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Production of antifungal recombinant peptides in *Escherichia coli*

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Abstract

Antifungal peptides derived from the human bactericidal/permeability-increasing protein (BPI) were produced in *Escherichia coli* as fusion proteins with human BoneD. Bacterial cultures transformed with the gene encoding the fusion protein were grown to a high cell density ($OD_{600} > 100$), and induced with L-arabinose to initiate product expression. Fusion protein accumulated into cytoplasmic inclusion bodies and recombinant peptide was released from BoneD by acid hydrolysis at an engineered aspartyl–prolyl dipeptide linker. Acid hydrolysis of purified inclusion bodies at pH < 2.6 followed Arrhenius kinetics and did not require prior inclusion body solubilization in detergents or denaturants. Surprisingly, at pH < 2.6 and 85°C, cell lysis and aspartyl–prolyl hydrolysis with concomitant peptide release occurred simultaneously. Bacterial cultures were, therefore, adjusted to approximately pH 2.6 with HCl directly in the bioreactor and incubated at elevated temperature. Peptide, which is soluble in the aqueous acidic environment, was separated from the insoluble material and purified using column separation techniques. Recombinant peptide was separated from the hydrolyzed bioreactor culture with $> 76\%$ recovery and a final peptide purity of $> 97\%$. Antifungal peptide prepared by recombinant and solid phase synthesis methods demonstrated similar activity against *Candida* sp. in a broth microdilution assay.

Keywords: Peptide; *Escherichia coli*; Antifungal; Aspartyl–prolyl cleavage; Bactericidal/permeability-increasing protein

1. Introduction

Although bioactive peptides can be produced chemically by a variety of synthesis strategies, recombinant production of peptides in the 5–50 amino acid size range offers the potential for large scale production at reasonable cost. Expression of very short polypeptide chains can sometimes be problematic in microbial systems such as *Escherichia coli*. Some peptides have been expressed as part of fusion proteins (Callaway et al., 1993; Piers et al., 1993). As part of a fusion protein, peptides may be directed to specific cellular compartments, i.e. cytoplasm, periplasm, or media, with the goal of achieving high expression yield and avoiding cellular degradative processes.

Preparation of a peptide from a fusion protein in pure form requires that the peptide be released and recovered from the fusion protein by some

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mechanism and then purified. Methods for cleaving fusion proteins have been identified. Each method recognizes a chemical or enzymatic cleavage site that links the carrier protein to the desired protein or peptide (Forsberg et al., 1992; Pilon et al., 1997; Park et al., 1998). Chemical cleavage reagents generally recognize single or paired amino acid residues which may occur at multiple sites along the primary sequence and, therefore, may be of limited utility for release of large peptides or protein domains which contain multiple internal recognition sites. However, recognition sites for chemical cleavage can be useful at the junction of short peptides and carrier proteins. Chemical cleavage reagents include cyanogen bromide, which cleaves at methionine residues (Piers et al., 1993; Haught et al., 1998; Lee et al., 1998), *N*-chloro succinimide (Forsberg et al., 1989) or BNPS-skatole (Dykes et al., 1988; Knott et al., 1988) which cleaves at tryptophan residues, dilute acid which cleaves aspartyl–prolyl bonds (Marcus, 1985; Gram et al., 1994), and hydroxylamine which cleaves asparagine–glycine bonds at pH 9.0 (Moks et al., 1987).

BPI is a 55 kDa protein present in human neutrophils (Weiss et al., 1978). A recombinant N-terminal BPI fragment of approximately 23 kDa, $rBPI_{23}$, possesses the bactericidal and lipopolysaccharide binding activities of the naturally-derived human holoprotein (Gazzano-Santoro et al., 1992; Weiss et al., 1992). Three separate functional domains within the recombinant protein have been defined by the activities of proteolytic cleavage fragments, overlapping 15-mer peptides, and other synthetic peptides (Little et al., 1994). Remarkably, peptides derived from these three rBPI domains have antibacterial, antifungal, endotoxin binding and neutralizing, as well as a heparin binding and neutralizing activity.

We have constructed a series of recombinant peptide expression vectors which encode antifungal peptide sequences derived from $rBPI_{23}$ linked as fusions to the human BoneD protein. An acid labile aspartyl–prolyl bond was positioned at the junction between the peptide and BoneD, a protein that readily forms inclusion bodies in *E. coli*.

