# Isolation and characterization of a protease deficient mutant of *Aspergillus niger*

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## Abstract

*Aspergillus niger* has been used as a host system to express many heterologous proteins. It has various advantages over other expression systems in that it is a small eukaryotic GRAS (Generally Recognized as Safe) organism with a capacity of secreting large amount of foreign proteins. However, it has been known that the presence of an abundant protease is a limiting factor to express a heterologous protein. The proteases deficient mutants of *A. niger* were obtained using UV-mutagenesis. A total of $1 \times 10^5$ spores were irradiated with 10-20% survival dose of UV, 600J/M2 at 280nm, and the resulting spores were screened on the casein-gelatin plates. Ten putative protease deficient mutants were further analyzed on the starch plates to differentiate the pro from the secretory mutant. An endogenous extracellular enzyme, glucose oxidase, was also examined to confirm that the mutant phenotype was due to the proteases deficiency rather than the mutation in the secretory pathway. The reduced proteolytic activity was measured using SDS-fibrin zymography gel, casein degradation assay, and bio-activity of a supplemented hGM-CSF (human Granulocyte-Macrophage Colony Stimulating Factor). Comparing with the wild type strain, less than 30 % of proteolytic activity was observed in the culture filtrate of the protease deficient mutant (pro -20) without any notable changes in cell growth and secretion.

## Introduction

Filamentous fungi are used in a variety of industrial processes, including the production of fermented foods, primary metabolites such as organic acids, vitamins, and secondary metabolites. Moreover, filamentous fungi are excellent producers of a broad spectrum of extracellular enzyme, and it has advantages over that other expression systems in that it is a small eukaryotic GRAS

(Generally Recognized as Safe) organism with a capacity of secreting a large amount of foreign proteins. However, yields of heterologus proteins have generally been low compared to those of homologous proteins, because the presence of an abundant protease is a limiting factor to express a heterologous protein. To improve the expression of heterologous proteins in *A. niger*, we isolated several protease deficient mutants using a UV- mutagenesis, and characterized the proteolytic activity of mutants comparing with impaired degradation of several protein substrates. Here we report a mutant which shows a considerable decrease of extracellular protease activity.

## Matreial and Methods

**Starins and Media.** *A. niger* ATCC2119 was used as a host strain in the UV-mutagenesis experiments. *A. niger* was grown on complete medium (CM) or on minimal medium (MM) with a composition according to Pontecorvo (1953).

**Mutagenesis.** Conidial suspension was made in Tween 20 (0.02%) with vigorous shaking to break conidial chains and to reduce conidial aggregation. These conidial suspension was filtered, inoculated at $2 \times 10^8$/ml, and incubated at 30。C on shaking incubator. A suspension of freshly harvested spores of *A. niger* ATCC2119, grown on CM, was exposed to 600J/$M^2$ UV light from a philips TUV lamp emitting UV-C light at 280 nm. UV-treated spores (survival rate 10-20%) were plated for single colonies on casein-gelatin medium containing 0.01% Triton X-100 to restrict colony diameter. After 72hr of incubation, a turbid halo is formed around the colonies due to extracellular proteolytic activities.

**Determination of proteolytic activity.** Proteolytic activities of mutants were determined by extracellular glucose oxidase (GO) activity, hGM-CSF assay, fibrin zymogrpahy gel assay (Choi and Kim, 2000), and casein hydrolysis (Mang et al., 2001).

## Results and discussion

**Isolation of mutants.** Several protease-deficient mutants were isolated by combination of UV mutagenesis and Casein hydrolysis as described on Materials and Methods. Ten putative protease deficient mutants were further analyzed on the starch plates to differentiate the pro from the secretory mutant. Out of surviving colonies, two pro mutants (#20, #4) were obtained. These mutants had

a reduced or no halo on milk plates and a normal halo on starch plates. #4 mutant showed reduced growth and/or sporulation rates (Fig. 1). An endogenous extracellular enzyme, glucose oxidase, was also examined to confirm that the mutant phenotype was due to the protease deficiency rather than the mutantion in the secertory pathway (Fig. 2).

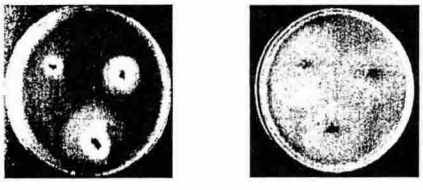


fig.1.A, Proteolytic activity of wild type strain and mutant strains on the casein gelatin plate. B, Cell growth and starch hydrolysis of wild type and mutant strains on starch plate. 1, pro--4; 2, pro--20; 3, wild type strain.

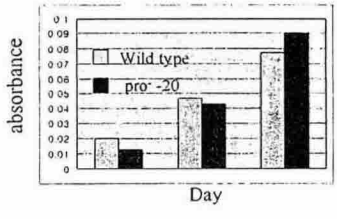


Fig. 2. Comparison of secretory enzyme, glucose oxidase (GO), activity of wild type and pro-20 strain. One unit of GO activity was defined as the amount of enzyme that will oxidize 1.0 $\mu$ mole of$\beta$ -D-gluconic acid and $H_2O_2$ per minute at pH 5.1 at 35 oC.

**Determination of protease activity.** Protease activity in culture filtrate of pro˙20 mutant was determined by measuring the degradation of fibrinogen on SDS-PAGE gel (Fig. 3), and by measuring the degradation of casein (Fig. 4). The protease activity of wild type was increased on the culture time, but pro-20 mutant was consistant. Comparing with the wild type strain, less than 30% of proteolytic activity was observed in the culture filtrate of the protease deficient mutant (pro -20) without any notable changes in cell growth and secretory mechanism.

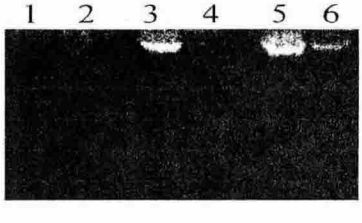


Fig 3. Fibrin zymology assay: SDS-fibrin PAGE gel for detection of ptotease analysis of wild type and pro-20. Lane1,2-wild type 1day, pro-20 1day, Lane3,4-wild type 3day, pro-20 3day Lane5,6-wild type 5day, pro-20 5day

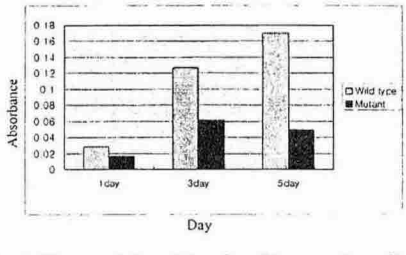


Fig. 4. The proteolytic activity of wild type and pro-20 strain on the casein hydrolysis assay . One unite of proteolytic activity of casein was defined as the amount of enzyme that will increase the absorbance at 280nm of 0.1.

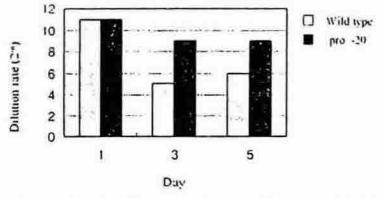


Fig.5. Comparison of proteolytic activity of wild type and pro- -20 strain with hGM-CSF assay. The proteolytic activiy was compared with the dilution rate having the significant cpm on hGM-CSF assay. The culture filtrates obtained from different culture time were incubated with serial diluted hGM-CSF, and the most dilution rate of culture filtrates supplying the hGM-CSF dependent cell line were represented

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