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A defined level of protein disulfide isomerase expression is required for optimal secretion of thaumatin by *Aspergillus awamori*

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Abstract Thaumatin, a 22-kDa protein containing eight disulfide bonds, is secreted by the filamentous fungus *Aspergillus awamori* at levels which are dependent upon the extent of overexpression of protein disulfide isomerase (PDIA). Additional copies of the PDIA-encoding gene *pdiA* were introduced into a strain of *A. awamori* that expresses a cassette encoding thaumatin. Transformants with different levels of *pdiA* mRNA and measured PDIA levels were chosen for examination of the impact that PDIA levels had on thaumatin secretion. The secretion of two native proteins, α-amylase and acid phosphatase, was also examined in relation to varying levels of PDIA. Over a range of PDIA levels of 1–8, relative to the native level in strains with just one copy of the *pdiA* gene, the fraction of α-amylase and acid phosphatase in the total secreted protein was unaffected. In contrast, a peak level of thaumatin, about 5-fold higher than in the strain with one copy of *pdiA*, was found in strains with a relative PDIA level of between two and four. Improved thaumatin production was confirmed in 5-l fermenters using a strain of *A. awamori* with six *pdiA* gene copies, containing 3.2-fold higher levels of PDIA than wild-type strains.

Keywords Protein secretion · Protein disulfide isomerase · Protein folding · Thaumatin production · *Aspergillus awamori*

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Introduction

There is still little information available on the limiting steps of the protein secretory pathway in filamentous fungi, despite the interest in using these microorganisms for the industrial production of extracellular enzymes (Archer and Peberdy 1997; Pedersen et al. 2000). Filamentous fungi possess an efficient secretory system (Gouka et al. 1997), although secretion of heterologous proteins is often limited and may result in the intracellular accumulation of foreign proteins that are diverted from the secretory system and degraded by intracellular proteolysis (Wiertz et al. 1996). Some of the heterologous proteins that are expressed as fusion proteins may not be processed properly or be misfolded (Punt et al. 1998; Spencer et al. 1998).

In a previous study (Moralejo et al. 1999) we observed that expression of a synthetic thaumatin gene (*tha*) with optimized codon usage in *A. awamori* led to production and secretion of this protein, but some of the thaumatin formed was retained intracellularly. The sweet-tasting protein thaumatin, produced in nature by the tropical plant *Thaumatococcus danielli*, is a 22-kDa protein that contains eight disulfide bonds (Witty and Higginbotham 1994). Therefore, folding and secretion of this protein in *A. awamori* may be significantly affected by the level and specificity of foldases which catalyze the formation of disulfide bonds.

Protein disulfide isomerase (PDIA) has both foldase and chaperone activities, and genes encoding PDIA have been cloned from *A. awamori* (Malpricht et al. 1996) and *A. niger* (Ngiam et al. 1997). A decrease in PDIA levels in *A. niger* led to a reduced yield of secreted glucoamylase but over-expression of the structural gene, *pdiA*, had no effect on secreted levels of either glucoamylase or the heterologous protein hen egg-white lysozyme (Ngiam et al. 2000). Thaumatin has more disulfide bonds than either lysozyme or glucoamylase and it is possible that the wild-type level of PDIA required for its proper folding is limiting for the production of export-competent protein.

We therefore decided to examine if PDIA levels had an effect on the secretion of thaumatin from *Aspergillus*. The aim was also to examine thaumatin secretion when PDIA levels were varied over a range of values rather than studying the effect of just one concentration. We report in this article that secretion of thaumatin in *A. awamori* is optimal at a defined level of protein disulfide isomerase in the cell, about three-fold higher than in the wild type. Very high levels of PDIA do not favour secretion of thaumatin.

Materials and methods

Transformation of protoplasts

Transformation of *A. awamori* TGDth4, a thaumatin-producing strain (Moralejo et al. 2000), was accomplished by PEG-mediated DNA uptake into protoplasts (Punt and van den Hondel 1987). The plasmids used in the transformation were pIGCPDI (Ngiam et al. 2000), which carries the PDIA-encoding *pdiA* cDNA under the transcriptional control of the *A. niger* glucoamylase (*glaA*) promoter, and pAN7-BlueI (Ngiam et al. 2000), which contains the hygromycin B resistance gene. The transformed protoplasts were poured onto minimal medium-agarose and allowed to regenerate overnight before the addition of hygromycin B in an agarose overlay to give a final plate concentration of 100 µg/ml. Resistance to hygromycin was then confirmed by testing any transformants for their ability to grow on slopes containing hygromycin concentrations of 200 µg/ml.

RNA and DNA isolation

Selected transformants and the parental strain were grown in 250-ml shake flasks containing 100 ml of ACMS/N/P (Archer et al. 1990) medium for 44 h at 150 rpm and 25°C. Mycelia were harvested by filtration through Miracloth (CalBiochem) and flash frozen in liquid nitrogen. The mycelia were ground under liquid nitrogen and RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. DNA was extracted using a DNeasy kit (Qiagen) according to the manufacturer's instructions. RNA (10 µg per lane) was fractionated on an agarose gel containing 7% formaldehyde (Sambrook et al. 1989). Transfer to Hybond XL nylon membrane (Amersham International) was accomplished using an Appligene vacuum blotter according to the manufacturer's instructions. For Southern blots, 10 µg of DNA per lane were electrophoresed on a 1% agarose-TAE gel (Sambrook et al. 1989) prior to vacuum transfer to Hybond XL membrane.

