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Expression and Characterization of Recombinant Human Secretory Leukocyte Protease Inhibitor (SLPI) Protein from *Pichia pastoris*

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Abstract

The human secretory leukocyte protease inhibitor (SLPI) has been shown to possess anti-protease, anti-inflammatory and antimicrobial properties. Its presence in saliva is believed to be a major deterrent to oral transmission of human immunodeficiency virus-1. The 11.7 kD peptide is a secreted, nonglycosylated protein rich in disulfide bonds. Currently, recombinant SLPI is only available as an expensive bacterial expression product. We have investigated the utility of the methylotrophic yeast *Pichia pastoris* to produce and secrete SLPI with C-terminal *c-myc* and polyhistidine tags. The posttransformational vector amplification protocol was used to isolate strains with increased copy number, and culturing parameters were varied to optimize SLPI expression. Modification of the purification procedure allowed the secreted, recombinant protein to be isolated from the cell-free fermentation medium with cobalt affinity chromatography. This yeast-derived SLPI was shown to have an anti-protease activity comparable to the commercially available bacterial product. Thus, *P. pastoris* provides an efficient, cost-effective system for producing SLPI for structure function analysis studies as well as a wide array of potential therapeutic applications.

Introduction

The human secretory leukocyte protease inhibitor (SLPI) gene encodes an 11.7 kD secreted inhibitor whose main role is to protect epithelial tissues from serine proteases such as trypsin, leukocyte elastase, and cathepsin G [1,2]. Furthermore, SLPI possesses an immunomodulatory role in that it can regulate the levels and activities of factors responsible for causing inflammation [1,3]. For example, SLPI decreases the activation of the transcription factor

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nuclear factor κ B (NF- κ B) and the production of interleukin-8 in human macrophages induced by bacterial lipopolysaccharide, in part due to its ability to bind certain promoter elements [4]. SLPI knockout mice exhibited greatly compromised wound healing, increased inflammation and increased activity of elastase, which degraded tissue [5]. Found in various bodily secretions including seminal plasma, cervical mucus, breast milk, tears, saliva, and bronchial secretions [2], SLPI appears to be a component of the innate host defense that maintains a balance between protective inflammatory responses and uncontrolled inflammation that can lead to tissue destruction and failure to heal [6].

Along with these properties, SLPI protein possesses broad-spectrum antimicrobial activity [7,8]. It has been shown to be active against Gram-positive and Gram-negative bacteria, viruses including human immunodeficiency virus-1 and herpes simplex viruses, and some fungi [1, 7,9-15]. In terms of preventing HIV transmission, SLPI blocks the interaction of the virus with the annexin II receptors on human macrophage cells [16]. In addition, it has been suggested that the cationic nature of SLPI allows it to disrupt the anionic plasma membranes of many bacteria. SLPI is also considered an antimicrobial 'defensin-like' peptide because its expression is upregulated by 'alarm signals' such as bacterial lipopolysaccharides, as well as cytokines such as interleukin-1 and tumor necrosis factor [1]. Thus, SLPI plays a role in anti-inflammatory, anti-proteolytic and antimicrobial mechanisms in the human body, making it an attractive candidate for many types of therapy.

It is difficult to comprehend how such a small protein can afford so many diverse functions. The SLPI structure has been shown to resemble a boomerang with the ends forming the *N*-terminal and *C*-terminal domains [17]. In the crystal, each domain is stabilized by four disulfide bonds with identical bridging patterns [17,18]. The protease inhibitory activity of SLPI has been shown to be located in the *C*-terminal domain while its antimicrobial activity resides in its *N*-terminal domain [19,20]. Although the protein is secreted by the cell, it is not glycosylated. Overall, SLPI has no hydrophobic core and contains little hydrogen-bonded secondary structure [21].

