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Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases

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Summary. In the present study, the extracellular protease activity in a strain of the filamentous fungus *Aspergillus niger* was investigated and mutant strains deficient in the production of extracellular proteases were isolated. The major protease, which is responsible for 80–85% of the total activity, is aspergillopepsin A, a protein of ca. 43 kDa, the activity of which is inhibited by pepstatin. In addition, a second protease, aspergillopepsin B, is produced, which is much less sensitive to inhibition by pepstatin. Several protease-deficient mutants were obtained by in vivo UV mutagenesis. In addition, a mutant lacking aspergillopepsin A was constructed by an in vitro gene replacement strategy. In this mutant, AB1.1, the entire coding region of the gene for aspergillopepsin A *(pepA)* is deleted. In three UV-induced mutants, aspergillopepsin A is also missing. One of these mutants, AB1.18, is mutated in the *pepA* gene, which is located on chromosome I. One of the other mutants, AB1.13, which has only 1–2% of the extracellular protease activity in the parent strain, is deficient in both aspergillopepsin A and aspergillopepsin B. The mutation involved, *prt-13,* has been localized to chromosome VI, and is probably a mutation in a regulatory gene. Another mutation involved in loss of protease function, *prt-39,* is located on chromosome VIII. Degradation of various heterologous proteins in culture media of the mutants is reduced but, even in strain AB1.13, not completely abolished.

Key words: *Aspergillus niger* – Extracellular proteases – Protease-deficient mutants – Parasexual analysis – Aspergillopepsin A – Heterologous protein degradation

Introduction

Several species of filamentous fungi have been used for many years in industrial processes for the production of metabolites and enzymes, such as citric acid and (gluco)-amylases (Barbesgaard 1977; Bennett 1985). To improve the production of homologous enzymes, classical mutagenesis and screening methods have been used. The recent development of recombinant DNA techniques for filamentous fungi (Timberlake 1991) has provided an alternative approach to the improvement of the production of homologous proteins, as well as the possibility to produce heterologous proteins in these fungi (van den Hondel et al. 1991). *Aspergillus* species, in particular *Aspergillus niger,* are attractive candidates for large-scale production of heterologous proteins, since they have the capacity to secrete large amounts of proteins into the culture medium. Yields of heterologous proteins have generally been low, however, compared to those of homologous proteins. In addition to problems affecting synthesis, proper glycosylation and secretion of these proteins, an important contributory factor may be degradation of heterologous proteins by native fungal proteases present in the culture medium (Cohen 1977; Thompson 1991).

To overcome the problem of protein degradation, it would be helpful to have *A. niger* strains available that are deficient in extracellular protease activity. Using an in vitro gene replacement strategy, Berka et al. (1990) constructed a mutant stain of *A. awamori* lacking the structural gene *(pepA)* coding for the aspartic protease aspergillopepsin A. The proteolytic activity in the culture medium was considerably reduced, as judged by a milk-clotting assay, and evidence was obtained for the existence of additional proteases (Ward 1991).

In the present paper the extracellular protease activity in the culture medium of an *A. niger* strain is investigated. In addition, the isolation and characterization of protease-deficient mutants, obtained either by an in vitro gene replacement strategy or by random in vivo mutagenesis, are described.

Materials and methods

Strains. A. niger strains used are derivatives of strain AB4.1 (van Hartingsveldt et al. 1987) which is a *cspA1 pyrG1* derivative of strain ATTC 9029 (Bos et al. 1988).

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The protease-deficient *(prt)* strains AB1.13 *prt-13,* AB1.18 *prt-18,* AB1.33 *prt-33* and AB1.39 *prt-39* were derived from AB4.1 by UV irradiation. In a second round of UV mutagenesis the double mutant AB1.18-25 *prt-18-25,* was obtained from AB1.18. Mutant strain AB1.1 *pepA1* was isolated by in vitro gene replacement. For complementation analysis a *niaD* derivative of strain AB1.1 was isolated by selection for chlorate resistance. Several colour mutants were isolated for genetic analysis: *fwn* mutants from strains AB4.1, AB1.13, AB1.18, AB1.33 and AB1.1; *niaD* and an *olv* mutant from strain AB1.18. The master strains N661 *cspA1 fwnA1 trpA1 argB2 leuA1 nicA1 pabA1* and N845 *cspA1 fwnA1 pdxA2 nicB5 trpB2* (Bos et al. 1988) were obtained from Dr C.J. Bos (University of Wageningen, The Netherlands).