We find, as have others (Gram et al., 1994), that peptide can be released from fusion protein inclusion bodies in dilute acid without prior solubilization, and that the released peptide is soluble in the aqueous acidic environment. Here we describe that the acidic condition used to hydrolyze aspartyl–prolyl bonds is sufficient to lyse bacterial cells and that cell lysis and aspartyl–prolyl bond hydrolysis can occur simultaneously. We thereby developed a simple peptide recovery process consisting of cell lysis and peptide release in a single step, followed by peptide purification by column chromatography. By this process, biologically active rBPI-derived peptides can be produced in *E. coli* at high yield.

2. Materials and methods

2.1. Chemicals and materials

SP Sepharose Fast Flow, Butyl Sepharose Fast Flow, and Superdex 30 were from Pharmacia (Piscataway, NJ). HCl (36.5–38%) was from J.T. Baker (Phillipsburg, NJ). Media components were from Spectrum (Gardena, CA) or J.T. Baker, except yeast extract which was from Red Star Speciality (Milwaukee, WI), L-arabinose which was from Pfanstiehl Laboratories (Waukegan, IL), and Biotin which was from American Biorganics (Niagara Falls, NY).

2.2. Construction of recombinant expression vectors

Three plasmid vectors were constructed for expression of BPI-derived peptides as part of a fusion protein with BoneD. The encoded fusion proteins had the following sequences at their C-terminus:

Tandem 16-mer + 15-mer
BoneD-[DP]-[KSKVGALIQLFHKK-DP]$_4$
-KSKVGALIQLFHKK
Single 15-mer
BoneD-[DP]-KSKVGALIQLFHKK
Single 14-mer
BoneD-[DP]-PKVGWLIQLFHKK

Oligonucleotide primers encoding the peptide amino acids, including DP, were synthesized with a Cyclone model 8400 DNA synthesizer (PerSeptive Biosystems, Bedford, MA) and cloned onto the 3′-end of the BoneD gene. DNA encoding the tandem peptide was cloned as a direct repeat of five peptide-encoding units separated by DP. The DNA sequence of the encoded peptide units in each vector was verified with Sequenase (US Biochemical, Cleveland, OH). The peptides liberated after acid hydrolysis for the three fusion proteins are PKSKVGALIQLFHKKD and PKSKVGALIQLFHKK in a 4:1 ratio for the tandem 16-mer + 15-mer, PKSKVGALIQLFHKK for the single 15-mer, and PPKVGWLIQLFHKK for the single 14-mer.

2.3. Expression of fusion protein in 14 or 35 l bioreactors

Bacterial cultures were grown in glycerol minimal salts medium (GMM) containing per liter: 12 g $(NH_4)_2SO_4$, 1.57 g KH_2PO_4, 14.1 g K_2HPO_4, 0.28 g $MgSO_4 \cdot 7H_2O$, 0.0012 g biotin, 18.5 g glycerol, 3 ml phosphoric acid, 1 ml $CaCl_2 \cdot 2H_2O$ (10% w/v), 0.1 ml thiamine HCl (10% w/v), 2 ml nicotinic acid (1% w/v), 4.6 g yeast extract, 1.0 ml antifoam (Ucaferm), plus 16 ml of trace element solution containing per liter: 3.24 g $FeCl_3 \cdot 6H_2O$, 0.84 g $ZnSO_4 \cdot 7H_2O$, 0.6 g $MnCl_2 \cdot 4H_2O$, 0.288 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $CuSO_4 \cdot 5H_2O$, 0.12 g $CoCl_2 \cdot 6H_2O$, 48 ml phosphoric acid, and 0.36 g boric acid. The medium at pH 6.0 was inoculated with bacteria harboring a plasmid encoding the fusion protein. Bacteria were grown at 32°C to limit the maximal growth rate. When the culture OD_{600} reached approximately 15–20 (approximately 20 h), a feed containing per liter: 10 g $MgSO_4 \cdot 7H_2O$, 0.01 g biotin, 700 g glycerol, 35 ml $CaCl_2 \cdot 2H_2O$ (10% w/v), 3.5 ml thiamine HCl (10% w/v), and 7 ml nicotinic acid (1% w/v) was added to maintain a dissolved oxygen concentration of approximately 20%. When the culture OD_{600} reached approximately 100 (approximately 45 h), a second feed solution (induction feed) containing L-arabinose was added. The induction feed was identical to the first feed solution except that it contained 60 g l^{-1} L-arabinose. Cell growth in the bioreactor was complete when all of the induction feed had been provided (approximately 25 h). Bacterial cells from the bioreactor were either used directly as described below or the cells were separated from the media by centrifugation at 11 000 × *g* for 20 min with a JA10 rotor in a Beckman J2-21 centrifuge, or filtration with a 0.2 μm hollow fiber cartridge with 1.0 m^2 of surface area (Spectrum, Laguna Hills, CA). For some experiments the cell paste was stored frozen at −20°C.

