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F. J. Moralejo · R. E. Cardoza · S. Gutiérrez
H. Sisniega · I. Faus · J. F. Martín

Overexpression and lack of degradation of thaumatin in an aspergillopepsin A-defective mutant of *Aspergillus awamori* containing an insertion in the *pep*A gene

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Abstract A gene encoding the sweet-tasting protein thaumatin (*tha*) with optimized codon usage was expressed in *Aspergillus awamori*. Mutants of *A. awamori* with reduced proteolytic activity were isolated. One of these mutants, named *lpr*66, contained an insertion of about 200 bp in the *pep*A gene, resulting in an inactive aspergillopepsin A. In vitro thaumatin degradation tests confirmed that culture broths of mutant *lpr*66 showed only a small thaumatin-degrading activity. *A. awamori lpr*66 has been used as host strain for thaumatin expression cassettes containing the *tha* gene under the control of either the *cah*B (cephalosporin acetylhydrolase) promoter of *Acremonium chrysogenum* or the *gdh*A (glutamate dehydrogenase) promoter of *Aspergillus awamori*. Residual proteolytic activities were repressed by using a mixture of glucose and sucrose as carbon sources and L-asparagine as nitrogen source. Degradation of thaumatin by acidic proteases was prevented by maintaining the pH value at 6.2 in the fermentor. Expression of cassettes containing the *gdh*A promoter was optimal in ammonium sulfate as nitrogen source, whereas transformants expressing the *tha* gene from the *cah*B promoter yielded higher thaumatin levels using L-asparagine as nitrogen source. Under optimal fermentation conditions, yields of 105 mg thaumatin/l were obtained, thus making this fermentation a process of industrial interest.

Introduction

Some filamentous fungi, including *Aspergillus niger*, *A. oryzae* and *A. awamori*, are able to secrete large amounts of extracellular proteins (Archer and Peberdy 1997). The production of heterologous proteins in *A. awamori* and other species of *Aspergillus* has in the last few years become an important research field. Several heterologous proteins secreted by species of *Aspergillus*, including chymosin (Dunn-Coleman et al. 1991), egg lysozyme (Jeenes et al. 1993) and lactoferrin (Ward et al. 1995) are products of commercial interest. Proteolytic degradation of heterologous proteins is a problem found frequently in filamentous fungi (Archer et al. 1992; Van den Homberg et al. 1997).

Thaumatin is a sweet protein (Witty and Higginbotham 1994; Faus et al. 1996, 1998) used as a sweetener in human food or as a feed additive (Faus 2000). We have previously described the expression in *A. awamori* of a synthetic gene (named *tha*) which encodes the sweet protein thaumatin (Moralejo et al. 1999). One of the problems observed in previous studies was the proteolytic degradation of the secreted thaumatin by culture broths of the fungus. Thaumatin has a compact tertiary structure (De Vos et al. 1985) and it was thought to be rather refractory to in vitro proteolysis, because of the extensive disulfide crosslinking of the molecule (van der Wel et al. 1984). Several proteolytic enzymes, such as trypsin or thermolysin, do not affect the structure or sweetness of thaumatin at neutral or basic pH values. However, an increased susceptibility of thaumatin to proteolytic degradation mediated by acid denaturation of the protein was observed at low pH values (Shamil et al. 1990).

When the *tha* gene is expressed in *A. awamori*, the thaumatin is secreted and folded, but part of the newly synthesized thaumatin is degraded by the host proteases during the secretion and folding processes (Moralejo et al. 1999). Therefore, a genetic modification of the host strain and an optimization of the culture conditions of the thaumatin-producing transformants is needed to avoid the formation of proteases that affect the stability of thaumatin. In this work, we describe the isolation and characterization of an aspergillopepsin A (PepA)-deficient mutant (named *lpr*66) containing an insertion in

F. J. Moralejo · R. E. Cardoza · S. Gutiérrez · J. F. Martín (✉)
Institute of Biotechnology INBIOTEC, Science Park of León,
Avda del Real 1, 24006 León, Spain
e-mail: degjmm@unileon.es
Tel.: +34-987-210308
Fax: +34-987-210388

H. Sisniega · I. Faus
Urquima S.A., Arnau de Vilanova 22-42,
08100 Sant Fost de Campsentelles, Barcelona, Spain

the *pep*A gene and its use as host for overexpression of the *tha* gene. In addition, the use of carbon and nitrogen sources under controlled conditions in the fermentors allowed us to obtain very high yields of thaumatin.