Media and culture conditions. Aspergillus minimal medium (MM) was as described by Pontecorvo (1953). Spores were inoculated at 2×10^6/ml and incubated for 24 h or 48 h in MM enriched with 0.1% casamino acids (Difco), a 1:2500 diluted vitamin solution (100 mg thiamine, 1000 mg riboflavin, 100 mg p-aminobenzoic acid, 1000 mg nicotinamide, 500 mg pyridoxine, 100 mg pantotheic acid and 2 mg biotin per litre) and 10 mM uridine. Skim milk plates contained: 50 mM Na_2HPO_4/NaH_2PO_4 buffer pH 5.3, 7 mM KCl, 2 mM $MgSO_4$, a trace element mixture (1:1000), 1% glucose, 1% skim milk (Difco), 0.1% casamino acids, 1:2500 vitamin mixture, 0.05% Triton X–100 and additional growth factors as required. Starch plates contained MM plus 0.1% glucose, 10 mM uridine and 1% corn starch (BDH). Spores were generated on potato dextrose agar (Difco).

Isolation of mutants. A suspension of freshly harvested spores of strain AB4.1 in saline (10^7 spores/ml) was exposed to 600 J/m^2 UV light from a Philips TUV lamp emitting 90% of its energy at 245 nm. The irradiated spores (survival rate 10–20%) were diluted and plated on 1% skim milk plates containing 0.05% Triton X–100, to restrict colony growth. After 24 h of incubation at 30° C a turbid zone develops around the growing colonies, due to precipitation of the casein in the milk caused by acidification of the medium. After ca. 48 h the whole plate becomes turbid and a clear halo, due to proteolysis of the casein, is formed around the colonies. The plates were inspected after 72 h and colonies with a smaller clear halo than parental colonies or no halo at all, were purified and tested again on milk plates as well as on 1% starch plates, in order to distinguish between general secretion-deficient mutants and specific protease mutants.

A mutant in which the total coding region of the gene coding for aspergillopepsin A *(pepA)* is deleted was constructed by in vitro gene replacement. The *pepA* replacement vector used, pUC4del AP-pyrG, (kindly supplied by R.M. Berka, Genencor Inc., San Francisco, USA) differed from the vector described by Berka et al. (1990) in that the internal *Sal*I fragment of pUC4del AP-argB, which encodes *argB,* was replaced by a fragment containing the *pyrG* gene from *A. nidulans.* Protoplasts of strain AB4.1 were transformed (van Hartingsveldt et al. 1987) with *Eco*RI-treated pUC4del AP-pyrG and transformants were selected for prototrophy. Pyr$^+$ transformants were screened by Southern and Western blotting for the absence of the *pepA* gene and aspergillopepsin A, respectively. As a probe for the Southern blots, a digoxygenin(DIG)-labelled 576 bp internal fragment of *pepA* (nucleotides 550–1126) was amplified by PCR from pBR322:AP (Berka et al. 1990). Hybridization and detection of the DIG-labelled probe were carried out with a kit (Boehringer Mannheim, UK) according to the manufacturer's instructions.

Protein analysis. SDS-polyacrylamide gel electrophoresis and Western blotting for detection of aspergillopepsin A were performed as described previously (Archer et al. 1990). Antiserum to purified aspergillopepsin A and a sample of authentic aspergillopepsin A were kindly provided by R.M. Berka. For detection of human interleukin-6 (hIL6), the Phast System (Pharmacia) was used according to the protocol supplied by the manufacturer. Antiserum to hIL6 as well as a sample of hIL6 were kindly provided by R. Contreras (State University of Gent, Belgium). Degradation of hIL6 in culture media was measured by incubation of 1 µl hIL6 solution (containing 0.4 µg hIL6) with 5 µl of culture medium at 30° C for 2 h or 18 h. Incubation was terminated by the addition of 2 µl 4× concentrated sample buffer (Pharmacia). After 5 min at 95° C, 1 µl samples were subjected to gel electrophoresis.

Protease assay. Proteolytic activity in 48 h culture media was determined by incubating medium samples with ^{3}H-labelled sperm whale myoglobin at pH 4.0 and measuring the radioactivity in the TCA-soluble fraction, as described by van Noort et al. (1991). Chromatography of culture filtrates on bacitracin-Sepharose was performed as described by van Noort et al. (1991).

Parasexual analysis. Formation of heterokaryons, isolation and haploidization of heterozygous diploids was performed as described by Bos et al. (1988), except that 0.7 µg/ml benomyl was used for haploidization of the diploids.

