RESEARCH ARTICLE

# Recombinant expression of ShPI-1A, a non-specific BPTI-Kunitz-type inhibitor, and its protection effect on proteolytic degradation of recombinant human miniproinsulin expressed in *Pichia pastoris*

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## Abstract

*Pichia pastoris* is a highly successful system for the large-scale expression of heterologous proteins, with the added capability of performing most eukaryotic post-translational modifications. However, this system has one significant disadvantage – frequent proteolytic degradation by *P. pastoris* proteases of heterologously expressed proteins. Several methods have been proposed to address this problem, but none has proven fully effective. We tested the effectiveness of a broad specificity protease inhibitor to control proteolysis. A recombinant variant of the BPTI-Kunitz protease inhibitor ShPI-1 isolated from the sea anemone *Stichodactyla helianthus*, was expressed in *P. pastoris*. The recombinant inhibitor (rShPI-1A), containing four additional amino acids (EAEA) at the N-terminus, was folded similarly to the natural inhibitor, as assessed by circular dichroism. rShPI-1A had broad protease specificity, inhibiting serine, aspartic, and cysteine proteases similarly to the natural inhibitor. rShPI-1A protected a model protein, recombinant human miniproinsulin (rhMPI), from proteolytic degradation during expression in *P. pastoris*. The addition of purified rShPI-1A at the beginning of the induction phase significantly protected rhMPI from proteolysis in culture broth. The results suggest that a broad specificity protease inhibitor such as rShPI-1A can be used to improve the yield of recombinant proteins secreted from *P. pastoris*.

## Introduction

The methylotrophic yeast *Pichia pastoris* is commonly used as a heterologous protein expression system. Its advantages include the strong, regulated, and inducible alcohol oxidase promoter, efficient post-translational modification, effective transformation and high cell density culture, and commercial availability of strains and vectors (Macauley-Patrick *et al.*, 2005). However, the degradation of heterologously expressed proteins in *P. pastoris* culture supernatants has been identified as a major problem for its use as an expression system (Cregg *et al.*, 1993).

It is generally thought that the cause of this degradation is the release into the culture medium of vacuolar proteases from dead cells (Sinha *et al.*, 2005). Several strategies have been developed to diminish the proteolysis of secreted heterologous proteins expressed in *P. pastoris*, such as cultivating at extreme pHs (Clare *et al.*, 1991), or temperatures 15–20 °C (Li *et al.*, 2001; Jahic *et al.*, 2003), or the addition of organic nitrogen sources (Clare *et al.*,

1991; Brankamp *et al.*, 1995). Another approach has been the development of protease A mutants (encoded by *PEP4* gene), responsible for the activation of other vacuolar proteases (Gleeson & Howard, 1994).

In addition, synthetic inhibitors have been added to culture broths to study and reduce proteolytic activity (Shi *et al.*, 2003). The use of class-specific inhibitors has revealed that nearly 95% of the proteolytic activity of culture supernatants (around pH 7) is due to serine and metalloproteases (Shi *et al.*, 2003; Sinha *et al.*, 2005). However, proteolytic activity is dependent on the fermentation pH, and its change could modify the relative activities of proteases in the culture media (Sinha *et al.*, 2005).

The most important *P. pastoris* vacuolar proteases that degrade extracellular proteins are carboxypeptidase Y (E.C 3.4.16.5), a serine protease active at acidic pH (Mortensen *et al.*, 1998), aspartic protease A or saccharopepsin-like protease (E.C 3.4.23.25), with optimum activity at pH 4.5, and protease B or cerevisin-like protease (E.C 3.4.21.48), a subtilase protease with maximum activity at pH 6.3 (Shi *et al.*, 2003; Sinha *et al.*, 2005).

Albeit the use of synthetic inhibitors might constitute a method for proteolytic control, some of them are toxic, as phenylmethylsulfonyl fluoride (PMSF) (Massicotte *et al.*, 1999). In addition, most have dissociation constant ($K_i$) values in the millimolar range, and their use in industrial-scale protein expression is prohibitive (Macauley-Patrick *et al.*, 2005). The alternative use of protease-deficient strains is of limited utility due to lower viability and transformation frequency, besides the presence of residual proteolytic activity (Shi *et al.*, 2003).

On the other hand, peptidic protease inhibitors are becoming very attractive, both for the study of structure-function relationship of their target proteases and because of their potential biotechnological and biomedical applications (Abbenante & Fairlie, 2005). Serine protease inhibitors belonging to bovine pancreatic trypsin inhibitor (BPTI)-Kunitz family (MEROPS: inhibitor family I2; Rawlings *et al.*, 2008) have been the most widely studied. However, the majority of the biochemical studies and applications in biomedicine and biotechnology have been restricted to BPTI itself.

A broad specificity protease inhibitor from the sea anemone *Stichodactyla helianthus* (ShPI-1) was isolated, purified, and characterized by our group (Antuch *et al.*, 1993; Delfín *et al.*, 1996). This molecule is a BPTI-Kunitz inhibitor (55 amino acid residues), but in contrast with other members of the family, ShPI-1 has an unusual specificity. It is a tight-binding inhibitor ($K_i \leq 10^{-7}$ M) of several serine proteases, as well as pepsin (an aspartic protease) and papain (a cysteine protease) (Delfín *et al.*, 1996). As a result of its inhibitory efficiency and broad specificity, ShPI-1 has been used in several protein purification processes and as an immobilized ligand in affinity chromatography (Delfín *et al.*, 1996). To produce large quantities of this inhibitor and to preserve the natural species, our group developed the expression of ShPI-1 in *Saccharomyces cerevisiae*. The recombinantly expressed protein, rShPI-1A, contains four additional amino acids at the N-terminus (EAEA) and two additional amino acids at the C-terminus (LG). The ShPI-1A synthetic gene was fused in frame to the *S. cerevisiae* prepro-α-factor secretion signal (ppαF), placed under the control of the *S. cerevisiae GAL7* promoter, and assembled into the expression pLJC6 vector. This system, however, produced very low yields (J. Díaz, unpublished results).

Herein, we describe the expression, purification, and characterization of rShPI-1A in *P. pastoris*. We also describe its ability to protect from proteolytic degradation during expression in *P. pastoris* of recombinant human miniproinsulin (rhMPI), a protein that is otherwise particularly prone to degradation (Mansur *et al.*, 2005; Mansur, 2007).