Materials and methods

Microbial strains

A. awamori ATCC 22342 was used as the parental strain for mutagenesis experiments. *Escherichia coli* DH5α was used for plasmid amplification and purification.

Mutagenesis and selection of mutants with low proteolytic activity

About 10^7 spores of *A. awamori* were swollen by overnight incubation in 100 ml Tris-maleate buffer 0.1 M, pH 9.0 at 30 °C and 250 rpm in a rotary G10 incubator (New Brunswick Scientific, New Brunswick, N.J.). Then, swollen spores were allowed to germinate synchronously by adding glucose (1.5% w/v) and ammonium sulfate (0.1% w/v; Martín et al. 1974). When the spores started to germinate, nitrosoguanidine (0.5 mM final concentration) was added and aliquots were taken at 0, 30, 60, 90, 120 and 150 min. The mortality reached at 60 min was 65%. Spore preparations mutagenized for 90–150 min suffered a high mortality (75–90%) and were not used because they resulted in crippled mutants (with multiple mutations).

To select mutants with low proteolytic activity, the mutagenized spores (at 60 min) were plated on 1.5% tryptic soy agar for 36 h at 30 °C and then replicated to the same medium with 1.5% non-fat dry milk. After 72 h incubation at 30 °C, the colonies formed a clear halo (due to proteolytic degradation of casein) with a diameter that correlated well to the total proteolytic activity as determined in liquid cultures. The mutants with proteolytic activity lower than the wild-type strain were named *lpr* (acronym for *l*ow *pr*oteolytic activity). To discriminate between mutations affecting proteolytic activity or mutants that may be impaired in secretion of several proteins (e.g. amylase), the mutants with proteolytic activity lower than the wild-type strain were tested for amylase activity. The mutants with proteolytic and amylolytic activities lower than the wild-type strain were considered to be secretion mutants and were not used in this study.

Southern blotting and hybridization

Total DNA from *A. awamori* ATCC 22342 and mutant *lpr*66 was obtained as described by Specht et al. (1982). Digestions with restriction endonucleases, agarose gel electrophoresis and transfer to nylon membranes were performed by standard techniques (Sambrook et al. 1989). A 2.4-kb *Sal*I fragment containing the complete *pep*A gene labelled with digoxigenin by the random-priming method was used as probe in hybridization experiments. The nylon filter was hybridized overnight at 42 °C in a buffer containing 40% formamide, 5× SSC, 0.1× sarcosine, 0.02% SDS and 2% blocking reagent, washed once with 2× SSC-0.1% SDS at 42 °C for 15 min and once more with 0.1× SSC-0.1% SDS at 42 °C for 15 min. Digoxigenin detection was carried out as described by the manufacturer (Roche Diagnostics) using BCIP/NBT as substrate.

PCR amplification of the *pep*A gene

A DNA fragment containing the *pep*A gene was amplified by PCR with the oligonucleotides P1 (5′-ATGGTCGTCTTCAGCAAAACCCGT-3′, corresponding to the eight initial codons of *pep*A gene) and P2 (5′-AGTGGAAAGTACTACTGGTTC-3′, corresponding to a region 241 bp downstream from the *pep*A gene; Berka et al. 1990). The amplification was carried out with BioTaq DNA polymerase (Bioline Ltd, UK) and the program used was 94 °C for 3 min, 35 cycles of 94 °C for 1 min, 50 °C for 45 seconds and 72 °C for 3 min, with a 10 min final extension at 72 °C. The amplification products were resolved in a 1% agarose gel.

Immunological methods

Quantification of the thaumatin secreted by *A. awamori lpr*66 transformants was performed by Western-blot and ELISA tests with anti-thaumatin polyclonal antibodies as described previously (Moralejo et al. 1999). Detection of PepA protein in selected mutants with lower proteolytic activity was made by growing them in SCM medium (Berka et al. 1990) for 120 h. A sample (12 µl) of supernatant was electrophoresed in a 12% SDS-PAGE gel and blotted on a polyvinylidene difluoride membrane. The PepA protein was detected by overnight incubation with a 1:5,000 diluted solution of anti-PepA polyclonal antibodies (kindly provided by M. Ward, Genencor International, Calif.) in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% Tween-20 (TBST) and 5% (w/v) non-fat dry milk and then 1 h incubation with a 1:10,000 dilution of anti-rabbit commercial alkaline phosphatase conjugate also in TBST. The membranes were developed with a BCIP/NBT solution until color was formed.