Results and discussion

Extracellular protease activity in strain AB4.1

Protease activity in culture medium of strain AB4.1, as well as the influence of pepstatin on this activity was determined by measuring the degradation of myoglobin. The result is shown in Fig. 1. It is clear that myoglobin is rapidly degraded. Pepstatin, an inhibitor of aspartyl proteases, strongly reduces this degradation. Degradation in culture medium of another heterologous protein, human interleukin-6 (hIL6), the expression and secretion of which is being studied in our laboratory (M. Broekhuijsen et al., manuscript in preparation) was determined by a Western blot assay. Partial degradation was observed after 2 h of incubation, while after 18 h no intact hIL6 could be detected in media from either 24 h and 48 h

cultures, even in the presence of pepstatin (results not shown). A third heterologous protein, porcine pancreatic lipase A2, is also rapidly degraded in culture medium of AB4.1 (results not shown).

In summary, considerable proteolytic activity is present in culture medium of *A. niger* AB4.1. In a previous investigation (van Noort et al. 1991), a single pH optimum for proteolytic degradation was found at pH 4.0, while at pH 6.5 or higher no detectable degradation of myoglobin occurred. Since pepstatin strongly reduces proteolytic degradation, these observations indicate that the major extracellular protease(s) of *A. niger* AB4.1 belong(s) to the class of aspartyl proteases.

To obtain more insight into the proteases present in the culture medium of AB4.1, and to investigate whether a protease similar to aspergillopepsin A from *A. awamori* is present, the proteases were concentrated and visualized using immobilized bacitracin and SDS–PAGE as described previously (van Noort et al. 1991). The result is shown in Fig. 2a (lane 2). A number of prominent as well as minor bands can be seen on the gel. The protein with a molecular weight of ca. 43 kDa is aspergillopepsin A, as it reacts with polyclonal antibodies against this enzyme (Fig. 2b, lane 2) isolated from the closely related species *A. awamori* (Berka et al. 1990). Some of the smaller bands may represent degradation products of aspergillopepsin A. The results suggest that other proteases besides aspergillopepsin are secreted into the culture medium, although it cannot be excluded that some bands represent proteins other than proteases (Van Noort et al. 1991).

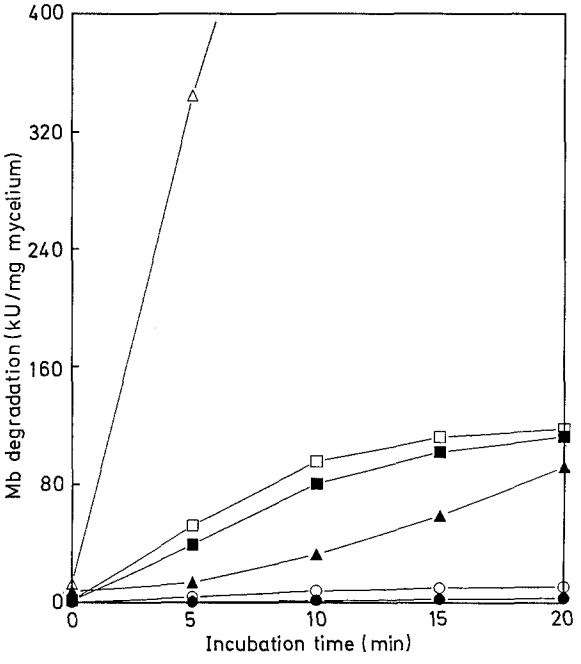

Fig. 1. Degradation of myoglobin in culture media of strains AB4.1, AB1.13 and AB1.18. *Open* symbols indicate assay in the absence, *closed* symbols assay in the presence of pepstatin (20 µg/ml). Triangles, AB4.1; circles, AB1.13; squares AB1.1

Isolation of protease-deficient mutants

Several protease-deficient *(prt)* mutants were isolated after in vivo mutagenesis by UV irradiation and screening for reduced degradation of casein in milk plates. To exclude secretion mutants, potential *prt* mutants were tested on starch plates for their ability to secrete (gluco)-amylases. Out of ca. 10 000 surviving colonies, 7 *prt* mutants were obtained. These mutants had a reduced or no halo on milk plates and a normal halo on starch plates. Some of these mutants showed reduced growth and/or sporulation rates. Four mutant strains, designated AB1.13, AB1.18, AB1.33 and AB1.39, were chosen for further study. A second round of UV mutagenesis was performed with strain AB1.18, which has a reduced halo on milk plates, in order to obtain double *prt* mutants. Several such mutants were obtained, most of them growing more slowly and showing reduced sporulation. One of the mutants that sporulated best, AB1.18–25, was chosen for further study. A strain specifically lacking aspergillopepsin A activity, AB1.1, was obtained by deleting the gene coding for aspergillopepsin A *(pepA)*, using a gene replacement strategy.