## Materials and methods

### Strains and molecular biology reagents

*Escherichia coli* strain DH5α was used during cloning. *Pichia pastoris* KM71H (*arg4*, *his4*, *aox1*::*ARG4*, *HIS4*) and C27 (*his4*, *HIS4*, *rhMPI*) strains were used as expression hosts of rShPI-1A and rhMPI (Mansur *et al.*, 2005), respectively. The C27 strain was also used as source of proteases. pPICZαA, pZErO-2.1, and pGAPαC vectors were obtained from Invitrogen (Carlsbad, CA). The plasmid pGAPHαC is a pGAPZαC-derived vector, where the zeocin resistance gene was replaced by the hygromycin B phosphotransferase gene (M. Mansur, unpublished results). Restriction enzymes, DNA ligase, alkaline phosphatase, *Taq* polymerase, and their buffers, were obtained from New England Biolabs (Beverly, MA) and were used following manufacturer's instructions.

### Cloning and construction of the rShPI-1A-producing strain

Two DNA bands were obtained by PCR-amplification of the *ppαF-rShPI-1A* gene from plasmid pLJC6 with oligonucleotides 1 and 2, and the *AOX1* promoter-ppαF gene from vector pPICZαA with oligonucleotides 3 and 4, respectively (Table 1). The DNA fusion *AOX1*-ppαF-rShPI-1A was obtained by overlapping PCR with oligonucleotides 1 and 4 (Table 1), using both fragments (*AOX1*-ppαF and ppαF-rShPI-1A) as templates. The product was cloned into the pZErO-2.1 vector, and the resultant vector was named pZAAS. The *AOX1*-ppαF-

**Table 1.** Oligonucleotides used for cloning and to construct rShPI-1A-producing strain

| Oligonucleotides | 5′→3′ |
|---|---|
| 1 | TTTCCTTCAATTTTTACTGCTGTT |
| 2 | CTCTAGATTACTACCCGAGCGC |
| 3 | TCAGATCTAACATCCAAAGACG |
| 4 | AGCTTTAGCCTCTCTTTTCTC |
| 5 | TTGCGACTGGTTCCAATTGACAAGC |
| 6 | GCAAATGGCATTCTGACATCCTC |

rShPI1A band was excised from pZAAS with the BglII/XbaI enzymes and inserted into pGAPHαC, previously treated with BglII/XbaI to remove the *GAPDH* promoter and the ppαF secretion signal. The final expression plasmid was named pBM301 (Fig. 1). This plasmid was linearized with SacI for integrative recombination and electroporated into the *P. pastoris* KM71H strain. The putative transformants were transferred to plates with increasing concentrations of hygromycin B (150–1200 mg $mL^{-1}$). Those clones resistant at higher antibiotic concentrations were screened with oligonucleotides 5 and 6 (Table 1), which hybridize with the *AOX1* promoter and terminator regions. The colony PCR technique (Pamfer, 1993) was used to confirm the presence of the rShPI-1A gene. The *P. pastoris* strain isolated at the higher antibiotic concentration was named SH1.

## Fed-batch fermentations

*Pichia pastoris* strains (either SH1, C27 or KM71H) were pre-cultured with shaking at 28 °C for 24 h in 300 mL YPD medium (yeast extract 10 g $L^{-1}$, peptone 20 g $L^{-1}$, and glucose 20 g $L^{-1}$). The cells were collected by centrifugation at 1000 ***g***, suspended in batch medium, and used to inoculate the starting volume (1 L of batch medium) in bioreactors. Fermentations were carried out in a computer-controlled 1.5-L working volume fermenter (B.E. Marubishi, Japan) with a computer-based process control. Fermentation pH was fixed at 5.5 with addition of 25% (m/v) ammonium hydroxide. The dissolved-oxygen concentration was maintained above 20% of saturation by controlling the stirrer speed between 500 and 1200 r.p.m. The temperature was controlled at 28 °C and the airflow was kept constant at 2 v.v.m. The batch medium contained 60 g $L^{-1}$ glycerol, 30 g $L^{-1}$ $(NH_4)_2SO_4$, 10 g $L^{-1}$ $KH_2PO_4$, 3.2 g $L^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.35 g $L^{-1}$ $CaCl_2{\cdot}2H_2O$, 0.2 mg $L^{-1}$ D (+)-biotin, and 1 mL $L^{-1}$ trace elements solution (65 g $L^{-1}$ $FeSO_4{\cdot}7H_2O$, 6 g $L^{-1}$ $CuSO_4{\cdot}5H_2O$, 20 g $L^{-1}$ $ZnSO_4{\cdot}7H_2O$, 3 mg $L^{-1}$ $MnSO_4{\cdot}H_2O$, 0.42 g $L^{-1}$ KI, 0.1 g $L^{-1}$ $H_3BO_3$, 1 g $L^{-1}$ $Na_2MoO_4{\cdot}2H_2O$, 0.5 g $L^{-1}$ $CoCl_2$, 10 mL $L^{-1}$ 98% $H_2SO_4$). After approximately 30 h of batch culture, a sharp increase in dissolved oxygen indicated the depletion of glycerol (the initial carbon and energy source), and the methanol feeding-phase was started by induction at 0.5% (v/v) methanol. Methanol concentration was kept constant at 0.7% (v/v) throughout the induction phase by a methanol concentration automatic control loop equipped with a TGS-822 (Figaro Engineering, Osaka, Japan) gas sensor. Cultures were stopped at 120 h, except where indicated.

