Short Technical Report

Buffered Non-Fermenter System for Lab-Scale Production of Secreted Recombinant His-Tagged Proteins in *Saccharomyces cerevisiae*

BioTechniques 33:1296-1300 (December 2002)

Chatri Ngamkitidechakul and Sally S. Twining

Medical College of Wisconsin,
Milwaukee, WI, USA

ABSTRACT

*Expression of recombinant proteins using a secretion system can minimize co-purification of contaminating host proteins. Production of His-tagged recombinant proteins in the yeast α -factor secretion system has previously required a fermenter system to control the growth conditions such as pH of the yeast culture. We describe an inexpensive non-fermenter system for the production of secreted recombinant His-tagged proteins in *Saccharomyces cerevisiae* that uses a buffered low peptone YP glycerol medium, which does not interfere with immobilized metal affinity chromatography. Maspin, a tumor suppressor serpin, was expressed as a secreted N-terminal His/FLAG[®]-tagged protein. Purification of the soluble active recombinant protein only requires centrifugation, concentration by ultrafiltration, and Ni²⁺ affinity chromatography. Purified protein yields of this system are 3–5 mg/L culture medium.*

INTRODUCTION

Recombinant proteins can be expressed at milligram/liter quantities in *Saccharomyces cerevisiae* and secreted using the α -factor peptide sequence fused to the N-terminus of the protein (1,6). In some cases, recombinant proteins are expressed with a fusion tag such as the FLAG[®] peptide (Sigma, St. Louis, MO, USA) (1,6) or a 6 $\times$ histidine (His-tag) (3) that can be used for purification and/or detection purposes. Because of the cost of chromatography resin with the FLAG antibody attached, large-scale purification using the FLAG epitope is impractical. The addition of a His-tag either on the N- or C-terminal end of proteins offers an efficient, cost-effective means of purification by immobilized metal affinity chromatography (IMAC). However, the only report combining the α -factor secretion system and IMAC utilizes costly and laborious procedures that require a fermentation apparatus to control growth conditions such as the pH of the system (3). Here we demonstrate the expression and purification of a recombinant His-tagged protein in a protease-deficient *S. cerevisiae* strain using the α -factor secretion expression system, a universal laboratory environmental incubator shaker, and standard culture flasks.

As an example, we produced yeast recombinant His/FLAG-tagged human maspin. Maspin is an antiangiogenic factor (9) and a tumor suppressor serpin (10). Because high doses (approximately 0.5 μ M) of maspin are required for activity (8,9), a system to economically produce high quantities of maspin was needed. Previous methods for maspin

expression include the production of GST-maspin fusion proteins in *E. coli* (7) or maspin as a cytoplasmic protein in yeast (5) or a baculovirus system (7). The presence of GST is not always desirable because GST-maspin differs in some properties from maspin (7). Removal of the GST by trypsin can inactivate maspin, since maspin is highly susceptible to this protease (5). The purification of maspin produced in the yeast and baculovirus systems requires multi-step purification protocols, which result in the loss of active protein (5,7). In our α -yeast secretion system, milligram quantities of soluble HIS/FLAG-tagged proteins are obtained directly from culture medium by a simple purification procedure involving only centrifugation, concentration, and one affinity chromatography step using a Ni²⁺ resin.

MATERIALS AND METHODS

Construction of Yeast Expression Vector YEpHF-Maspin

The yeast expression vector YEpHF was constructed from the YEpFLAG-1 vector (Sigma) by adding a 6 $\times$ His-tag between the α -factor and the FLAG sequences (Figure 1). Two separate PCR products were amplified using *PfuTurbo*[®] Hotstart DNA Polymerase (Stratagene, La Jolla, CA, USA) and primers obtained from Invitrogen (Carlsbad, CA, USA). Primer 1: 5'-CGACTACAAGGATGACGATGAC-3'; Primer 2: 5'-CGTGGTGGTGGTGGTGGTGTCTTTTATCCAAAGGTACCC-3'; Primer 3: 5'-ACACCACCACCACCA-

CCACGACTACAAGGATGACGATGA-3'; Primer 4: 5'-GACCGAGAACTAGTGCGAAGT-3'. Using an antisense primer in the α -factor sequence, the His-tag was incorporated at the 3'-end of the α -factor (Figure 1). The nucleotide sequences of the His-tag on the sense primer were inserted at the 5'-end of the FLAG sequence. The PCR products were gel-purified using a QIAquick® Gel Extraction kit (Qiagen, Valencia, CA, USA).

