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Molecular characterization of a PDI-related gene *prpA* in *Aspergillus niger* var. *awamori*

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Abstract A gene (*prpA*) homologous to the protein disulfide isomerase gene was isolated from *Aspergillus niger* by Southern hybridization using the *pdi1* gene isolated from *Trichoderma reesei* as a DNA probe. The corresponding cDNA of the *prpA* gene has also been isolated from an *A. niger* var. *awamori* cDNA library. The *prpA* gene does not belong to any currently recognized family of protein disulfide isomerases since it contains only a single conserved thioredoxin domain at the *N*-terminus of the protein. The *C*-terminal two-thirds of the protein has no homology to any known proteins in the database. The PRPA protein contains an ER retention signal (HDEL) at its *C*-terminal end suggesting that it is located in the ER. Southern hybridization at high stringency showed that it was present as a single copy in the genome. Northern hybridization indicated that the transcript level of the *prpA* gene was higher if the cells were secreting a heterologous protein, bovine prochymosin. However, over-expression of the *prpA* gene from a multicopy integrated vector had little effect on chymosin secretion. A strain containing a deletion of the *prpA* gene was viable. However, deletion of the *prpA* gene appeared to cause a reduction of bovine chymosin production.

Key words *Aspergillus niger* · Protein disulfide isomerase · Bovine prochymosin · *prpA*

Introduction

Protein disulfide isomerase (PDI, EC5.3.4.1) has been subjected to extensive study in both lower and higher eucaryotic cells since it was first discovered in the early 60s (Freedman et al. 1994). Molecular analysis and biochemical studies indicated that the PDI protein possesses disulfide-bond exchange properties in vitro. More importantly, due to its ER location, it has been proposed that the PDI protein, in conjunction with other chaperone proteins, is required for the proper folding of soluble ER proteins in vivo (Freedman et al. 1994). It has been demonstrated that protein disulfide isomerase is essential for normal cell growth since gene deletion causes loss of viability in *Saccharomyces cerevisiae* (Farquhar et al. 1991). Genes, or closely related genes, of the PDI protein have been isolated from a number of different organisms such as fungi, plants and mammals. There are four major groups in the family defined respectively as PDI, *ERp72*, *ERp60* and *ERp5* (Freedman et al. 1994). Each protein contains two conserved domains centered on the *WCGHCK* sequence except for the *ERp72* family proteins which contain three such regions (Mazzarella et al. 1990) and Mpd1 from *S. cerevisiae* which contains only one conserved domain (Tachikawa et al. 1995). Genes encoding PDI have been isolated from several filamentous fungi such as *Humicola insolens* (Kajino et al. 1994), *Aspergillus oryzae* (Hjort 1994) and *Aspergillus niger* (Malpricht et al. 1996; Ngiam et al. 1997). A gene (*tigA*) encoding a PDI-related protein of the Erp60 family has also been cloned from *A. niger* (Jeenes et al. 1997).

Purified PDI protein has not only been shown to possess disulfide-bond exchange activities but also to be able to enhance in vitro folding of proteins such as chymosin (Tang et al. 1994). Over-expression of the PDI protein has been found to selectively increase the secretion of human PDGF and *Schizsaccharomyces pombe* acid phosphatase in *S. cerevisiae* (Robinson et al. 1994). It has also been shown that improvement of the production of mammalian Fab′ antibody fragments in the periplasm of *Escherichia coli* could be achieved by co-expression of human protein disulfide isomerase (Humpherys et al. 1996) as could increased yield of the bovine pancreatic trypsin inhibitor by co-expression of

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rat protein disulfide isomerase (Ostermeier et al. 1996). Heterologous protein production in *A. niger* has been improved through classical mutagenesis and screening, and sufficient progress has been made to obtain commercial yields of bovine chymosin (Dunn-coleman et al. 1991). Although these approaches can improve protein production, the mechanism of improvement is not well understood and the yields of mammalian proteins are still much lower than for fungal proteins. We are now trying to address the problem by identifying individual components of the secretion pathway and attempting to identify the limiting steps in the production of heterologous proteins. We have isolated a *pdiA*-related gene (*prpA*) from *A. niger* by Southern hybridization and subsequent screening of a genomic DNA library using the *pdi1* gene isolated from *Trichoderma reesei* as a probe (Saloheimo et al. 1999). The corresponding cDNA of the *prpA* gene has been isolated from a cDNA library of *A. niger* var. *awamori*. Molecular characterization of the *prpA* gene is reported here.

Materials and methods

Strain constructions

The *A. niger* var. *awamori* UVK143f strain, a derivative of NRRL 3112, was obtained through several rounds of UV mutagenesis and screening for increased glucoamylase production (Ward et al. 1990). The *A. niger* 2663 E strain was derived from a separate lineage but was also improved for glucoamylase production after mutagenesis and screening. The GCΔAP4 strain was derived from UVK143f by deleting the major aspartic proteinase (Berka et al. 1990). *A. niger* var. *awamori* strain dgr246p2 (*pyrG*-) is deleted for the aspartic proteinase gene and was improved for chymosin expression by several rounds of mutagenesis and screening (Ward et al. 1993). The dgr246p2:trp- strain was isolated from a dgr246p2 strain in which the *Apa*I to *Xho*I fragment of the *trpC* coding region (Adams and Royer 1990) was deleted from the genome by homologous recombination and replaced by the *A. niger pyrG* gene. The *pyrG* gene was subsequently removed by selecting for 5-FOA resistance and the loss of the *pyrG* gene was confirmed by Southern analysis (Ward et al. 1993).