Northern and Southern hybridizations

Probes for both Southern and Northern blots were labelled using the Megaprime kit and γ-^{32}P dATP (both from Amersham International) according to the manufacturer's instructions. The *pdiA* probe was a 302-bp fragment corresponding to co-ordinates +63 to +365 in the sequence of the *pdiA* gene (N-terminal region of PDIA) (Ngiam et al. 1997), while the actin probe was a 765-bp fragment corresponding to co-ordinates +889 to +1654 in the γ-actin gene (the C-terminal region of γ-actin) of *A. nidulans* (Fidel et al. 1988). Both of the probes were amplified by PCR from *A. niger* genomic DNA. Blots were pre-hybridized at 65°C for 30 min. Hybridization was carried out overnight at 65°C using Hyb9 hybridization solution (Puregene). The blots were washed twice in low-stringency buffer (2×SSC, 0.1% SDS) for 15 min at 65°C and once in high-stringency buffer (0.1×SSC, 0.1% SDS) for 30 min at 65°C. Bands were visualized and the band intensities quantified using a FujiFilm BAS1500 phosphorimager. Expression levels for the *pdiA* gene and cDNA were calculated for each transformant tested relative to the expression in the parental strain, normalised using the corresponding actin expression levels. The copy number of pIGCPDI in each of the transformants was calculated as a ratio of the quantified band intensities from the parental (endogenous) *pdiA* gene and the integrated *pdiA* cDNA copies; the parental strain has a single copy of the gene that is detected as a larger fragment in DNA digested with *Eco*RI.

SDS-polyacrylamide gel electrophoresis and Western analysis

Detection of the PDIA protein was performed using a microsomal fraction obtained as described previously (Ngiam et al. 2000). Equal amounts of protein were loaded on two SDS-9% polyacrylamide gels. After electrophoresis, one gel was stained with Coomassie R-250 and the duplicate was transferred to a polyvinylidene difluoride membrane (PVDF) by using a Minitransblot electroblotting system (BioRad). The PDIA protein was detected by overnight incubation with a 1:5000 dilution of anti-PDIA polyclonal antibodies in 50 mM TRIS-HCl pH 8.0, 150 mM NaCl, 0.2% Tween-20 (TBST), followed by a 30-min incubation with a 1:10000 dilution of a commercial anti-rabbit alkaline phosphatase conjugate also in TBST. The membranes were developed with a BCIP/NBT solution until bands appeared.

Quantification of total PDIA protein

PDIA protein levels in microsomal fractions were quantified using an enzyme-linked immunoabsorbent assay (ELISA). The first lanes of plates (Nunc immunoplates) were coated with 5 µg of microsomal proteins and dilutions (1:10 to 1:640) were adsorbed to successive lanes. The plates were washed three times with phosphate-buffered saline (PBS) (NaCl, 8 g/l; KCl, 0.2 g/l; Na_2HPO_4, 1.15 g/l; KH_2PO_4, 0.2 g/l) plus 0.1% Tween-20 (PBS-T), blocked with PBS-T containing 5% dry milk for 1 h, and washed three times with PBS-T. The PDIA was measured by addition of a 1:2500 dilution of the rabbit anti-PDIA antiserum for 1 h followed by a 1:5000 dilution of a commercial goat anti-rabbit alkaline phosphatase conjugate for 30 min. The antigen-antibody complexes were quantified by using a stabilized substrate solution for alkaline phosphatase (Sigma) and scanning at 405 and 620 nm in a Scanning Autoreader and Microplate Workstation (Ceres 900C; Biotek Instruments). For comparative purposes, the absorbance of a 1:320 dilution (in the linear range of measurements) of samples of TGDth-4 transformant was normalized as value 1, and compared with absorbances obtained from the other samples at the same dilution.

Fermenter studies and quantification of thaumatin

For thaumatin production studies in shake flask cultures, *A. awamori* strains were grown in triplicate in MDFA medium with different carbon sources as described previously (Moralejo et al. 1999).

In fermenter studies of AA100 and TGDth-4 transformants, seed cultures were grown for 40 h in 500-ml shake-flasks containing 100 ml of CM medium at 28°C and 250 rpm in a rotary incubator. A 350-ml sample of the seed culture was used to inoculate a 5-l Biostat B fermenter (B. Braun Biotech Intlernational) containing 3.5 l of MDFA medium with 2% sucrose and 12% dextrin as carbon source and 80 mM L-asparagine as nitrogen source. The pH value was maintained at 6.2.

Quantification of thaumatin was carried out by ELISA as described previously (Moralejo et al. 1999).

α-Amylase and acid phosphatase activities

α-Amylase activity was measured by the method of García-González et al. (1991). Acid phosphatase activity was measured as described by Robinson et al. (1996).