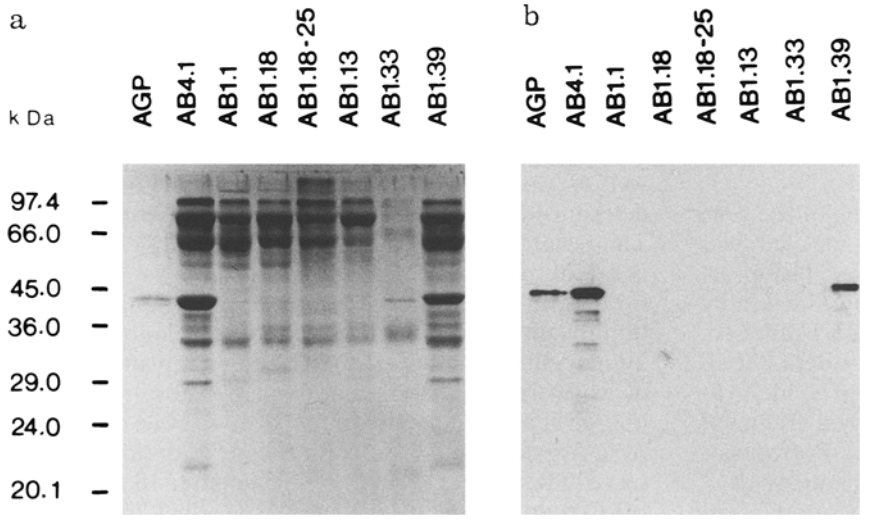

Fig. 2a, b. Protease profile of AB4.1 and *prt* mutants. **a** Coomassie blue staining, **b** Western blot using aspergillopepsin A (AGP)-specific antiserum. The strain used as source of culture medium for each lane is indicated

Table 1. Genetic analysis of *prt* mutants

Chromosome	?	I	II	III	III	IV	V	VI	VI	VII	VIII
N661	+	*fwnA*	*trpA*	*argB*		*leuA*	*nicA*	*pabA*			
N845	+	*fwnA*							*pdxA*	*nicB*	*trpB*
AB1.13	*prt-13*				*pyrG*						
%		52	39	55	53	39	53	23	11	55	58
AB1.18	*prt-18*	*olvA*			*pyrG*						
%		<1	64	46	46	53	54	48			
AB1.33	*prt-33*				*pyrG*						
%		32	50	37	32	46	37	29	19	41	23
AB1.39	*prt-39*				*pyrG*						
%		32	39	42	38	47	64	50	47	48	2

prt, protease deficiency; *fwnA, olvA*, fawn- or olive-coloured conidiospores; *trpA, trpB, argB, pyrG, leuA, nicA, nicB, pabA, pdxA*, requirements for tryptophan, arginine, uridine, leucine, nicotinic acid, p-aminobenzoic acid, pyridoxine, respectively; +: wild-type allele
The percentages of recombination between the various *prt* loci and the markers in the two master strains N661 and N845 are given

Biochemical characterization of prt *mutants*

The protease profiles of the mutant strains were determined by SDS–PAGE after passage of culture medium over immobilized bacitracin. A representative example of such an experiment is shown in Fig. 2 (lanes 3–8). Various differences are observed in the intensity of the different protein bands. It is difficult, however, to draw definitive conclusions about the presence or absence of specific proteases without further information, e.g. reaction with antibodies against the individual enzymes. It is clear, however, that the 43 kDa band, representing aspergillopepsin A, is missing in the *pepA* deletion strain AB1.1 as well as in all UV-induced protease mutants, except strain AB1.39.