Southern analysis of the *A. niger* genomic DNA and library screening

A 1.3-kb *Eco*RI to *Xho*I fragment was purified from the *Trichoderma pdi1* cDNA clone (Saloheimo et al. 1999) and used as a DNA probe in Southern-hybridization experiments. The genomic DNA from the *A. niger* var. *awamori* strain UVK143f was isolated by repeated phenol/chloroform extraction (Hynes et al. 1983), digested with restriction enzymes, electrophoresed on a 1% agarose gel with TBE buffer and then transferred to a nitrocellulose membrane and hybridized with a radioactively labeled DNA probe in a buffer containing 25% formamide, 5× SSPE, 0.5% SDS and 0.1 mg/ml of denatured herring-sperm DNA. After hybridization, the DNA blot was washed twice with 2× SSC, 0.1% SDS, once with 0.5× SSC, 0.1% SDS and once with 0.1× SSC, 0.1% SDS. Each wash was for 30 min at 50 °C. The same conditions were used to screen an *A. niger* (2663E strain) genomic DNA library which was constructed by Clontech with approximately 15-kb inserts, generated by partial *Sau*3A digestion, in a lambda *DASH*II vector (T. Fowler, personal communication). DNA fragments from isolated phage were then subcloned into plasmids for sequence analysis.

Northern analysis

Fungal strains were first grown on plates at 37 °C. An agar plug containing growing mycelium was transferred to CSL medium (Dunn-coleman et al. 1991) and cultured for 2 days at 37 °C. The growing mycelium was then transferred to 50 ml of CSM medium (Ward et al. 1990). Two-day old CSM cultures were used for RNA purification by phenol/chloroform extraction (Ward et al. 1990). RNA was electrophoresed in a 1% agarose-formaldehyde gel, transferred to Nytran membranes and probed with radiolabeled DNA. The blots were washed at 65 °C in 0.1× SSC, 0.1% SDS.

Cloning of *Aspergillus* cDNA

Total RNA was extracted from the *A. niger* var. *awamori* UVK143f strain after the mycelium was grown in four different media containing either glucose, starch or beet pulp as the sole carbon source or else under nitrogen starvation. The cDNA library was constructed from a mixture of all above four RNAs in the yeast expression vector pYES2.0 (Invitrogen). The cDNA of the *prpA* gene was isolated after six rounds of sib-selection. The cDNA was completely sequenced on both strands.

Construction of expression vectors

The glucoamylase-prochymosin fusion-protein expression vector pGAMpR-II (Baliu and Wang 1995) was used as a control plasmid (see Fig. 4 a). The pGAMpR-II plasmid is similar to the pGAMpR plasmid (Ward et al. 1990) except that the starch-binding domain of glucoamylase was removed (codons 502–610 of mature glucoamylase). The plasmid pGCprpA constructed to co-express the *prpA* and glucoamylase-prochymosin genes was made by two steps: first, a 2800-bp *Bgl*II to *Xba*I fragment containing the *prpA* genomic DNA was inserted into the *Bam*HI to *Xba*I sites of pBH2 (Ward et al. 1989). A 2400-bp *Sma*I to *Nsi*I fragment of the *prpA* gene containing a polylinker sequence of the pBH2 plasmid was then inserted into the *Ssp*I site of pGAMpR-II (see Fig. 4 b).

Deletion of the *prpA* gene

Deletion of the *prpA* gene was done by transforming the dgr246p2 strain with a linear *Hin*dIII to *Xho*I DNA fragment within which the coding region of the *prpA* gene from *Pst*I to *Sal*I was substituted by the *Xho*I to *Sca*I fragment of the *A. niger pyrG* gene (see Fig. 6 a). After screening ten transformants by PCR, one strain was found containing a *prpA* gene disruption which was then confirmed by Southern analysis (see Fig. 6 b). The pGAMpR-II plasmid was co-transformed into this disrupted strain with pOBT which contains the *Aspergillus nidulans oliC* promoter driving the bleomycin resistant gene isolated from *Escherichia coli* and the *A. nidulans trpC* terminator in a pUC-based plasmid. The transformants were selected on plates overlaid with 50 μg/ml of phleomycin.

Fungal transformation

All plasmids used for transformation were purified on DNA affinity columns from Qiagen and transformed into fungal protoplasts by standard procedures of PEG-calcium transformation (Ward et al. 1990). The transformants were selected on minimal plates without uridine or with plates containing phleomycin. Chymosin expression was assayed by measuring the milk-clotting activity (Dunn-Coleman et al. 1991).

