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High-efficiency heterologous expression, purification, and activity analysis of the fusion protein NusA-hRI postprint

Authors: Fu Dawei, Sun Yingying, Xu Wei

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Abstract

Human ribonuclease inhibitor (hRI) is an acidic cytoplasmic protein capable of regulating ribonuclease activity. Recombinant expression vectors containing fusion tags including SUMO, IF2, GST, NusA, MsyB, Trx, and MBP were constructed, and auto-induction (AI) expression was performed using Escherichia coli BL21(DE3) as the host strain, thereby enhancing the expression level of hRI. MagNi magnetic bead purification and electrophoretic analysis were utilized to assess hRI expression status, and high-purity protein was obtained through RNase/Sepharose affinity chromatography. The concentration of the purified fusion protein was 2960.513 mg/L. Compared with hRI activity from other companies, the enzymatic activity was determined to be approximately 50 U/ L, and it was successfully employed for RNA protection, providing a theoretical basis for the application of NusA-hRI.

Full Text

Efficient Heterologous Expression, Purification and Activity Analysis of Fusion Protein NusA-hRI

FU Dawei, SUN Yingying, XU Wei (College of Food Engineering, Harbin University of Commerce, Harbin 150076, Heilongjiang, China)

Abstract

Human ribonuclease inhibitor (hRI) is an acidic cytoplasmic protein that regulates ribonuclease activity. In this study, recombinant expression vectors containing various fusion tags—including SUMO, IF2, GST, NusA, MsyB, Trx, and MBP—were constructed and expressed in *E. coli* BL21(DE3) using auto-induction (AI) to enhance hRI expression levels. The expression status of hRI

was analyzed via MagNi magnetic bead purification and electrophoresis, and high-purity protein was obtained through RNase/Sepharose affinity chromatography. The purified fusion protein concentration reached 2960.513 mg/L, with enzyme activity of approximately 50 U/ L when compared with commercial hRI products. The protein was successfully applied for RNA protection, providing a theoretical basis for NusA-hRI applications.

Keywords: human ribonuclease inhibitor; fusion tags; auto-induction; magnetic bead purification; affinity chromatography

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Human ribonuclease inhibitor (hRI) is one of a family of related ribonuclease inhibitors found in the cytoplasmic extracts of many mammalian tissues, with a molecular weight of 50 kDa [1]. Comprising 460 amino acid residues, hRI is rich in leucine and cysteine, with at least 30 cysteine thiol groups existing in reduced form—essential for maintaining the inhibitor's normal biological activity [2]. Due to its inhibitory effects, hRI finds extensive applications in preserving RNA integrity during in vitro transcription and translation reactions.

This study constructed eight recombinant expression vectors containing different fusion tags (SUMO, IF2, GST, NusA, MsyB, Trx, and MBP) [3], using *E. coli* BL21(DE3) as the host strain for auto-induction expression [4]. Through MagNi magnetic bead purification [5] and RNase/Sepharose affinity chromatography, the high-expression recombinant strain NusA-hRI was ultimately selected. Single-factor and orthogonal experiments were conducted to optimize induction conditions. Preliminary tests confirmed its ability to inhibit RNase A activity and prevent RNA degradation [6-8], providing a theoretical foundation for the production and application of ribonuclease inhibitors.

Materials and Methods

1.1 Materials and Reagents

E. coli BL21(DE3) and Trans I strains were provided by our laboratory. HiFi DNA polymerase, DNA markers, and protein markers were purchased from Beijing TransGen Biotech. T4 DNA ligase and restriction enzymes BamH I and Not I were from NEB. DTT, ampicillin (Ampr), and Tris were from Taraka. NP40 was from Sigma. Lysozyme was from Amresco. RNase A was from Beijing Qinyuan Huizhi Biotech. MagNi Protein Purification Kit was from Beijing Noble Biotech.

All plasmids used in this study were constructed in our laboratory with the following characteristics: pNBE I–VII contain Ampr resistance, N-terminal His-Tag and C-terminal His-Tag, and carry fusion tags SUMO (13 kDa), IF2 (19.6 kDa), MBP (40 kDa), NusA (55 kDa), MsyB (16.1 kDa), GST (32 kDa), and Trx (17.3 kDa), respectively. pNBE VIII contains Ampr resistance, carries the MBP fusion tag (40 kDa) with signal peptides, and has a C-terminal His-Tag [3].

