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Secretion of Recombinant Pro- and Mature Fungal α-Sarcin Ribotoxin by the Methylotrophic Yeast *Pichia pastoris:* The Lys–Arg Motif Is Required for Maturation

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α-Sarcin is a ribosome-inactivating protein from the mold *Aspergillus giganteus*. The methylotrophic yeast *Pichia pastoris* has been transformed with two plasmids (pHILD2preαS and pHILS1preαS), which contain the complete α-sarcin cDNA, including its original fungal leader peptide, under the control of yeast alcohol oxidase promoter. The second one is indeed fused to the signal sequence of *P. pastoris* acid phosphatase. The transformed yeasts secreted both mature and pro-α-sarcin. The presence of this pro-α-sarcin in the yeast extracellular medium is due to an inefficient recognition of the pro-sequence by a putative Kex2p-like endopeptidase. A third plasmid accounting for a single mutation of the α-sarcin leader peptide was designed to produce a more efficient Kex2p recognition motif. This approach resulted in the extracellular production of only the mature protein, suggesting the existence of a two-step mechanism for processing its leader peptide. This recombinant α-sarcin is identical to the original fungal protein, according to activity and spectroscopic criteria. In addition, pro-α-sarcin, which has been characterized for the first time, also exhibits ribonucleolytic activity as the mature protein does. Therefore, protection of the producing cells against this kind of ribotoxins may depend on an efficient recognition of the signal sequence followed by translocation of the nascent polypeptide to the endoplasmic reticulum.

Secreted eukaryotic proteins are synthesized as preproproteins, with an NH_2-terminal targeting domain, or signal sequence, which promotes their translocation into the rough endoplasmic reticulum (1). These signal sequences show a number of conserved features that are essential for protein export (2), although displaying different interactions with the translocation machinery (3). The late secretory steps involve specific proteolysis of the proproteins to yield the corresponding secreted mature forms, Kex2p protease being the best known member of a family of homologous eukaryotic endopeptidases involved in such steps (4).

α-Sarcin is a cytotoxic protein, active against several human tumor cell lines (5), secreted by the mold *Aspergillus giganteus* MDH 18894 (6). This cytotoxin belongs to a family of highly similar proteins from different *Aspergillus* spp. (restrictocin, mitogillin, AspF1, clavin, and gigantin). Their cytotoxicity is due to their ribonucleolytic activity against a single phosphodiester bond of the larger rRNA, resulting in the so-called α-fragment of about 400 nt length (7), which leads to inhibition of protein biosynthesis (8). α-Sarcin is able to hydrolyze this bond in ribosomes from all eukaryotes and prokaryotes tested so far (9–12) because the region around the cleavage site is evolutionary conserved. Therefore, α-sarcin could be used as a good model for protein secretion, since inefficient export of this protein would potentially lead to cell death. In this context, the posttranslational processing, to render extracellular mature toxin, and the self-protection of the toxin-producing ribosomes are intriguing questions for this kind of cytotoxins. Although the *sar* gene has been cloned and efficiently expressed in *Escherichia coli* (13), a host eukaryote system needs to be used to answer these questions. Restrictocin, from *Aspergillus restrictus,* has been produced in *A. nidulans* and *A. niger,* by using both its own gene promoter (14) and the promoter of

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glucoamylase A from *A. awamori* (15), but in a much lower yield than by the original mold. Production of restrictocin in *Saccharomyces cerevisiae* failed because the cells died (12), although this protein has no cytotoxic effect on yeast when present in the medium (16).

The methylotrophic yeast *Pichia pastoris* has been used to produce heterologous proteins in extremely high yields (17, 18). Many of the secretion products used either their own signal peptide or other signal peptide efficiently recognized by the yeast, as a leader sequence for the *S. cerevisiae* α-mating factor (18–23). Thus, we have transformed *P. pastoris* cells using different expression cassettes containing the cDNA of α-sarcin with its own leader peptide (*preproαS*). Indeed, one of them is fused to the sequence encoding the signal peptide of *P. pastoris* acid phosphatase. Under these conditions, both mature and pro-α-sarcin are secreted by the yeast; however, a selected mutation of the leader peptide resulted in an efficient processing of the synthesized protein. Thus, several goals have been achieved: characterization of the pro-toxin (which has also resulted in an active ribonuclease), analysis of the specificity of posttranslational processing enzymes in *P. pastoris*, and production of native α-sarcin in high yield.