Results and discussion

Cloning of the *A. niger pdi1*-related gene *prpA*

Using the isolated *pdi1* gene from *T. reesei* as a DNA probe (Saloheimo et al. 1999), several bands in restric-

tion digests of *A. niger* var. *awamori* genomic DNA were detected on a Southern blot by low-stringency hybridization, suggesting that multiple homologous genes may exist. Similar conditions were subsequently used to isolate several phage clones from an *A. niger* genomic DNA library. Restriction mapping of phage DNA inserts indicated that they belonged to two distinct groups of clones. The genomic DNA of both clones was then analyzed by sequencing and the corresponding cDNAs were isolated from an *A. niger* var. *awamori* cDNA library and completely sequenced. Molecular characterization of the first *pdi1*-related gene revealed that it was the same as the *tigA* gene (Jeenes et al. 1997). The second gene was a distinct type of *pdi1* related gene which was then named *prpA* (Fig. 1). A TC-rich region (from −73 to −109) was found in the promoter region of the *prpA* gene which is similar to the TC-rich region found in the promoter of the *pdiA* gene of *A. niger* (Ngiam et al. 1997). There is a TATA-like sequence (TATTA) located 58 bp upstream of the TC-rich region (from −168 to −172), and several putative CAAT boxes were identified in the promoter region in both directions (data not shown). The genomic DNA of the *prpA* gene contains two introns located near the 5′ end of the coding sequence. The *prpA* gene encodes a protein of 464 amino acids. The translated protein sequence contained only one activation site (APWCGHCQNLKP) near the N-terminus of the protein. Sequence comparison showed that the N-terminal 150 amino acids contain homology to the protein disulfide isomerase while the C-terminal two-thirds of the protein did not match any sequences in the database. It does, however, contain an ER retention signal (HDEL) at the C-terminal end suggesting that it is an ER resident protein. Recently a yeast gene homologous to protein disulfide isomerase (MPD1) has been isolated as a high-copy suppressor of a deletion of the yeast PDI1 gene and it contains only one active site (Tachikawa et al. 1995). Searching of the database revealed that Mpd1 is one of the PDI-related proteins that shared highest homology to the PRPA protein. Figure 2 shows a sequence comparison of the thioredoxin domain of the two proteins.

Fig. 1 The genomic DNA sequence of the *A. niger prpA* gene. In the promoter region, the TC-rich region is *underlined*. Two introns are written in *lower case letters*. The translated protein sequence is also shown under the DNA sequence. The translation stop codon is represented by an *asterisk*. The DNA sequence was submitted to Genbank under the accession number AF095899

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AGA TCT CCC TGG GCA GAA GGG CCA TGC CCT TGC CTT CAG ATG GGG ATG CAA TGG ACA TGT TCG TTT GTA ATT TGA GGA TTG GTG AGG CGG GGA AGT GCC TGG CTT CCG AGC ACT AAG AAA -469

GAG ACC CTC GAT TAG TAC GGA GCA GGT AGT AAC TAA TCT AGT CTA ACG CTT AAG TAG ATA GGT AAG GGC TCC GCA TAA ACA AAG GAT CTT ATG GGC CCG GAG CTA CGG AGC TCG TGC TCA -349

TGG AGG CTT CCC GGA AGA CAG GAT TGC CGA CGC CAT GGA TTT TGC AGA ATA GTT AAC TGA ATC CAA TCC CGT AGA GAA TGG ATG CCA TTC ATG GGG GGA TGA ACA ATG GAC TGG TGT GAT -229

GGC GGG GCA ACA TCG AAC GGA CGG ATC CGC CGC CAT TCC AAA TCC ATA CAA TTC AAT ATT ACT TCT TAA GAC ATT TCG CGT CAC ATG CCA AGA GCT TCA GGA CAC CTT GCT TCT ATC TAC -109

TTC TTT CTG TCC TCT CTT CTC CCT CTC TTC TTC CTC GAT TCA TCC CCG CTG GGT GAT GCT TTA GCT GCT ACT CTT GGA TCC CCT CTC GCA TCT TCC TTA CCC GCA ATC ATG CTG CAG CCC  12
                                                                                                                                                    met leu gln pro

AGC TCT GCG TTG CTT TTC GTC ACG TCG CTT CTG GCG GCG TTG CCC GTC AAC GCC GAT GGA TTG TAT ACG AAG AAG TCC CCC GTC TTG CAG GTC AAC CAG AAG AAC TAC GAC CAG CTC ATT 132
ser ser ala leu leu phe val thr ser leu leu ala ala leu pro val asn ala asp gly leu tyr thr lys lys ser pro val leu gln val asn gln lys asn tyr asp gln leu ile

GCA AAC TCC AAT CAC ACT TCG gta agt aca gct gtg cag gtt att aca att gcc tac aga caa gtc taa taa gct ctc cta gAT CGT AGA gta agc cat cga tca ccc tac cca tct acc 252
ala asn ser asn his thr ser                                                                                                    ile val glu

tcc cac aat cct aaa cct ccc cgc tct ccc tct agA TTC TAC GCT CCC TGG TGC GGC CAC TGC CAG AAC CTA AAG CCC GCC TAC GAA AAA GCC GCA ACT AAT CTC GAC GGC CTG GCC AAA 372
                                               phe tyr ala pro trp cys gly his cys gln asn leu lys pro ala tyr glu lys ala ala thr asn leu asp gly leu ala lys

