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Short communication

Effect of Deglycosylation on the Aminopeptidase Isolated from *Aspergillus flavus*

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A leucine aminopeptidase has been isolated from the culture medium of the soil fungus, *Aspergillus flavus*. The enzyme was found to be a glycoprotein, as judged by electrophoresis analysis and the subsequent staining by the periodic acid-Schiff's reagent. Carbohydrate moieties could be cleaved by N-glycosidase, but not by O-glycosidase, indicating that the glucans are linked to the asparagine residue in the protein. Removal of N-glucans was observed without prior denaturation of the protein, implying that the N-glycosidic linkage is exposed and accessible to glycosidase. When the activity of native or deglycosylated enzyme was measured in the presence of various metal ions, removal of carbohydrates increased the aminopeptidase activity of the enzyme.

Keywords: Aminopeptidase, N-glycosylation, Glycoprotein, Metalloprotease.

Leucine aminopeptidases (LAP) catalyze the hydrolysis of the peptide bond adjacent to free alpha-hydrophobic amino acids such as leucine or methionine. They are widely distributed in nature, and a variety of LAPs from many different types of tissues and organs have been isolated and characterized (Taylor, 1993). All the enzymes obtained from organisms such as *Escherichia coli* (Vogt, 1970), *Aeromonas proteolytica* (Prescott and Wilkes, 1976), *Streptomyces griseus* (Spungin and Blumberg, 1989), and *Penicillium citrinum* (Kwon *et al.*, 1996) have been reported to be metalloproteases. In spite of variable enzyme sources, a common feature found in most aminopeptidases is the binding of one or two zinc atoms per monomer, while some aminopeptidases require cobalt ion for full enzyme activity (Spungin and Blumberg, 1989; Guenet *et al.*, 1992). LAPs from microbial sources have been used for *in vitro* N-terminal methionine processing of the heterologous eukaryotic proteins expressed in bacterial systems (Ben-Bassat *et al.*, 1987; Taylor, 1993).

We have previously isolated a 70 kDa LAP from the culture media of *Aspergillus flavus* and demonstrated that it efficiently processed the N-terminal methionine of the recombinant human growth hormone expressed in bacteria (Cho *et al.*, 1997). From this study, we showed that the LAP from *Aspergillus flavus* was a glycoprotein, and examined the effect of the carbohydrate moiety and metal ions on the activity of the enzyme.

Materials and Methods

Periodic Acid-Schiff's (PAS) staining PAS staining for the glycoprotein was carried out as described by Kapitany *et al.* (1973). After SDS-PAGE, the gel was fixed in 12.5% trichloroacetic acid for 1 h, incubated in 1% periodic acid (Sigma, St. Louis, USA) for 2 h, and washed with 15% acetic acid for 2 h. The periodic reaction was performed by adding Schiff's reagent (Sigma, St. Louis, USA) and the gel was stored at 4°C for 2 h in the dark. Destaining with 10% acetic acid developed the light purple color for glycoproteins.

Aminopeptidase assay Aminopeptidase activity was determined spectroscopically at 405 nm using a synthetic substrate, L-Leucine-*p*-nitroanilide (LpNA; Sigma) (Lin and van Wart, 1982). In 0.9 ml of 0.1 M Tris·HCl buffer (pH 8.0), 0.1 ml of the enzyme solution was mixed with 1 mM LpNA, and then incubated at 37°C for 10 to 30 minutes. The reaction was stopped by adding 0.1 ml of 70% acetic acid, and the absorbance at 405 nm was measured. One unit of aminopeptidase was defined as the amount of enzyme required to produce 0.1 mmoles of *p*-nitroanilide per minute.

Deglycosylation of aminopeptidase Deglycosylations of the purified aminopeptidase were carried out in the absence or the presence of SDS using N-glycosidase F or O-glycosidase (Boehringer Mannheim, Germany). Aminopeptidase was denatured by heating at 100°C for 5 min in the presence of 0.05%

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SDS, 0.1 M Hepes, pH 7. Subsequently, Triton X-100 was added in a 10-fold excess of SDS and 0.8 units of N-glycosidase or 1 milliunit of O-glycosidase was added per μg of aminopeptidase, and then incubated at 37°C overnight. Deglycosylation of aminopeptidase under nondenaturing conditions was performed by incubation of 1 μg aminopeptide with 0.8 U of N-glycosidase F, overnight at 37°C.

Results and Discussion

The 70 kDa enzyme used in this study was isolated from *Aspergillus flavus* as described previously (Cho *et al.*, 1997). It was shown to be a typical glycoprotein since it could bind to Con A sepharose and stained with PAS on electrophoresis gel (Fig. 1). The carbohydrate moieties were cleaved by N-glycosidase (Fig. 2), but not by O-glycosidase (data not shown), indicating that the carbohydrates may be linked to an asparagine residue by N-glycosylation. Since the carbohydrates were removed without prior denaturation of the aminopeptidase, the linkage between Asn and glycans appears to be exposed and accessible to the N-glycosidase (Fig. 2). After deglycosylation, the molecular weight of the enzyme reduced from 70 kDa to 55kDa which is similar to the 50 kDa aminopeptidase from the fungus *Penicillium* (Kwon *et al.*, 1996).