Because the native protein is found at the physiological concentration of 4–24 μ g/ml in saliva, recombinant expression is required to produce sufficient amounts for detailed biochemical characterization and biomedical applications. *E. coli* has previously been utilized as a host for heterologous production of recombinant SLPI, which is commercially available (R&D systems, Minneapolis, MN). This recombinant SLPI has been used for both structure-function studies and therapeutic applications [8,11,22,23]. Clinical trials have been carried out with the bacterial-produced SLPI to investigate its ability to accelerate wound healing [24]. Recently, recombinant SLPI has been tested as a treatment for inflammatory lung conditions, ischemia/reperfusion injury in cardiac transplantation, and tuberculosis [12,22,25]. Although SLPI can be produced in *E. coli*, potential contamination with LPS limits its use in anti-HIV studies because the bacterial endotoxin alone suppresses HIV infection [26]. In addition, expression of the disulfide rich protein in *E. coli* results in a non-native, inactive product that requires reductive denaturation and oxidative refolding to recover the native, active conformation [19,27,28] contributing to its high cost (245 dollars per 100 μ g). Some contradictory findings on SLPI by different research groups have also been attributed to variations in the preparation of the bacterially-produced peptide [15,29,30]. Concerns have been raised about the possibility that oxidation of the recombinant protein may affect its biological activity [31]. In one report, the ability of recombinant SLPI to reduce HIV-1 infectivity in differentiated human monocytic THP-1 cells depended on its source [32].

These issues associated with recombinant SLPI synthesized in bacteria indicate a need for an alternative expression host which is able to consistently provide SLPI in its native conformation at a reasonable cost. Insect cell culture has been used to prepare biologically active SLPI;

however, it is a rather expensive host system with relatively poor yields [33]. We have chosen to develop the methylotrophic yeast *Pichia pastoris* for this purpose. The use of *P. pastoris* as a heterologous expression host offers many advantages, compared to other expression systems [34,35]. First, *P. pastoris* products are not susceptible to the endotoxin problems associated with bacterial products or the viral contamination problem of proteins produced in animal cell cultures. Second, the methylotroph can be manipulated on the molecular genetic level in ways similar to *Saccharomyces cerevisiae*. Third, *P. pastoris* can express proteins at high levels, intracellularly or extracellularly, at fairly low costs. Fourth, unlike prokaryotes, it is capable of performing many eukaryotic posttranslational modifications, such as glycosylation and disulfide bond formation. Furthermore, it is able to reach high cell densities because of its preference for respiratory growth over fermentation. Finally, the *P. pastoris* expression system uses the methanol-induced alcohol oxidase (AOX1) promoter, an extremely strong, regulated promoter, to promote high yields of heterologous protein, from milligrams to grams per liter [36].

Based on this multitude of strengths, *P. pastoris* appears to be an ideal choice for heterologous expression and extracellular secretion of SLPI, a protein with specific structural requirements. We have constructed an efficient *Pichia* expression system for production of recombinant SLPI. The yeast-derived SLPI has been isolated and its biological activity was compared with the *E. coli*-derived recombinant peptide as a standard. Our results demonstrate that the yeast system is able to produce biologically active SLPI suitable for a multitude of applications.

Material and methods

Media, strains, and microbial techniques

Pichia pastoris strain yJC100 (wild-type) is a derivative of the original wild-type *P. pastoris* strain NRRL Y11430 (North Regional Research Laboratories, US Department of Agriculture, Peoria, IL) and has been described previously [37]. yJC100 cells were cultured in either YPD medium (1% yeast extract, 2% peptone, 2% glucose), BMGY (buffered minimal medium with 1% glycerol, 2% peptone, and 1% yeast extract) or BMMY (buffered minimal medium with 0.5% methanol, 2% peptone, and 1% yeast extract). Cultivation of *P. pastoris* strains was at 30°C unless otherwise indicated. For *P. pastoris*, Zeocin was added to a final concentration of 100 µg/ml for selection of transformants.