2.4. Isolation and cleavage of fusion protein

For some experiments the cells were mechanically disrupted prior to isolation of inclusion bodies or acid hydrolysis (to cleave the aspartyl–prolyl bond separating the peptide from the carrier protein). Either frozen cell paste or bacterial culture from a bioreactor was used. In the former case the frozen cell paste was thawed overnight at 4°C and diluted to 50% solids with water. Resuspension of the cells was aided by stirring for 20 min. The cell suspension was passed through several layers of cheese cloth to remove the larger clumps and this filtered material was further diluted to 10% solids. This diluted cell paste or bacterial culture directly from the bioreactor was passed through a microfluidizer (model M110Y, Microfluidics, Newton, MA) at 15 000 psi. Inclusion bodies and other insoluble material were separated from the soluble components by centrifugation at approximately 20 000 × *g* for 40 min in a Beckman J2-21 centrifuge using a JA 14 rotor, and suspended to various weight/volume ratios in water. Concentrated HCl was added to the desired final concentration. The acidified inclusion body slurry was incubated at 37, 55, or 85°C to cleave the fusion protein. After acid hydrolysis, any peptide contained in the supernatant was separated from the insoluble material by centrifugation at 22 000 × *g* for 40 min with a JA 14 or JA 20 rotor.

2.5. Hydrolysis of bacterial cultures

For simultaneous cell lysis and acid hydrolysis of the aspartyl–prolyl bond, 10% HCl was added

directly to a 35 l bioreactor until the pH was approximately 2.3. The bioreactor was heated to 85°C using a steam controlled heating jacket and stirred at 1000 rpm.

2.6. Recovery and purification of peptide from acid hydrolyzed cells

After acid hydrolysis of the bacterial culture (cells plus media) at 85°C for 5.5 h the soluble material was isolated by centrifugation and adjusted to pH 3.0 with 500 mM sodium citrate. An aliquot of this clarified acid hydrolysate was loaded onto an SP Sepharose Fast Flow column (2.5×4.4 cm) equilibrated in 10 mM sodium citrate, pH 3.0. The column was washed with 10 mM sodium phosphate, pH 7.0 (buffer A) until the effluent pH reached 7.0. Next the column was washed with buffer A containing 150 mM NaCl, followed by elution with buffer A containing 800 mM NaCl. The eluate was diluted with one volume of buffer A containing 3 M $(NH_4)_2SO_4$ and loaded onto a Butyl Sepharose Fast Flow column (1.0×4.0 cm) equilibrated with buffer A containing 1.5 M $(NH_4)_2SO_4$. After washing the column with buffer A containing 1.1 M $(NH_4)_2SO_4$ until the absorbance at 280 nm reached baseline, the column was eluted with buffer A containing 0.4 M $(NH_4)_2SO_4$. A Superdex 30 gel filtration column (1.6×53 cm) was used as a final purification and buffer exchange step. The column was run in 5 mM sodium acetate, 150 mM NaCl, pH 5.0.

2.7. Reverse phase HPLC

Reverse phase HPLC was used to quantitate peptide concentration using a Beckman HPLC system (110A pumps, 421A controller, and 160 UV detector) with a Shimadzu auto injector (SIL-10A). The column was a 0.46×25 cm C18 with a 5 μM particle size (Vydac, 218TP54). The solvents were 10% acetonitrile/ 0.1% TFA (solvent A) and 90% acetonitrile/0.1% TFA (solvent B). The column was equilibrated in 15 or 20% B and 100 μl of sample was loaded onto the column. Elution was accomplished with a 20 min gradient from 15–35% B or 20–40% B at a flow rate of 1 ml min^{-1}. Detection was at 229 nm. A standard curve was prepared by serial dilution of a peptide standard of known concentration (determined by amino acid composition analysis). The peak was related to peptide standard concentration by linear regression. The concentration of unknown samples was determined by the linear regression equation.