Overlap extension PCR was used to generate the PCR product containing the His-tag between the α -factor and the FLAG regions (Figure 1). After purification, the PCR product was double-digested with the restriction enzymes *Kpn*I and *Spe*I (New England Biolabs, Beverly, MA, USA) and then inserted into the YEFLAG-1 vector using T4 DNA ligase (New England BioLabs). The ligation reaction product was transformed into competent *E. coli* cells, JM 109 (Promega, Madison, WI, USA), by the heat-shock method. The sequence of the YEepHF vector was determined,

confirming the His-tag was in-frame (MCW Protein & Nucleic acid Facilities, Milwaukee, WI, USA).

The full-length cDNA of the human maspin gene was obtained from human corneal epithelial cells as described previously (4). The cDNA was then subcloned into the YEepHF vector using *Eco*RI and *Bgl*II sites available on the multiple cloning site of the vector (Figure 1). This yeast expression vector containing maspin cDNA was then called YEepHF-Maspin.

Expression and Purification of Yeast Recombinant His/FLAG-Tagged Maspin

The YEepHF-Maspin vector was transformed into the protease-deficient *S. cerevisiae* strain BJ3505 (*pep4::HIS3 prb-Δ1.6 HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can1*) (Sigma) using the modified lithium acetate method described by Gietz et al. (2). The cells were plated on selective SC agar medium (0.67% yeast nitrogen base,

0.1% yeast synthetic drop-out media supplement without tryptophan, and 2% bacteriological agar) and grown at 30°C for 3–5 days.

A colony of YEepHF-Maspin was inoculated into 400 mL SC medium without tryptophan and grown at 30°C for two days. The cells were harvested by centrifugation at 2000× g for 5 min and resuspended in 1 L buffered YP expression medium (1% glucose, 3% glycerol, 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.4). Two 500-mL cultures in 2-L baffled flasks were grown on a rotary platform shaker at 175 rpm at 30°C for 72 h. After 72 h incubation, the cultures were centrifuged at 8000× g for 20 min at 4°C, and the supernatant fractions were concentrated 5- to 6-fold using an Amicon™ ultrafiltration device with BIOMAX® YM-30 membrane (Millipore, Bedford, MA, USA) at 65 psi and 4°C. The pH of the concentrated medium was adjusted to 8.0 with NaOH, and PMSF (Sigma) was added to a final concentration of 1 mM to protect against proteolysis.

The recombinant HIS/FLAG-tagged maspin was purified using Ni-NTA™ agarose resins (Qiagen). Five milliliters of resin were equilibrated in 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl and then incubated with the concentrated medium with gentle rocking motion overnight at 4°C. The charged resin was transferred to a 10-mL gravity flow column and washed with 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole until the UV absorbance baseline at 280 nm became stable (Figure 2B). The column was washed, and the protein was partially eluted with 10 bed-volumes of 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl and 20 mM imidazole. The His-tagged maspin was then eluted with 10 bed-volumes of 50 mM phosphate buffer, pH 7.0, containing 300 mM NaCl and 250 mM imidazole. Five-milliliter fractions were collected, and their UV absorbance was monitored at 280 nm. Fractions containing recombinant maspin were pooled and concentrated using a 30-kDa cutoff centrifugal spin concentrator (Ultra-free™; Millipore). Imidazole was removed by dialysis in PBS, pH 7.4, at 4°C. To confirm cleavage of the α -factor

Figure 1. Schematic representation of YEepHF and YEepHF-Maspin vector construction by PCR. The sequence for six histidine residues was inserted into the YEFLAG-1 vector between the α -factor and the FLAG peptide sequences by overlap extension PCR. This PCR product was then inserted into the original YEFLAG-1 at *Kpn*I and *Spe*I sites. The new vector with the His-tag was named YEepHF. The cDNA of full-length human maspin was inserted into the multiple cloning sites at *Eco*RI and *Bgl*II.

Proteomic Technologies

Table 1. Summary of Protein Yield

Step	Volume (mL)	Total Protein (mL)	Total Maspin (mg)	Maspin (mg)/ Total Protein (mg)	Purification (fold)	Recovery (%)
Clarified Media	1000	530	4.6	0.01	1	100
Concentrate	200	160	4.1	0.03	3	89
Ni ²⁺ Resin Eluate (Pooled Fraction)	70	3.5	3.3	0.94	94	72

leader peptide and the presence of the histidine tag at the N-terminus of recombinant protein, the purified recombinant protein was sequenced (MCW Protein & Nucleic acid Facilities).

Analyses of Recombinant His/FLAG-Tagged Maspin

Protein fractions were analyzed by SDS-PAGE and Western immunoblotting techniques (5). Yeast recombinant His/FLAG-tagged maspin was detected using either nickel-conjugated HRP:INDIA™ HisProbe™-HRP (Pierce Chemical, Rockford, IL, USA) or primary monoclonal mouse-anti human maspin antibody (BD Biosciences, San Diego, CA, USA) with HRP-conjugated goat anti-mouse IgG (Pierce Chemical) as the secondary antibody. The HRP-labeled bands were detected using the ECL™ detection system (Amersham Biosciences, Piscataway, NJ, USA).