Recombinant DNA manipulations were carried out in *Escherichia coli* strain TOP10 (Invitrogen Corp., Carlsbad, CA). TOP10 cells were cultured in LB medium (0.5% yeast extract, 1% glucose, 0.5% NaCl) at 37°C. Antibiotics were added to LB medium at the following final concentrations: 100 µg/ml ampicillin and 25 µg/ml Zeocin for plasmid selection. Transformation and other standard recombinant DNA techniques used in this study for *E. coli* follow techniques described previously [38]. Bacterially produced recombinant SLPI protein was purchased from R&D Systems (Minneapolis, MN).

Recombinant DNA methods were performed essentially as described in Sambrook *et al.* (1989). Plasmid DNA was purified from *E. coli* cultures using a QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA). Restriction enzymes were purchased from MBI Fermentas (Hannover, MD). DNA digested with restriction enzymes was resolved on TBE agarose gels. DNA fragments were purified from agarose gels by using the GeneClean II kit (Qbiogene, Carlsbad, CA). Chromosomal DNA from *P. pastoris* transformants was prepared using the OmniPrep™ kit from GenoTechnology, Inc. (St. Louis, MO). All ligation junctions in newly synthesized vectors were confirmed by DNA sequencing (Geneway Research, Hayward, CA). Oligonucleotides were synthesized by Sigma Genosys (Plano, TX).

Vector construction

pABU1 was constructed by amplifying an approximately 320 bp SLPI coding sequence from pET24a-SLPI [32] using the primers slpi5ecor1 CAGGAATTCACATGTCTGGTAAAAGC and slpi3not1 CTGGCGGCCGCTGCTTTTACCGGGGA. The PCR product was digested with *EcoRI* and *NotI* and ligated into the *EcoRI-NotI* digested pPICZalphaB vector backbone. The ligation mix was then transformed into competent *E. coli*, and the resulting transformants were selected for Zeocin resistance. pABU1 was verified by nucleotide sequence analysis of the insert.

Pichia pastoris transformation

P. pastoris was electrotransformed as previously described [39,40]. Briefly, pABU1, as well as pPICZalphaB as a control, were linearized at the *MphI*103I site within the *AOX1* promoter, purified with the QIAprep Spin PCR Purification Kit (Qiagen, Valencia, CA) and electroporated into competent yJC100 cells. Transformed cells were allowed to recover for one hour in 1 ml of a 50% 1M sorbitol/50% YPD solution at 30°C and then plated on selective medium containing Zeocin (100 ug/mL). Transformed colonies were then purified by streaking for isolated colonies on selective medium, and rapid colony PCR was used to determine the presence of the SLPI coding sequence in pABU1-transformed cells [41]. Posttransformational vector amplification was carried out as previously described [42].

Real-time PCR

Real-time PCR reactions were composed of 10µl of 2X reaction buffer from the DyNAmo SYBR Green qPCR kit distributed by New England BioLabs (Beverly, MA), 10ng of genomic DNA, and 0.3µM primers in a 20µl total reaction volume. PCR reactions were carried out in a MJ MiniOpticon (BioRad Corp, Hercules, CA) with the following parameters (1 cycle of 95°C for 5 min., 40 cycles of 94°C for 1 min., 59°C for 1 min., 72°C for 1 min.). Primers used to amplify the SLPI target gene were allieslpirtfow ttgataccccaacccgactc and allieslpirtrev ttgccacacataccatacagc. Primers used to amplify the *MET2* [41] reference gene were mets100 cgtctcgcgaactcttcgaa and metas100 caatggcatcagttatgacggaag. All samples were performed in duplicate, and independently evaluated in separate experiments. *Q-gene* software was utilized to analyze real-time PCR data [43].

Small scale SLPI expression

Cultures were first grown overnight in YPD medium to stationary phase. On the second day, the optical density was measured, and 1.0 OD₆₀₀ units of each culture were pelleted for 30 seconds at 2000×g at room temperature. The cells were suspended in 5 mls of BMMY medium in a 50 ml conical centrifuge tube. The cultures were induced for 48 and 72 hours at 30°C with shaking (225 rpm), adding methanol every 24 hours to compensate for losses from evaporation and metabolism. At each time point, the OD₆₀₀ of each culture was measured, and 1.0 ml was spun down at 10,000×g for 1 minute to separate cells from extracellular supernatant. The supernatants were transferred to a new microcentrifuge tube, and Protease Arrest protease inhibitor (G Biosciences, St. Louis, MO) was added. Pellets and supernatants were immediately frozen and stored at -80°C.