The two different BPI peptides released from the 5-peptide tandem were not resolved from each other under the conditions described here. Mass spectrometry demonstrated both peptides were present at the expected ratios.

2.8. Peptide activity assay

Candida albicans (ATCC # 14053) and *Candida glabrata* (ATCC # 2001) cultures were tested for susceptibility to peptide. Fungal cells were grown overnight at 30°C in YPD media (1% yeast extract, 2% peptone, and 2% dextrose). A 400-fold dilution of the cultures in YPD was then made and grown at 30°C for 8 h. Each culture (3 ml) was collected by centrifugation and suspended in 0.9% NaCl to an OD_{600} of 0.3. The cultures were further diluted to 1×10^4 CFU ml^{-1} in Sabouraud dextrose broth (6 ml). Recombinant peptide was in 5 mM sodium acetate, 150 mM NaCl, pH 5.0, at a concentration of about 2 mg ml^{-1}. Synthetic peptide was at 1 mg ml^{-1} and was produced using FMOC solid phase synthesis with an Advanced Chemtech model 357 MPS synthesizer using the methods of Merrifield et al. (1966). Samples were serially diluted and added to microtiter plates containing the cultures. The plates were incubated at 30°C for 48 h.

3. Results

3.1. Bacterial expression of recombinant peptide

Three DNA segments encoding BPI-derived peptides were cloned onto the 3′-end of a gene encoding the mature subunit D of human osteogenic protein (BoneD); BoneD is identical to OP-1 (Ozkaynak et al., 1990). The encoded peptides were cloned downstream of the BoneD gene either

singly or as part of a tandem repeat of five peptide units. At the junction between the BoneD and peptide segments, DNA encoding an aspartic acid–proline dipeptide was included. The DNA encoding each BoneD-peptide fusion was linked to the *pelB* leader sequence (Lei et al., 1987), and cloned under the transcriptional control of the *Salmonella typhimurium araB* promoter (Johnston et al., 1985). After induction with L-arabinose, the expressed BoneD-peptide fusion protein accumulated intracellularly into inclusion bodies even though a secretory leader sequence had been included.

To prepare sufficient fusion protein, bacterial cultures were grown to a high cell density ($OD_{600} > 100$) in 14 or 35 l bioreactors. Recombinant fusion protein typically accumulated intracellularly to greater than 40% of total cell protein

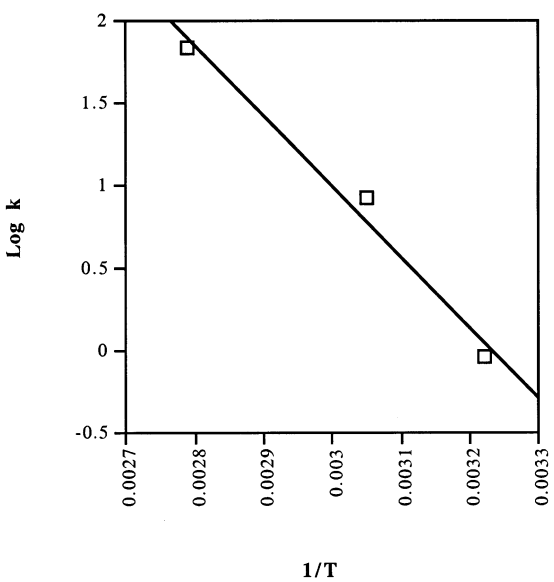

Fig. 1. Arrhenius plot for fusion protein hydrolysis. Inclusion bodies from cells containing a tandem 16-mer + 15-mer fusion protein were isolated from mechanically disrupted *E. coli* and suspended in 30 mM HCl to a final weight/volume ratio of 10%. The slurry was incubated in three aliquots at 85, 55, and 37°C and the samples were removed at various times. The peptide in the supernatant of each aliquot was quantitated by HPLC. Initial reaction rates followed zero order kinetics. Rate constants, k, were determined for each temperature by the slope of the line relating concentration to time. Units for the rate constant k are $\mu g\ ml^{-1}\ h^{-1}$. T is absolute temperature in K.

(data not shown). At the high cell densities achieved in these experiments, up to 75 g dry cell weight per liter accumulated.

3.2. Release of peptide from fusion protein

Since aspartyl–prolyl bonds are acid labile, we evaluated whether peptides could be liberated from the fusion proteins by incubation in dilute HCl. For each of the three fusion proteins tested we found that peptide release in dilute acid proceeded with inclusion body suspensions, and that solubilization of fusion protein with detergents or denaturants was not required.