Submerged culture fermentations

For thaumatin production, seed cultures of *A. awamori* transformants were grown for 40 h in 500-ml shake-flasks containing 100 ml of CM medium (Moralejo et al. 1999) at 28 °C and 250 rpm in a rotary incubator. A 400-ml sample of the seed cultures were transferred to a 5-l Bioflo II fermentor (New Brunswick Scientific, New Brunswick, N.J.) containing 4 l MDFA medium (Shen et al. 1984). The fermentor was equipped with control systems for antifoam, dissolved oxygen, pH and stirring.

Results

Mutants with lower proteolytic activity showed decreased thaumatin degradation

Initial results indicated that thaumatin is susceptible to proteolytic degradation by *A. awamori* culture filtrates. To avoid the degradation of thaumatin, mutants with lower proteolytic activity were selected among survivors of the nitrosoguanidine mutation. After testing 2,322 mutants, nine clones with reduced proteolytic activity on non-fat dry milk were selected. These nine mutants were grown in SCM medium for 120 h and their culture filtrates were incubated with 340 ng of pure thaumatin for 1 h at 30 °C. The wild-type *A. awamori* degraded thaumatin, giving four major fragments (Fig. 1). All selected mutants, except strains *lpr*132 and *lpr*331, degraded thaumatin to a lower extent than the parental strain. The mutants *lpr*6 and *lpr*66 showed only minor thaumatin degradation. Mutant *lpr*6 sporulated very poorly. Therefore, mutant *lpr*66 was selected for further analysis and expression studies.

*A. awamori lpr*66 contains a modified *pep*A gene

In order to characterize whether this mutation in *A. awamori lpr*66 was affecting the *pep*A locus, genomic

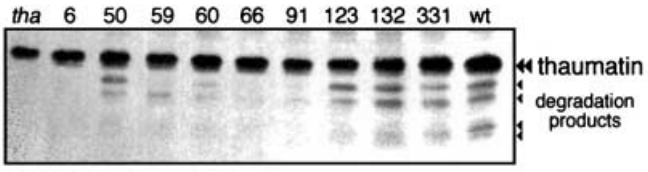

Fig. 1 Immunoblot detection of pure thaumatin after 1 h incubation with culture filtrates of several *lpr* mutants with reduced proteolytic activity. Thaumatin (340 ng) was incubated with 2.5 µg of extracellular protein (culture broth). The numbers above each lane indicate extracts from the different *lpr* mutants. The proteins in the incubation mixture were resolved by SDS-PAGE, the gel was blotted and the thaumatin was visualized with polyclonal anti-thaumatin antibodies (see text for details). Note the low thaumatin degradation by strains *lpr*6 and *lpr*66. *tha*, Control pure thaumatin (340 ng; without culture broth), *wt*, wild-type *Aspergillus awamori* strain

DNA of the *lpr*66 strain was digested with *Apa*I, *Cla*I and *Pst*I restriction enzymes and hybridized with a 2.4-kb *Sal*I fragment containing the complete *pep*A gene (encoding aspergillopepsin A). The hybridization results with the *lpr*66 mutant showed a change of the restriction pattern in the *pep*A locus as compared to the wild-type strain (Fig. 2A, B). This result suggests that the mutation resulted in a DNA rearrangement with an insertion of about 200 bp in the DNA fragment corresponding to the *pep*A gene (Fig. 2). Western-blot analysis of culture filtrates from several *lpr* mutants grown in SCM medium showed the presence in the *lpr*66 mutant of a 53-kDa protein (instead of the normal 43-kDa PepA protein) that reacted with the anti-PepA antibodies (Fig. 2C). This result indicates that the insertion in the *pep*A locus does not interrupt the open reading frame of *pep*A and does not affect the promoter of this gene, since *pep*A expression still occurs.

To confirm that the *pep*A locus has suffered a rearrangement, the region containing the *pep*A gene was amplified by PCR using as primers oligonucleotides P1 and P2 as indicated in Materials and methods. The results of the PCR amplification showed clearly that a band of 1.8 kb was formed, instead of the 1588 bp band existing in the wild type, confirming that an insertion of about 0.2 kb has been introduced in the *pep*A gene.