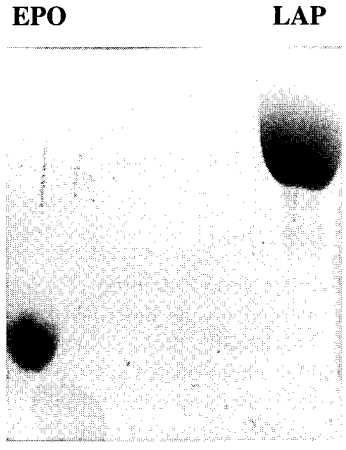

Fig. 1. PAS staining of aminopeptidase. Leucine aminopeptidase (LAP) is shown along with erythropoietin (EPO).

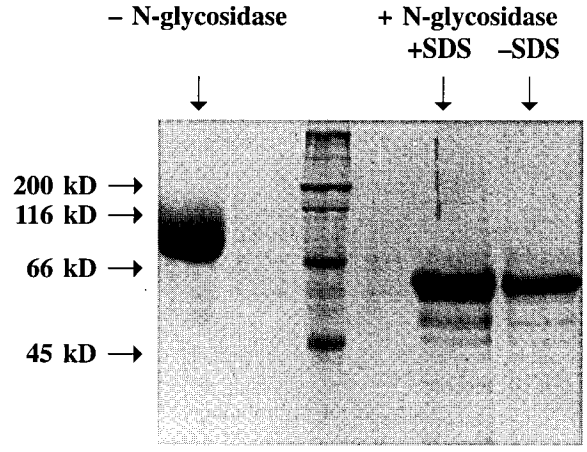

Fig. 2. Deglycosylation of Aminopeptidase. Molecular weights of markers are 200 kDa (myosin), 116 kDa (β-galactosidase), 66 kDa (serum albumin), and 45 kDa (ovalbumin). Aminopeptidase incubated without N-glycosidase (– N-glycosidase) is shown. N-glycosidase digestions of the aminopeptidase (+ N-glycosidase) were done with the denatured (+SDS) and the native (–SDS) aminopeptidase.

Deglycosylation under denaturing conditions completely abolished the enzyme activity, but the removal of N-glycans in the absence of SDS appeared to increase the enzyme activity almost twice (Table 1). The effect of Zu, Mn, and Co ions on the activity of the native or deglycosylated enzyme has been examined. The activity of both forms of the enzyme was not affected by 1 mM of Zn or Mn. However, Co ion increases the activity of the deglycosylated and native enzyme by 2.6 to 3.2 fold, respectively (Table 1). When the metal component of the native enzyme was examined by inductively-coupled plasma atomic emission spectroscopy (ICP-AES), the enzyme was found to contain 2.5 zinc atoms per protein

Table 1. Exopeptidase activity of aminopeptidase in the presence of various metal ions after deglycosylation.

Metal Ions[a]	Relative Activity(%)[b]					
	N-glycosidase –	SDS –	N-glycosidase +	SDS -	N-glycosidase +	SDS +
None	100		192		5	
Co^{++}	326		499		1	
Zn^{++}	86		96		1	
Mn^{++}	118		179		5	

[a] Enzyme in the reaction buffer was preincubated with 1 mM of the respective metallic ion at 37°C for 15 min and then the substrate was added to the final concentration of 1 mM.

[b] Deglycosylation with or without SDS was carried out as described in Materials and Methods.

monomer. Replacement of zinc by the cobalt ion increases the enzyme activity, as observed in other aminopeptidases (Spungin and Blumberg, 1989; Guenet *et al.*, 1992). On the other hand, addition of extra metal ions did not restore the SDS-denatured deglycosylated enzyme.

We have shown that the purified aminopeptidase specifically cleaves the N-terminal methionine of the recombinant human growth hormone expressed in bacteria, and can be used for the efficient conversion of unnatural recombinant proteins to their natural form (Cho *et al.*, 1997). In order to produce the aminopeptidase in large quantities for industrial use, the enzyme needs to be cloned and produced as a recombinant enzyme. If the N-glycosylation of the enzyme was important, the necessity of further glycosylation would limit the host of the enzyme production to eukaryotes. Our data supports the conclusion that the deglycosylated aminopeptidase is active and may be used for the removal of the N-teminal methionine of proteins. To our knowledge, the *Aspergillus* aminopeptidase characterized in this study is the first microbial aminopeptidase which contains carbohydrates. The reason for the glycosylation or the particular role of the carbohydrate in this enzyme remains to be investigated further.

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