MATERIALS AND METHODS

Microorganisms

P. pastoris GS115 *his4* strain (Invitrogen Corp.) was used as host for transformation. The bacterial strains used for DNA manipulations were *E. coli* DH5αF′ [F′ *endA*1 *hsdR*17 (r_K^- m_K^+) *supE*44 *thi*-1 *recA*1 *gyrA* (Nal^R) *relA*1 Δ(*lacZYA-argF*)U169 *deoR* ϕ*80dlac*Δ*(lacZ)*M15] and TG1 [F′Δ(*lac-proAB*) *supE thi* Δ(hsd^M-*mcrB*)5(r_K^- m_K^+ $McrB^-$) *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]. Single-stranded dU-substituted template for mutagenesis was produced in *E. coli* BW313 [*HfrKL*16 *po*/45 [*lysA*(61-62)] *dut*1 *ung*1 *thi*1 *relA*1] subsequent to its infection with f1 helper phage.

Media and Growth Conditions

P. pastoris GS115 strain was cultured on complete medium YPD[2] (24). Minimal media used for selection and maintenance of His^+ (*His4* genotype) transformants were composed of 13.4 g/liter yeast nitrogen base with ammonium sulfate, 0.4 mg/liter biotin, and 20 g/liter dextrose (MD medium), or 0.5% (v/v) methanol (MM medium) as carbon source. For solid cultures, all these media were supplemented with 15 g/liter agar. All cultures were carried out at 30°C.

For the production of α-sarcin, selected (His^+ Mut^s) transformed strains (slow methanol utilization phenotype) were cultured for 48 h in BMGY medium (10 g/liter yeast extract, 20 g/liter peptone, 0.4 mg/liter biotin, 13.4 g/liter yeast nitrogen base with ammonium sulfate, 0.1 M potassium phosphate, pH 6.0, and 1% v/v glycerol). Cells were then collected by centrifugation and resuspended in 1/5 of the original volume of BMMY (same as BMGY, but 0.5% v/v methanol instead of glycerol) for induction of the *AOX1* promoter. This culture was maintained for 2 or 4 days and daily supplemented with 2.5 ml of methanol per liter of culture.

Construction of α-Sarcin Expression Vectors

Recombinant DNA methods other than those reported below were performed according to standard procedures (25). The codifying region of the *sar* gene, including its leader sequence (26), was amplified by PCR using the plasmid pOMPAαS (13) as template and two primers, 5′-GGAATTCATGGTTGCAATCAAAAAC-3′ and 5′-GGAATTCCTAATGAGAGCAGAGCTT-3′, which respectively hybridized with the 5′ and 3′ ends of the protein-encoding region. In addition, both primers included an *Eco*RI site to facilitate the cloning of the amplified DNA into the expression vectors described below. The enzyme employed was Vent DNA polymerase (New England Biolabs). The DNA fragment purified from the PCR (*preproαS* cDNA) was digested and inserted into the unique *Eco*RI site of plasmids pHIL-D2 and pHIL-S1 (Invitrogen Corp.), under the control of the *P. pastoris AOX1* promoter (Fig. 1). This type of control is extremely convenient for a toxic protein as α-sarcin because the regulation is exerted at the level of transcription, involving a repression/derepression mechanism. In addition, pHIL-S1 contains the secretion signal sequence of *P. pastoris* acid phosphatase (*PHO1*) for the extracellular production of the recombinant protein. The in-frame arrangement of both signal sequences in pHILS1preαS was established after performing the DNA sequence of this plasmid region.

[2] Abbreviations used: ACE_T, testicular angiotensin-converting enzyme; *Amp*, ampicillin resistance gene; *AOX1*, alcohol oxidase gene; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; BPTI, bovine pancreatic trypsin inhibitor; dU, deoxiuridine; *HIS4*, histidinol dehydrogense gene; His^+, histidinol dehydrogenase activity phenotype; His^-, deficient histidinol dehydrogenase activity phenotype; MD, minimal dextrose medium; MM, minimal methanol medium; Mut^+, methanol utilization phenotype; Mut^s, slow methanol utilization phenotype; nt, nucleotide(s); PCR, polymerase chain reaction; pHILD2preαS, pHILD2 plasmid containing *preproαS* cloned into the *Eco*RI site; pHILD2preαS(A-2K), plasmid identical to pHILD2preαS with the only difference that the sequence corresponding to Ala in position −2 of *preproαS* has been changed to Lys; pHILS1preαS, pHILS1 plasmid containing *preproαS* cloned into the *Eco*RI site and fused in-frame to the signal sequence of *PHO1; PHO1*, DNA encoding the *P. pastoris* acid phosphatase; poly(A), polyadenylic acid; pOMPAαS, plasmid containing *preproαS* fused to the outer membrane protein A signal peptide; *preproαS*, DNA encoding prepro-α-sarcin, including its start and stop codons; *sar*, gene encoding α-sarcin; YPD, yeast peptone dextrose medium.