In vitro thaumatin-degrading activity of the *lpr*66 mutant

To gain insight into the possible inactivation status of PepA produced by the *lpr*66 mutant, pure thaumatin was incubated in vitro with 8 µl of culture filtrates from this mutant and from the wild-type strain for 6 h in the presence and absence of pepstatin A, an inhibitor of aspergillopepsin A and other aspartic proteinases (Fig. 3). The incubation of thaumatin with supernatants

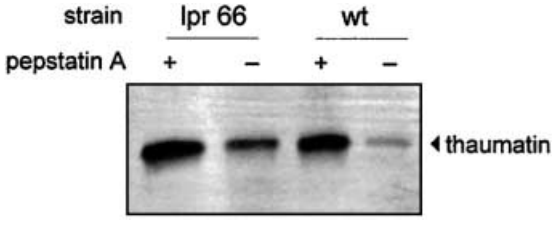

Fig. 3 Degradation of pure thaumatin (100 ng) after incubation with culture filtrates (2.5 µg of protein) of wild-type *A. awamori* (*wt*) and the mutant *lpr*66 grown in SCM medium, with or without pepstatin A (10 µg/ml), as shown by immunoblotting of the thaumatin with anti-thaumatin antibodies

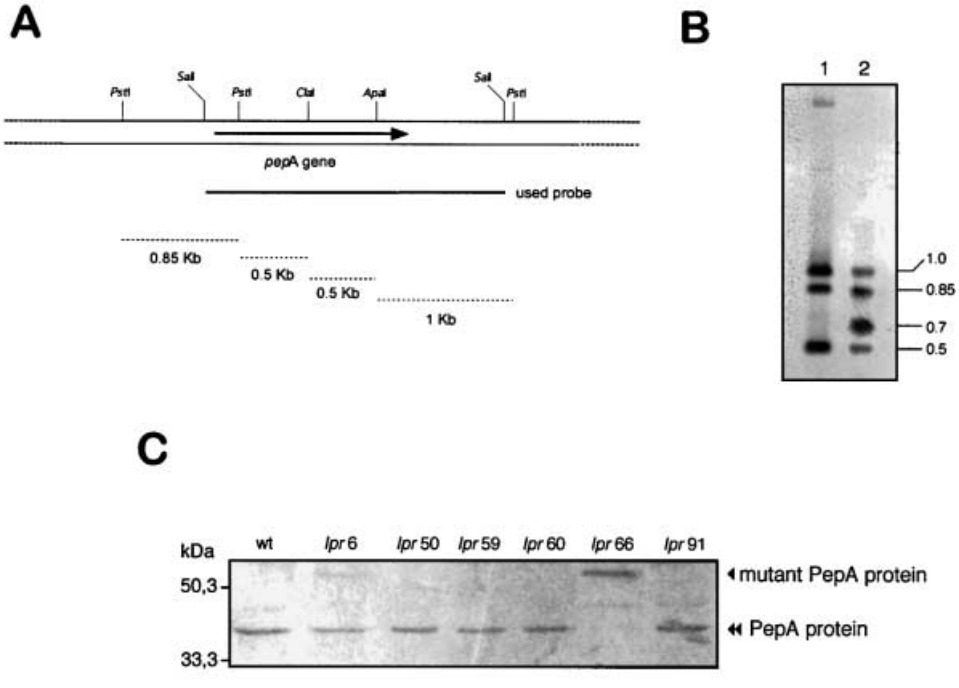

Fig. 2A–C Molecular analysis of mutant *lpr*66. **A** Physical map of the DNA region containing the *pep*A gene; *dashed lines* indicate the expected pattern of hybridization fragments using the indicated 2.4-kb *Sal*I probe. **B** Southern blot hybridization of *Apa*I + *Cla*I + *Pst*I-digested total DNAs from wild-type *A. awamori* and the *lpr*66 mutant with a digoxigenin-labeled 2.4-kb *Sal*I probe containing the *pep*A gene. The size (in kb) of the fragments detected is indicated on the right. *Lane 1 A. awamori* wild-type strain, *lane 2 lpr*66 mutant. **C** Immunoblot detection of PepA protein secreted by the wild-type *A. awamori* strain and several *lpr* mutants with reduced proteolytic activity

of the wild-type *A. awamori* strain resulted in thaumatin degradation, which was inhibited by the addition of pepstatin A (10 μg/ml), indicating that PepA plays an important role in degradation of thaumatin. However, the proteolytic degradation of thaumatin by the *lpr*66 mutant was clearly smaller in all tests.