For quantitative analysis, immunodot blot assays were performed using a 96-well dot blot apparatus (Schleicher & Schuell, Keene, NH, USA), and the blots were processed as given above. The data were analyzed using an ELISA plate reader with a 550-nm filter (EL380 Microplate Reader; Bio-Tek Instruments, Winooski, VT, USA). The total protein amounts of each fraction were determined using Coomassie® Plus Protein Assay Reagent (Pierce Chemical) with BSA as standard. Linear standard curves of BSA and maspin were used to determine the amount of total protein and total maspin, respectively.

To determine the activity of recombinant maspin, a cell-fibronectin adhesion assay was employed as previously described using fibronectin precoated Cytomatrix™ cell adhesion strips (Chemicon, Temecula, CA, USA) (4). Before the assay, subconfluent mammary gland carcinoma MDA-MB-231 cells (ATCC, Manassas, VA, USA) were pretreated

overnight with either 0.5 or 1 μM yeast recombinant maspin or 0.5 μM bacterial recombinant maspin (a gift from Dr. PA Pemberton, LXR, Richmond, CA, USA) as a positive control.

RESULTS AND DISCUSSION

The YEpFLAG-1 yeast expression system provides an excellent tool based on the α-factor secretion mechanism to express high amounts of recombinant protein. For a more economical and simpler purification of the secreted recombinant proteins, we constructed the YEpHF vector that contains a His-tag between the α-factor and the FLAG peptide sequence (Figure 1). To test this system, the coding sequence of the serpin maspin was placed into the vec-

Table 2. Effect of Yeast Recombinant His/FLAG Maspin on MDA-MB-231 Carcinoma Cell Adhesion to Fibronectin

Pretreatment	% Cell-Fibronectin Adhesion ($\bar{x} \pm \text{SD}$)
Control	100 ± 7
0.5 μM bMaspin ^a	135 ± 10
0.1 μM yMaspin ^b	143 ± 15
0.5 μM yMaspin	159 ± 22

^aBacterial recombinant GST-maspin
^bYeast recombinant His/FLAG maspin

tor between the *Eco*RI and *Bgl*II sites. The type of medium used to grow the yeast for expression of the protein was critical. Medium containing high

Figure 2. Maspin expression and purification. (A) Time-course of protein expression. Coomassie Brilliant Blue-stained SDS polyacrylamide gel (left) and Western blot probed with mouse anti-human maspin antibody (right). (B) Elution profile of yeast recombinant His/FLAG maspin purified by Ni²⁺ affinity chromatography. W, wash fraction; E, elute fraction. (C) Coomassie Brilliant Blue-stained SDS polyacrylamide gel, (D) Western blots probed with maspin antibody, and (E) HRP-conjugated Ni²⁺. BC, before concentration; AC, after concentration; NA, non-absorbed.

Proteomic Technologies

amounts of peptone (8%) resulted in maspin production of up to 10 mg/L of non-fractionated medium; however, there was a high level of background proteins, and a compound was present in the conditioned medium that strongly interfered with the binding of His-tagged maspin to the Ni²⁺ resin and stripped the Ni²⁺ from the IMAC resin (data not shown). Removal of the compound either by dialysis or repeated ultrafiltration was time consuming and resulted in degradation of the protein. In addition, contaminating metal binding proteins were co-purified. Only 0.5 to 1 mg/L active maspin was obtained by this method.

YP expression medium containing less peptone (2%) could potentially solve the purification problem; however, the medium becomes acidic (pH 4.2 or lower) at 48 h incubation. Under these conditions, protein yields are typically 5-fold less than that in the high peptone medium and the protein produced often is denatured. To overcome these problems in our expression system, we stabilized the pH of the YP medium at pH 6.4 by adding potassium phosphate buffer to a final concentration of 100 mM. The protein is detected in the medium at 48 h with increasing amounts present at 72 h (Figure 2A, right). Two bands are observed that react with the maspin monoclonal antibody; the upper band represents about two-thirds of the maspin. The lower band is likely a recombinant protein containing an incomplete histidine tag, as the band is undetectable after protein purification (Figure 2C). Typically, 5 mg recombinant maspin are present per liter of the buffered low peptone culture medium at 72 h (Table 1). Higher amounts of the protein (10 mg/L) are obtained at 96 h; however, more contaminating proteins are present that co-purify with maspin (data not shown).