The site specific hydrolysis of the aspartyl–prolyl bonds between BoneD and peptide was temperature dependent and followed Arrhenius kinetics assuming a zero order initial reaction rate (Fig. 1). We followed the reaction at three temperatures: 85, 55, and 37°C (Fig. 2A). Cleavage was complete after 3 and 48 h at 85 and 55°C, respectively. At 37°C hydrolysis of aspartyl–prolyl bonds was still incomplete at 312 h (data not shown). The concentration of intact peptide decreased after reaching a maximum at both 85 and 55°C. Reverse phase HPLC revealed a closely related peptide species after extended hydrolysis. N-terminal amino acid analysis of this peak demonstrated that the single glutamine residue had been deamidated to glutamic acid. At 85°C, approximately 5% of recovered peptide contained glutamic acid after 3 h.

To limit process volumes for scale-up, it may be desirable to hydrolyze a high concentration slurry of inclusion bodies. Therefore, additional experiments were performed in 30 mM HCl to investigate conditions for complete acid cleavage of fusion protein from inclusion bodies using various weight/volume concentrations of inclusion bodies. As the percent solids in the slurry was increased from 10 to 50% the reaction rate decreased significantly (Fig. 2B). In particular, with samples containing 30 and 50% solids, little or no peptide was released. When the pH of each of the acid-treated inclusion body suspensions was subsequently measured we discovered that the pH varied dramatically (pH 2.6, 3.7, 4.4, 5.1 for acid treated inclusion body suspensions of 10, 20, 30, and 50%, respectively).

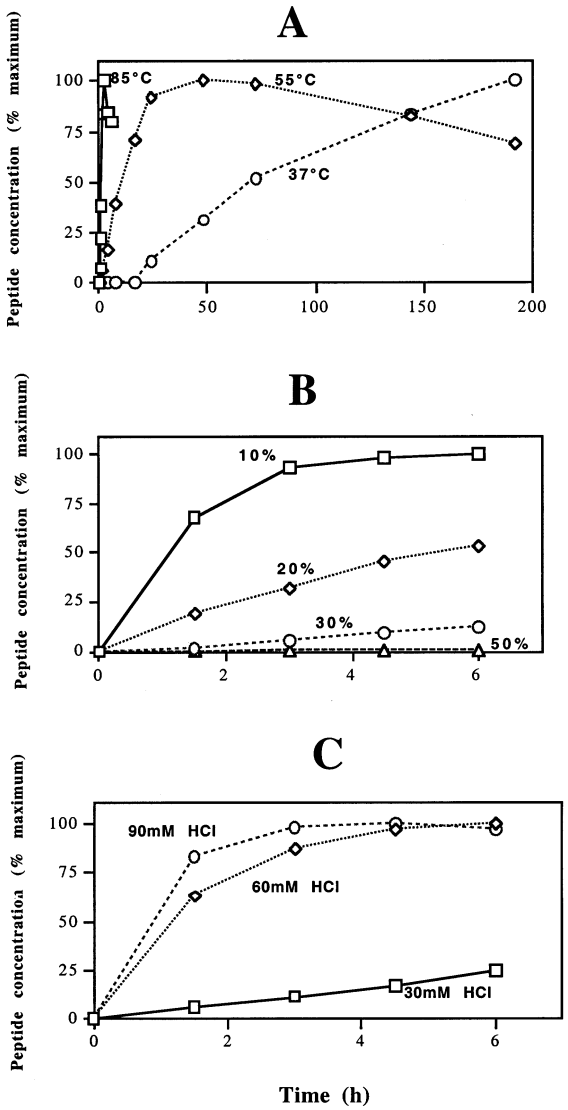

Fig. 2. (A) Acid hydrolysis time course. Inclusion bodies from cells containing a tandem 16-mer + 15-mer fusion protein were isolated from mechanically disrupted *E. coli* by centrifugation and suspended in 30 mM HCl to a final weight/volume ratio of 10%. The slurry was incubated at 85, 55, or 37°C and aliquots were removed at various times. The supernatant in each aliquot was assayed for peptide by HPLC. (B) Acid hydrolysis as a function of weight/volume ratio of inclusion bodies. Inclusion bodies from cells containing a single 15-mer fusion protein were suspended to several weight/volume ratios in water. Concentrated HCl was added to 30 mM. Samples were incubated at 85°C in a shaking water bath. Aliquots were taken at various times and the amount of peptide in the supernatants was determined by HPLC. (C) Acid hydrolysis as a function of HCl concentration. Inclusion bodies from cells containing a single 15-mer fusion protein were suspended in a 30% weight/volume slurry in water. Concentrated HCl was added to achieve the desired final HCl concentration (30, 60, or 90 mM). The samples were incubated at 85°C in a shaking water bath. Aliquots were taken at various times and the peptide in the supernatant was quantified by HPLC.