Utilization of *A. awamori lpr*66 as host results in high thaumatin production

The *lpr*66 mutant was used as host strain for transformation with expression cassettes containing the *tha* gene under the control of efficient fungal promoters. Several of the transformants obtained reached more than 10 mg thaumatin/l in shake-flask cultures, whereas in initial studies using wild-type *A. awamori* strains, the yield was only of 1–2 mg/l. TB2b1-44 transformants containing the *tha* gene under the control of either the *cah*B (cephalosporin acetylhydrolase) promoter of *Acremonium chrysogenum* (Velasco et al. 2000), or TGDTh-4, which expressed the *tha* gene under the control of the *gdh*A (glutamate dehydrogenase) gene of *Aspergillus awamori* (Cardoza et al. 1998) were selected for further studies, since they resulted in higher thaumatin yields than constructions with the promoter of the glucoamylase (*gla*A) gene. Both transformants had a similar copy number of the expression cassette (5–6 copies for TB2b1-44 and 8–9 copies for TGDTh-4) and produced similar thaumatin levels in shake-flasks. Moreover, the production of thaumatin in shake-flasks showed, in both strains, a reduction after 48–72 h when the pH turned acidic, suggesting that some proteolytic degradation of thaumatin by other acid proteases in the culture filtrates still occurred.

To optimize thaumatin production and to reduce protease expression, strain TB2b1-44 was grown in a 5-l fermentor with a mixture of 6% sucrose and 2.4% glucose as carbon sources [which repress protease gene expression (Jarai and Buxton 1994)] and L-asparagine as nitrogen source. The pH of the medium was maintained at 6.2 with NaOH 2.5 N, to avoid proteolytic degradation by acidic proteases different from PepA. Under these controlled conditions, a reduced acidic protease activity was observed and thaumatin production reached 70 mg/l, about seven times more than in uncontrolled shake-flasks (Fig. 4). The maximal level of thaumatin was reached at 96 h; between 72 h and 96 h, only a small increase of thaumatin was observed, correlating with a reduced level of residual carbon source.

Effect of carbon source and daily additions of sucrose on thaumatin production

Substitution of sucrose (6%) by a mixture of sucrose (3.6%) and glucose (2.4%) gave a reduction of 30% in the production of thaumatin by strain TB2b1-44. In contrast, addition of 1% sucrose after 36, 48, 60 and

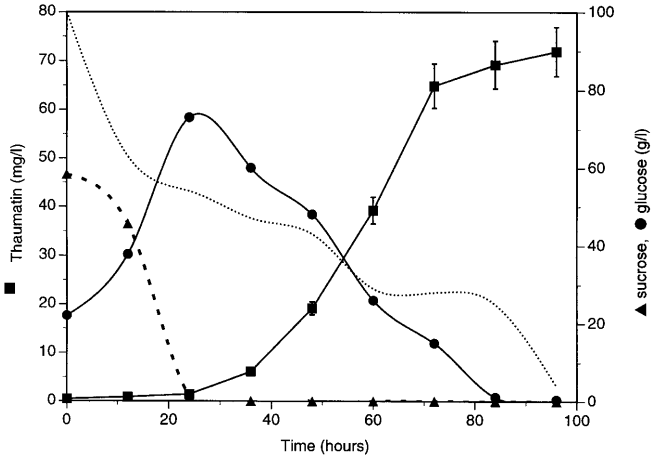

Fig. 4 Production of thaumatin by transformant TB2b1-44 in a 5-l Bioflo II fermentor in MDFA medium with 6% sucrose and 2.4% glucose as carbon source, and 1.2% L-asparagine as nitrogen source. ■ thaumatin production (mg/l, left scale), ● glucose concentration (g/l, right scale), ▲ sucrose concentration (g/l, right scale). Note that production of thaumatin under these conditions is limited by glucose depletion at 84 h. The *dotted line* indicates the dissolved oxygen (as a percentage of initial saturation) as recorded directly by the dissolved oxygen electrode (right scale)

72 h fermentation to cultures with an initial 6% sucrose and 2.4% glucose resulted in a 40% increase of production, yielding 100 mg thaumatin/l at 84 h, without reaching a plateau in production (Fig. 5A).