## Purification of rShPI-1A

The SH1 fermentation broth was centrifuged at 10 000 ***g*** for 30 min at 4 °C, and the pH of the supernatant was adjusted to 7.5. This supernatant was filtered and applied on a Streamline™ Direct HST-1 cation-exchange column (2.4 × 5.3 cm), previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5 using an ÄKTA Prime Liquid Chromatography Purification System (GE Healthcare, Uppsala, Sweden). The recombinant inhibitor was eluted using a linear gradient of 0–100% of 20 mM Tris-HCl buffer, pH 9.0 containing 1.3 M NaCl, and with a linear flow of

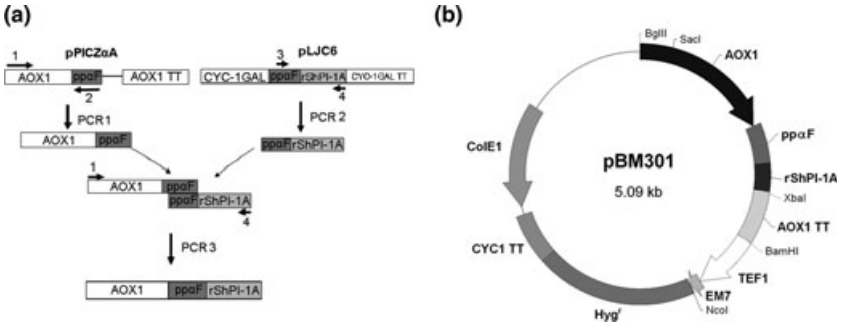


**Fig. 1.** Cloning strategy for rShPI-1A expression in the *Pichia pastoris* KM71H strain. (a) Schematic representation of the PCR amplifications used to the AOX1-ppαF-rShPI-1A fusion DNA. Two DNA fragments containing the *AOX1* promoter fused to ppαF, and the rShPI-1A gene fused to ppαF, were PCR-amplified from pPICZαA and pLJC6 vectors, respectively. The *AOX1-ppαF*-rShPI-1A fusion DNA was obtained by overlapping PCR using both *AOX1*-ppαF and ppαF-rShPI-1A fragments as templates. (b) Map of the expression pBM301 vector.

40 cm $h^{-1}$. The elution profile was monitored at 280 nm and with an assay of inhibitory activity against bovine pancreatic trypsin, using *N*-benzoyl-arginine-p-nitroaniline (Bz-Arg-pNA) (Sigma Chemical Co.) as substrate (Erlanger *et al.*, 1961). Purified rShPI-1A was analyzed using SDS-PAGE (Laemmli, 1970) and RP-HPLC with a C8 column (5.4 × 250 mm; Grace-Vydac, Hesperia, CA) and a linear gradient from 5% to 35% of solution B (0.05% TFA in acetonitrile) at a flow rate of 0.8 mL $min^{-1}$.

## Functional and molecular characterization of rShPI-1A

### Specificity

Proteases were incubated with rShPI-1A for 10–30 min at 25 °C before substrate addition. The activities of the serine proteases bovine pancreatic trypsin (EC 3.4.21.4; 0.2 μM) (Sigma Chemical Co.), human neutrophil elastase (HNE) (EC 3.4.21.37; 0.2 μM) (Calbiochem-Novabiochem), *Bacillus licheniformis* subtilisin A (EC 3.4.21.62; 0.6 μM) (Calbiochem-Novabiochem), bovine pancreatic chymotrypsin (EC 3.4.21.1; 60 μM) (Sigma Chemical Co.), and porcine pancreatic elastase (EC 3.4.21.36; 0.15 μM) (Calbiochem-Novabiochem) were measured with the synthetic substrates 1.0 mM Bz-Arg-pNA (1.0 $K_M$, Erlanger *et al.*, 1961) (Sigma Chemical Co.), 1.0 mM MeO-Suc-Ala-Ala-Pro-Val-pNA (7.1 $K_M$, Nakajima *et al.*, 1979) (Calbiochem-Novabiochem), 0.43 mM Suc-Ala-Ala-Pro-Phe-pNA (2.0 $K_M$, Estell *et al.*, 1986) (Bachem AG), 1.5 mM Suc-Phe-pNA (2.1 $K_M$, Erlanger *et al.*, 1966) (Sigma Chemical Co.), and 1.0 mM Suc-Ala-Ala-Ala-pNA (0.27 $K_M$, Nakajima *et al.*, 1979) (Sigma Chemical Co.), respectively. The activity of *Carica papaya* papain (EC 3.4.22.2; 1.5 μM) (Calbiochem-Novabiochem) was measured with the substrate 1.0 mM Bz-Arg-pNA (1.0 $K_M$, Berger & Schechter, 1970). In addition, the proteolytic activities of 150 μM papain and 36 μM porcine pancreatic pepsin (EC 3.4.23.1) (Sigma Chemical Co.) were determined with bovine hemoglobin (20 g $L^{-1}$) as a protein substrate (Anson, 1938).

### Determination of dissociation constant ($K_i$)

The concentration of active trypsin was determined by titration with a standard solution of 9.4 mM p-nitrophenyl-p′-guanidinium benzoate (Sigma Chemical Co.) (Chase & Shaw, 1967). The active concentration of rShPI-1A purified by ion exchange chromatography (IEC) was determined by titration with a constant active concentration of trypsin. The $K_i$ value against trypsin was determined using the methodology recommended by Bieth (1974), which was previously used for natural ShPI-1 (Delfín *et al.*, 1996). Different initial concentrations ($E_0$) of active trypsin (18–24 nM) were assayed against a constant concentration of active rShPI-1A (33.7 nM) and the substrate 0.24 mM benzoyl-arginine-ethyl-ester (55.0 $K_M$, Trautschold & Werle, 1961) (Sigma Chemical Co.). Initial rates were determined in absence ($v_0$) and presence ($v_i$) of the inhibitor, and data were plotted as $E_0\ (1 - v_i/v_o)$ vs. $(v_o - v_i)/v_i$, following a linearization of the general tight-binding inhibition equation (Bieth, 1974).

### Molecular characterization

The N-terminal amino acid sequence of rShPI-1A from RP-HPLC (2 nmol) was determined by automatic Edman degradation using a PPSQ-23 protein sequencer (Shimadzu, Tokyo, Japan). The molecular mass was determined using MALDI-TOF mass spectrometry analysis with a Bruker Biflex spectrometer (Bruker Daltonics Inc., Billerica, MA).

## Secondary structure of native and denatured rShPI-1A

### Denaturation of rShPI-1A

Native rShPI-1A (0.4 mg $mL^{-1}$) was dissolved in 20 mM Tris-HCl buffer, pH 7.5. Its concentration was determined by measuring absorbance at 280 nm using the extinction coefficient previously reported for the natural inhibitor ($\xi^{1\%,\ 280\ nm} = 5.2$) (Delfín *et al.*, 1996). Samples were prepared by mixing rShPI-1A with a stock solution of urea to obtain a fixed inhibitor concentration and different concentrations of urea, ranging from 0 to 6.0 M. The effect of 5 mM dithiothreitol (DTT) and 10 mM sodium bisulfite (BiNa) on native and denaturant-treated proteins was also analyzed. The reagents were mixed by vortexing and incubated overnight at 4 °C.