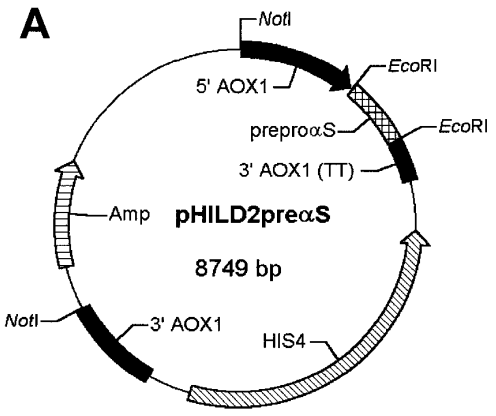

FIG. 1. (A) pHILD2preαS expression plasmid. *Amp,* ampicillin resistance gene. *HIS4, P. pastoris* histidinol dehydrogenase gene to complement the defective *his4* genotype in *Pichia* GS115 host strain. *5′AOX1,* segment of about 1000 bp, including the alcohol oxidase promoter. *3′AOX1*(TT), segment of about 260 bp with the alcohol oxidase transcriptional terminating sequence. *3′AOX1,* segment of the alcohol oxidase locus which is necessary for gene replacement. The pHILS1preαS plasmid is similar to pHILD2preαS with the following main differences: *Not*I sites have been replaced by *Bgl*II, and the *5′AOX1* region is followed by the *PHO1* (acid phosphatase) secretion signal sequence in-frame with the *preproαS* DNA. (B) Nucleotide and amino acid sequences at the leader peptide and mature α-sarcin boundary for pHILD2preαS and pHILS1preαS (I), and pHILD2preαS(A-2K) (II). Ala* is the NH_2-terminal residue of the mature α-sarcin.

The third plasmid constructed was based on pHILD2preαS, by replacing the encoding Ala by Lys at position (−2) of the α-sarcin leader peptide, by oligonucleotide site-directed mutagenesis (Fig. 1B) as previously described (27, 28). Single-stranded wild-type DNA template was prepared after subcloning the α-sarcin DNA *Eco*RI fragment within the plasmid pEMBL18(+) (29). The mutagenic oligonucleotide employed was 5′-AGGTCACCGCGCG**TTT**CTCGAGAGGCGAG-3′. The mutation was confirmed by completely sequencing the mutated α-sarcin DNA, which was subcloned again in the pHIL-D2 *Eco*RI site, rendering the plasmid pHILD2preαS(A-2K) (Fig. 1).

Transformation of P. pastoris GS115

Plasmids pHILS1preαS and pHILD2preαS were digested with either *Bgl*II or *Not*I, respectively. The purified larger fragments (1 μg), containing the α-sarcin expression cassettes (Fig. 1), were used to transform GS115 cells by electroporation on a Bio-Rad Gene-Pulser apparatus as described (30). After a prepulse incubation of 5 min at 4°C, the cells were subjected to a pulse (1.5 kV, 25 μF, 200 Ω) in 0.2-cm cuvettes. Cells were immediately diluted with 1 ml of cold 1 M sorbitol and plated on MD medium containing 1 M sorbitol. Incubation at 30°C was performed for 5–7 days until colonies appeared. Screening for gene replacement of the *sar* construct by homologous recombination at the *AOX1* locus, rendering a (His^+ Mut^s) phenotype, was performed by patching the His^+ colonies on two different types of His-deficient media: one containing glucose (MD) and another one with methanol (MM) as the only carbon source. Among these transformants, containing the *sar* gene under the control of the *AOX1* promoter, the best producer was selected according to the results of small-scale pilot expression experiments in the media described above. Transformation of *P. pastoris* with pHILD2preαS and pHILS1preαS rendered 26 and 71 His^+ colonies for each construct, respectively. When tested for their methanol utilization phenotype only 5 of the first and 11 of the second group of colonies resulted Mut^s. For pHILD2preαS(A-2K), 16 colonies were obtained when plated on His-deficient media, and 7 resulted to be Mut^s.