An advantage of the buffered low peptone YP medium is the low level of background proteins, which facilitates the ultrafiltration process (Figure 2A, left). The concentrated medium adjusted to pH 8.0 can be incubated with the Ni²⁺ resin without additional manipulation. Binding of the His-tagged protein to the resin is greater than 95% (Figure 2, D and E, lanes AC vs. NA). A portion of the bound His-tagged maspin is

eluted with 20 mM imidazole; however, higher levels of imidazole (250 mM) are required to efficiently release the protein from the IMAC resin (Figure 2B). Removal of most of the nonspecific protein is accomplished by washing the resin with buffer containing 10 mM imidazole, which does not elute the specifically bound His/FLAG-tagged maspin (Figure 2C, lanes E3 and E12).

Purified recombinant His/FLAG-tagged maspin reacts with both an antibody to maspin (Figure 2D) and HRP-conjugated Ni²⁺ (Figure 2E) on a Western blot, indicating the presence of immunoreactive maspin and the His-tag. N-terminal sequencing confirmed the presence of six histidine residues, followed by the FLAG peptide sequence and then the N-terminus of recombinant maspin. The major purified product (Figure 2, D and E) represents the higher molecular weight maspin band observed in the yeast-conditioned medium (Figure 2A, right). In addition, the purified yeast recombinant His/FLAG-tagged maspin is biologically active, as it is able to induce an increase in adhesion of mammary carcinoma cells to a fibronectin matrix (Table 2). The total yield of purified protein is about 3 mg/L culture medium with 70% recovery (Table 1). The yield range for different preparations was 3–5 mg/L purified maspin, which represents a 3- to 10-fold increase in the amount of purified maspin over that for the high peptone system. If required, then the His-Tag and the FLAG sequence can be removed by the specific protease, enteropeptidase, which does not cleave within the reactive site loop of maspin.

In summary, we have established a yeast expression/IMAC purification system that allows easy, rapid production of purified His-tagged recombinant proteins such as maspin. This system could also become an affordable tool for lab-scale production of other proteins.

ACKNOWLEDGMENTS

This work was supported in part by EY12731 and EY14168 (S.S.T.) and P30-EY01931 from the National Eye Institute of the National Institutes of Health and a grant from the Wisconsin Breast Cancer Showhouse (S.S.T.).

REFERENCES

1. Clarke, H.R., M.F. Wolfson, C.T. Rauch, B.J. Castner, C.-P. Huang, M.J. Gerhart, R.S. Johnson, D.P. Cerretti, et al. 1998. Expression and purification of correctly processed, active human TACE catalytic domain in *S. cerevisiae*. *Protein Expr. Purif.* 13:104-110.
2. Gietz, D., A. St. Jean, R.A. Woods, and R.H. Schiestl. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* 20:1425.
3. Gozar, M.M.G., V.L. Price, and D.C. Kaslow. 1998. *S. cerevisiae*-secreted fusion protein Pfs25 and Pfs28 elicit potent *Plasmodium falciparum* transmission-blocking antibodies in mice. *Infect. Immun.* 66:59-64.
4. Ngamkitidechakul, C., J.M. Burke, W.J. O'Brien, and S.S. Twining. 2001. Maspin: synthesis by human cornea and regulation of in vitro stromal cell adhesion to extracellular matrix. *Invest. Ophthalmol. Vis. Sci.* 42:3135-3141.
5. Pemberton, P.A., D.T. Wong, H.L. Gibson, M.C. Kiefer, P.A. Fitzpatrick, R. Sager, and P.J. Barr. 1995. The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. *J. Biol. Chem.* 270:15832-15837.
6. Ramjee, M.K., J.R. Petithory, J. McElver, S.C. Weber, and J.F. Kirsch. 1996. A novel yeast expression/secretion system for the recombinant plant thiol endoprotease propapain. *Prot. Eng.* 9:1055-1061.
7. Sheng, S., P.A. Pemberton, and R. Sager. 1994. Production, purification and characterization of recombinant maspin protein. *J. Biol. Chem.* 269:30988-30993.
8. Sheng, S., J. Carey, E.A. Seftor, L. Dias, M.J. Hendrix, and R. Sager. 1996. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc. Natl. Acad. Sci. USA* 93:11669-11674.
9. Zhang, M., O. Volpert, Y.H. Shi, and N. Bouck. 2000. Maspin is an angiogenesis inhibitor. *Nat. Med.* 6:196-199.
10. Zou, Z., A. Anisowicz, M.J. Hendrix, A. Thor, M. Neveu, S. Sheng, K. Rafidi, E. Seftor, and R. Sager. 1994. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science* 263:526-529.

Received 26 March 2002; accepted 24 June 2002.

Address correspondence to:

Dr. Sally S. Twining
Department of Biochemistry
Medical College of Wisconsin
Milwaukee, WI 53226, USA
e-mail: stwining@mcw.edu

For reprints of this or
any other article, contact
Reprints@BioTechniques.com