Effect of the culture pH on extracellular thaumatin production

In order to prevent residual proteolytic degradation of thaumatin, the pH of one of two twin fermentors inoculated with the same inoculum was increased to 6.5 and the other was maintained at pH 6.2. A reduction of 20% in the production of thaumatin at pH 6.5 was observed, when compared with cultures maintained at pH 6.2 (Fig. 5B); this result is in agreement with the preference of *A. awamori* for growth in acidic medium (see Discussion).

Effect of the nitrogen source on thaumatin production from cassettes containing the *tha* gene under different promoters

A comparison between strains TGDTh-4 and TB2b1-44 (containing expression cassettes with the *gdh*A and *cah*B promoters, respectively) was performed using a nitrogen source selected according to the known regulation of each promoter. The carbon source was in all cases 6% initial sucrose and 2.4% glucose with four additions of 1% sucrose (every 12 h). The pH was kept at 6.2. The *gdh*A promoter was induced by ammonium. Using 80 mM ammonium sulfate as nitrogen source, a production of 105 mg thaumatin/l was achieved at 75 h in

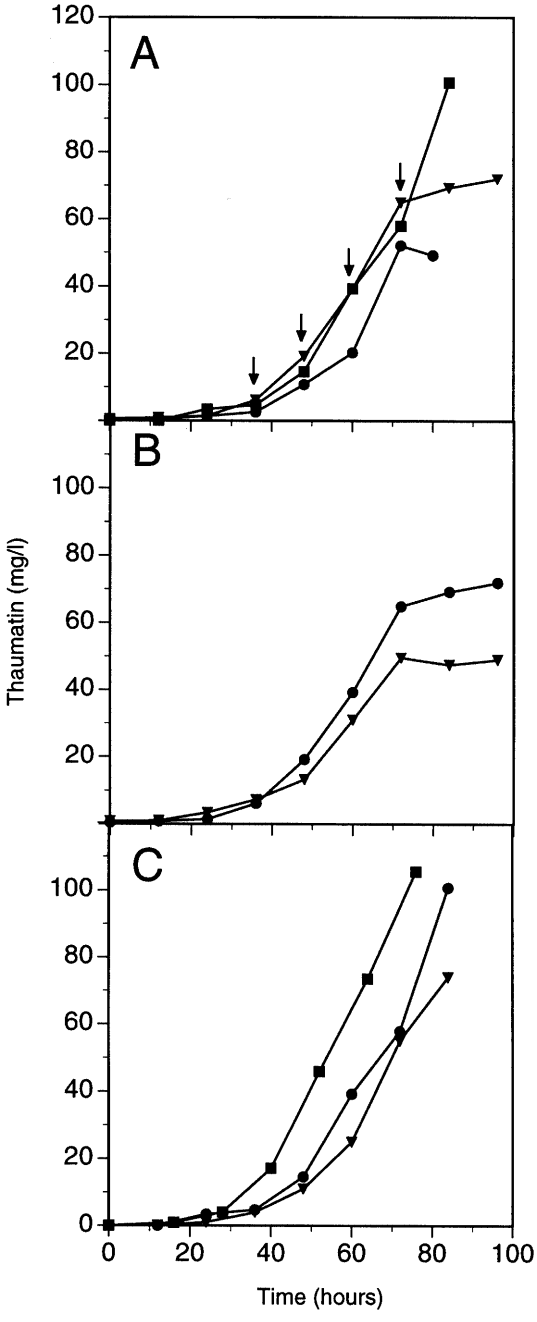

Fig. 5 **A** Effect of carbon source concentration on thaumatin production by transformant TB2b1-44 in 5-l fermentors. ● medium with 3.6% sucrose and 2.4% glucose, ▼ medium with 6% sucrose and 2.4% glucose, ■ medium with 6% initial and 2.4% glucose and 1% additional sucrose added at the time indicated by *arrows*. **B** Effect of pH on thaumatin production by strain TB2b1-44. ● pH 6.2, ▼ pH 6.5. **C** Effect of nitrogen source on thaumatin secretion by transformant TB2b1-44 in MDFA medium (Shen et al. 1984) with L-asparagine (●) or $SO_4(NH_4)_2$ (▼), and by transformant TGDTh-4 with $SO_4(NH_4)_2$ (■) as nitrogen source