### Circular dichroism (CD) experiments

Far-UV CD (190–250 nm) spectra of native and denatured rShPI-1A were recorded at 20 °C using a Jasco J-600 spectro-polarimeter equipped with a temperature-regulated sample chamber. Analyses of CD spectra to estimate secondary structural composition were done using the CDSSTR algorithms available at the DICHROWEB web server (Sreerama & Woody, 2000). The results were compared with DSSP (Define Secondary Structure of Proteins) assignment of secondary structure, considering the NMR structure of natural ShPI-1 (PDB code: 1SHP; Antuch *et al.*, 1993) as well as the CD spectrum and crys-

tal structures of BPTI (Kosen *et al.*, 1981) and rShPI-1A (PDB code: 3OFW).

### Protection of rhMPI from proteolytic degradation in *P. pastoris* supernatant

#### Production of rhMPI

The recombinant protein was obtained in the conditions established by Mansur *et al.* (2005). Briefly, the C27 strain was cultured in the conditions described above, and the culture was stopped 96 h after methanol induction.

#### Quantitation of rhMPI

Several concentrations (0–600 μg $mL^{-1}$) of an external human insulin standard (NIBSC 83/500) were applied on a C4 RP-HPLC column (4.6 × 150 mm; YMC, Kyoto, Japan); the chromatographic parameters were similar to those described for RP-HPLC of the rShPI-1A, with a 20–40% linear gradient of solution B in 40 min. The concentration of rhMPI was deduced from the correlation between the area-under-curve of eluted peak and the mass of applied protein, and allowed establishing the protein concentration (Mansur *et al.*, 2005).

#### *In vitro* assay

To study the influence of rShPI-1A on the hydrolysis of rhMPI by proteases released from *P. pastoris*, a supernatant from a batch culture of the C27 strain was incubated at pH 5.5 without (rhMPI 130 mg $mL^{-1}$, control) and with different levels of rShPI-1A (30, 75 and 150 μM) at 28 °C. Aliquots were taken at time 0 and 30 h, and the remaining rhMPI concentrations were estimated.

#### Influence of rShPI-1A addition during rhMPI fermentation

The rhMPI-producing C27 strain was fermented as described above. Group A ($n = 3$) refers to C27 strain fermentations without inhibitor (control). Group B ($n = 3$) refers to fermentations with rShPI-1A (30 μM) added after 20 h of induction. Samples were taken at induction time 0, and every 24 h until 96 h. The rhMPI concentration ($P$) and cell wet weight (biomass, $X$) were determined to obtain the product per biomass yield ($Y_{P/X}$).

#### Statistical analyses

For the protection assays, we performed an ANOVA with the software GRAPHPAD PRISM ver. 5.00 (2007) to determine the statistical significance of differences between experimental values. A *post-hoc* analysis with the Tukey–Kramer multiple comparisons test or the Bonferroni test provided by the same software was conducted to average values comparison and homogeneous group assignments.

## Results

### Cloning, expression and purification of rShPI-1A

The cloning strategy used to obtain the final expression vector (pBM301) is shown in Fig. 1a. The fusion DNA fragment *AOX1*–ppαF–rShPI-1A was excised from the pZAAS plasmid and inserted into pGAPHαC, to obtain the expression vector pBM301 (Fig. 1b). A KM71H clone resistant to the highest antibiotic concentration used (SH1) was isolated for benchtop fermentations.

A trypsin inhibitory activity was detected in SH1, but not in KM71H or C27 fermentation broths. The inhibitor rShPI-1A was separated from other proteins secreted by *P. pastoris* by IEC of the cell-free SH1 supernatant. Active fractions were only eluted by increasing both pH and ionic strength (Fig. 2a). The procedure led to an 11-fold purification of rShPI-1A, with a 96% yield (Table 2). A value of 1.2 g $L^{-1}$ was estimated of the starting rShPI-1A concentration in the culture supernatant, according to the mass balance analysis in the IEC eluated. Analysis by RP-HPLC and SDS-PAGE showed a single symmetric peak (Fig. 2b) and a single protein band (Fig. 2c), respectively, indicative of high protein purity.

### Molecular characterization of rShPI-1A

As expected, the N-terminal amino acid sequence of purified rShPI-1A was EAEASIXSEPKKVGR. X mean indicates that no phenylthiodantoin-amino acid was detected, consistent with the presence of a Cys residue at this position. This sequence corresponds to the EAEA tag followed by the N-terminus sequence of natural ShPI-1 (Delfín *et al.*, 1996). The molecular mass determined using MALDI-TOF mass spectrometry was 6681.6 Da (Fig. 2d), consistent with the expected molecular mass from sequence (6680.5 Da).

The far-UV CD spectrum of native rShPI-1A (Fig. 3a) was similar to that of BPTI (Kosen *et al.*, 1981). CD spectra analysis using the CDSSTR algorithm generated the most accurate result with a normalized root-mean-square deviation (NRMSD) value of 0.021 and a sum of fractions of 0.99, indicative of a good fit of the CD spectrum (Fig. 3a). The spectrum of the native recombinant inhibitor had a small positive band at 190 nm, a sharp negative