GTC GCC GCC GTC AAT TGC GAC TAT GAC GAC AAC AAA CCC TTC TGC GGC CGC ATG GGC GTC CAG GGC TTC CCT ACC CTC AAG ATC GTC ACC CCC GGC AAG AAA CCC GGC AAG CCC CGC GTG 492
val ala ala val asn cys asp tyr asp asp asn lys pro phe cys gly arg met gly val gln gly phe pro thr leu lys ile val thr pro gly lys lys pro gly lys pro arg val

GAA GAC TAC AAG GGC GCA CGA AGT GCC AAA GCG ATT GTC GAG GCA GTC GTC GAC CGG ATT CCC AAC CAT GTG AAG CGC GCA ACA GAC AAG GAC CTT GAC ACT TGG CTC GCG CAG GAT GAG 612
glu asp tyr lys gly ala arg ser ala lys ala ile val glu ala val val asp arg ile pro asn his val lys arg ala thr asp lys asp leu asp thr trp leu ala gln asp glu

GAA TCC CCC AAG GCC ATC CTC TTC ACG GAG AAA GGC ACC ACC AGC CCA CTC CTC CGC GCC CTG GCC ATC GAC TTC CTC GGC TCC ATC CAA GTC GCT CAA GTC CGC AAC AAG GAA ACC GAA 732
glu ser pro lys ala ile leu phe thr glu lys gly thr thr ser pro leu leu arg ala leu ala ile asp phe leu gly ser ile gln val ala gln val arg asn lys glu thr glu

GCC GTC GAG AAA TTC GGC ATC ACC GAG TTC CCA ACC TTC GTC CTA CTC CCA GGA GGC GGC CAA GAC CCC ATC GTC TAC GAC GGC GAA CTG AAG AAG AAG CCC ATG GTC GAA TTC CTC AGC 852
ala val glu lys phe gly ile thr glu phe pro thr phe val leu leu pro gly gly gly gln asp pro ile val tyr asp gly glu leu lys lys lys pro met val glu phe leu ser

CAA GCC GCT GCT CCT AAC CCG GAT CCT GCT CCC AAG GGC TCG ACC GCG CCC CGC GAT AAC AAC AAG AAG AAA TCC ACC GAA CCT TCT CCA GAC TCC AAG ATT GTC TCG GAC GAG GCC AAA 972
gln ala ala ala pro asn pro asp pro ala pro lys gly ser thr ala pro arg asp asn asn lys lys lys ser thr glu pro ser pro asp ser lys ile val ser asp glu ala lys

CCC GCC AGT GTG CCC ATT CCG GCT CCC CCC ATT GGT ACC CTG CCC ACT GCG GAA GCC CTC GAG GCT GCT TGT CTG ATG CCG AAA TCC GGT ACC TGT GTG CTG GCT CTC CTC CCT GAA CCG 1092
pro ala ser val pro ile pro ala pro pro ile gly thr leu pro thr ala glu ala leu glu ala ala cys leu met pro lys ser gly thr cys val leu ala leu leu pro glu pro

AGT GAG CCG GAC GCA GAG CTC CCG GCT CCG GCC AAG GAC GCC CTC CTC AGT CTC GCT GAG ATC TCG CAC AAG CAC GCA GTC CGT AAG AGC AAG CTC TTC CCG TTC TAC AGT GTC CCG GCT 1212
ser glu pro asp ala glu leu pro ala pro ala lys asp ala leu leu ser leu ala glu ile ser his lys his ala val arg lys ser lys leu phe pro phe tyr ser val pro ala

ATC AAT AGC GGA GCT AAG ACC CTC CGC GCT GGG CTT GGT CTG CCT GAG GAT AAC TCG GTG GAG ATC GTT GCT GTG AAT GGA CGC CGT GGC TGG TGG CGC CGG TAT GAC TCG GTT GAG GGC 1332
ile asn ser gly ala lys thr leu arg ala gly leu gly leu pro glu asp asn ser val glu ile val ala val asn gly arg arg gly trp trp arg arg tyr asp ser val glu gly

GCA GAG TAC GGC CAG GAG CGT GTC GAG GCT TGG ATT GAT GCG ATC AGG CTG GGT GAG GGT GAG AAG CAG AAG TTG CCT GAT GGC GTT GTC GTT GAA GAG GTA GTT GAG GAG AAG GTC GAA 1452
ala glu tyr gly gln glu arg val glu ala trp ile asp ala ile arg leu gly glu gly glu lys gln lys leu pro asp gly val val val glu glu val val glu glu lys val glu

GAG AAG GTC GAG GAA GTG GTT GAA GAA CCC GTC GAG GAG AAG CCG GCG GTC GAC CAC GAC GAA TTG TAA AAC ATA TGG TCC GTA TGG AGT GCA TGA ATT TGT TTA TTA GCA CAG GTG TTT 1572
glu lys val glu glu val val glu glu pro val glu glu lys pro ala val asp his asp glu leu  *

ATC AGG TCA AAT AAG TAC TAC TAG CTG GTT CCC CAT ATC GAG TAT CAA AAG CAT ACA TAT CAT CTA CTG TCA GCT ACT TCA ATT CCA CTA ATC GGG ATG AAC TTG TAT TGG AAC ACT CAT 1692