Results

Overexpression of the *pdiA* gene in *A. awamori*

To test whether the *pdiA* gene product has an effect on folding and secretion of thaumatin in *A. awamori*, an expression cassette containing the *pdiA* gene under the control of the inducible promoter of the glucoamylase gene (P*glaA*) was introduced into the thaumatin production strain *A. awamori* TGDth-4 (Moralejo et al. 1999) in which the thaumatin gene is expressed from the *gdhA* gene promoter. Six transformants were selected at random. Southern analysis showed that the six transformants contained from 1 to 28 additional copies of the *pdiA* gene.

The six transformants were compared with the parental strain (untransformed TGDth-4) by Northern analysis and ELISA to assess the levels of the *pdiA* transcript and intracellular PDIA protein, respectively (Table 1).

The results indicated that all six transformants showed increased expression of the *pdiA* gene and a higher level of the PDIA protein than the parental strain. The highest *pdi*A transcript level and PDIA protein concentration was observed in an extract of AA096, a transformant that contained nine copies of the *pdiA* gene. There was no strict correlation between *pdiA* gene copy number and the transcript level. The *pdiA* transcript levels in five of the six transformants examined were 6-to 8-fold higher than in the untransformed strain. One transformant (AA096) has a higher (16-fold) relative *pdi*A transcript level and also expresses the highest level of PDIA protein. Transformant AA096 did not, however, have the highest *pdiA* gene copy number: that was found in AA084. We presume that in AA084 some of the *pdiA* copies are either incomplete or have integrated in a genomic locus that shows low transcriptional activity.

PDIA protein levels in the different transformants

To test whether there was a correlation between the PDIA levels determined by ELISA and the protein observed in immunoblots, the PDIA protein in cell-free extracts of all the transformants was visualized by Western analysis using anti-PDIA antibodies. Cultures of all transformants and the parental untransformed TGDth-4 strain were grown in two media: (1) MDFA containing glucose (1%) and sucrose (3%), or (2) MDFA containing glucose (1%) and maltose (3%). Glucose (1%) was used in both media to stimulate growth during the rapid growth phase, and maltose (but not sucrose) was used as an inducer of the *glaA* promoter. Glucose is a moderate inducer of the *glaA* promoter, whereas maltose is a strong inducer.

Immunoblots of the microsomal fraction from all the different transformants showed a clear PDIA-specific band at 56 kDa (Fig. 1). Overexpression of a heterologous protein (thaumatin) did not substantially affect the total PDIA protein levels since bands of the same intensity were observed by Western analysis in the parental *lpr*66 strain and the TGDth-4 strain containing the thaumatin expression cassette (lanes 1 and 2 in Fig. 1). It is possible that the levels of free PDIA, i.e. the PDIA not bound to protein (Ngiam et al. 2000) may have been affected by thaumatin. We can not exclude at this time the possibility that the accumulated thaumatin may trigger the unfolded protein response. This phenomenon is being investigated in more detail.

In sucrose-containing medium there were some differences in the amounts of PDIA observed in the different strains. All the transformants showed higher PDIA levels than the endogenous level of the control untransformed strains (lanes 1 and 2, Fig. 1C). There was some degradation of the accumulated PDIA protein that was not observed in the control strains. The highest PDIA level was found in transformants AA016, AA084 and AA096.

In MDFA medium containing maltose instead of sucrose, a clearly higher level of PDIA was observed in some of the transformants, particularly in AA084 and AA096. In these two transformants, the high overexpression of the PDIA protein allowed direct visualization in the Coomassie blue-stained gels of a 56-kDa protein that corresponds in size to PDIA (Fig. 1B) and to the bands detected by Western blotting (Fig. 1D).

Table 1 Analysis of *pdiA* copy number, transcript level and PDIA activity in *A. awamori* TGDth-4 strains transformed with the *pdiA* gene

Strains	Number of additional copies of the *pdiA* gene[a]	Transcript level[b]	PDIA protein[c]
*A. awamori lpr*66 (untransformed)	–	n.d.	0.9 ± 0.1
TGDth-4	–	1	1
AA016	2	8	1.7 ± 0.1
AA064	4–5	6	3.6 ± 0.4
AA068	1	8	2.3 ± 0.3
AA084	28	6	5.4 ± 0.4
AA096	9	16	7.9 ± 0.7
AA100	2–3	6	3.2 ± 0.3

[a]Not counting the endogenous gene. The estimate is based on Southern hybridization data
[b]Relative transcript values determined by slot-blot hybridization. n.d., not determined
[c]Relative values of PDIA with respect to that in the TGDth-4 strain, as determined by ELISA