1.2 Instruments and Equipment

PCR amplification was performed using a T100™ Thermal Cycler (Bio-Rad, USA). Gel imaging was conducted with a Gds8000 system (UVP, USA). Centrifugation used a H2050R benchtop centrifuge (Xiangyi Laboratory Instruments, Hunan). Incubation equipment included a DK-8D electro-thermostatic water bath and DHP-9162 incubator (Shanghai Yiheng Technology). Shaking incubation used a DZP-102 constant temperature oscillator (Harbin Donglian Electronics).

1.3 Methods

1.3.1 hRI Gene Amplification Primers were designed based on the hRI gene sequence reported in GenBank and the vector sequences used. BamH I and Not I restriction sites were inserted at the 5' ends of the forward and reverse primers, respectively. The forward primer P1 was 5' -TCCAGGGGCCCCTGGGATCCATGAGCCTGGACATCCAGAGC-3' , and the reverse primer P2 was 5'-TGGTGCTCGAGTGCGGCCGCTCAGGAGATGACCCTCAGGGATG-3' . Primers were synthesized by Shanghai Sangon Biotech. Using the full-length synthetic hRI gene as template, PCR amplification was performed with primers P1 and P2. Products were detected by 1% agarose gel electrophoresis, recovered from the gel, digested with BamH I and Not I, and purified by column chromatography.

1.3.2 Recombinant Expression Vector Construction The digested hRI gene fragment was ligated with BamH I/Not I-digested pNBE3C I–VIII expression vectors using T4 DNA ligase to generate recombinant vectors pNBE3CI-VIII-hRI. These were transformed into *E. coli* Trans I competent cells, plated on Ampr-resistant agar, and incubated at 37°C overnight. Positive clones were screened by colony PCR and restriction enzyme digestion, then sent to Shanghai Sangon Biotech for sequencing verification.

1.3.3 Auto-Induction Expression of Recombinant Strains The eight verified recombinant vectors were heat-shock transformed into expression host *E. coli* BL21(DE3), plated on Ampr-resistant agar, and single colonies were selected. Each colony was inoculated into 1 mL Ampr-containing LB medium and cultured at 37°C with shaking for 16 h. A 1:1000 dilution was then used to inoculate 10 mL auto-induction medium [9], followed by incubation at 37°C, 150 rpm for 6 h, then at 20°C, 150 rpm for 24 h. Cells were harvested by centrifugation [10] and protein expression was analyzed by 12% polyacrylamide gel electrophoresis.

1.3.4 Soluble Expression Analysis of Recombinant Proteins One milliliter of culture from Section 1.3.3 was centrifuged at 12,000 rpm for 5 min to collect the pellet. After lysis and overnight freezing, samples were thawed and purified using MagNi magnetic beads according to the manufacturer' s

protocol. The pellet, supernatant, flow-through, and eluate [3] were analyzed by 12% polyacrylamide gel electrophoresis, and protein expression levels were quantified using Bandscan software to identify high-expression strains.

1.3.5 Single-Factor Optimization of Fusion Protein hRI Auto-Induction Conditions Four factors influencing auto-induction expression—temperature, pH, inoculum size, and culture volume—were investigated through single-factor experiments. Each experiment was performed in triplicate, and average values were used to determine optimal conditions [11].

1.3.5.1 Temperature Optimization: Four 10 mL aliquots of sterile auto-induction medium supplemented with four induction components and Ampr were inoculated at 1:1000 ratio. When OD_{600} reached approximately 0.2 at 37°C, cultures were transferred to shakers at 16, 20, 25, and 30°C for 24 h to determine the optimal temperature.

1.3.5.2 Initial pH Optimization: Auto-induction medium was adjusted to pH 5, 6, 7, 8, and 9, sterilized, supplemented with four induction components and Ampr, and inoculated at 1:1000 ratio. Cultures were incubated at 37°C, 150 rpm for 6 h, then at 20°C, 150 rpm for 24 h to identify the optimal pH.

1.3.5.3 Inoculum Size Optimization: Four 10 mL aliquots of sterile auto-induction medium were inoculated at 1‰, 2‰, 5‰, 10‰, and 20‰ (v/v) ratios, then incubated at 37°C, 150 rpm for 6 h followed by 20°C, 150 rpm for 24 h to determine the optimal inoculum size.

1.3.5.4 Culture Volume Optimization: Four 250 mL flasks containing 25, 50, 75, 100, and 150 mL of sterile medium were supplemented with four auto-induction components and Ampr, inoculated at 1:1000 ratio, and incubated at 37°C, 150 rpm for 6 h, then at 20°C, 150 rpm for 24 h to determine the optimal culture volume.