GTA GAA ATA AGC TCT CTA AAG ATT CAA TTA AGA CTT TTT CAA ACC CAC CCA CAT CAT ATA TAC AAT ACA ACT ATA CAT CAT TCA AAA CAA GAT TCA                                 1788
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                    10        20        30        40        50        60
prpA          MLQPSSALLFVTSLLAALPVNADGLYTKKSPVLQVNQKNYDQLIANSNHTSIVEFYAPWCGHCQNLKP
                  :  :  |      | |   |      :  :  | :|  | | | ||:||||||||||  |
MPD1           MLFLNIIKLLLGLFIMNEVKAQNFYDSDPHISELTPKSFDKAIHNTNYTSLVEFYAPWCGHCKKLSS

                    70        80        90       100       110       120       130
prpA          AYEKAATNLDGLAKVAAVNCDYDDNKPFCGRMGVQGFPTLKIVTPGKKPGKPRVEDYKGARSAKAIVE
                 : |||  |||:  |||||||   ||  | :  |:||||| :  | |      ::  |   ||:|
MPD1          TFRKAAKRLDGVVQVAAVNCDLNKNKALCAKYDVNGFPTLMVFRPPKIDLSKPIDNAKKSFSAHANEV
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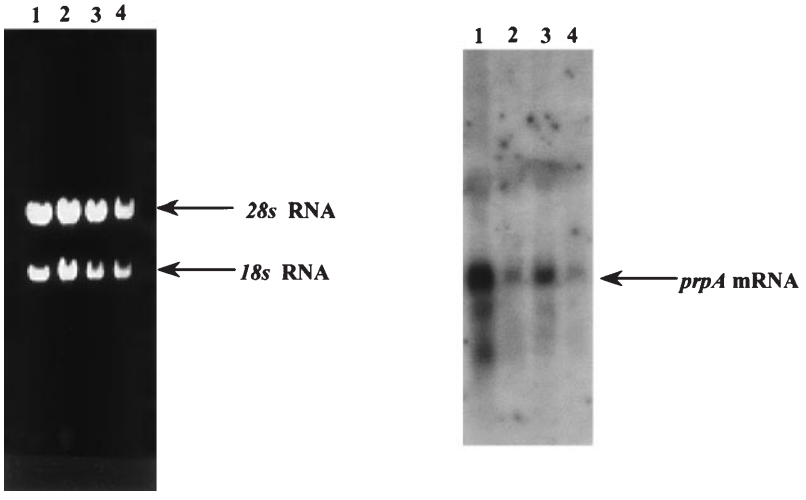
Fig. 2 Sequence alignment of the *N*-terminal 136 amino-acid sequence of the *A. niger* var. *awamori PRPA* protein with the *S. cerevisiae* Mpd1 protein sequence. Identical residues are marked by *vertical lines* and conserved changes are indicated by *two dots*. There are 51 identical amino acids (37.5%) and 14 conserved amino acids (10.3%) between the two proteins

Northern analysis

We have obtained and analyzed many transformants from strain dgr246p2 (a strain improved for chymosin production) containing four different expression plasmids. Two transformants, selected for Northern analysis, contained a similar copy number of multicopy integrated plasmids expressing either the preproglucoamylase catalytic domain (aa 1–501 of the mature glucoamylase) or the preproglucoamylase catalytic domain fused to bovine prochymosin. The other two strains selected for Northern analysis contained only a single plasmid integrated at the glucoamylase locus expressing either the catalytic domain of glucoamylase or the glucoamylase-prochymosin fusion protein. Northern hybridization was performed to determine the effects of the above expression vectors on the expression levels of the *prpA* gene. The results clearly showed that the level of transcription of the *prpA* gene increased when cells were expressing fusion proteins containing chymosin (Fig. 3 a, lanes 1 and 3). The highest increase of mRNA can be seen in lane 1, as compared to lane 2, when the chymosin was expressed as a fusion protein in a multicopy-integrant. Ethidium bromide staining indicated that slightly more RNA was loaded in lane 2. A modest increase in mRNA level was also evident in lane 3, as compared to lane 4, when the chymosin was expressed as a fusion, but only a single expression plasmid was integrated at the glucoamylase locus. Ethidium bromide staining demonstrated that very similar amounts of RNA were loaded in lanes 3 and 4. The mRNA level of the *prpA* gene was similar in an untransformed control strain (data not shown) compared to the two strains which did not produce chymosin (lanes 2 and 4). The mRNA of the *prpA* gene was also induced when the bovine chymosin was expressed as a fusion to the catalytic domain of the glucoamylase in a multicopy integrant in strain GCΔAP4 (see lanes 1 and 2 of Fig. 5). We have also observed a similar induction of the *pdiA* and the *tigA* genes (data not shown). Therefore, the expression of heterologous proteins affects the expression of all three *pdi*-related genes in *A. niger*.