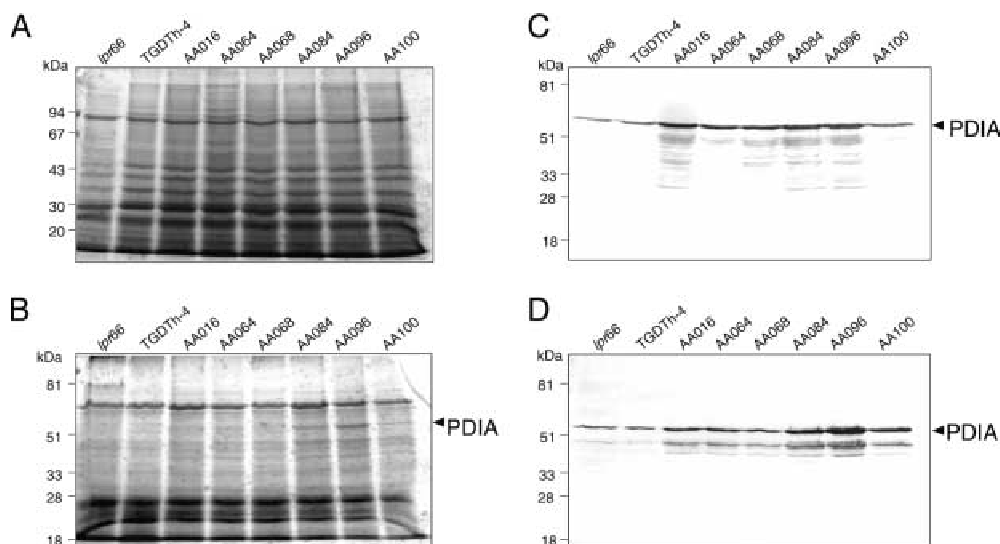

Fig. 1A–D PDIA levels in six transformants of *A. awamori* containing the *pdiA* gene under the control of the *glaA* promoter integrated in several copies (see Table 1). For **A** and **C**, cells were grown in MDFA medium with sucrose as carbon source; for panels **B** and **D**, cells were grown in MDFA medium with maltose as carbon source. **A** and **B** show total protein; **C** and **D**, immunoblots with anti-PDIA antibodies. Lanes 1, *A. awamori lpr*66 (untransformed); lane 2, *A. awamori* TGDth-4 containing the thaumatin expression cassette pGDTh. Lanes 3–8, transformants AA016, AA064, AA068, AA084, AA096 and AA100, respectively. The PDIA band is indicated by the *arrowhead*. Note that the PDIA band is observed directly in Coomassie blue-stained gels of extracts of transformants AA084 and AA096 (lanes 6 and 7)

In all the transformants grown in maltose as carbon source there was a second immunoreactive band (revealed by antibodies against PDIA) that was also detectable in sucrose-grown cells, suggesting that proteolytic processing of PDIA occurs particularly in maltose-grown cells.

The levels of PDIA, as quantified by ELISA, in transformants overexpressing the *pdiA* gene were between 1.7- and 7.9-fold higher than in the parental control strain TGDth-4 (Table 1). Transformant AA096 gave the highest level of PDIA in ELISA determinations and this correlated very well with the intensity of the PDIA band in Western blots.

Effect of *pdiA* amplification on secretion of thaumatin and extracellular proteins in *A. awamori*

Thaumatin production was determined in supernatants of the same cultures used to quantify the intracellular PDIA protein in the extracts. As shown in Fig. 2, in sucrose-grown cultures, there were some differences in thaumatin production levels in the transformants with respect to the parental strain TGDth-4 but the differences were much more significant in maltose-grown cultures, in which expression of the *pdiA* gene is strongly induced.

In the presence of both carbon sources, but particularly in maltose-grown cultures, the best thaumatin production was achieved by transformants AA064, AA068 and AA100, yielding levels of 11, 12 and 19 mg/l, respectively. This is 2–4 times the level of thaumatin obtained in the parental strain. There was no strict correlation between *pdiA* copy number and *pdiA* transcript levels with the amounts of thaumatin secreted (see below).

To establish if the increase in PDIA content affected the secretion of endogenous extracellular proteins, α-amylase and acid phosphatase (both of which are well expressed under the slightly acid conditions of the culture) were quantified in each transformant. As shown in Fig. 3A, there were no significant differences in total extracellular protein between cultures of the different transformants, except for transformant AA084 which appears to show a clear reduction in protein secretion at 48 h and 72 h of incubation. All transformants showed similar growth kinetics. α-Amylase secretion was not significantly affected by the PDIA content. Transformants AA084 and AA096, both of which are also poor thaumatin secretors, showed delayed amylase secretion (Fig. 3B). Similarly, there was no correlation between the PDIA content and the extracellular acid phosphatase levels when data for levels of phosphatase at 72 h are used to calculate the PDIA effect.

An interesting observation was found when the levels of extracellular thaumatin, α-amylase and acid phosphatase per mg of extracellular protein were plotted against the relative concentration of PDIA in each transformant. As shown in Fig. 4, the best secretion of thaumatin was observed in strains with intermediate levels of PDIA (3- to 4-fold that of the parental strain) whereas poor thaumatin secretion was obtained in the transformants showing very high intracellular levels of PDIA. This pattern of PDIA-dependent secretion was not observed with the endogenous α-amylase or acid phosphatase activity, both of which were largely independent of the PDIA levels in the cell (Fig. 4).