To test whether the variation in pH caused the reduced release, and if so to determine the pH range needed to achieve complete hydrolysis, additional experiments were performed with 30% inclusion body suspensions using 30, 60, or 90 mM HCl. As shown in Fig. 2C, maximal peptide release was achieved at 60 and 90 mM HCl. The pH of the 30, 60, and 90 mM treated suspension was 3.5, 2.4, and 1.5, respectively. We conclude that a low pH (≤ 2.6) and a high temperature (85°C) was critical to achieve rapid peptide release.

3.3. Simultaneous cell lysis and fusion protein hydrolysis

For the experiments described in the previous section, inclusion bodies were isolated by centrifugation after mechanical cell disruption. Since the optimal conditions for rapid release of the peptide, pH < 2.6 at 85°C, may be considered harsh, we evaluated whether inclusion bodies needed to be isolated from cells prior to acid hydrolysis, i.e. would the reaction conditions of low pH and elevated temperature simultaneously disrupt cells and release peptide. In the first experiment bacterial cells were separated from the majority of culture supernatant with a hollow fiber cartridge and frozen. Upon thawing the cell paste was suspended in water and the pH was adjusted to 2.5 with HCl. The slurry was incubated at 85°C. Remarkably, the conditions were sufficient for quantitative peptide release from the BoneD protein (data not shown). In the second experiment, a sample of bacterial culture (cells plus media) was adjusted to pH 2.15 with HCl and incubated at 85°C to evaluate the time course of peptide release from cells that had not previously

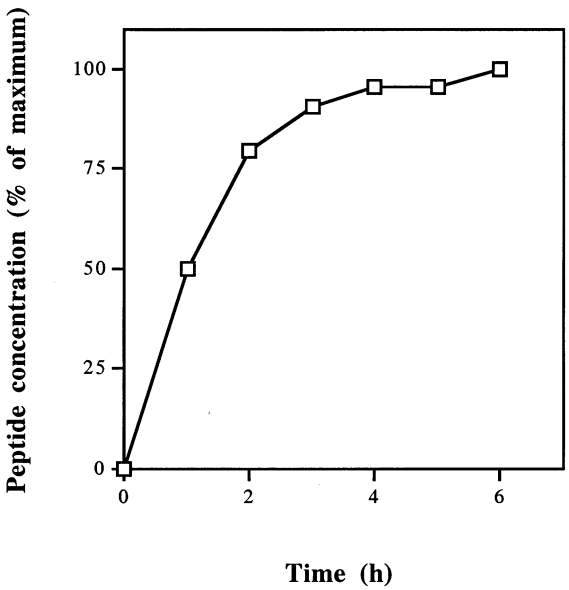

Fig. 3. Simultaneous cell lysis and hydrolysis of aspartyl–prolyl bonds. After growth of *E. coli* containing a single 14-mer fusion protein in a bioreactor, 10% HCl was added directly to the vessel until the pH was 2.4. The reactor was heated to 85°C and stirred for 6 h. Samples were taken every hour and the peptide in the supernatant was quantitated by HPLC.

been concentrated and frozen. Similarly these conditions were also sufficient for peptide release. Additional experiments also demonstrated that dilute sulfuric and nitric acid could release soluble peptide from bacterial cells in cell culture media at 85°C.

Since peptide release was possible from cell culture in one step at low pH, we evaluated peptide release directly in a bioreactor. A bacterial culture was grown in a 35 l bioreactor and induced with L-arabinose. Approximately 20 h after initiation of the induction feed HCl was added to a final pH of 2.4. The reactor was heated to 85°C and samples were taken every hour to evaluate the time course of peptide release. The results shown in Fig. 3 demonstrate that peptide was released directly in the bioreactor and that the hydrolysis reaction was complete at 4 h.