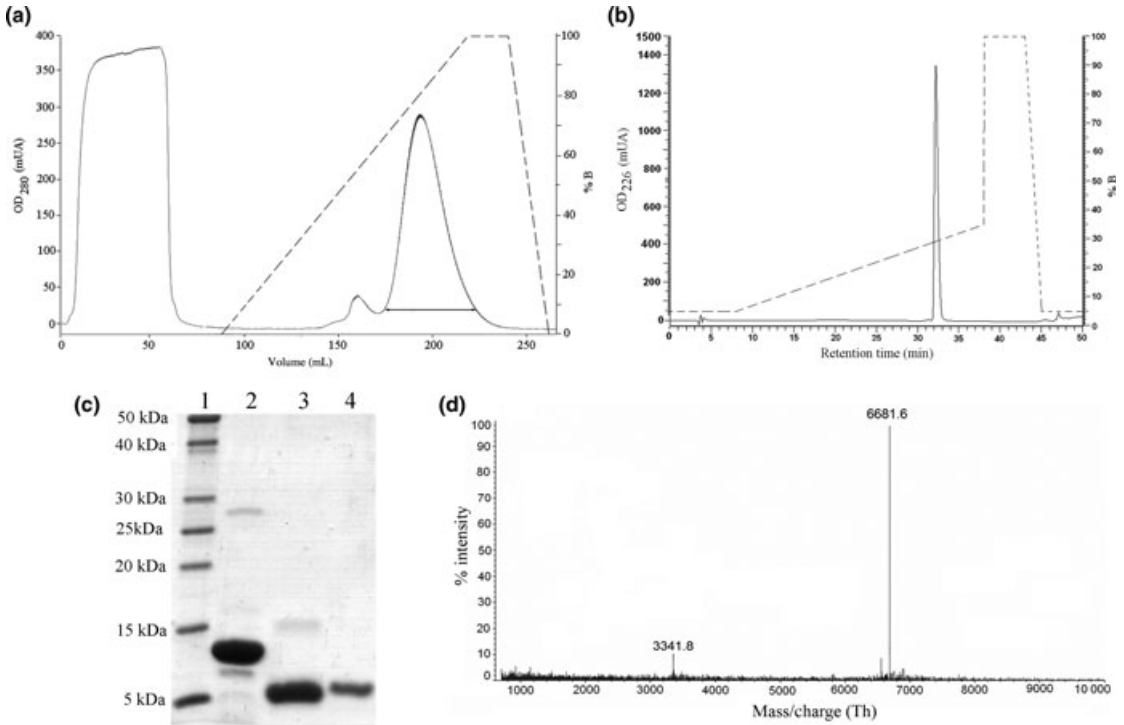


**Fig. 2.** Purification of rShPI-1A expressed in *Pichia pastoris*. (a) IEC of rShPI-1A cell-free fermentation supernatant. A typical cation-exchange chromatography profile of a SH1 strain culture broth is showen ($N = 15$). A 100 mL sample of supernatant was applied on a Streamline™ HST-1 column. The inhibitor was eluted with 0–100% linear gradient of 20 mM Tris-HCl, 1.3 M NaCl, pH 9 buffer (broken line) at a linear flow of 40 cm $h^{-1}$. The elution profile was monitored by measurements of $OD_{280\ nm}$ (gray, solid line) and inhibitory activity using trypsin as target protease (bold bar). (b) RP-HPLC of purified rShPI-1A. Fifty microliters of the fraction isolated from IEC were applied on a C8 column. A linear gradient (broken line) from 5% to 35% of solution B (0.05% TFA in acetronitrile) was applied at a flow rate of 0.8 mL $min^{-1}$. Elution profile (solid line) was monitored by measuring $OD_{226\ nm}$. (c) SDS-PAGE (17%) of rShPI-1A from IEC. Lane 1: molecular weight markers from 5.0 to 50 kDa, lane 2: lysozyme (14.5 kDa), 3: BPTI (6.5 kDa), 4: rShPI-1A. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. (d) MALDI-TOF MS of purified rShPI-1A. Two micrograms rShPI-1A purified by IEC and RP-HPLC were mixed with the matrix α-cyano-4-hydroxycinnamic acid.

**Table 2.** Typical purification process of rShPI-1A produced by *Pichia pastoris*

| Step | Inhibitory activity* ($\times 10^6$ U) | Protein† (mg) | Specific activity‡ (U $mg^{-1}$) | Purification§ (fold) | Yield¶ (%) |
|---|---|---|---|---|---|
| SH1 culture supernatant | 13 671 | 10 600 | 1285 | 1.0 | 100.0 |
| Ion exchange chromatography | 13 196 | 850 | 14 979 | 11.7 | 96.5 |

*Inhibitory activity: one inhibitory unit (U) was defined as the amount of inhibitor needed to inhibit 1 U of trypsin enzymatic activity. One unit of trypsin activity is the enzymatic activity that hydrolyzes 1 mmol of substrate per min under specified conditions.

†Protein concentration was estimated by OD at 280 nm assuming an extinction coefficient equal to 1.00 for culture broth proteins, and to 0.52 for the purified recombinant inhibitor, based on the value of the natural inhibitor.

‡Specific activity was expressed in U of inhibitor per mg of total protein.

§Purification factor was calculated using the specific activity data.

¶Yield was given as percentage of isolated active material.

band at 202 nm, and a shoulder at ~220 nm. This analysis allowed estimation of secondary structural content (table in Fig. 3a).

We also studied the conformational stability of rShPI-1A using different concentrations of urea as denaturing agent. A full secondary structure analysis was not possible, as the absorption by urea and chloride ions precluded measurements below 200 nm (Griko *et al.*, 2001). The secondary structure was partially affected by the addition of urea (Fig. 3b). Even at 6 M urea, the denaturation of