Fig. 3 A,B Northern analysis of expression levels of the *prpA* gene in transformants of *A. niger* var. *awamori* strain dgr246p2. **Panel A** shows the ethidium bromide staining picture of the RNA gel. The 28S and 18S ribosomal RNA bands are indicated by *arrows*. **Panel B** shows the Northern hybridization result using the *prpA* gene as probe (the 747-bp *Xba*I to *Xho*I genomic DNA fragment of the *prpA* gene). The mRNA transcripts of the *prpA* gene are indicated by an *arrow*. *Lane 1* contains RNA extracted from a multicopy integrant transformant with pGAMpR-II. *Lane 2* contains RNA extracted from a multicopy integrant transformant with a version of $pGAM_pR$-II lacking the prochymosin sequence. *Lane 3* contains RNA extracted from a strain with a single copy of plasmid pGAMpR-II integrated at the glucoamylase locus. *Lane 4* contains RNA extracted from a strain with a single plasmid integrated at the glucoamylase locus expressing the glucoamylase catalytic domain only



Effect of over-expression of the *prpA* gene

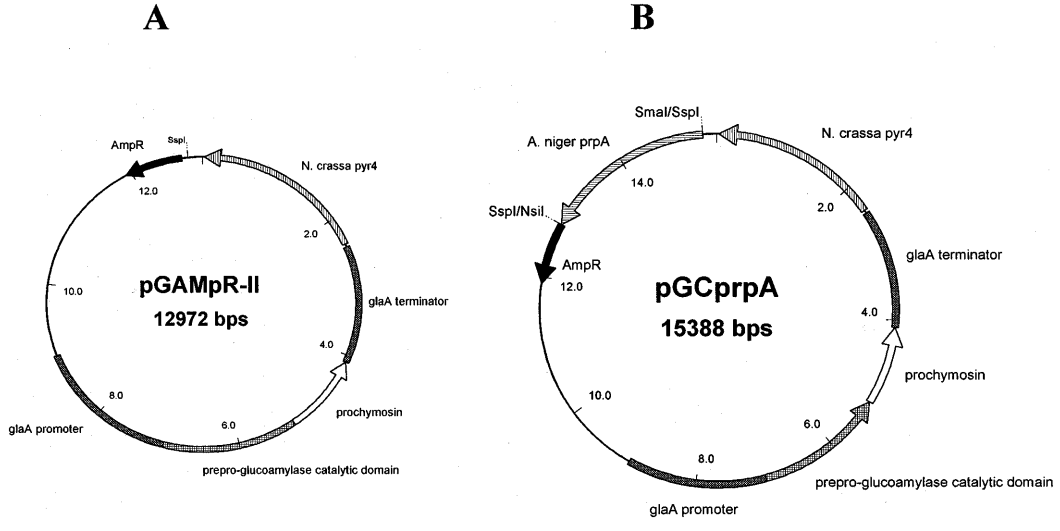
Since the expression of a heterologous protein in *A. niger* var. *awamori* clearly induced expression of the *prpA* gene, we decided to test the effects of further over-expression of the *prpA* gene on bovine prochymosin production. We first constructed a vector (pGCprpA) designed to co-express the preproglucoamylase catalytic domain-prochymosin fusion with the *prpA* gene in the same plasmid (Fig. 4 b). The plasmid, pGAMpR-II (Fig. 4 a), which did not contain the *prpA* gene, was used as control. The expression vectors were transformed into strain GCΔAP4. Twenty transformants with each vector were isolated, grown in shake flasks and assayed for chymosin activity. Table 1 shows the results obtained from the three highest chymosin producers obtained with each plasmid. The level of chymosin produced by the control transformants without over-expression of the *prpA* gene (pGAMpR-II transformants) was similar to the transformants with over-expression of the *prpA* gene (pGCprpA transformants). Therefore, there was no effect of over-expression of the *prpA* gene on chymosin production. The difference in chymosin expression level between strains presumably results from integration of the expression plasmids at different chromosomal loci or at different copy numbers. We showed by Northern hybridization that the messenger levels of the *prpA* gene were indeed increased in two of three pGCprpA transformants (lanes 4 and 5 of Fig. 5 a). The smearing of the *prpA* transcript in these two lanes may result from abnormal transcription initiation or termination when over-expressed. In the control experiment, the messenger level of the *pgk* gene was similar in all five strains (lanes 1–5 of Fig. 5 b). However, we have not been able to confirm over-expression at the protein level due to the lack of corresponding antibodies and activity assays of the PRPA protein. Similar results, showing a lack of effect of *prpA* over-expression, were obtained with strain dgr246p2 which is capable of producing higher levels of chymosin than the GCΔAP4 strain.

Table 1 Assay data for chymosin expression from transformant strains containing the expression plasmids. The first column lists the names of the plasmids that were used for each transformation. The second column shows the designation of the three best transformants among 20 independent transformants picked from each transformation. The third column indicates the chymosin expression level of each construct in chymosin milk-clotting activity units

Integrated plasmid	Transformant	Chymosin units
pGAMpR-II	Q33-3	12.9
	Q33-4	9.9
	Q33-16	13.1
pGCprpA	Q57-2	18.3
	Q57-4	9.7
	Q57-6	12.0

A previous report (Jeenes et al. 1997) showed induction of the *pdiA* and *tigA* mRNAs when cells were treated with tunicamycin. Here, we have clearly demonstrated that the *prpA* mRNA levels (and those of *pdiA* and *tigA*, data not shown) increased as a result of the production of the heterologous protein prochymosin in *A. niger* var. *awamori*. Several reports have suggested that over-expression of protein disulfide isomerase will enhance heterologous protein production extracellularly in *S. cerevisiae*, especially for those proteins that contain multiple disulfide bonds (Robinson et al. 1994). However, over-expression by an increased copy number of the *prpA* gene in *A. niger* var. *awamori* did not stimulate extracellular chymosin production significantly. Similar results were obtained when the *pdiA* or *tigA* genes were over expressed (data not shown). No improvement was

