopeptidase from *Penicillium citrinum*

Purification step	Volume (ml)	Proteins (mg)	Total activity (U)	Sp activity (U mg^{-1})	Purification fold
Homogenates	2200	2000	88	0.044	1
30% $(NH_4)_2SO_4$ supernatant	700	650	112.7	0.17	3.86
S-Sepharose Fast Flow	24	5.85	27.6	4.73	107.5
Gel filtration	4	0.25	12	48.6	1105

Effects of pH and temperature

Under the conditions described in Materials and Methods, the enzyme showed the highest activity at pH 8.5, where the enzyme activity was three-fold higher than that at pH 7.0 (data not shown). Beyond pH 8.5, however, the enzyme activity decreased. In the temperature effect study, maximal

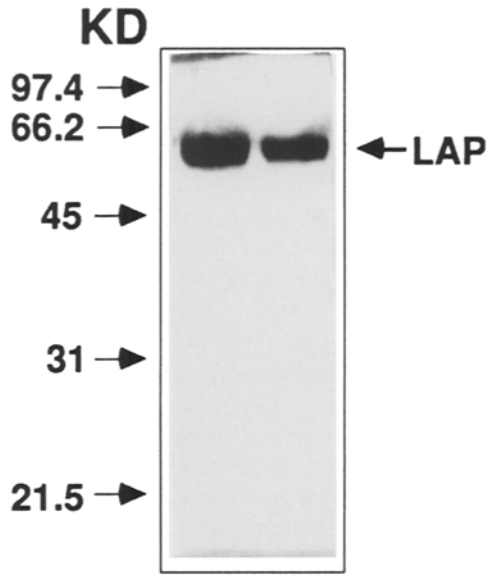

Figure 1 SDS-polyacrylamide gel electrophoresis of the purified *Penicillium citrinum* aminopeptidase. The protein bands were visualized by the silver-staining method [12]. The purified leucine aminopeptidase and half amount of the enzyme (10 μg) was loaded in the left and right lanes, respectively.

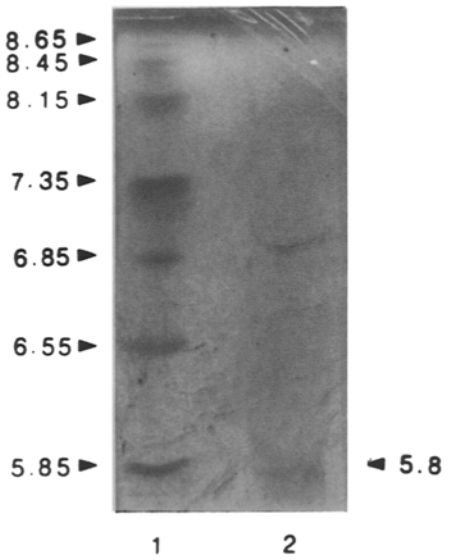

Figure 2 Isoelectrofocusing of the purified *Penicillium citrinum* aminopeptidase. A smile-looking band in the middle of lane 2 is not a true band but a trace mark of the sample loading site.

activity was found within the temperature range of 45–50°C, but the enzyme activity decreased above 55°C (data not shown).

Effects of protease inhibitors

As shown in Table 2, the aminopeptidase was completely inhibited by 1 mM bestatin, which is generally known as an inhibitor of aminopeptidases. Strong inhibition was also observed in the presence of 1,10-phenanthroline (2 mM) and dithiothreitol (2 mM). These results indicate that *Penicillium citrinum* aminopeptidase is a metalloprotease and that intact disulphide bond(s) are required for catalytic activity. Cysteine protease inhibitors such as leupeptin, antipain dihydrochloride, and iodoacetamide had no effect on enzyme activity. This strongly suggests that cysteine may not be localized at the active center of the enzyme. The enzyme was also resistant to aprotinin and lima bean trypsin inhibitor. However, other serine protease inhibitors such as TPCK and 3,4-dichloroisocoumarin inhibited enzyme activity significantly. In addition, the partial inhibition by pepstatin A indicates that Asp may be involved in the reactivity of the enzyme.