1.3.6 Orthogonal Optimization of Culture Conditions An $L9(3^4)$ orthogonal array was used to optimize temperature, pH, inoculum size, and culture volume, with each experiment performed in triplicate to determine optimal culture conditions [11].

1.3.7 Cell Lysis and MagNi Magnetic Bead Purification One hundred milliliters of sterile auto-induction medium was supplemented with four induction components and Ampr, inoculated at 1:1000 ratio, cultured at 37°C for 6 h, then at 20°C for 24 h. Cells were harvested by centrifugation, and the pellet was resuspended in lysis buffer containing 50 mmol/L Tris-HCl (pH 8.0), 1% Triton-X 100, 4‰ NP40, and 1 mg/mL lysozyme [12,13]. After overnight freezing at -20°C, samples were thawed at 37°C for 30 min, then 1% 500 mM $CaCl_2$ and 1% 1 mg/mL DNase were added, mixed, and incubated at 37°C for 30 min. Subsequently, 1/10 volume of 5 M NaCl was added [12], and the mixture was

centrifuged at 12,000 rpm for 5 min. The supernatant was collected for MagNi magnetic bead purification.

1.3.8 RNase/Sepharose Affinity Chromatography Purification [14] Crude protein obtained from magnetic bead purification was dialyzed twice for 6 h each against 45 mM potassium phosphate buffer containing 5 mM DTT, 1 mM EDTA, pH 6.4. The affinity column was equilibrated with 20 column volumes of the same buffer, then the dialyzed protein was loaded. Flow-through fractions were collected, and non-specific proteins were washed with 20 column volumes of buffer containing 0.5 M NaCl. Target protein was eluted with 100 mM acetate buffer (pH 5.0) containing 3 M NaCl, 5 mM DTT, 1 mM EDTA, and 15% glycerol. Eluted fractions were analyzed by SDS-PAGE.

1.3.9 Concentration Determination of Purified hRI Purified protein was concentrated using PEG 2000, desalted by dialysis, and quantified using the BCA protein assay method. Protein storage solution was added, and samples were stored at -20°C.

1.4.0 Activity Assay of Fusion Protein NusA-hRI NusA-hRI activity was determined by its ability to inhibit RNase-mediated RNA hydrolysis [3]. Using commercial RI from Company M as a control, varying volumes of RI fusion protein were added to reaction mixtures containing 50 mM Tris-Cl buffer (pH 7.4), 50 mM NaCl, and 10 mM DTT [15]. After adding 5 ng RNase A and incubating at room temperature for 10 min, 1 g of extracted plant total RNA was added. The 20 L mixture was incubated at room temperature for 10 min, then analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. Electrophoresis bands were analyzed for similarity using Bio-1D software.

Results

2.1 Target Gene Amplification

PCR amplification using the full-length synthetic hRI gene as template yielded a clear band of approximately 1383 bp on 1% agarose gel electrophoresis [Figure 1: see original paper], consistent with the expected fragment size.

2.2.1 Colony PCR

After digesting pNBE I–VIII expression vectors [Figure 2: see original paper] and the amplified hRI gene with BamH I and Not I, ligation with T4 DNA ligase, and transformation into *E. coli* Trans I, cells were plated on Ampr-resistant agar and incubated at 37°C overnight. Four positive colonies for each vector were selected for colony PCR screening [Figure 3: see original paper]. Bands of approximately 1383 bp were observed for all pNBE I–VIII recombinant vectors, confirming successful construction.

2.3 Auto-Induction Expression of Recombinant Strains

Verified recombinant plasmids were transformed into expression host *E. coli* BL21(DE3). After inoculation and auto-induction culture, harvested pellets were analyzed by 12% polyacrylamide gel electrophoresis [Figure 4: see original paper]. Fusion proteins expressed by recombinant strains BL21(DE3)/pNBE I-hRI, BL21(DE3)/pNBE III-hRI, BL21(DE3)/pNBE IV-hRI, and BL21(DE3)/pNBE V-hRI showed molecular weights of 63, 90, 105, and 66.1 kDa, respectively, matching expected sizes.

