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Selective removal of N-terminal methionine from recombinant human growth hormone by an aminopeptidase isolated from *Aspergillus flavus*

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An aminopeptidase was isolated from a soil fungus, which specifically cleaves the unnatural N-terminal methionine in recombinant human growth hormone. Reaction mixtures with different ratios of aminopeptidase to recombinant methionyl human growth hormone showed that the removal of N-terminal methionine was complete at 1:200 (w/w), and more than 90% complete at ratios up to 1:2000 (w/w) when incubated for 24 h at 37°C. The data indicate that the aminopeptidase we have purified can be used for the efficient conversion of unnatural recombinant proteins to their natural form.

Keywords: aminopeptidase; N-terminal methionine; recombinant protein; recombinant human growth hormone

Recombinant DNA technology has permitted the large-scale production of eucaryotic proteins expressed in bacterial systems. In eucaryotes most of the proteins lack an N-terminal methionine residue due to post-translational processing. In procaryotes, however, the recombinant proteins are massively overproduced and N-terminal methionine processing is incomplete. Therefore, the production of heterologous proteins in procaryotic systems often results in the presence of a foreign N-terminal methionine residue [1]. When the recombinant protein is administered to eucaryotes, the extra methionine residue at the N-terminus may cause an immune reaction [4]. Therefore, it would be desirable to remove the N-terminal methionine, and to produce the mature eucaryotic protein. A number of aminopeptidases have been isolated from microbial sources and tested for N-terminal methionine processing of recombinant proteins [6]. *Aeromonas* aminopeptidase purified by Prescott and Wilkes [5] appears to remove N-terminal methionine, but many other residues are also cleaved in addition to the methionine. Aminopeptidase from *Aeromonas proteolytica* was a suitable enzyme for N-terminal methionine processing of recombinant methionyl human growth hormone (met-rhGH) when the aminopeptidase was used at the amount of 1/400 of processed protein [2]. We have isolated *Aspergillus flavus* from soil, and purified an aminopeptidase from it. In this study we demonstrate that the purified aminopeptidase is an efficient enzyme for the N-terminal methionine processing of met-rhGH.

Materials and methods

Microorganism and culture conditions

Aspergillus flavus isolated from soil was used as a source of the enzyme. For culturing cells, mycelium tips of *Aspergillus flavus* in 1.5% (w/v) agar (Difco; Detroit, MI, USA) were inoculated into a medium containing 0.3% (w/v) yeast extract, 0.3% (w/v) peptone, and 0.1% (w/v) KH_2PO_4. The culture was incubated for about 80 h at 30°C with shaking at 250 rpm. The culture was filtered through Whatman No.1 paper, and the filtrate was collected for the purification of aminopeptidase.

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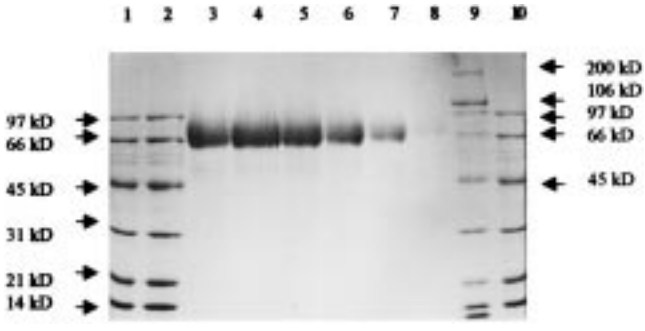

Figure 1 SDS-PAGE of purified aminopeptidase. Micrograms (15, 12, 10, 7, 5 and 2) of purified aminopeptidase were loaded in lanes 3–8. Molecular weight markers (lanes 1, 2, 9 and 10) are also shown.