3.4. Purification of antifungal peptide

The peptide released by acid hydrolysis of cells was purified to assess its antifungal activity in vitro. Separation by centrifugation of the insoluble cellular material, which contained most bacterial proteins, from the soluble peptide resulted in a large initial purification. Peptide accounted for approximately 12% of the total protein in the clarified sample. The peptide-containing supernatant was purified using column separation techniques. A process consisting of sequential SP Sepharose Fast Flow, Butyl Sepharose Fast Flow, and gel filtration using Superdex 30 (S-30) effectively removed the remaining protein impurities

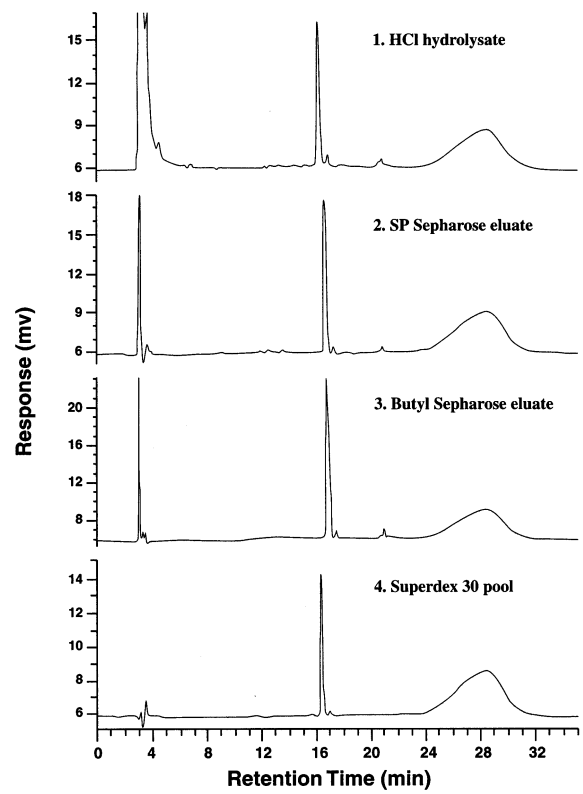

Fig. 4. Purification of peptide by column chromatography. HPLC was used to evaluate the purity of recombinant peptide following each step in the purification process. The clarified acid hydrolysate from a bacterial culture containing a single 14-mer fusion protein was purified using SP Sepharose Fast Flow followed by Butyl Sepharose Fast Flow, and then Superdex 30. Recombinant peptide elutes at 16–17 min.

Table 1
Purification of antifungal peptide

Sample	Recovery (%)	Purity (%)
HCl hydrolysate	100.0	12
SP-Sepharose eluate	86.1	80.1
Butyl Sepharose eluate	76.6	87.2
Superdex 30 pool	76.6	97.4