Table 2 Effects of protease inhibitors on the enzyme activity

Inhibitors	Concentration[a]	Relative activity (% of control)
None	–	100
Iodoacetamide	1 mM	113
TPCK	1 mM	51
Pepstatin A	50 μM	54
	10 μM	79
Chymostatin	10 μg ml^{-1}	89
Aprotinin	50 μg ml^{-1}	99
Lima bean, trypsin inhibitor	5 μg ml^{-1}	106
Dithiothreitol	2 mM	4
Bestatin	1 mM	0
	0.1 mM	8
Leupeptin	10 μg ml^{-1}	102
Antipain dihydrochloride	10 μg ml^{-1}	105
3,4-Dichloroisocoumarin	1 mM	53
L-Leucinethiol, oxidized	50 μg ml^{-1}	101
Elastatinal	100 μg ml^{-1}	98
Phosphoramidon	100 μg ml^{-1}	105
NEM	2 mM	108
PCMB	2 mM	115
1,10-Phenathroline	2 mM	6

[a]Concentration of inhibitors in the preincubation mixture. After the mixture including indicated inhibitors and 0.4 μg of the enzyme was preincubated at 37°C for 20 min, L-Leu-*p*NA substrate was added to the final 1-mM concentration.
Abbreviations: TPCK (tosylphenylalanine chloromethylketone); NEM (N-ethylmaleimide); PCMB (*p*-chloromercuribenzoate).

Effects of chemical reagents

As shown in Table 3, the enzyme failed to catalyze the hydrolysis of substrates when incubated at acidic pH (1% or 5% acetic acid). This may be caused by metal-ligand instability of the enzyme under these conditions. The low activity in acidic conditions appears to be similar to leucine aminopeptidases obtained from many bacteria studied in our laboratory (unpublished results). A 6-M chaotropic agent (urea) or solvents such as 10% (v/v) ethanol and methanol had no effect on enzyme activity. However, increasing the concentration of these alcoholic solvents to 50% inhibited the enzyme activities. The aminopeptidase was readily inactivated by EDTA or 1,10-phenanthroline treatment. The addition of 1 mM Co^{2+} to EDTA-treated enzyme restored the activity up to 90% of control. The activity of EDTA-treated enzyme was not restored by addition of Zn^{2+}, but reconstitution with Ca^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+} restored enzyme activity up to 11%, 11%, 10% and 7%, respectively.

Table 3 Effects of chemical reagents on enzyme activity[a]

Chemicals	Final concentration	Relative activity (% of control)
None	–	100
PMSF	1 mM	102
	2 mM	108
EDTA	1 mM	41
Mercaptoethanol	1%	4
SDS	0.1%	99
Urea	6 M	94
Acetic acid	1%	4
	5%	3
Ethanol	10%	107
	50%	49
Methanol	10%	94
	50%	70
Acetonitrile	5%	103
	10%	109

[a]A 0.5-μg of sample aminopeptidase was preincubated with the chemicals indicated overnight at 4°C. The remaining activity in mixture was determined in 100 mM Tris (pH 8.0) at 37°C. PMSF (phenylmethylsulphonylfluoride) was dissolved in DMSO (dimethylsulfoxide).

Substrate specificity

Para-nitroanilide (*p*NA) derivatives of a series of L-amino acids were used to measure the relative activity of *Penicillium citrinum* aminopeptidase. Table 4 compares the hydrolyzing rates of seven *p*NA derivatives of amino acids. The highest rate of hydrolysis was observed with L-Leu-*p*NA. The aminopeptidase cleaved the hydrophobic side chains (L-Met-*p*NA and L-Ala-*p*NA) with higher efficiency (33% and 27%, respectively) than other amino acid derivatives. It is noteworthy that the aminopeptidase was much less favorable for bulky nonpolar amino acids such as Phe than for linear hydrophobic residues. The K_m values for two synthetic substrates, L-Leu-*p*NA and L-Met-*p*NA were determined by using a Lineweaver–Burk plot and were found to be 2.7 mM and 1.8 mM, respectively.

Because *Penicillium citrinum* aminopeptidase has a strong affinity for hydrophobic amino acids, we applied the enzyme in order to eliminate N-terminal methionine from recombinant Met-hGH. FPLC chromatography using a Mono-S column allowed for an efficient separation of hGH products from the reaction mixture. The first amino acid of the aminopeptidase treated-hGH was Phe and the second was Pro (data not shown). Therefore, the affinity of the enzyme for L-Met-*p*NA was confirmed by the N-terminal processing of recombinant Met-hGH which starts with Met-Phe-Pro-Thr.