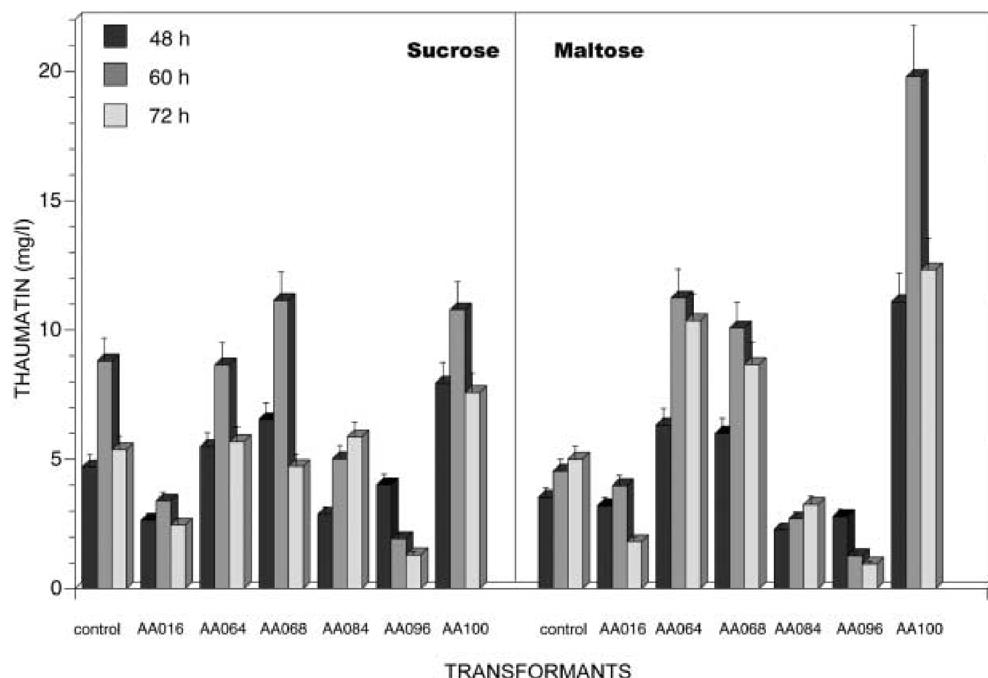

Fig. 2 Effect of *pdiA* amplification on secretion of thaumatin. Levels of extracellular thaumatin in the control *A. awamori* TGDth-4 strain and in the indicated transformants at 48, 60 and 72 h grown in MDFA medium containing sucrose or maltose as carbon source. The *vertical bars* indicate standard deviations of the means

Production of thaumatin by transformant AA100 in submerged culture

It is likely that the increase in thaumatin production by transformant AA100 in maltose-containing medium is due to the overexpression of the *pdiA* gene from the *gla*A promoter.

To improve the efficiency of the expression system, cultures of this transformant and the parental TGDth-4 strain were compared in two identical 5-liter fermenters using 6% dextrin (a good inducer of the *glaA* promoter) as the carbon source and controlled pH conditions (constant pH value of 6.2). As shown in Fig. 5A, the parental TGDth-4 strain produced 42 mg/l of thaumatin at 60 h under these conditions, whereas transformant AA100 reached 56 mg/l under the same conditions. Addition of 1% sucrose at 48, 60, 72 and 84 h did not affect growth significantly (Fig. 5B) (which is probably limited by other factors) but production of thaumatin continued at a good rate until 96 h. The same results were observed when the production was expressed as micrograms of thaumatin per mg dry weight (Fig. 5C). The increase in thaumatin production by transformant AA0100 as compared to the parental strain was about 50 to 70% at 36 and 48 h of cultivation (Fig. 5C), although there was a decrease in thaumatin yields in this strain after 60 h that was prevented by daily additions of sucrose. However, the biomass accumulated in the fermenters with sucrose as carbon source was lower than expected.

Additional fermentations were performed under the same conditions, except that 12% dextrin was used. As shown in Fig. 6A, growth and thaumatin production under these conditions by the AA100 transformant continued until 72 h giving a three-fold increase in yield with respect to the parental TGDth-4 strain. Thaumatin accumulation peaked at 96 h. However, when 1% sucrose was added at 48, 60, 72 and 84 h accumulation of thaumatin continued until at least 120 h, reaching levels of 150 mg per liter.

In summary, amplification of the *pdiA* gene results, in some strains, in a significantly improved capacity to secrete the sweet-tasting protein thaumatin. Growth in high concentrations of dextrin (a strong inducer of the *gla* promoter) is very favourable for thaumatin secretion.

Discussion

A relatively large amount of thaumatin accumulates intracellularly, relative to the secreted levels (Moralejo et al. 2000; F. J. Moralejo and J. F. Martín, unpublished) despite the proven ability of *A. awamori* to secrete extracellular enzymes, suggesting that there is a specific problem in the secretory pathway for the secretion of thaumatin. We have demonstrated that overexpression of the *pdiA* gene from the *glaA* promoter results in high levels of the PDIA protein in a strain of *A. awamori* producing thaumatin. Several of the *A. awamori* transformants with the *pdiA* gene showed increased secretion of thaumatin in maltose-containing MDFA medium.