Table 1 Properties of the purified aminopeptidase preparation

Property	Aminopeptidase	Endoprotease
Substrate	Leucine-pNA	resorufin-casein
Max absorption λ	405 nm	574 nm
ε (M^{-1} cm^{-1})	9900	66000
Absorbance $Unit^{-1}$	1.36	0.00015
Relative activity	9067	1

Aminopeptidase assay

Aminopeptidase activity was measured spectroscopically at 405 nm using a synthetic substrate, l-Leucine-*p*-nitroanilide (LpNA; Sigma) [3]. Enzyme solution (0.1 ml) was

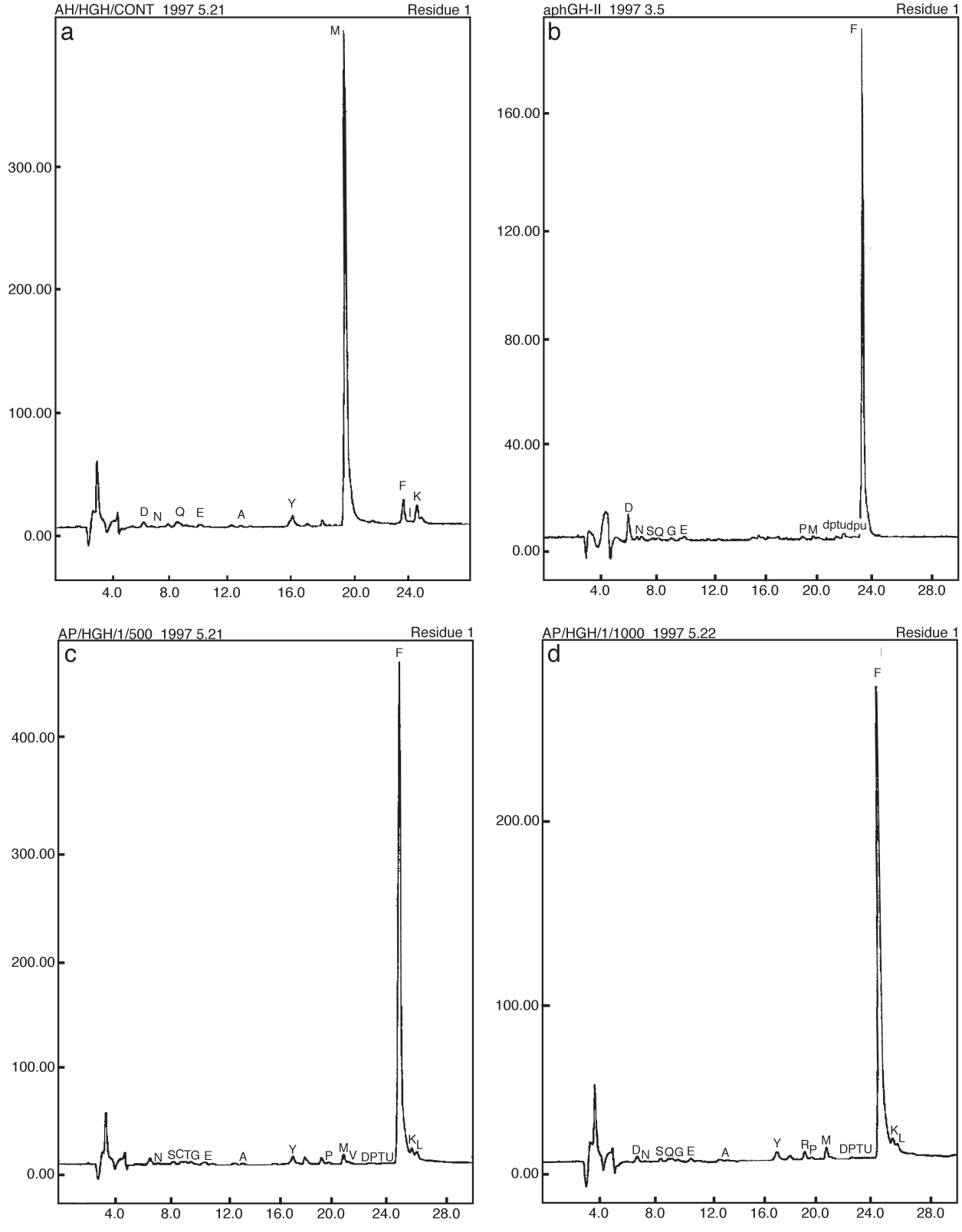

Figure 2 N-terminal sequencing of rhGH: (a) met-rhGH; (b) met-rhGH incubated with aminopeptidase with a ratio of aminopeptidase: rhGH = 1:200; (c) 1:500; (d) 1:1000; (e) 1:1500; (f) 1:2000 for 24 h. The first cycle of untreated met-rhGH shows a single methionine peak, whereas that of met-rhGH digested by aminopeptidase is phenylalanine.