The proteolytic activity towards myoglobin in culture media of the *prt* mutants AB1.13 and AB1.18 in comparison with the parent strain AB4.1 is given in Fig. 1. It is apparent that the proteolytic activity towards myoglobin is reduced considerably in both mutants. With mutant AB1.18 approximately 15–20% and with AB1.13 approximately 1–2% of the activity in the culture medium of the parent strain AB4.1 remains. Reduction in proteolytic activity similar to that found for strain AB1.18, was observed for strain AB1.1, in which the *pepA* gene has been deleted (results not shown). This result indicates that 80–85% of the total protease activity in the parental strain AB4.1 is due to the presence of aspergillopepsin A. The effect of pepstatin on the residual proteolytic activity in AB1.13 and AB1.18 is also shown in Fig. 1. The residual protease activity in AB1.18 is reduced only slightly by pepstatin. This indicates the existence of another protease (designated aspergillopepsin B) in *A. niger* strain AB4.1, which is relatively insensitive to inhibition by pepstatin. The residual proteolytic activity in culture medium of AB1.13 is almost completely inhibited in the presence of pepstatin. This indicates that strain AB1.13 is deficient in at least two proteases, aspergillopepsin A and aspergillopepsin B. The residual proteolytic activity in strain AB1.13 might be due to the presence of a low level of aspergillopepsin A, not detectable on the gel (Fig. 2) or to the activity of another pepstatin-sensitive protease. The loss of two protease activities in AB1.13 might be due to the presence of mutations in two genes. Alternatively, a regulatory mutation may have been induced, affecting the expression of the genes coding for both proteases. The results of the genetic analysis of the mutations, presented in the following section, indicate that the latter possibility is more likely.

The proteolytic activity of the mutants against two other heterologous proteins, human interleukin-6 and porcine pancreatic phospholipase A2, was also determined. Degradation of hIL6 in culture media of strain AB1.13, AB1.18, AB1.33 and AB1.39 was determined by Western blotting after SDS–PAGE. Compared to the parent strain, the rate of degradation was lower in all mutants, most notably in AB1.13, especially in the presence of pepstatin (results not shown). Phospholipase A2, which is rapidly degraded in culture medium of AB4.1, is degraded at a slightly lower rate in medium of AB1.1, while degradation is practically absent in culture medium of AB1.13 (results not shown). In conclusion, degradation of heterologous proteins is reduced to various extents in culture medium of the mutants and the degree of reduction depends on the specific protein investigated.

Genetic characterization of prt *mutants*

The chromosomal locations of the *prt* mutations in AB1.13, AB1.18, AB1.33 and AB1.39 were determined using classical genetic recombination procedures (Bos et al. 1988). Two master strains were used: N661, in which chromosomes I–VI are marked and N845, in which chromosomes VI–VIII are marked. For each analysis, 100–120 haploid segregants were analysed. The results, expressed as the percentage of recombination between each of the *prt* loci and the markers of the master strains, are shown in Table 1. Three *prt* genes can be mapped unambiguously: *prt-13* on chromosome VI, *prt-18* on chromosome I and *prt-39* on chromosome VIII. The data for *prt-33* do not show a clear linkage pattern; location in linkage group II, IV, V and VII can be excluded and the best linkage is found with chromosomes VI and VIII.

Further analysis is required to obtain a definitive localization. The *pepA* gene has been localized by electrophoretic analysis (Debets et al. 1990) and shown to be located on chromosome I (J. Verdoes, personal communication).

Complementation of the various *prt* mutations by the wild-type alleles was determined in diploid strains by assaying for halo formation on milk plates. It was found that the *prt-18, prt-33* and *prt-39* mutations are recessive to their wild-type alleles, while *prt-13* is semi-dominant. Analysis of complementation between *prt-18* and *pepA*, both located on chromosome I, showed that these mutations do not complement each other. This indicates that both mutations are allelic, *prt-18* being located either in the coding region or in the regulatory region of the *pepA* gene. Strain AB1.13 is probably a regulatory mutant as at least two proteases, including aspergillopepsin A, are missing from the culture medium, while genetic data indicate the presence of a single semi-dominant mutation, not linked to the *pepA* gene.

In conclusion, several mutant strains of *A. niger* have been obtained which have a reduced proteolytic activity in the culture medium. The use of protease-deficient mutants has practical advantages for the efficient production of heterologous proteins, which are easily degraded in *Aspergillus*. Even in culture media of AB1.13, however, which has lost ca. 98% of its proteolytic activity, proteins like sperm whale myoglobin and human interleukin-6 are still degraded, although at a much lower rate compared to the parent strain. This points to the desirability of obtaining multiply deficient mutants, which could be accomplished by parasexual combination of suitable single mutations, or by cloning and disruption of the genes coding for each of the various proteases by in vitro genetic manipulation.

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