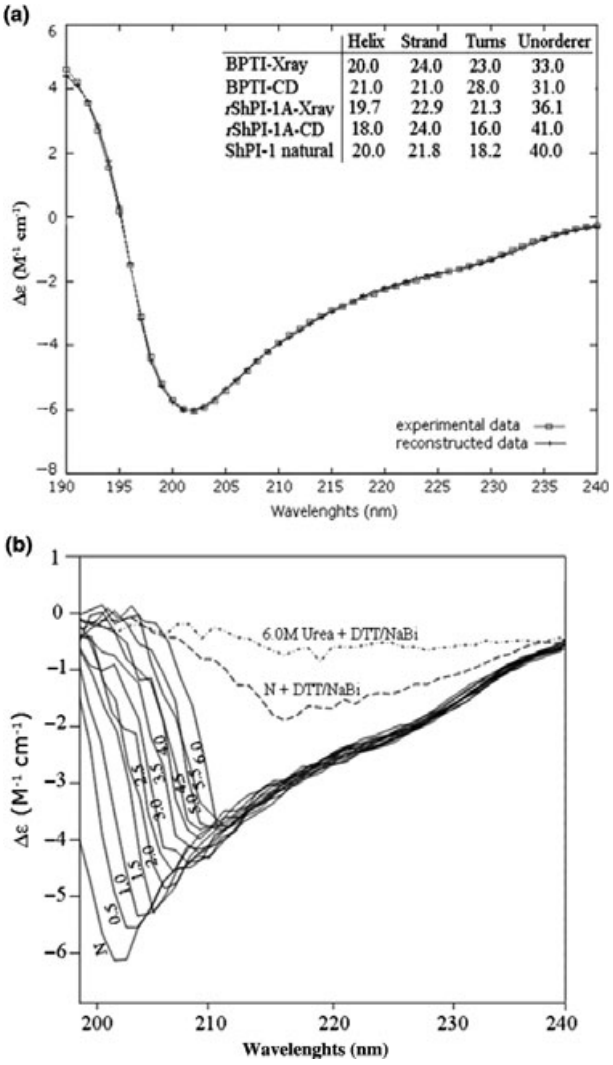


**Fig. 3.** CD analysis of rShPI-1A. (a) Far-UV CD spectra of rShPI-1A. Spectra were recorded in 20 mM Tris-HCl, pH 6.5 buffer. CD spectra were acquired at a scan speed of 50 nm $min^{-1}$ and a wavelength step resolution of 0.1 nm. Three individual scans were averaged in each experiment, and the background spectra were subtracted. A comparison of the experimental and calculated spectra using the CDSSTR algorithm was performed. The inserted table shows the percentage of secondary structure elements calculated considering the previously reported CD spectra deconvolution and DSSP analysis of rShPI-1A and BPTI X-ray structures. (b) Effect of denaturing and reducing agents on the CD spectrum of rShPI-1A. The urea concentration used in each experiment is indicated close to the spectrum. The CD spectrum of the native protein is labeled as 'N'. Dashed lines show spectra recorded after addition of 5.0 mM DTT and 10 mM NaBi on the native (N) or 6.0 mM urea treated inhibitor.

rShPI-1A was only partial. Full denaturation could only be achieved by addition of 5 mM DTT and 10 mM BiNa in 6 M urea. The conformational stability of rShPI-1A was also affected by the addition of both reducing agents even in the absence of urea (Fig. 3b), suggesting that the reduction of the three disulfide bonds is the most critical factor contributing toward the stability of the inhibitor.

## Functional characterization of rShPI-1A

Table 3 displays the protease specificity of rShPI-1A. It inhibited the serine proteases trypsin, HNE, and chymotrypsin, but neither subtilisin A nor porcine pancreatic elastase. It also inhibited the cysteine protease papain (when hemoglobin was used as a substrate) and the aspartic protease pepsin. However, we did not detect inhibition of papain if a small synthetic substrate was used, even with a 30-fold molar excess of inhibitor vs. enzyme. A similar behavior was also observed in the natural inhibitor (Delfín *et al.*, 1996). The $K_i$ value of rShPI-1A against trypsin ($5.6 \times 10^{-10}$ M) was similar to that reported for the natural inhibitor ($1.3 \times 10^{-10}$ M) (Delfín *et al.*, 1996).

## rShPI-1A protects rhMPI from proteolysis

Incubation of cell-free C27 culture supernatants with rShPI-1A significantly increased the remnant rhMPI concentration (Fig. 4). However, the protection level of rhMPI was not linearly correlated with the inhibitor concentration, and at the highest rShPI-1A concentration tested (150 μM), we still detected rhMPI degradation. Subsequently, we carried out fermentations of the C27 strain with or without 30 μM rShPI-1A, the lowest concentration used in the *in vitro* experiments. Table 4 shows that rShPI-1A enhanced two-fold the rhMPI concentration and $Y_{P/X}$ at 45 h, whereas the effect was lower at 69 h. The inhibitor enhanced rhMPI concentration by 1.3 fold at the end of the fermentation, a result consistent with the *in vitro* experiments.

RP-HPLC profiles of control C27 fermentation supernatants (at 69 h) showed several molecular species with hydrophobicity distinct from that expected for rhMPI (Fig. 5a). These peaks were not detected in chromato-

**Table 3.** Inhibitory profile of rShPI-1A against various proteases

| Enzyme | Substrate | [Io] : [Eo] | Inhibition |
|---|---|---|---|
| Trypsin | Bz-Arg-pNa | 1 : 100 | ++ |
| HNE | Suc-Ala-Ala-Ala-pNa | 1 : 100 | ++ |
| Chymotrypsin | Suc-Phe-pNa | 1 : 1.5 | ++ |
| Subtilisin A | MeO-Suc-Ala-Ala-Pro-Phe-pNa | 30 : 1 | − |
| Papain | Bz-Arg-pNa | 30 : 1 | − |
| | Hemoglobin | 1 : 4 | + |
| Pepsin | Hemoglobin | 1 : 1 | + |

[Io] : [Eo], relation between initial active inhibitor concentration [Io] and enzyme concentration [Eo]; ++, total inhibition (residual activity $a < 0.2$); +, partial inhibition ($0.2 < a < 0.8$); −, no inhibition ($a > 0.8$).

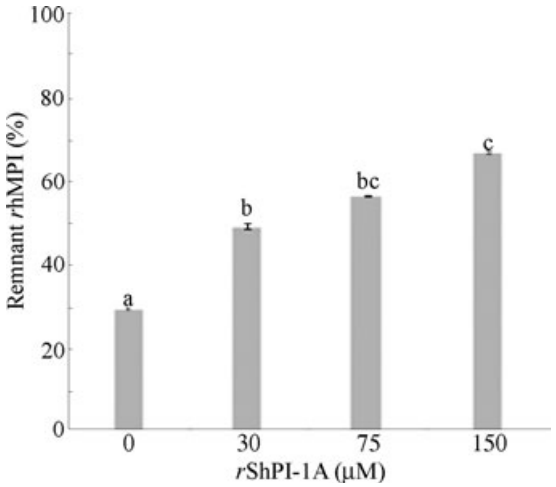


**Fig. 4.** Protection of rhMPI degradation in the presence of rShPI-1A. A C27 strain culture supernatant obtained from a fermentation induced for 96 h (pH 5.5, $T = 30$ °C) containing 130 mg $L^{-1}$ of rhMPI was incubated without or with different concentrations of rShPI-1A (30, 75 and 150 μM) at 28 °C. Samples taken at 0 and 30 h were applied on the C4 column ($n = 3$). The rhMPI concentration was estimated by interpolating the peak area against a standard curve made with a human insulin standard. Each letter represents assignations to homogeneous groups based on statistical significance analysis of mean values.