These results support previous evidence indicating that the level of PDIA in the cell is important for protein folding and secretion. Reduction of PDIA levels by antisense RNA in *A. niger* reduced glucoamylase secretion by 70% (Ngiam et al. 2000). Furthermore, disruption of the *prpA* gene of *A. awamori* (which belongs to the *pdiA* family) reduced secretion of chymosin by up to 80% (Wang and Ward 2000). However, the secretion of

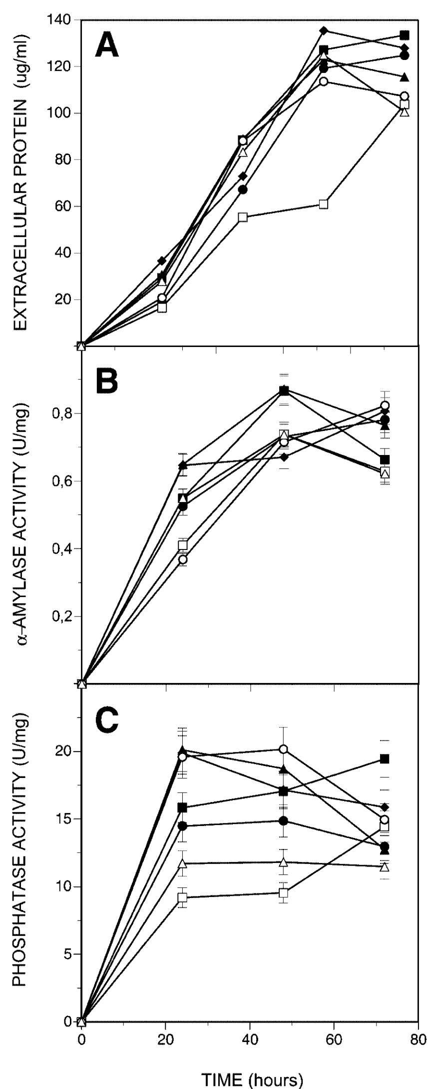

Fig. 3A–C Effect of amplification of the *pdiA* gene on secretion of extracellular enzymes and total protein in *A. awamori*. **A** Total extracellular protein. **B** α-Amylase. **C** Acid phosphatase. Symbols: *filled squares*, the parental TGDth-4 transformant; *filled circles*, AA016; *filled triangles*, AA064; *filled diamonds*, AA068; *open squares*, AA084; *open circles*, AA096; *open triangles*, AA100

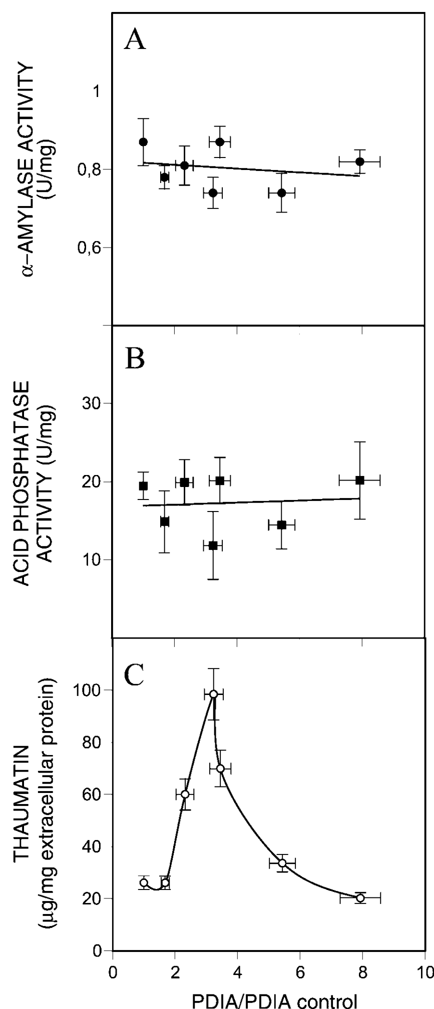

Fig. 4A–C Correlation between the levels of PDIA in the different *A. awamori* transformants and the levels of extracellular α-amylase (**A**), acid phosphatase (**B**) and thaumatin (**C**) they produce. The levels of PDIA are expressed as relative values with respect to the PDIA in the control strain. Note that extracellular α-amylase and acid phosphatase levels are relatively independent of the PDIA levels in the transformants, whereas optimal thaumatin secretion is highly dependent on the PDIA level

lysozyme, glucoamylase (or chymosin) was not improved by enhanced PDIA levels (Ngiam et al. 2000; Wang and Ward 2000).

It is interesting that the PDIA effect is observed on heterologous proteins such as thaumatin but not on endogenous *A. awamori* extracellular enzymes such as α-amylase or phosphatase, proteins with either no, or a small number of, disulfide bonds. Enhanced levels of PDIA in *Saccharomyces cerevisiae* have improved the secretion of disulfide bond-rich proteins such as antistasine (Schultz et al. 1994), antibodies (Shusta et al. 1998) or the platelet-derived human growth factor (Robinson et al. 1994), although in these systems a detailed study of relative PDIA levels was not done. Our results suggest that there is an optimal level of PDIA for thaumatin secretion and it is conceivable that the optimal level of PDIA will be different for other secreted proteins.