High density fermentation

Large scale fermentation of *P. pastoris* was performed according to standard procedures [44, 45], with slight modifications. An overnight culture of the *Pichia pastoris* expression strain in 3×100 ml BMGY medium was inoculated into 5 L of FM22 minimal medium (1) supplemented with PTM4 trace metals solution in a New Brunswick Scientific Bio-Flo 300 bioreactor equipped with a 14 L fermentation vessel. The initial glycerol batch cultivation (approximately 20 hours) was performed with vigorous agitation (500 rpm) while sparging the solution with

an air/O₂ mixture to maintain 40% dissolved oxygen level. The pH of the culture medium was maintained at pH 4.5 by addition of 5 N NH₄OH. A methanolic solution of antifoam (5% KFO 673 antifoam (Kabo Chemicals Co., Jackson Hole, WY) was delivered as required to control foaming. The temperature was maintained at 29°C using a Neslab refrigerated circulator (CFT-33). After the glycerol batch phase was completed (as evidenced by a decreased O₂ requirement and reduced acidification of the medium) the glycerol fed-batch phase was started by delivery of 400 ml of 50% glycerol at a rate of 15 ml/L/h. When the addition was complete, the temperature was adjusted to 25°C and recombinant expression was induced by methanol feeding. Filter sterilized methanol containing 4 mL/L of PTM4 trace metals solution and 0.4 mg/L biotin was delivered using a continuous-duty Watson-Marlow peristaltic pump at a constant rate of approximately 15 mL/h for 3 days, for a total of 1 L of methanol. At the end of the fermentation run, the cell mass was removed by centrifugation (approximately 5500×g, 20 min) and the protein-containing supernatant was separated from the yeast pellet.

Purification of SLPI using cobalt affinity chromatography

The purification protocol was performed according to manufacturer specifications of the Pierce HisPur Cobalt Spin Columns (Rockford, IL) with some modifications. The fermentation supernatant was thawed in a 37°C water bath, and the required amount of Protease Arrest (G Biosciences, St. Louis, MO) was added to inhibit sample degradation. The supernatant was adjusted to pH7.4 with 10N NaOH. After the neutralized solution was sterilized using a 0.22 µm cellulose filter unit, approximately 1 ml of 10X equilibration buffer was added to 9 ml of the supernatant. The mixture included was maintained on ice until the column was ready. A HisPur column (1 ml resin volume) was equilibrated to room temperature and centrifuged at 700×g, 2 minutes at 4°C to remove storage buffer in column. The column was then washed with 2ml 1X equilibration buffer without imidazole and spun at 700×g for 2 minutes at 4°C. The ice cold supernatant was loaded onto the column by gravity feed. The column was then centrifuged two times as described above, and the flow-through was collected. The column was washed 3 times with 2ml 1X equilibration buffer and centrifuged. Finally, the SLPI^{myc}-his6 protein was eluted with three 1ml volumes of elution buffer. The elutant fractions were analyzed for SLPI by western analysis, and those containing purified SLPI were pooled and dialyzed for 24 hours against 1×PBS. For larger volumes of supernatant, this protocol was scaled up by using the HisPur columns containing 3 mls of cobalt resin.

Polyacrylamide gel electrophoresis and western analysis

Protein concentrations were determined using the Pierce (Rockford, IL) Bicinchoninic Acid (BCA) Protein Assay kit with bovine serum albumin as a standard. Either equivalent amounts of proteins or volumes of extracellular medium from equivalent numbers of cells were loaded onto stacked sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) for electrophoresis. Gels were either stained for total protein visualization using either the Gelcode Blue (Pierce, Rockford, IL) or utilized in western analysis. For westerns, proteins were transferred onto nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Immunoblots were done with polyclonal antibodies to the antigen, and the results were visualized with the Western-Star Protein Detection Kit (Tropix, Bedford, MA). The anti-*myc* and anti-SLPI antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies, conjugated to alkaline phosphatase, were purchased from Tropix (Bedford, MA). Chemiluminescent signals resulting from alkaline phosphatase activity were captured with a ChemiImager 5500 (Alpha Innotech, San Leandro, CA).