cultures of the TGDTh-4 transformant. A similar thaumatin production was obtained at 84 h with the TB2b1-44 strain, using L-asparagine (80 mM) as nitrogen source. However, the later strain yielded only 76 mg/l of thaumatin when it was grown with ammonium sulfate as nitrogen source. These results indicate that ammonium sulfate is an excellent nitrogen source to express the thaumatin gene from the *gdh*A promoter whereas L-asparagine is preferred to overexpress the thaumatin gene from the cephalosporin esterase (*cah*B) promoter.

Discussion

A. awamori is widely used for heterologous protein production because of its ability to secrete large amounts of proteins to the culture medium. However, proteolytic degradation of the heterologous proteins has been a major problem in obtaining high yields of the desired products. Several proteolytic enzymes are secreted to the culture medium by species of *Aspergillus* (Van den Homberg et al. 1997); one of them, aspergillopepsin A (PepA) is considered to be the major extracellular proteolytic activity involved in the degradation of heterologous proteins (Mattern et al. 1992).

In this work, we isolated an aspergillopepsin A-deficient mutant *A. awamori lpr*66. Molecular analysis of this mutant indicated that it contains an insertion of approximately 200 nucleotides in the *pep*A gene without disruption of its open reading frame, resulting in an inactive aspergillopepsin A larger than the natural one. The mutant PepA protein showed immunoreactivity with anti-PepA antibodies and was secreted by the *lpr*66 mutant.

The *lpr*66 mutant showed considerably less thaumatin degrading activity than the wild type in a long incubation time (6 h) and was therefore used as host for thaumatin overproduction using expression cassettes containing a synthetic *tha* gene with optimized codon usage (Moralejo et al. 1999). Very high levels of thaumatin were obtained with two of these constructions, reaching values of 80–105 mg/l, whereas cultures with transformants using the wild-type *A. awamori* yielded very low levels of thaumatin (5–15 mg/l).

Some thaumatin degradation was still observed in shake-flask cultures, especially after 84 h, when nutrients were depleted from the medium. This residual degradation of thaumatin was correlated with the presence in the broths of proteolytic activities other than aspergillopepsin A. Therefore, an optimized culture medium was developed to avoid all proteolytic activity by using sucrose and glucose as carbon sources and L-asparagine or ammonium sulfate as nitrogen sources, nutrients that are known to repress expression of proteases in *A. niger* (Jarai and Buxton 1994). In addition, the culture pH was kept above 6.0, for inactivation of other acid proteases, like aspergillopepsin B (Takahashi 1995), that also degrade thaumatin (Moralejo et al., unpublished). However the allowed pH range for optimal thaumatin production was quite narrow (optimal pH was 6.2) since at higher pH values (e.g. pH 6.5) a considerable decrease of secretion was observed, perhaps due to a reduction of the proton gradient across the

membrane. Under optimal conditions in the fermentors, a seven-fold increase in thaumatin production by strain TB2b1-44 with respect to uncontrolled shake-flask cultures was observed.

Comparison between strains TGDTh-4 and TB2b1-44 in fermentor cultures confirmed the results obtained in shake-flask experiments. The promoter of the *gdh*A gene (glutamate dehydrogenase) of *A. awamori* (Cardoza et al. 1998) is very efficiently expressed in this filamentous fungus; and this also occurs also with the *gdh*A promoters of *Penicillium chrysogenum* (Gutiérrez et al. 1997; Díez et al. 1999). The *cah*B promoter of *A. chrysogenum* that governs expression in the TB2b1-44 cassette is very efficiently expressed in media with sucrose as carbon source (Velasco et al. 2000). The *gdh*A promoter is better expressed during the rapid growth phase and therefore fermentations with TGDTh-4 are 12 h shorter than fermentations with transformants containing the TB2b1-expression cassette. These two promoters gave better thaumatin yields than the promoter of the glucoamylase of *A. niger* in comparative fermentation studies.

In summary, these results provide convincing evidence that removing the aspergillopepsin A and other proteolytic activities of *A. awamori* by genetic and regulatory manipulations results in very important increases in thaumatin yields, making this process industrially attractive.

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