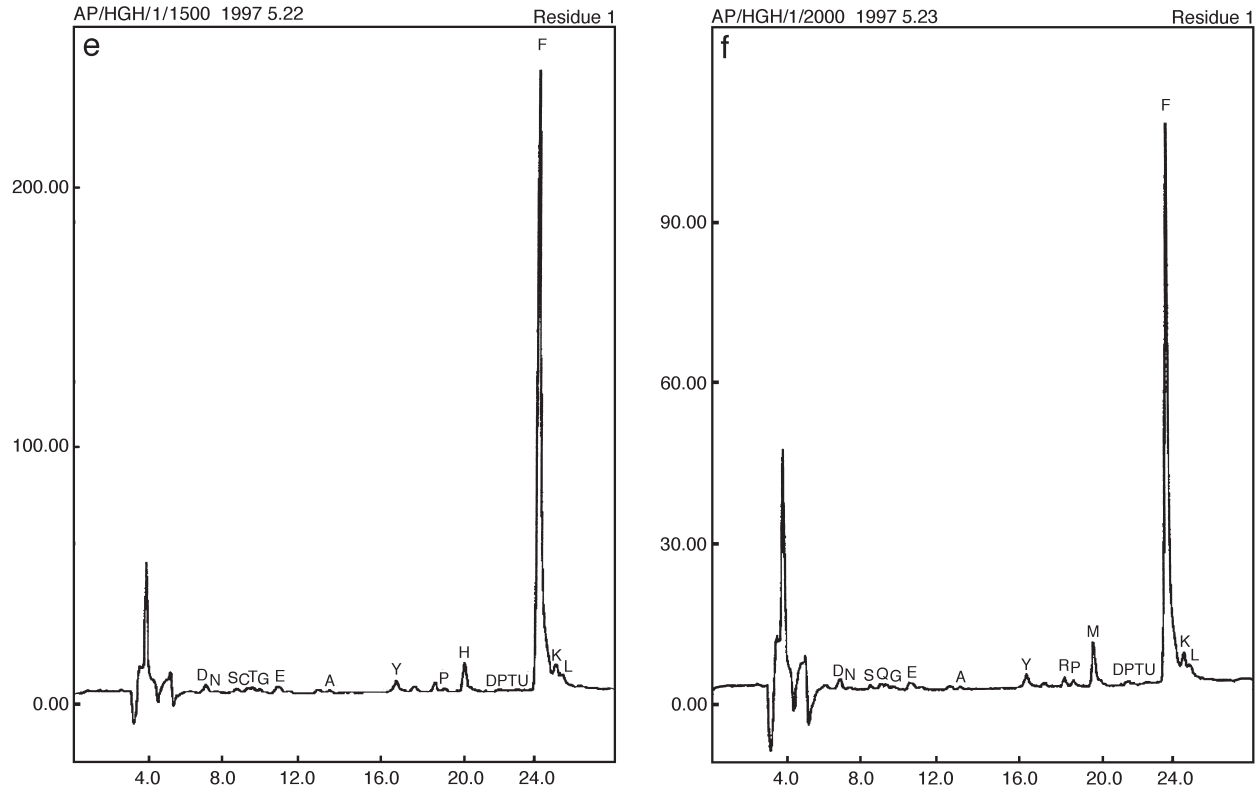

Figure 2 Continued.

mixed with 1 mM LpNA in 0.9 ml of 0.1 M Tris-HCl, pH 8.0 buffer and incubated at 37°C. The reaction was stopped by adding 0.1 ml of 70% (w/v) acetic acid. One unit of aminopeptidase is defined as the amount of enzyme required to produce 0.1 μmoles of *p*-nitroanilide per minute.

Endoprotease assay

The resorufin-casein assay [7] was used to measure endoprotease activity. Fifty microliters of 0.4% (w/v) resorufin casein (Boehringer Mannheim; Germany) were mixed with 100 μl enzyme solution and 50 μl of 0.2 M Tris-HCl, pH 7.8 buffer containing 0.02 M $CaCl_2$, and incubated at 37°C for 30 min to 6 h depending on the purity of the enzyme solution. The reaction was stopped by adding 0.48 ml of 5% (w/v) trichloroacetic acid (TCA), and incubating the mixture at 37°C for 10 min. The reaction mixture was centrifuged for 5 min, 0.4 ml of the supernatant phase was mixed with 0.6 ml of 0.5 M Tris-HCl, pH 8.8 buffer, and the absorbance was measured at 574 nm.