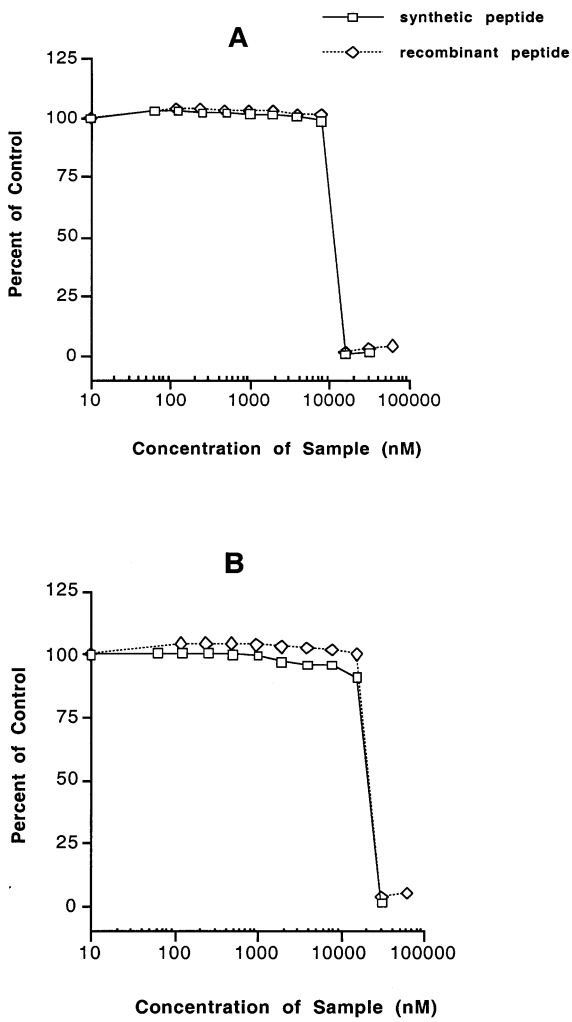

Fig. 5. Broth microdilution assay of purified peptide. Peptide was incubated in cultures of *C. albicans* (panel A) and *C. glabrata* (panel B) in Sabouraud dextrose broth for 48 h. Percent of control is relative to optical density at 600 nm. Synthetic peptide has the amino acid sequence K V G W L I Q L F H K K–NH_2. Recombinant peptide has the sequence P P K V G W L I Q L F H K K–COOH.

(Fig. 4). Peptide purity was approximately 80% after elution from SP Sepharose Fast Flow. Incremental improvements in purity were achieved with Butyl Sepharose Fast Flow and S-30 to a final purity of greater than 97%. Overall recovery was $>76\%$ (Table 1). Improvements in the purification process may be possible which would further remove trace amounts of the deaminated product.

Purified peptide displayed antifungal activity against *C. albicans* and *C. glabrata* in a broth microdilution assay (Fig. 5). The activity of recombinant purified peptide against these microbial species was equivalent to that of a similar chemically synthesized peptide. These two peptides differed slightly, however, since the synthetic peptide was amidated at its carboxy-terminus and lacked the two N-terminal proline residues.

4. Discussion

The production of peptide for preclinical and clinical evaluation often requires multigram quantities (Kelley, 1996), and production of recombinant peptides at this scale from microbial fermentation can potentially be economical. Downstream processing steps for the production of peptides and proteins from bacteria, however, can often contribute a significant fraction of the total production cost. Initial recovery of peptide from bacterial inclusion bodies, for example, generally requires three distinct steps: cell lysis, solubilization of inclusion bodies in denaturant or detergent, and separation of peptide and carrier protein. In an effort to produce antifungal peptides derived from BPI we have developed a method which precludes the need for solubilization and unexpectedly accomplishes cell lysis and release of peptide in a single step.

Gram et al. (1994) demonstrated that by employing an acid labile aspartyl–prolyl peptide bond, target peptide could be released from the carrier protein by incubation in dilute HCl without solubilizing the inclusion bodies in denaturant or detergent. Our method provides several significant improvements. By employing the higher temperature of 85°C we greatly reduced the reaction

time to 3–4 h. In addition, we found that cells could be efficiently lysed at 85°C in dilute acid. Accordingly, lysis and release of peptide was accomplished in a single step, and could be done directly in the bioreactor after cell growth. Hydrolysis of aspartyl–prolyl bonds was efficient at a pH below 2.6 and followed Arrhenius kinetics.

Deamidation of asparagine and glutamine residues to aspartic acid and glutamic acid, respectively, can occur under the reaction conditions used here (Wright, 1991). The antifungal peptides contain an internal glutamine which was subject to deamidation. Glutamic acid was present in approximately 5% of the peptide molecules when inclusion bodies were incubated in dilute HCl at 85°C after 3 h.

Recombinant peptides generated by the method described here will have an N-terminal proline and a free carboxy-terminus. The peptides produced here from fusion proteins with a single peptide contain a lysine at their C-terminus, while those from fusion proteins with a tandem repeat contain a 4:1 ratio of molecules with aspartic acid and lysine at their C-termini. Additional steps may be required to produce molecules with a unique C-terminus using dilute acid hydrolysis of tandem repeat fusion proteins, even though production of peptide units from tandem repeats may enhance product yield (Shen, 1984; Kempe et al., 1985; Lennick et al., 1987). If an N-terminal proline is disadvantageous, others have shown that it can be removed with dipeptidyl peptidase IV (Gram et al., 1994). If a recombinant peptide requires an amidated C-terminus for activity, this modification can be introduced by a variety of chemical and enzymatic methods (Bongers et al., 1992a,b; Ray et al., 1993; Merkler, 1994).

The antifungal peptide was purified directly from the acid hydrolysate using conventional purification media. Peptide purity exceeded 97% with a yield of 77%. These media are rigid beads which can be operated at relatively high flow rates with small pressure drops, making them compatible for large scale purification. The general production and purification strategy described here is simple and can be utilized to produce any desired recombinant peptide.

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