Spot western

The spot western technique has been described in detail [46]. Whatman paper and nitrocellulose were sized to fit into the Spot Blotter unit (Topac, Bristol, United Kingdom). The Whatman was fitted into the base of the unit followed by the nitrocellulose which had been soaked in sterile PBS. The lid was then secured over the membrane and screwed in. After vacuum was applied to the unit, the desired amount of extracellular supernatant (originating from equivalent cell numbers) was deposited into the wells in the cover of the unit. The liquid samples were drawn through, capturing the total protein from each sample as uniform spots on the nitrocellulose membrane. The nitrocellulose was removed from the disassembled unit, dried in a 55°C oven for 5 minutes and then processed as using the Western-Star Protein Detection Kit (Tropix, Bedford, MA), as noted above.

Glycosylation analysis

Purified SLPI, and RNase B (New England Biolabs, Beverly, MA) as a control, were digested with PNGaseF according to manufacturer's specifications (New England Biolabs, Beverly, MA) for 4 hours and the products resolved on 15% SDS-PAGE gels. The gels were then stained using the GelCode Blue (Pierce, Rockford, Ill) to visualize the proteins.

ELISA

SLPI quantitation was performed using the Quantikine Human SLPI ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's procedure. Sample absorption was monitored at 450nm using an ELx800 Universal Microplate Reader (Bio-Tek, Inc, Winooski, VT).

SLPI Anti-protease Fluorescence Assay

The Protease Fluorescent Detection Kit (Sigma, St. Louis, MO) was modified to measure SLPI anti-protease activity. Briefly, for the blank sample, 30 µL of incubation buffer (20 mM sodium phosphate with 150 mM sodium chloride, pH 7.6) and 20 µL of FITC-casein substrate were added to a microcentrifuge tube. For the control sample, 25 µL of incubation buffer and 5 µL of trypsin solution in incubation buffer were added to a microcentrifuge tube. The amount of bacterial and yeast-derived SLPI used in these assays was determined by ELISA and diluted appropriately. For each SLPI test sample, 20 µL incubation buffer, 5 µL SLPI in PBS and 5 µL of trypsin in incubation buffer were added to a microcentrifuge tube. Then 20 µL of FITC-casein substrate was added to the tubes, mixed gently and incubated at 37°C in the dark for 60 minutes. After incubation, 150 µL of the 0.6 N trichloroacetic acid solution was added to each tube. The solution was mixed gently and incubated at 37°C in the dark for 30 minutes. After the tubes were spun for 10 minutes at 10,000×g, 5 µL of the supernatant was added to 500 µL of assay buffer (500 mM Tris buffer, pH 8.5) and mixed gently. 70 µL of the mixture was transferred to a minicell and the fluorescence intensity was measured with excitation at 485 nm and emission wavelength of 535 nm with a TBS-380 Mini-Fluorometer (Turner Biosystems, Sunnyvale, CA). Three trials were performed with both bacterial and yeast derived SLPI for each amount of trypsin used (20, 40, 60, 80 and 100 ng).

Results

Isolation of SLPI expression strains of *P. pastoris*

P. pastoris cells were transformed with an expression vector, pABU1, which encoded a secreted form of SLPI. Mature SLPI, lacking its native secretion peptide, was cloned in frame with the *N*-terminal *Saccharomyces cerevisiae* Matalpha prepro peptide leader and a *C*-terminal region encoding the *c-myc* epitope and polyhistidine tag. The modified coding sequence was under the control of the *AOX1* promoter, which is tightly repressed under glucose