The best thaumatin-producing strain, AA100, contained intermediate levels of protein disulfide isomerase but, interestingly, transformants AA084 and AA096, which contain very high levels of PDIA, showed clearly lower levels of thaumatin secretion. An excess of PDIA accumulation in the cell, as occurs in transformants AA084 and AA096, may be unfavourable, perhaps because it modifies the folding equilibrium and leads to the synthesis of non-secretable forms of thaumatin. The PDIA protein also has a chaperone activity and at high levels it promotes the aggregation of immature proteins (Quan et al. 1995), independently of the number of disulfide bonds they contain (the so-called anti-chaperone effect) (La Mantia et al. 1991; Klappa et al. 1997). The decrease in protein export in transformants

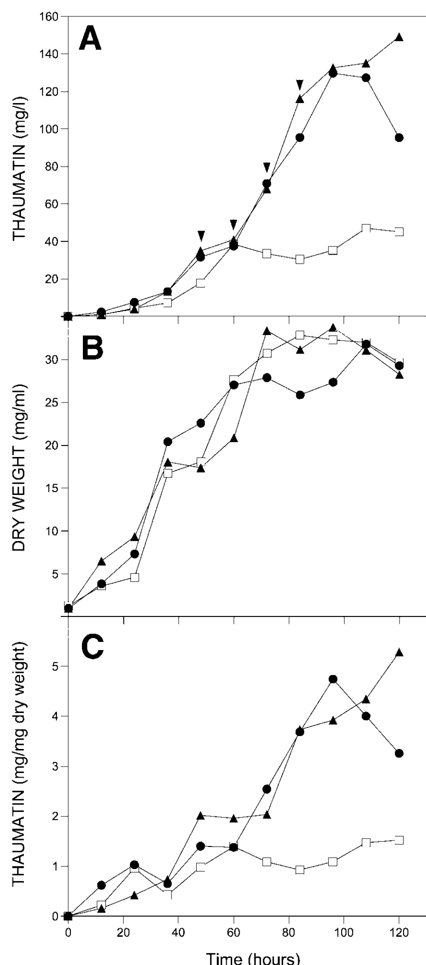

Fig. 5A–C Production of extracellular thaumatin in 5-l fementer cultures of the parental strain *A. awamori* TGDth-4 (*open squares*) and transformant AA100 (*filled circles*, *filled triangles*) in MDFA medium using dextrin (6%) as carbon source with (*filled triangles*) or without (*filled circles*) addition of 1% sucrose at the times indicated by the *vertical arrows*. **A** Volumetric production of thaumatin (mg/l). **B** Dry weight of mycelium (mg/l). **C** Specific yield of thaumatin (mg thaumatin/mg dry weight)

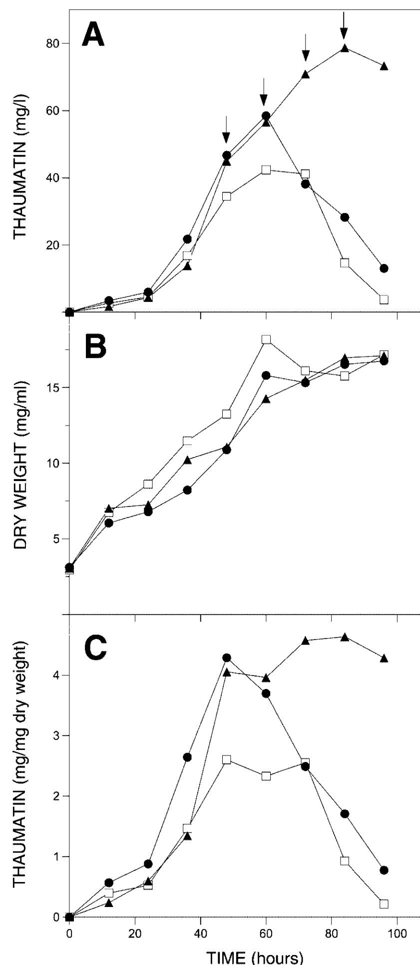

Fig. 6 Production of extracellular thaumatin in 5-l fermenter cultures of the parental strain *A. awamori* TGDth-4 (*open squares*) and transformant AA100 (*filled circles*, *filled triangles*) in MDFA defined medium containing increased amounts (12%) of dextrin as carbon source with (*filled triangles*) or without (*filled circles*) addition of 1% sucrose at the time indicated by the *vertical arrows*. **A** Volumetric production of thaumatin (mg/l). **B** Dry weight of mycelium (mg/l). **C** Specific yield of thaumatin (mg thaumatin/mg dry weight)

AA096 and AA084 may also be due to this antichaperone effect of the excess PDIA. In this regard, modulation of *pdiA* gene expression from a regulable promoter is important to avoid excessive accumulation of PDIA.

Using transformant AA100, very high levels of extracellular thaumatin were produced in submerged culture in controlled 5-l fermenters using dextrins as a carbon source. Dextrins are good inducers of the *glaA* promoter that drives expression of the *pdiA* gene. Under optimal conditions, the levels of thaumatin produced reached 150 mg/liter, a level that is of industrial interest.