Protein Production and Purification

Large-scale production of recombinant α-sarcin was carried out using the best producing colony transformed with pHILD2preαS or pHILD2preαS(A-2K). After 2 days of incubation at 30°C, with strong aeration, 2 liters of BMGY culture was centrifuged; the pelleted cells were resuspended in 400 ml of BMMY and further incubated for two more days at the same temperature. Then, the extracellular medium was removed by centrifugation, and the cells were induced again in 400 ml of fresh BMMY medium for an additional 2 days. This 800 ml of collected extracellular material was used as the starting material for purification of recombinant α-sarcin as previously described (13, 31). First, a cation exchange column on Amberlite IRC 50 was employed (6). Then, the fractions containing the desired proteins were pooled, concentrated, and chromatographed again on a Biogel P10 column.

Characterization of the Recombinant Proteins

Fungal α-sarcin, used as a control in these experiments, was purified from *A. giganteus* MDH 18894 cultures as described previously (31). Polyacrylamide electrophoresis of proteins (32), Western immunoblots with anti-α-sarcin rabbit serum (10 ng of fungal protein could be detected under these conditions), HPLC fractionations, protein hydrolyses, amino acid analyses, and automatic Edman degradation were carried out as

described previously (13, 33). The specific ribosome-inactivating activity of α-sarcin was detected by the release of the 400-nt α-fragment from the 28S RNA of eukaryotic ribosomes (7), by using a cell-free rabbit reticulocyte lysate (Promega) as substrate (13, 14, 33). Activity of the purified proteins against poly(A) (9) was assayed as previously described (33, 34). Briefly, standard SDS–PAGE (32) was performed on a gel containing 0.3 mg/ml poly(A). Afterward, the gel was incubated at 37°C for 3 h and stained with 0.2% toluidine blue. Proteins exhibiting ribonucleolytic activity appear as colorless bands after proper destaining treatment.

Culture media of the α-sarcin-producing strains were analyzed for the presence of glycosylated proteins by reaction with concanavalin A (35) after SDS–PAGE separation and transference to an Immobilon membrane as described (36).

Absorbance measurements were carried out on an Uvikon 930 spectrophotometer. Circular dichroism spectra were obtained on a Jasco J-715 spectropolarimeter. All these determinations were performed under conditions described elsewhere (37).

RESULTS

The codifying region of the *sar* gene, including its leader sequence (26), was amplified by PCR using the plasmid pOMPAαS (13) as a template for the two primers described under Materials and Methods. Initially, two different constructs were made. The first, pHILD2preαS, contained the complete *preproαS* cDNA, including its original ATG initiation codon (Fig. 1). In the second, pHILS1preαS, the signal sequence of the acid phosphatase was fused to the full-length *preproαS* cDNA, including both its start and stop codons. Therefore, this second construction should potentially be able to secrete the immature form of α-sarcin, since yeasts in general initiate translation at the ATG nearest to the 5′ end of the mRNA. However, this process appears to be different in this system (see below).

Neither pro- nor mature α-sarcin was detected in any of the intracellular lysates corresponding to the (His^+ Mut^s) colonies transformed with pHILD2preαS or pHILS1preαS. However, two α-sarcin-immunoreactive electrophoretic bands were observed in the extracellular media of all the (His^+ Mut^s) obtained. One of them comigrated with the mature fungal protein, the second being slightly larger (Fig. 2). These observations were independent of the expression vector employed, although the colonies transformed with pHILD2preαS produced sufficient quantities of both extracellular recombinant proteins to be detected by Coomassie blue staining.

Although the original fungal α-sarcin is not glycosylated (6), the larger extracellular immunoreactive band

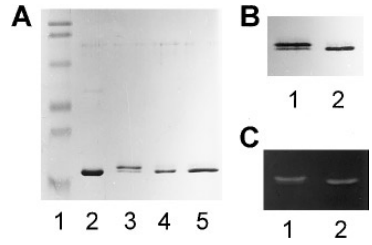

FIG. 2. Characterization of the purified proteins secreted by *P. pastoris*, when transformed with pHILD2preαS or pHILD2preαS(A-2K). (A) SDS–PAGE Coomassie blue staining: (1) Prestained molecular weight standards (Bio-Rad); (2) 1.0 μg of fungal α-sarcin as a control; (3) 0.5 μg of the proteins isolated from the *P. pastoris* culture transformed with pHILD2preαS; (4) 0.5 μg and (5) 1.0 μg of the purified protein when the plasmid employed was pHILD2preαS(A-2K). (B) Western-blot immunostaining with α-sarcin antiserum of samples (3) and (4) in part A: (1) 0.15 μg and (2) 0.08 μg, respectively. (C) Ribonucleolytic activity against poly(A) (0.5 μg of each purified protein was assayed): (1) mixture of pro- and mature α-sarcin and (2) mature recombinant α-sarcin.

