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CLONING, EXPRESSION AND RAPID PURIFICATION OF RECOMBINANT CHICKEN INTERFERON-ALPHA

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ABSTRACT

The poultry industry in Vietnam is vulnerable to many viral diseases. Since antibiotics and vaccination provide inadequate protection, it will be beneficial to have alternative immunostimulants to boost non-specific immunity in poultry. Chicken interferon-alpha (ChIFNα) has been described previously with antiviral activity against many pathogens. Therefore, the purpose of this study is to clone and express recombinant ChIFNα in *Escherichia coli*. The ChIFNα gene was successfully cloned into the pET32a(+) vector, then expressed in the *E. coli* Rosetta strain. Different expression conditions were tested for the best yield of the expressed protein. Results showed that recombinant ChIFNα was expressed in Rosetta *E. coli* as inclusion bodies with a yield of 30 mg/100 L culture after induction with 0.5 mM IPTG in 4 hours at 37 ^{o}C. The recombinant protein was purified using affinity column chromatography under denaturing conditions with the purity > 94%. Western blot analysis indicated that recombinant ChIFNα reacted specifically with its antibody. Further studies will be carried out to characterize the biological activities of recombinant ChIFNα and its application in the poultry industry.

Keywords: Chicken interferon-alpha, *E. coli*, recombinant, insoluble, expression, immunity.

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INTRODUCTION

The poultry industry in Vietnam is under constant threats of viral diseases such as influenza, Marek, Gumboro or Newcastle. High risk of transmission among farms and ineffective treatments using antibiotics can lead to great economic loss. Currently, vaccination is the best approach to prevent and control infections by promoting immunity among poultry. Nevertheless, vaccine efficacy may be reduced due to viral mutations and high adaptation to hosts. For example, vaccines against Marek disease can only prevent symptoms development but failed to prevent infection and viral transmission (Davison & Nair, 2005; Read et al., 2015). In the long term, highly-virulent strains that survived post-vaccination pose a major threat to breeding farms. Therefore, the poultry industry will greatly benefit from a bio-product that can boost the immunity to defense against multiple viral pathogens.

Interferons (IFNs) are a large family of glycoproteins produced by immune cells and with antiviral, anti-tumor, and immunomodulatory functions (Zhao et al., 2019). Type I IFNs may work by promoting the transcription of IFN-stimulating genes (ISGs), leading to the production of antiviral proteins such as myxovirus resistance protein (Mx) and 2'–5 ' oligoadenylate synthetases (2',5'-OAS) (Katze et al., 2002). Chicken type I IFN (ChIFN) has been studied and proven to inhibit the replication of Newcastle disease virus (NDV), infectious bronchitis virus (IBV), Marek's disease virus (MDV), Rous sarcoma virus (RSV) and the avian influenza A/H9N2 virus in poultry (Davison & Nair, 2005; Jarosinski et al., 2001; Meng et al., 2011; Mo et al., 2001). Large-scale production of ChIFN alpha (ChIFNα) may thereby have enormous potential and economic impact on the poultry industry.

To save the mass-production cost yet retain high productivity, the choice of expression system is crucial. In general, recombinant proteins can be expressed in prokaryotes such as *Bacillus subtilis* and *Escherichia coli*, in eukaryotes such as yeast, insect or animal cells, or *in vitro* on cell lines (Tripathi & Shrivastava, 2019). Among them, many proteins used in the treatment of human diseases have been successfully expressed, such as IntronA® (IFN α2b) for cancer and hepatitis treatment, or Roferon®-A (IFN) α2a) for leukemia treatment (Baeshen et al., 2015). Thanks to their fast growth and easy genetic manipulation, *E. coli* was selected as the expression system in this study. This study aims to clone and express the recombinant ChIFNα in *E. coli* Rosetta bacterial strain, which can contribute to the production of a biological product that provides non-specific immunity against many harmful pathogens in poultry. The vector design as well as expression conditions were optimized for the highest yield and best solubility of the recombinant protein.

MATERIALS AND METHODS

Construction of recombinant chicken interferon alpha (ChIFNα) expression plasmid

The ChIFNα gene (585 bp) (Genbank AM049251) was optimized for codon usage in the *E. coli* expression system, synthesized by GeneScript then inserted into pET32a(+) vector via enzyme recognition sites EcoRI and HindIII. T4 ligase was used for the ligation experiment.

Cloning and expression of recombinant ChIFNα

The ligation product was first transformed into *E. coli* DH5α competent cells following the heat-shock method at 42 ^{o}C in 45 seconds, then plated on Luria-Bertani agar containing ampicillin (100 µg/mL) and grown at 37 ^{o}C in 16 hours. To check that the transformant colonies contain the recombinant pET32a(+)/ChIFNα vector, plasmid extraction and excision with EcoRI and HindIII were done before DNA electrophoresis on agarose gel 1%.