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References

Archer DB, Peberdy JF (1997) The molecular biology of secreted enzyme production by fungi. Crit Rev Biotechnol 17:273–306

Archer DB, Jeenes DJ, MacKenzie DA, Brightwell G, Lambert N, Lowe G, Radford SE, Dobson CM (1990) Hen egg white lysozyme expressed in, and secreted from, *Aspergillus niger* is correctly processed and folded. Biotechnology 8:741–745

Fidel S, Doonan JH, Morris NR (1988) *Aspergillus nidulans* contains a single actin gene that has unique intron locations and encodes gamma actin. Gene 70:283–293

García-González MD, Martín JF, Vigal T, Liras P (1991) Characterization, expression in *Streptomyces lividans*, and processing of the amylase of *Streptomyces griseus* IMRU 3570: two

different amylases are derived from the same gene by an intracellular processing mechanism. J Bacteriol 173:2451–2458
Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. Appl Microbiol Biotechnol 47:1–11
Klappa P, Hawkins HC, Freedman RB (1997) Interactions between protein disulphide isomerase and peptides. Eur J Biochem 248:37–42
La Mantia ML, Miura T, Tachikawa H, Kaplan HA, Lennarz WJ, Mizunaga T (1991) Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast. Proc Natl Acad Sci USA 88:4453–4457
Malpricht S, Thamm A, Khanh NQ (1996) Cloning of cDNA for the protein disulfide isomerase from *Aspergillus niger* strain NRRL3 using PCR. Biotechnol Lett 18:445–450
Moralejo F-J, Cardoza R-E, Gutiérrez S, Martín JF (1999) Thaumatin production in *Aspergillus awamori* by use of expression cassettes with strong fungal promoters and high gene dosage. Appl Environ Microbiol 65:1168–1174
Moralejo F-J, Cardoza RE, Gutiérrez S, Sisniega H, Faus I, Martín JF (2000) Overexpression and lack of degradation of thaumatin in an aspergillopepsin-A defective mutant of *Aspergillus awamori* containing an insertion in the *pepA* gene. Appl Microbiol Biotechnol 54:772–777
Ngiam C, Jeenes DJ, Archer DB (1997) Isolation and characterization of a gene encoding protein disulphide isomerase, *pdi*A, from *Aspergillus niger*. Curr Genet 31:133–138
Ngiam C, Jeenes DJ, Punt PJ, van den Hondel CAMJJ, Archer DB (2000) Characterization of a foldase, protein disulfide isomerase A, in the protein secretory pathway of *Aspergillus niger*. Appl Environ Microbiol 66:775–782
Pedersen H, Beyer M, Nielsen J (2000) Glucoamylase production in batch, chemostat and fed-batch cultivations by an industrial strain of *Aspergillus niger*. Appl Microbiol Biotechnol 53:272–277
Punt PJ, van den Hondel CAMJJ (1987) Transformation of filamentous fungi based on hygromycin and phleomycin resistance markers. Methods Enzymol 216:447–457
Punt PJ, Van Gemeren IA, Drint-Kuijvenhoven J, Hessing JGM, Van Muijilwijk-Harteveld GM, Beijersbergen A, Verrips CT, van den Hondel CAMJJ (1998) Analysis of the role of the gene *bipA*, encoding the major endoplasmic reticulum chaperone protein, in the secretion of homologous and heterologous proteins in black *Aspergilli*. Appl Microbiol Biotechnol 50:447–454
Quan H, Fan GB, Wang CC (1995) Independence of the chaperone activity of protein disulfide isomerase from its thioredoxin-like active site. J Biol Chem 270:17077–17079
Robinson AS, Hines V, Wittrup KD (1994) Protein disulphide isomerase overexpression increases secretion of foreign proteins in *Saccharomyces cerevisiae*. Biotechnology 12:381–384
Robinson AS, Bockhaus JA, Voegler AC, Wittrup KD (1996) Reduction of BiP levels decreases heterologous protein secretion in *Saccharomyces cerevisiae*. J Biol Chem 271:10017–10022
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Schultz LD, Markus HZ, Hofmann KJ, Montgomery DL, Dunwiddie CT, Kniskern PJ, Freedman RB, Ellis RW, Tuite MF (1994) Using molecular genetics to improve the production of recombinant proteins by the yeast *Saccharomyces cerevisiae*. Ann NY Acad Sci 721:148–157
Shusta EV, Raines RT, Plückthun A, Wittrup KD (1998) Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. Nat Biotechnol 16:773–777
Spencer JA, Jeenes DJ, MacKenzie DA, Haynie DT, Archer DA (1998) Determinants of the fidelity of processing glucoamylase-lysozyme fusions by *Aspergillus niger*. Eur J Biochem 258:107–112
Wang H, Ward M (2000) Molecular characterization of a PDI-related gene *prpA* in *Aspergillus niger* var. *awamori*. Curr Genet 37:57–64
Wiertz EJHJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, Rapoport TA, Ploegh HL (1996) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 384:432–438
Witty M, Higginbotham JD (1994) Thaumatin. CRC Press, Boca Raton