Fig. 4 A,B Circular maps of two plasmids used in the co-expression of chaperone genes with a prepro-glucoamylase catalytic domain-bovine prochymosin fusion. **A** shows the pGAMpR-II plasmid which was used as a control for prochymosin expression without any chaperone gene. **B** is pGCprpA containing the *A. niger* var. *awamori* *prpA* gene cloned into the *Ssp*I site of pGAMpR-II. Plasmids are not drawn to the same scale



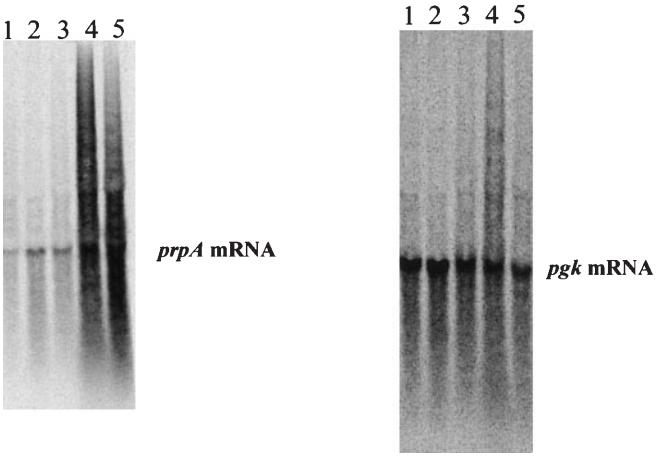


Fig. 5 A,B Northern analysis of *prpA* over-expression in strain GCΔAP4. **Panel A** the Northern blot was probed with the *prpA* genomic DNA (the 747-bp *Xba*I to *Xho*I genomic DNA fragment of the *prpA* gene). *Lane 1* shows the *prpA* mRNA level in the parental strain (GCΔAP4). *Lane 2* shows the *prpA* mRNA level in GCΔAP4 transformed with plasmid pGAMpR-II. *Lane 3* shows the *prpA* mRNA level in the GCΔAP4 strain containing pGCprpA (the transformant Q57-2). *Lane 4* shows the *prpA* mRNA level in the GCΔAP4 strain containing pGCprpA (the transformant Q57-4), and *lane 5* shows the *prpA* mRNA level in the GCΔAP4 strain containing pGCprpA (the transformant Q57-6). **Panel B** shows Northern hybridization of an identical blot using the *pgk* probe (the 2.3-kb *Cla*I to *Pst*I genomic DNA fragment of the *pgk* gene)

also observed for chymosin production when we used another strain, dgr246p2:trp- (a strain improved for glucoamylase-prochymosin production by mutagenesis and selection, and having a *pepA* gene disruption). In this case, the glucoamylase-prochymosin fusion and the *prpA* gene were expressed from separate plasmids (data not shown). It seems that the *A. niger* var. *awamori* ER chaperones are well regulated to meet the requirement for high-level expression of some heterologous proteins. The fact that over-expression of the *prpA, pdiA* or *tigA* genes did not improve chymosin production may suggest that expression of these chaperones is not the limiting step. However, it is possible that over-expression of these genes may enhance the production of other heterologous proteins. We cannot eliminate the possibility that over-expression of the chaperone genes may increase the intracellular chymosin level, without affecting extracellular levels, by promoting proper folding in the ER. This was apparently the effect on IL-6 production since over-expression of the *bipA* gene increased the intracellular human interleukin-6 level, but not the extracellular protein level in *A. niger* (van den Hondel et al. 1997). It will be important to determine the effect of the combination of over-expression of the *prpA* gene with other ER chaperones, such as the *bipA* gene, on hetero- logous protein production in filamentous fungi

Fig. 6 A,B Scheme for the deletion of the *prpA* gene and Southern analysis of the deletion strain. **Panel A** shows the restriction map of the wild-type *prpA* genomic DNA (top) and a restriction map of the *prpA* gene deletion (bottom). The ΔprpA strain was made by replacing the *Pst*I to *Sal*I fragment of the *prpA* gene (most of the coding region) with *A. niger pyr*G gene. **Panel B** shows the Southern hybridization result for the *prpA* gene deletion. DNA isolated from the wild-type strain and the deletion strain are indicated on top of the Southern blot. The DNA probe used for Southern analysis is indicated on top of the restriction map in **Panel A**. A 3.7-kb *Xho*I fragment detected in the wild-type strain is representative of the undisrupted *prpA* gene. However, in the deletion strain, a larger *Xho*I fragment (about 7 kb) is present due to insertion of the *A. niger pyr*G gene and loss of an internal *Xho*I site. Two weak hybridization bands in each lane may result from partial *Xho*I digestion of the genomic DNA