growth conditions but induced over 1000-fold under methanol growth conditions. The *P. pastoris* strain yJC100 was transformed with linearized pABU1 and colonies were selected on YPD medium containing Zeocin. Several colonies were re-streaked for purification and analyzed by colony PCR to confirm the presence of the SLPI gene in the genome. Positive clones were grown in 5 mls of BMGY medium (a rich complex medium containing glycerol) to accumulate biomass and then shifted to BMMY medium containing methanol as a sole carbon source. The methanol in this medium also functions as an inducer, driving expression of the SLPI under the *AOX1* promoter. After growth in methanol-based medium, the amount of SLPI secreted by various strains was compared by spot western analysis. Aliquots of extracellular supernatant, corresponding to equivalent numbers of cells, were spotted onto nitrocellulose and probed with a primary mouse anti-*myc* antibody followed by a secondary goat anti-mouse antibody-alkaline phosphatase conjugate. A representative spot western is shown in Figure 1. As expected, the extracellular medium of a strain transformed with the empty vector (pPICZalphaB) gave no signal while a commercially available *myc*-tagged protein control produced a strong signal. Varying amounts of *myc* tagged recombinant protein were detected in the extracellular media from strains containing the SLPI gene. Similar results were obtained using a primary polyclonal SLPI antibody (data not shown). Real time PCR indicated that all strains tested contained approximately 1 copy of SLPI gene/cell (data not shown). A SLPI detection ELISA indicated that strains produced between 1 and 2 mg/L of the recombinant protein under small scale expression conditions. Based on the results of several spot westerns, four strains, designated yJC100:pABU1-A, yJC100:pABU1-D, yJC100:pABU1-6, yJC100:pABU1-7, showed the highest level of SLPI-*myc*-his6 expression in the small scale assay at 72 hours of induction.

Optimization of SLPI expression conditions and strains

These four isolates were then cultured under varied conditions to optimize SLPI expression. Typically, *P. pastoris* expression strains are grown at 30°C on medium containing 0.5% methanol for heterologous protein production. However, past studies have shown that modification of temperature, increase in methanol concentration, and the addition of 10 mM CaCl₂ can enhance the secretory expression of some recombinant proteins [46]. These variations were explored using the top-producing strains, and an SLPI detection ELISA was used to quantify the amount of the recombinant peptide in the extracellular medium. The highest level of SLPI expression by the most productive strain, pABU1-A, was estimated by SLPI ELISA to be approximately 3.8 mg/L when cultivated at 30°C in growth medium containing higher (1%) methanol (Table 1).

This expression level is fairly low compared to other heterologous proteins secreted by *P. pastoris* although yields ranging from mg/L to g/L have been reported [34]. In order to further boost SLPI productivity, strain yJC100:pABU1-A was submitted to the posttransformational vector amplification procedure (PTVA). Briefly, in PTVA, the SLPI-*myc*-his6 expression colonies were streaked onto gradually higher levels of Zeocin in order to select for plasmid duplication and higher SLPI copy number [42]. Progeny and parental strains were assayed as in small scale analysis above. Spot westerns indicated that that one progeny strain, pABU1-A1.1, consistently secreted approximately 2 fold higher levels (~7.5 mg/L) of SLPI-*myc*-his6 than its parent strain, pABU1-A, and other PTVA progeny strains. Real time PCR analysis indicated that the pABU1-A1.1 strain contained two copies of the SLPI expression cassette (data not shown). This strain was selected for large-scale expression.

High level expression and purification of SLPI

10 liter fed-batch fermentation was carried out with the yJC100:pABU1-A1.1 strain in order to generate sufficient SLPI protein for further analysis. This high cell density procedure yielded approximately 26 mg/L of the SLPI fusion protein in the extracellular medium according to

our SLPI ELISA. Purification methods were then optimized to recover SLPI-*myc*-his6 from the fermentation broth. Because of the presence of the polyhistidine tag, we initially used an immobilized ion affinity chromatography (IMAC) resin containing nickel to isolate the recombinant peptide. After centrifuging the medium and filtering (0.22 μ m) to remove particulates, the supernatant was incubated with resin, washed extensively and eluted with imidazole. However, very little purified SLPI was recovered from this process because the protein did not bind efficiently to the nickel IMAC resin (data not shown). Replacement of cobalt for nickel did not significantly improve the results. However, adjusting the pH of the supernatant to pH 7.4 from the original pH of 4.5 by addition of sodium hydroxide allows the SLPI protein to bind efficiently to the cobalt IMAC resin. The protein was released from the column when imidazole was added (Table 2).