2.4 Soluble Expression Analysis of Recombinant Proteins

Pellets from Section 1.3.3 were purified using MagNi magnetic beads to assess soluble expression. Analysis by 12% polyacrylamide gel electrophoresis [Figure 5: see original paper] revealed that recombinant strains BL21(DE3)/pNBE I-hRI, BL21(DE3)/pNBE III-hRI, BL21(DE3)/pNBE IV-hRI, and BL21(DE3)/pNBE V-hRI showed eluted protein bands matching expected sizes, with expression levels of 37.17%, 32.99%, 42.63%, and 29.18%, respectively. The remaining four strains showed no soluble protein expression. Recombinant strain BL21(DE3)/pNBE IV-hRI exhibited the fewest contaminating protein bands and highest expression of the target protein NusA-hRI at approximately 105 kDa, consistent with the expected molecular weight. This strain was selected for subsequent culture condition optimization.

2.5.1 Single-Factor Experiments

Four factors significantly affecting cell growth—temperature, pH, inoculum size, and culture volume—were investigated through single-factor experiments. While cell density increased with temperature, protein expression and yield decreased, establishing 20°C as the optimal induction temperature with a protein yield of 437.113 mg/L. Larger culture volumes reduced oxygen availability, making 50 mL/250 mL the optimal volume with a protein yield of 587.243 mg/L. Inoculum size and pH had relatively minor effects, with maximal yields of 546.339 mg/L at 2‰ inoculum and 542.011 mg/L at pH 7.

2.5.2 Orthogonal Optimization

Analysis of variance showed RA > RD > RC > RB, indicating the order of factor influence on protein yield as: temperature > culture volume > inoculum size > pH. Variance analysis revealed temperature had extremely significant effects ($P < 0.01$), while culture volume and inoculum size had significant effects ($P < 0.05$). The orthogonal design identified the optimal combination as A2D2C3B3, which was not included in the original experimental matrix. A supplementary experiment using these conditions (20°C, pH 8, 5‰ inoculum, 50 mL/250 mL culture volume) yielded 723.337 mg/L protein. The comprehensive optimal auto-induction conditions were thus determined to be: 20°C, pH 8, 5‰ inoculum, and 50 mL/250 mL culture volume.

2.5.3 MagNi Magnetic Bead Purification

Ten milliliters of supernatant from lysed and thawed cells was purified using magnetic beads. Analysis by 12% polyacrylamide gel showed minimal hRI in the flow-through and virtually no protein in wash fractions, indicating efficient protein binding to the beads. Eluted protein showed a single clear band at 105 kDa, consistent with expectations, demonstrating high purification efficiency [Figure 6: see original paper].

2.5.4 RNase/Sepharose Affinity Chromatography Purification

Eluted fractions from each stage were analyzed by 12% polyacrylamide gel electrophoresis, confirming the second peak as containing the target protein [Figure 7: see original paper].

2.5.5 Concentration Determination of Recombinant Protein NusA-hRI

After PEG concentration and desalting, the purified fusion protein NusA-hRI was quantified by the BCA method at a concentration of 2960.513 mg/L.

2.5.6 Activity Assay of Fusion Protein NusA-hRI

Using commercial RI from Company M as a control, 1% agarose gel electrophoresis showed that RNA remained intact when >1 L of commercial RI was added, while purified NusA-hRI prevented RNA degradation at volumes >0.8 L. Bio-1D software analysis revealed similarity indices of approximately 18%, 30%, 30%, 98%, 98%, 30%, 50%, 98%, and 98% for lanes 2–10 compared to lane 1. Based on comparison with the commercial RI standard, the enzyme activity was determined to be approximately 50 U/ L [Figure 8: see original paper].

Discussion

This study successfully constructed recombinant expression vectors containing SUMO, IF2, GST, NusA, MsyB, Trx, and MBP fusion tags. Soluble expression was evaluated by magnetic bead purification [3], leading to selection of the high-expression strain BL21(DE3)(pNBE IV-hRI). Single-factor and orthogonal experiments established optimal auto-induction conditions: 37°C for 6 h followed by 20°C for 24 h, pH 8, 5‰ inoculum, and 50 mL/250 mL culture volume. High-expression fusion protein NusA-hRI was obtained through magnetic bead purification and RNase/Sepharose affinity chromatography, with a final concentration of 2960.513 mg/L after concentration and desalting. Activity assays demonstrated approximately 50 U/ L enzyme activity.

To achieve soluble expression of NusA-hRI, this study employed fusion tag technology [15] and low-temperature induction using BL21(DE3) as host [16]. The results demonstrate that temperature exerts extremely significant effects on

protein induction and inclusion body formation, with low-temperature induction promoting soluble expression. However, due to varying protein properties, methods for maximizing soluble expression require further investigation for hRI.

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