Purification of the aminopeptidase

The aminopeptidase was purified by anion-exchange chromatography followed by two gel-exclusion chromatographies. The filtered medium was loaded onto a Q-Sepharose (Pharmacia; Uppsala, Sweden) 2.5×10 cm column (Bio-Rad; Hercules, USA) which had been equilibrated with 20 mM phosphate, pH 7.0 buffer, followed by an extensive washing with the same buffer. Elution was carried out with the same buffer containing 0.5 M NaCl. The fractions having enzymatic activity were collected, concentrated using a YM10 membrane in a stirred cell (Amicon; Beverly, MA, USA), and loaded onto a Sephacryl S-200 (Pharmacia) 1.5×100 cm column (Bio-Rad) which had been equilibrated with 0.05 M Tris-HCl, pH 8.0 containing 0.15 M NaCl solution. The enzyme fractions eluted with the same buffer were collected and reloaded onto a Sephacryl S-200 column (1.5×75 cm), and the last step was repeated.

N-terminus removal by aminopeptidase

Recombinant methionyl human growth hormone (met-rhGH) expressed in yeast was used as substrate for N-terminus methionine cleavage by the aminopeptidase. N-terminal methionine was removed from met-rhGH by mixing aminopeptidase and rhGH at the ratios, 1:200, 1:500, 1:1000, 1:1500 and 1:2000 (w/w), and incubating the mixture in 0.05 M Tris-HCl, pH 8.0 containing 0.1 M NaCl at 37°C for 24 h. The cleavage reaction was stopped by adding trichloroacetic acid (final concentration, 10% w/v). The precipitate was collected by centrifugation and the degree of methionine removal was assayed by N-terminal sequencing using an Automatic Sequencer 471A (Applied Biosystems; Foster City, USA).

Results and discussion

The aminopeptidase was purified using three conventional chromatographic columns. Figure 1 shows the purity of the homogenous enzyme. In order to be used for N-terminal methionine processing of a recombinant protein, the purified aminopeptidase preparation should lack endopeptidase activity. Table 1 compares the aminopeptidase and endo-

Table 2 Cleavage of N-terminal methionine from met-rhGH by the purified aminopeptidase

Aminopeptidase:met-rhGH (w/w)	Methionine cleavage (%)
1:200	100
1:500	98
1:1000	97
1:1500	95
1:2000	91

protease activities of the purified aminopeptidase preparation. The endopeptidase activity of the purified aminopeptidase was negligible. In addition SDS-PAGE of rhGH incubated with the purified aminopeptidase showed that the aminopeptidase preparation did not cause the internal cleavage of rhGH (data not shown).

Sequencing of rhGH incubated with the aminopeptidase indicated a single N-terminus amino acid, phenylalanine. As shown in Figure 2a, the N-terminus amino acid of untreated rhGH is methionine, whereas that of rhGH incubated with the aminopeptidase is phenylalanine (Figure 2b–f). Sequencing data also indicate that the N-terminal processing reaction stopped after the complete removal of the N-terminal Met residue and did not proceed to cleave the next Phe residue of met-rhGH. The degree of N-terminus methionine cleavage was calculated from the molar ratios in phenylalanine and methionine peaks. Table 2 summarizes the degree of methionine cleavage of met-rhGH by different amounts of aminopeptidase. Cleavage was more than 90% complete at an aminopeptidase to met-rhGH ratio of 1:2000 (w/w), and complete at the ratio of 1:1000 (w/w).

In summary, our data indicate that the aminopeptidase purified from *Aspergillus flavus* is an efficient enzyme for the cleavage of the N-terminal methionine of proteins. At this moment, it is not clear why the enzyme is specific for the cleavage of Met residue and does not cleave Phe, the first residue of the native hGH. It may be that the enzyme does not use N-terminus Phe as a substrate, or Pro at the second residue acts as a stop signal for the cleavage as in the case of *Aeromonas* enzyme [8].

Acknowledgements

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