Figure 2 shows SDS-PAGE analysis of samples taken during purification analyzed with Coomassie stain (to visualize all proteins, Figure 2A) or immunoblotted with the anti-*myc* antibody (to specifically detect the SLPI fusion, Figure 2B). The anti-*myc* antibody, followed by a secondary antibody-alkaline phosphatase conjugate, detected a high level of the recombinant protein in the original supernatant, a low level in the flow-through, no detectable antigen in the wash, and an increased level in the eluted fractions (Figure 2B). In a complementary fashion, Figure 2A demonstrates that the Coomassie stain is unable to detect any SLPI in the wash fractions but reveals purified SLPI, as a doublet of approximately 15–16 kD, in the elutant fractions. The doubling of the protein band may reflect formation of some intramolecular disulfide cross-links during electrophoresis, resulting in a fraction of the polypeptide chains exhibiting higher mobility on the gel. A small amount of degradation product, detected by the anti-*myc* antibody, is also present in the eluted fractions. After elution from the column, the SLPI fusion was dialyzed thoroughly before testing for protease inhibitory activity.

Glycosylation analysis of purified SLPI

The SLPI-*myc*-his protein has a predicted molecular weight of approximately 15 kD, owing to the size of the SLPI (11.7 kD), the linker region from cloning (0.6 kD) and the *myc*-his6 C-terminus extension (2.5 kD). The SDS-PAGE data shows that the purified peptide exhibits the expected molecular weight near 15 kD, indicating minimal glycosylation of the protein (Figure 2A). In human cells, SLPI is secreted but not glycosylated. In order to confirm that the recombinant protein was not glycosylated by *P. pastoris*, the purified protein was digested with PNGase F to remove any N-linked glycan. Digestion of the glycosylated control protein, RNase B, with PNGaseF significantly changed its electrophoretic mobility. However, digestion of the purified SLPI protein under identical conditions did not change its mobility, indicating that the protein is not N-glycosylated by the yeast (data not shown). We cannot rule out the possible presence of a small amount of O-glycan. However, it is clear that the protein is not hyperglycosylated, a common problem for human proteins produced in fungal hosts [34, 35].

Comparative analysis of the biological activity of SLPI

To confirm that the purified recombinant SLPI was correctly folded into a biologically active form, we assayed the protease inhibitory activity of the *P. pastoris* derived SLPI, using the commercially available, bacterial-derived SLPI as a comparison. Increasing amounts of trypsin, a serine protease, were added to equivalent quantities (200 ng) of both *P. pastoris* SLPI (PpSLPI) and *E. coli* -produced peptides (EcSLPI). As seen in figure 3, the *P. pastoris* derived SLPI inhibited trypsin to a comparable degree as the commercially available, bacterially-produced peptide. These results suggest that the *P. pastoris* – produced peptide has the required 3D structure to perform at least one of its multi-faceted biological functions.

Discussion

Our studies indicate that *P. pastoris* can serve as a suitable expression host for efficient production of functionally active human SLPI. The recombinant protein was directed to the extracellular medium, which dramatically facilitates purification because *P. pastoris* secretes relatively few of its own proteins. Fusion to a polyhistidine tag allows the SLPI protein to be easily purified from the extracellular supernatant by affinity techniques, but only after neutralization of the supernatant solution.

The recombinant protein does not appear to be N-glycosylated. This is an advantage for its potential use in therapeutic applications since yeast glycans can occasionally trigger an antigenic response, in addition to their effects on protein stability and function [47]. Once isolated and dialyzed, the *P. pastoris*-synthesized SLPI, containing *myc* and *his6* regions at its C-terminus, was shown to have a similar anti-protease activity level as the recombinant SLPI derived from *E. coli* in a functional assay. Thus, the presence of the C-terminal groups did not affect its function.