could be related to a glycosylated form, since it is well known that secretion pathways in yeast are closely related to glycosylation events (17, 38). However, this possibility should be discarded because the N-glycosylation consensus sequence is absent in α-sarcin (38, 39), and none of the two recombinant proteins bound to concanavalin A, thus indicating that mannose modification of Thr or Ser residues did not occur.

Defective proteolytic processing might also explain the larger immunoreactive band. In fact, amino-terminal sequencing of the mixture of the two proteins, which copurified using the standard isolation procedure described for α-sarcin, revealed the presence of the mature protein and another form containing 6 extra amino acid residues from its leader peptide (Fig. 3A). In addition, both purified proteins displayed ribonucleolytic activity against poly(A) (Fig. 2). This deficient processing could be explained by saturation of the yeast secretion pathway due to an overproduction of pro-α-sarcin. This might occur at the processing step of pro-protein by convertases, such as a Kex2p-like endopeptidase. However, a similar misprocessing of pro-BPTI by Kex2p protease was observed in *S. cerevisiae*, and the yield of properly secreted BPTI was not increased when yeast mutants overproducing this peptidase were used (40). A more convincing explanation would be a failure of pro-sequence recognition by a *P. pastoris* Kex2p-like endopeptidase. Actually, removal of the leader sequence by the Kex2p protease is not a prerequisite for protein secretion in *S. cerevisiae* (4). This enzyme usually cleaves the peptide bond involving the carboxyl group of the arginine residue at the dibasic recognition site Lys–Arg (41) and, although the α-sarcin pro-sequence is very similar to the efficiently cleaved COOH-terminal pro-sequence of *S. cerevisiae*

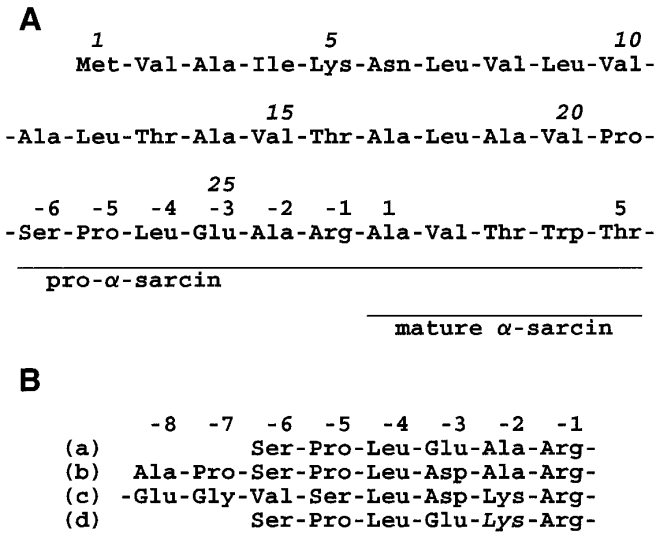

FIG. 3. (A) Amino acid sequence of the 21-residue α-sarcin leader peptide (sequence numbers in italics) and NH_2-terminal residues of the mature protein. Residues of the pro- sequence are identified as negative numbers preceding the NH_2-terminal residue of the fungal mature protein, position 1. The sequences found for the two purified proteins (pro-α-sarcin and mature α-sarcin) produced by transformed strains of *P. pastoris* are underlined. (B) Amino acid sequences of the pro-region of: (a)α-sarcin from *A. giganteus* when produced by *P. pastoris;* (b) restrictocin from *A. restrictus,* as predicted (46); (c), α-mating factor from *S. cerevisiae* (a fragment is only shown, since the pro-sequence is much larger) (47); (d) mutated pro-sequence of α-sarcin to render the dibasic Lys–Arg motif included within the recognition sequence of *Saccharomyces* Kex2p-endopeptidase.