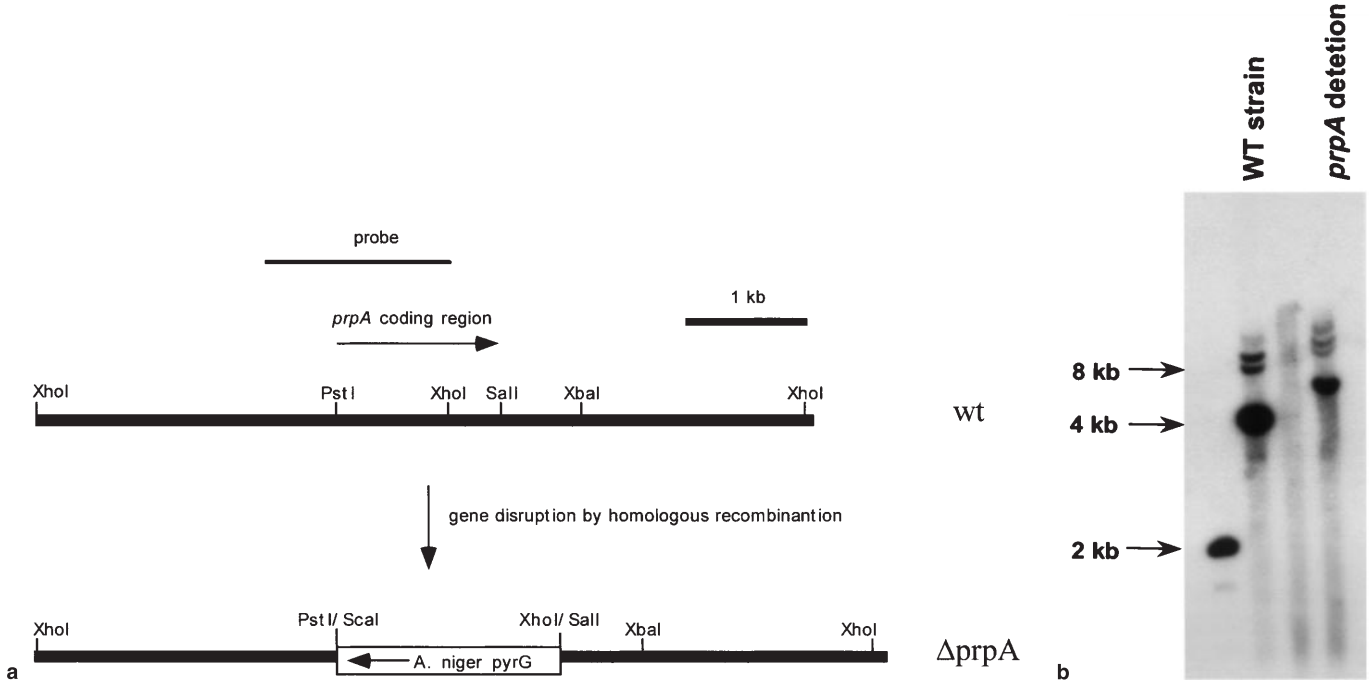


Table 2 Assay data for chymosin expression from the wild-type strain and the *prpA* deletion strain containing the expression plasmids. The expression levels of the top three producers are shown in the wild-type strains from 15 randomly picked transformants and in the *prpA* deletion strain from 19 randomly picked transformants

Strain used for transformation	Wild-type strain	Deletion strain
Plasmid used for transformation	pGAMpR-II	pGAMpR-II and pOBT
Chymosin units from top three producers	58 units (CS5-6) 64 units (CS5-7) 52 units (CS5-14)	12.8 units (Q6971-1) < 3.2 units (Q6971-11) 8.2 units (Q6971-19)

since an additive effect has been reported in *Saccharomyces* (Shusta et al. 1998).

Effect of *prpA* gene disruption

We decided to test the effects of disruption of the *prpA* gene of *A. niger* var. *awamori* on strain viability and chymosin expression. Southern hybridization indicated that the *prpA* gene was present in only a single copy in the genome (data not shown). We disrupted the *prpA* gene in strain dgr246p2 by replacement with the *pyrG* gene of *A. niger* through homologous recombination (Fig. 6 a). The disrupted transformant was first identified by PCR and Southern hybridization was used to confirm this deletion (Fig. 6 b). It is a non-essential gene since we have not detected any noticeable growth difference compared to an undeleted strain. Unexpectedly, we were unable to obtain stable uridine auxotrophic derivatives of this deleted strain by 5-FOA selection. Therefore we co-transformed pGAMpR-II with the plasmid, pOBT, which contains the bacterial phleomycin resistance gene under the control of an *A. nidulans oliC* promoter and *trpC* terminator (see Materials and methods), into the *prpA* deleted strain. The transformation efficiency was very low for this strain, but 19 colonies were obtained from phleomycin-containing plates, of which only three transformants produced bovine prochymosin. The production level of bovine prochymosin was lower than expected for three randomly isolated transformants (Table 2), which may result from *prpA* gene deletion. However, we cannot rule out that this simply reflects the integration sites or copy number of the expression plasmid since only three transformants expressing chymosin were analyzed. We have also deleted the *tigA* gene but were unable to detect any noticeable effect of the deletion on cell growth under normal laboratory growth conditions or on chymosin production (data not shown).

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