P. pastoris offers several important advantages over the bacterial host system for the production of SLPI. As a eukaryote, the yeast has the capacity to carry out posttranslational modifications needed by many human proteins, including a secretory network which provides the intracellular environment to promote correct disulfide bridge formation, an important requirement for proper SLPI since it contains eight disulfides [34]. The presence of protein disulfide isomerase (PDI) in the *P. pastoris* endoplasmic reticulum may play a role in the secretion of SLPI from the yeast cell [48]. PDI not only catalyzes the oxidation and isomerization of disulfide bridges in newly synthesized proteins but also promotes correct folding of proteins, acting as a chaperone. In contrast, the *E. coli*-derived SLPI is not secreted by the heterologous host cell. Localized to the cytoplasm, the bacterially produced SLPI requires denaturation and renaturation to recover functional activity [27,28]. This processing is not only technically demanding, but also costly and may introduce some variability in the product. [29]. By comparison, *P. pastoris* allows for SLPI to be made efficiently and economically. This alternative source of recombinant SLPI may be expected to benefit the diverse efforts to utilize SLPI for therapeutic applications and will also facilitate studies to understand how this small protein can have such a wide array of important biological activities.

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Figure 1. Representative spot western of pABU1 strains expressing SLPI-myc-his6 protein

Strains containing pABU1 were grown in BMGY glycerol medium and then induced in BMMY methanol medium, as described in the Methods. Aliquots of culture supernatant, corresponding to equivalent numbers of cells (based on OD₆₀₀), were spotted onto nitrocellulose membrane and probed with a primary mouse anti-myc antibody followed by a secondary goat anti-mouse antibody conjugate to quantify the level of secreted SLPI-myc-his6. “+” represents the positive control sample containing purified myc epitope-tagged protein (Invitrogen, Carlsbad, CA). “a” represents extracellular supernatant from strains containing the empty plasmid pPICZalphaB. “b-f” represent extracellular supernatants of various strains transformed with pABU1.

Figure 2. Coomassie stain and western analysis of SLPI purification

Samples from the various steps in the purification of the SLPI from fermentation medium were subjected to SDS-PAGE and then visualized with either Coomassie stain (A) or analyzed by western blot using an anti-*myc* antibody (B). Lane 1:fermentation supernatant; lane 2:first flow-through; lane 3:second flow-through; lane 4:first wash; lane 5:second wash; lane 6: third wash; lane 7:first elution; lane 8:second elution; lane 9:third elution; lane 10: molecular weight ladder.

Figure 3. Inhibition of trypsin activity by *P.pastoris* and *E. coli* derived SLPI

Increasing amounts of trypsin were added to equivalent quantities (200 ng) of either *P. pastoris* SLPI (PpSLPI) or *E. coli* -produced SLPI (EcSLPI) and the amount of trypsin activity remaining was recorded. The values represent an average of three trials.

Table 1**Growth conditions for optimized SLPI expression**

Strain yJC100:pABU1-A was grown under different conditions, and the amount of secreted SLPI was determined by ELISA.

Growth conditions	SLPI concentration (µg/ml)
BMMY (0.5% methanol) 22°C	2.1
BMMY (0.5% methanol) 30°C	2.5
BMMY (1.0% methanol) 30°C	3.8
BMMY (2.0% methanol) 30°C	2.3
BMMY (0.5% methanol) 30°C 10 mM CaCl ₂	2.5

Table 2**Summary of purification of recombinant SLPI**

The values are based on a starting volume of 10 mls of filtered fermentation supernatant. The total protein calculation was based on a BCA assay while the SLPI protein measurement was determined by ELISA.

Purification Step	Total Protein (μg)	SLPI protein (μg)	Recovery (%)	Purity (%)
Filtered supernatant	14, 200	188	100	1.32
Cobalt Affinity Column	99	51.0	27.1	51.5