Discussion

Fungi are classified phylogenically between procaryotes and higher eukaryotic organisms. It can be assumed that a fungal enzyme can have a variety of intermediate physicochemical properties, some aspects of which are equivalent to those of lower or higher organisms. We found that *Penicillium citrinum* leucine aminopeptidase was a monomer with 63 kDa molecular mass, relatively large in size compared to those of bacterial aminopeptidases but smaller than those of mammals [22].

The *Penicillium citrinum* aminopeptidase requires Co^{2+} for activity. Although most of the known aminopeptidases are metalloproteases with zinc-binding sites, there are some exceptions [7,13]. Direct evidence supporting the view that *Penicillium citrinum* aminopeptidase is a Co^{2+}-requiring enzyme comes from the metal reconstitution study. The enzyme was reconstituted with a variety of metal ions after being incubated with 10 mM EDTA overnight at 4°C, a high enough concentration for chelating. The aminopeptidase activity is directly dependent upon Co^{2+} when compared to other metal ions (data not shown). The addition of Co^{2+} after complete removal of the endogenous metal ions by EDTA treatment allowed the aminopeptidase to regain the activity up to 90% of the maximum activity. These results support the hypothesis that Co^{2+} plays an important role in the catalysis or stability of the *Penicillium citrinum* aminopeptidase, as zinc plays a similar role in other leucine aminopeptidases. In contrast, the zinc treatment completely destroyed the activity of *Penicillium citrinum* aminopeptidase. Similar zinc inhibition was observed with methionine aminopeptidase from yeast [7] and in another metalloprotease, carboxypeptidase A [15]. There have been few reports regarding the aminopeptidase inhibition by zinc *per se*. In most of the aminopeptidases studied, zinc ions increase enzyme activity or stabilize the protein structure. Laren *et al* suggested that enzyme inhibition by zinc might result from the formation of stable hydrogen bonding between Zn^{2+} and the catalytic metal ion [15].

Table 4 Substrate specificity of the *Pencillium citrinum* aminopeptidase

Synthetic peptides	Relative rate (%) of hydrolysis (*p*-nitroanilide)
L-Leu-*p*NA	100
L-Met-*p*NA	33
L-Ala-*p*NA	27
Gly-Phe-*p*NA	22
L-Lys-*p*NA	19
Gly-*p*NA	18
Gly-Pro-*p*NA	13
L-Phe-*p*NA	8
L-Pro-*p*NA	6

The Michaelis–Menten constant shows that *Penicillium citrinum* aminopeptidase maintained a 50% higher affinity for Met-*p*NA than that for Leu-*p*NA. However, the hydrolyzing rate of Leu-*p*NA is about three-fold faster than that of Met-*p*NA (Table 4). From the combination of these results, we conclude that *Penicillium citrinum* aminopeptidase has a much faster turnover rate (Kcat) with Leu-*p*NA than with Met-*p*NA.

The *Penicillium citrinum* aminopeptidase can be used in the production of Met-free recombinant proteins. The removal of the N-terminal methionyl residue from Met-hGH has been an important subject with which bioindustry has dealt. This study shows that the *Penicillium citrinum* aminopeptidase cleaves the N-terminal methionine residue of Met-hGH selectively and efficiently. To achieve a high yield of N-terminal methionine removal, the reaction mixture was buffer-exchanged once with fresh reaction buffer. This treatment was particularly useful for reducing product inhibition due to the accumulation of free methionine molecules. N-terminal sequencing data revealed that the first amino acid of aminopeptidase-treated hGH is Phe and the second residue is Pro (data not shown), indicating no further amino acid cleavage by the enzyme. In *in vitro* N-terminal processing experiments, the reaction ratio of the enzyme *vs* the protein was 1 : 10 (w/w). Thus, *Penicillium citrinum* aminopeptidase has a high specificity for the methionyl residue of the protein and is able to recognize the Xaa-Pro sequence which is a stop signal for leucine aminopeptidases. These results are consistent with the substrate specificity of aminopeptidase shown in Table 4. This study demonstrates that *Penicillium citrinum* leucine aminopeptidase is applicable for the production of authentic recombinant proteins.

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