α-mating factor, there is Ala instead of Lys at the cleavage site (Fig. 3B). Therefore, this change might explain the deficient recognition and processing.

A third plasmid, pHILD2preαS(A-2K), was used to confirm this hypothesis. It was constructed by replacing the Ala at position (−2) of the α-sarcin leader peptide by Lys (Fig. 1B). The ribotoxin pro-sequence was thus converted into a very similar motif to that of the Kex2p-endopeptidase recognition sequence (4) included within the *S. cerevisiae* α-mating factor (Leu–Asp–Lys–Arg). In fact, the main difference between both sequences is the Asp residue at position (−3) of the *Saccharomyces* protein substituted by Glu in the mutated α-sarcin pro-sequence (Fig. 1). No pro-form of α-sarcin was detected in any of the (His^+ Mut^s) new transformants thus obtained, proving that the deficient recognition by a Kex2p-like peptidase of *P. pastoris* was the reason for anomalous production of pro-α-sarcin.

One of the transformed colonies was selected to isolate the protein (about 1 mg per liter of BBMY culture, final yield). This preparation was more than 98.0% homogeneous according to its electrophoretic (Fig. 2) and HPLC behavior, as well as by the presence of a single NH_2-terminal amino acid sequence, which was identical to the one described for the mature fungal α-sarcin. The ribosome-inactivating activity (production of the α-fragment in a cell-free retyculocyte lysate) (Fig. 4), as well as amino acid composition and spectroscopic properties (UV-absorption and far- and near-UV CD spectra; Fig. 5), were also coincident with those of the fungal α-sarcin.

DISCUSSION

P. pastoris cells transformed with either pHILS1preαS or pHILD2preαS secrete both defectively processed pro-α-sarcin and its mature form. The existence of pro-α-sarcin has been previously proposed to explain self-protection in *A. giganteus* against the toxicity of the mature protein (42, 43); but this is the first time that pro-α-sarcin has been characterized, since it is not secreted by the α-sarcin-producing mold and has not been purified. The results obtained strongly suggest that the expression system herein employed recognizes the initiation codon of the *sar* gene even when the region encoding for the *P. pastoris* acid phosphatase signal peptide is present. Similar results have also been obtained using pHILS1 with the in-frame-fused testicular angiotensin-converting enzyme (ACE_T) signal sequence (44). It can be argued that yeast transformed with pHILS1preαS might translate both yeast and *Aspergillus* signal sequences, recognizing the longer chimeric leader peptide. However, this would result in an extremely long and unusual signal peptide (2). Indeed, the extremely low levels of protein produced using the pHILS1preαS construct also suggest that the tandem arrangement of two different signal sequences results in a very inefficient system.

It is also clear from the results presented here that the signal sequence of the fungal α-sarcin is recognized in *P. pastoris.* This would explain why the protein is not toxic for this yeast and why a single mutation in the α-sarcin pro-region produces large amounts of re-

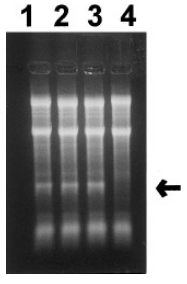

FIG. 4. Specific α-sarcin ribonucleolytic activity of the purified protein secreted by *P. pastoris* cells harboring the plasmid pHILD2preαS(A-2K). The assay is based on the production of the RNA α-fragment (indicated by an arrow) on a cell-free reticulocyte lysate (see Materials and Methods). (1) 60 ng of fungal α-sarcin; (2) 60 ng and (3) 120 ng of the recombinant mature protein, respectively; (4) no α-sarcin added.