**Table 4.** Effect of rShPI-1A on the production yield of rhMPI in *Pichia pastoris*

| Group | Induction time (h) | rhMPI concentration (mg $L^{-1}$)* | $Y_{P/X}$* |
|---|---|---|---|
| A† | 0 | 4.98 ± 4.00[a] | 0.030[a] |
| | 20 | 37.31 ± 3.00[bc] | 0.120[b] |
| | 45 | 46.64 ± 4.27[c] | 0.137[b] |
| | 69 | 105.37 ± 3.70[e] | 0.282[c] |
| B‡ | 0 | 2.50 ± 3.00[a] | 0.030[a] |
| | 20 | 26.00 ± 5.00[b] | 0.120[b] |
| | 45 | 92.23 ± 2.78[d] | 0.320[cd] |
| | 69 | 138.41 ± 7.65[f] | 0.343[d] |

*rhMPI concentration and product-biomass yield ($Y_{P/X}$) values were analyzed using an ANOVA test with a *post-hoc* comparison using a Tukey–Kramer multiple comparisons test. Different letters in each column represent assignation to homogeneous groups based on statistically significant differences in mean values.

†Group A: rhMPI-producing C27 strain fermentations without inhibitor (control group).

‡Group B: rhMPI-producing C27 strain fermentations with 30 μmol $L^{-1}$ of rShPI-1A added at 20 h of induction.

graphic profiles of control fermentations with other *P. pastoris* strains (e.g. KM71H or GS115) (data not shown), and might thus be attributed to rhMPI degradation products in the C27 strain culture broth. However, addition

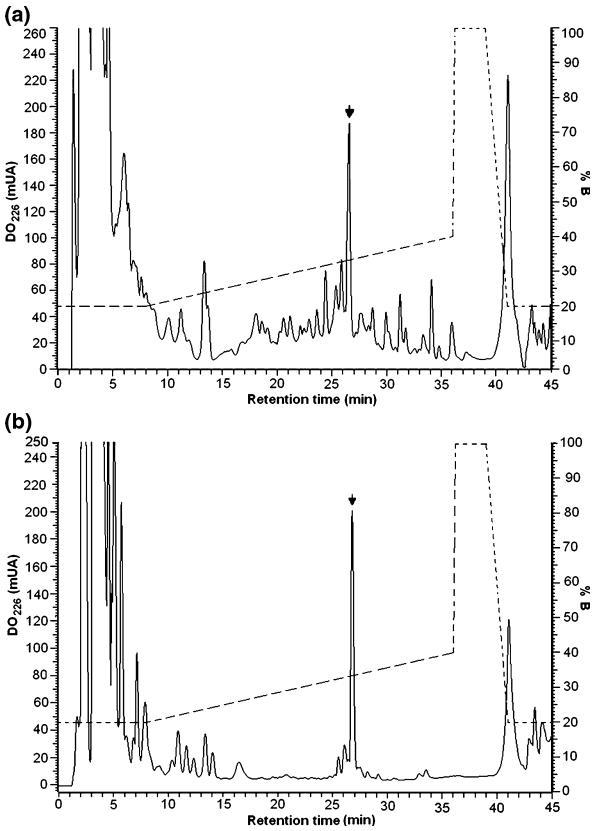


**Fig. 5.** Effect of rShPI-1A on the RP-HPLC profile of rhMPI-producing C27 fermentation supernatants. Typical C4 RP-HPLC profiles of 100 μL fermentation supernatants of rhMPI-producing C27 strain (at 69 h of induction) cultivated without (a) or with 30 μM of rShPI-1A (b). The linear gradient (broken line) was from 20% to 40% of 0.05% TFA in acetonitrile, in 40 min, with a constant flow of 0.8 mL $min^{-1}$. Elution profile (solid line) was monitored by $OD_{226\ nm}$. The black arrow shows the rhMPI peak.

of rShPI-1A during fermentation reduced these undesired peaks (Fig. 5b).

## Discussion

The presence of proteases in supernatants of *P. pastoris* fermentations has been extensively documented (Gleeson & Howard, 1994; Jahic *et al.*, 2003; Shi *et al.*, 2003; Macauley-Patrick *et al.*, 2005; Sinha *et al.*, 2005). These are mainly vacuolar proteases belonging to different mechanistic classes. This may justify the use of protease inhibitors to increase expression levels of heterologous proteins secreted by *P. pastoris*. The use of synthetic protease inhibitors is not economically viable (Shi *et al.*, 2003). In addition, not only does synthetic protease inhibitors typically have wider activity spectra but also have higher $K_i$ values (in the order of $10^{-6}$ M) than protein inhibitors of proteases (Abbenante & Fairlie, 2005).

Therefore, they have to be used at high molar concentrations in culture supernatants.

These considerations motivated us to test the protective effect of ShPI-1, a broad specificity inhibitor, on the proteolysis of recombinant proteins produced in *P. pastoris*. The heterologous protein rhMPI, a precursor of recombinant human insulin, was selected as a model to study the protective effects of rShPI-1A. It has been shown that rhMPI is degraded during induction, and its accumulation in *P. pastoris* culture supernatant is arrested, presumably because the synthesis rate equals the degradation rate. Moreover, rhMPI concentration decreases rapidly when the protein is incubated in a cell-free supernatant for 4 h (Mansur, 2007).

The design of the rShPI-1A gene, which encodes a variant of ShPI-1, included an EAEA tag at the N-terminus (J. Díaz *et al.*, unpublished results), to mimic the $P'_n$ sites of the target sequence of Golgi Kex2p subtilase. This tag has usually improved the secretion of heterologous proteins in yeast and allows homogenous processing at the N-terminus (Vedvick *et al.*, 1991; Kjeldsen *et al.*, 1996). As rShPI-1A expression levels were low in *S. cerevisiae* (J. Díaz, unpublished results), we selected the more potent *P. pastoris AOX1* promoter (Cereghino & Cregg, 2000; Romanos *et al.*, 2001). The selection of putative transformants, based on the resistance to hygromycin B (Gritz & Davies, 1983), allowed us to obtain SH1 strain at higher antibiotic levels, which generally bear a higher number of copies of the recombinant expression cassette (Scorer *et al.*, 1994; Sauer, 2001).

Final expression levels of rShPI-1A (1.2 g $L^{-1}$) are higher than the average productivity (in absolute value) for the *P. pastoris* expression system (Macauley-Patrick *et al.*, 2005). The one-step purification procedure of the recombinant inhibitor, carried out on a Streamline™ Direct HST-1 cation-exchange matrix, is especially designed for efficient protein capture at high ionic strength and can be effectively used for handling culture supernatants from high cell density *P. pastoris* cultures (www.gelifesciences.com). On the other hand, the pI value of the inhibitor (around 8.3) allowed binding of the inhibitor from the SH1 culture supernatant at pH 7.3 on the cation-exchange matrix, whereas most of the contaminant proteins did not bind to the matrix. The simultaneous increase of pH up to 9.1 and ionic strength to 1.3 M of NaCl allowed elution of rShPI-1A with a high protein purity.

The N-terminal sequence and experimental molecular mass of rShPI-1A confirmed the expected differences with the natural protein. The ShPI-1A CD spectrum, similar to that previously observed for BPTI (Kosen *et al.*, 1981), corroborates the correct folding of the expressed protein. The secondary structure estimation agrees well with the NMR structure of natural ShPI-1 (Antuch *et al.*, 1993), as well as with the X-ray structure of rShPI-1A (R. García-Fernández *et al.*, unpublished results). The unusual minimum around 202 nm is a feature of BPTI-Kunitz domains (Kosen *et al.*, 1981), and it is associated with the rigid conformation of Tyr23 and Phe24 within the hydrophobic core of the properly folded domain (Sreerama *et al.*, 1999). This hydrophobic pair is conserved in the family, including Tyr19 and Phe20 of ShPI-1 (Tyr23 and Phe24 of rShPI-1A). Estimation of the α-helix fraction from the ellipticity value at 222 nm (Chen *et al.*, 1972) indicated that strong denaturing conditions are unable to completely denature rShPI-1A. A shift in the minimum ellipticity at 202 nm was detected with increasing urea concentrations; this could be due to local changes in the secondary structure around the hydrophobic core of rShPI-1A. Reduction of disulfide bridges affected the conformation of the recombinant inhibitor, as expected because the disulfide bonds contribute to overall stability (Otlewski *et al.*, 2005). Thus, rShPI-1A is a very stable protein, as described for other Kunitz-BPTI inhibitors; mainly due to the hydrophobic core and three disulfide bridges (Krowarsch *et al.*, 2003).

In addition, the recombinant inhibitor specificity and the $K_i$ value against trypsin were similar to those of the natural protein (Delfín *et al.*, 1996). Functional and structural studies of rShPI-1A demonstrated that the additional residues do not influence the overall folding and specificity of the protein. This was expected, as the rShPI-1A additional residues are outside of the primary and secondary binding sites. In the case of BPTI-Kunitz inhibitors, these sites interact with serine proteases (Otlewski *et al.*, 2005). Consequently, this recombinant variant of ShPI-1 may be used for structure–function relationship studies as well as biotechnological and biomedical applications.

The protection capacity of rShPI-1A against the degradation of rhMPI was studied in conditions simulating a potential industrial use. Simultaneously, we simplified the system to exclude the effect of the inhibitor on the concentration of rhMPI due to a direct effect on the cells. The *in vitro* assays demonstrated that rShPI-1A, even at a low concentration, is able to protect rhMPI against degradation by proteases, although high concentrations of the recombinant inhibitor were unable to eliminate the proteolytic activity in the C27 supernatant completely. On the other hand, under fermentation conditions, we used the lower effective concentration of rShPI-1A (30 μM), to reduce the fermentation costs. Although at the end of the fermentation, the protective capacity of the recombinant inhibitor slightly diminishes, our results demonstrate that it is possible to the reduce rhMPI degradation rate with rShPI-1A treatment.

The co-expression of the protease inhibitor rShPI-1A with rhMPI in *P. pastoris* cells could be a technological variant, but several considerations should be taken into account when one of the proteins is intended for therapeutic use in humans. There are many successful examples of co-expression of different proteins for the improvement in post-translational modification of heterologous proteins (Ruohonen *et al.*, 1997; Huo *et al.*, 2007; Payne *et al.*, 2008). These proteins play an important role in removing bottlenecks in certain expression/secretion systems, but these proteins are first, native yeast proteins, and second, intracellular proteins. Thus, while the desired protein is secreted, the enhancer protein remains intracellular and therefore does not contaminate the desired product. Some authors suggest that overproduction of heterologous proteins in the same host could generate a metabolic burden that reduces growth rate as well as final biomass yields (Berges *et al.*, 1996; Martínez-Alonso *et al.*, 2010). This phenomenon can be amplified when increasing gene copy number, a strategy used during the current expression of rhMPI (Mansur *et al.*, 2005; Mansur, 2007) and rShPI-1A. Hence, co-expression of both proteins would not necessarily enhanced rhMPI production. Nevertheless, co-expression could be attempted in future experiments.

On the other hand, *P. pastoris* proteases have been partially characterized. Among them, many are active between acid and neutral pH values (range 4.0–7.0) (Sinha *et al.*, 2005), similar to the pH value used in fermentations of the C27 strain, at which a high degradation rate of rhMPI was observed. Considering the broad specificity of rShPI-1A, we hypothesized that saccharopepsin-like aspartic proteases or others proteases as yet undescribed could be inhibited after its addition in C27 culture supernatants. The remaining proteolytic activity observed in rhMPI-producing C27 supernatants treated with a high concentration of rShPI-1A could be due to subtilisin-like and/or carboxypeptidase-like proteases, which are not inhibited by rShPI-1A. In fact, while this work was being completed, Salamin *et al.* (2010) reported a new subtilase Sub2, which is secreted in *P. pastoris* KM71 fermentations on methanol medium. Thus, the addition of more than one inhibitor(s) to the culture supernatant could decrease the remaining proteolytic activity. Although further studies may demonstrate the protective effect of other inhibitors, our results show the effectiveness of a broad specificity inhibitor against the proteolytic degradation of a heterologous protein secreted by *P. pastoris*.

## Conclusions

The broad spectrum Kunitz-type inhibitor, rShPI-1A, is secreted at high concentrations by the *P. pastoris* C27 strain. It is functionally active, with folding and specificity patterns very similar to those of the natural inhibitor. The use of rShPI-1A diminishes rhMPI degradation in *P. pastoris* culture broth, and concomitantly increases rhMPI accumulation. This is the first report to show that a peptidic protease inhibitor improves heterologous protein expression in the *P. pastoris* system. These results open new perspectives to the use of this recombinant inhibitor (and/or its mutants) for obtaining higher concentrations of rhMPI and other recombinant proteins expressed in this system. Our results demonstrate that rShPI-1A could be a biotechnological tool for the optimization of the production of proteins secreted by *P. pastoris*.

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