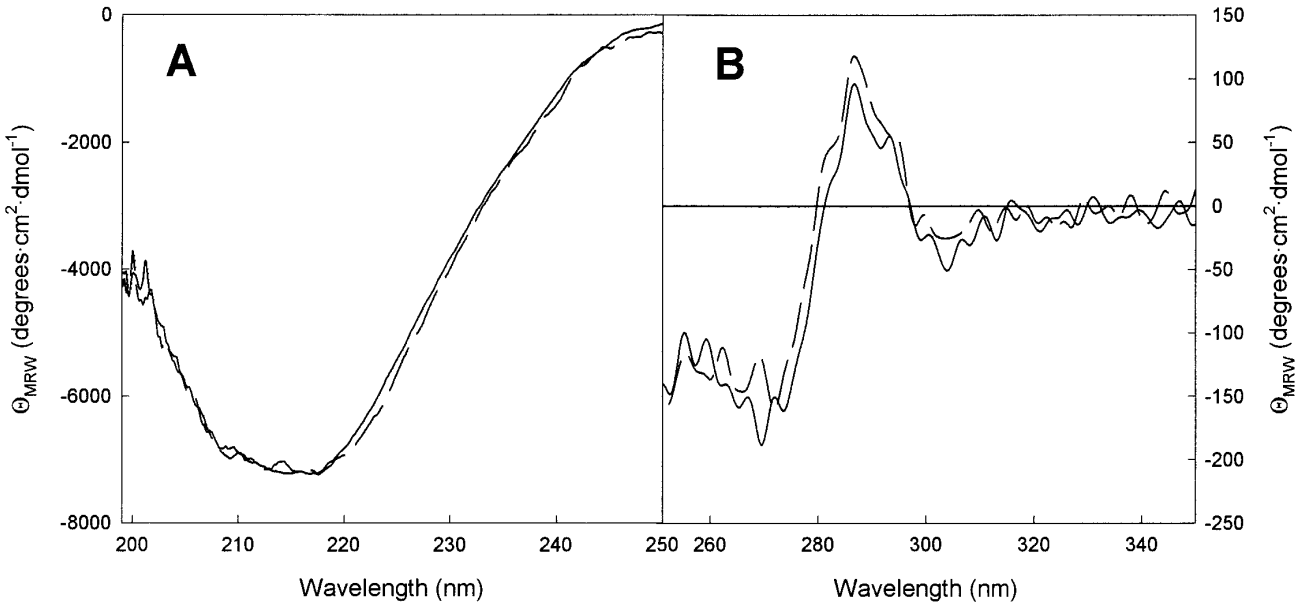

FIG. 5. (A) Far-UV and (B) near-UV circular dichroism spectra of the fungal α-sarcin (continuous line) and the species purified from the extracellular medium of *P. pastoris* (dashed line) when transformed with the plasmid pHILD2preαS(A-2K).

combinant mature α-sarcin. This is remarkable considering both the highly toxic character of this ribosome-inactivating protein and the eukaryotic nature of the producing organism. Based on these results, *S. cerevisiae* and *P. pastoris* apparently display signal sequence recognition mechanisms different enough to explain the production of α-sarcin in *P. pastoris* while restrictocin (the homologous protein from *A. restrictus*) is toxic for *Saccharomyces*, although leader peptides in both α-sarcin and restrictocin are almost identical (14, 26).

Protection of the α-sarcin-producing mold against its own ribotoxin remains controversial. It seems clear from the present results that defective recognition of the α-sarcin pro-sequence by a processing endopeptidase in *P. pastoris* is the reason that both pro- and mature α-sarcin are detected in the extracellular medium. Therefore, the proteolytic processing of the α-sarcin precursor to the mature protein must involve at least two steps in *P. pastoris*. The first proteolytic cleavage is catalyzed by the signal peptidase and renders pro-α-sarcin. Cleavage occurs at an unusual site (i.e., at the peptide bond between Pro-21 and Ser-22 of the precursor form; Fig. 3A) compared to predictive signal peptide cleavage sites (45). The second step involves the cleavage of the pro-sequence by a proprotein convertase. Two-step processing has also been suggested for restrictocin as a protective mechanism against self-toxicity, supposing that pro-restrictocin would be enzymatically inert (14, 46). However, we show here that pro-α-sarcin is an active ribonuclease (Fig. 2). An inhibitor has also been proposed as the protective mechanism in *A. giganteus* (14). Ribotoxins such as α-sarcin are not produced in yeasts. Therefore, specific protection against them is not needed in *P. pastoris* and such a putative inhibitor must not be present. It seems that protection against these ribosome-inactivating proteins arises from an efficient recognition of the signal sequence and subsequent translocation of the nascent polypeptide to the endoplasmic reticulum in the *Aspergillus* spp. and also in *P. pastoris*, but not in *S. cerevisiae* (12).

Finally, the results obtained also allow us to propose the presence of a Kex2p-like protease in *P. pastoris* and an equivalent convertase of different specificity in *A. giganteus*, based on the sequence similarity between the processing site of pro-α-sarcin and that recognized by the *S. cerevisiae* Kex2p protease. In addition, we have shown how a leader peptide sequence from *Aspergillus* can be used, with minor modifications, to efficiently produce extracellular proteins in certain yeasts.

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