For expression of the recombinant protein, *E. coli* Rosetta 1 (Novagen) was used. With the designed vector, the recombinant ChIFNα protein will be

expressed in fusion with Trx and His tags. In brief, the recombinant plasmid pET32a(+)/ChIFNα was transformed into Rosetta competent cells as described above. The transformant colony was cultured at 37 ^{o}C in LB broth and multiplied to the density of OD_{600} = 0.6. To optimize the level of expression and solubility of the recombinant protein, the strains were first induced isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM final concentration and cultured at 15 ^{o}C in 16 hours, 25 ^{o}C and 30 ^{o}C in 10 hours, and 37 ^{o}C in 4 hours (Table 1). Further trials were performed at 0.5 mM IPTG, 37 ^{o}C in 4 hours and 15 ^{o}C up to 72 hours after induction. The bacteria were harvested by centrifugation at 4,000 rpm/min for 10 minutes at 4 ^{o}C. The supernatant was discarded and the cell pellets were stored at -20 ^{o}C for further experiments.

Table 1. Different expression conditions of the recombinant ChIFNα

	Temperature (^{o}C)	IPTG concentration (mM)	Induction time (hour)
Experiment 1	15	1	16
	25	1	10
	30	1	10
	37	1	4
Experiment 2	15	0.5	24
	15	0.5	48
	15	0.5	72
Experiment 3	37	0.2	4
	37	0.5	4

Purification of the expressed recombinant ChIFNα protein

To separate the soluble proteins and the inclusion bodies, the cells were resuspended in 20 mM Tris-HCl pH 8 and subjected to sonication with a pulse 10 seconds and interval 10 seconds, amplitude 30% for 15 minutes. The lysate was then centrifuged at 10,000 rpm/min for 10 minutes. The supernatant was collected as the soluble fraction. The precipitate or insoluble fraction was washed once with Tris-HCl pH 8 and then resuspended in Denaturing Binding buffer (8M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8). The His-tagged recombinant ChIFNα protein was then purified using the Ni^{2+} affinity Probond^{TM} Purification system (Invitrogen, Life Technologies) according to the manufacturer's guideline with minor modifications. In short, the purification column was loaded with 2 mL resin then balanced with the Denaturing Binding buffer. Next, the insoluble fraction suspended in the buffer was loaded onto the resin column. After that, the column was washed with Wash buffer 1 (8M urea, 20 mM sodium phosphate, 500 mM NaCl pH 6), then by Wash buffer 2 (50 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 8). Elution buffer (50mM NaH_2PO_4, 500 mM NaCl, 400 mM imidazole, pH 8) was used to elute the recombinant protein. For SDS-PAGE analysis, 20 µL sample was added per lane, which corresponded to about 0.8 µg per lane.

Identification of recombinant ChIFNα by Western blot analysis

After SDS-PAGE gel electrophoresis, the protein samples were electrically transferred to an Immuno-Blot^{TM} PVDF membrane Cat. 162-0177 (Bio-Rad, Hercules, California, USA). After that, the membrane was blocked with blocking buffer (5% skim milk) in 2 hours at room temperature, followed by incubation with a mouse monoclonal antiserum against Trx at 4 ^{o}C in 15 hours. Finally, the membrane was incubated with a secondary human anti-rabbit IgG antibody (Sigma-Aldrich) prior to the addition of horseradish peroxidase (HRP) (Bio-Rad,

Hercules, California, USA) for color observation.

RESULTS AND DISCUSSION

Construction of recombinant pET32(+)/ChIFNα vector

The ChIFNα gene sequence optimized by GeneScript was ligated into the pET32a(+) vector. The ligation product was then transformed into *E. coli* DH5α competent cells. The colonies grown on an LB agar plate containing ampicillin were selected to do plasmid extraction and cut with restriction enzymes. Results obtained from agarose gel 1% electrophoresis showed two DNA bands of approximately 6,000 and 585 bp, which corresponded to the estimated sizes of the pET32a(+) vector and recombinant ChIFNα after plasmid excision with EcoRI and HindIII. These results indicate that the recombinant pET32a(+)/ChIFNα vector has been successfully constructed and cloned into *E. coli* DH5α strain (Fig. 1).

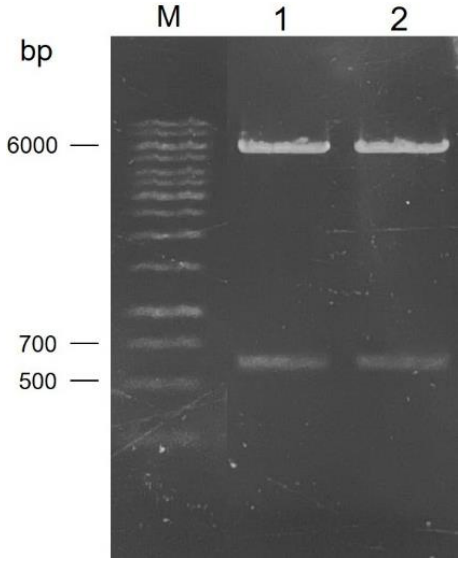

Figure 1. Agarose gel 1% electrophoresis of the pET32a(+)/ChIFNα recombinant plasmid digested by restriction enzymes EcoRI and HindIII after transformation into *E. coli* DH5α strain. M: Marker 1kb Thermo Scientific™ Gene Ruler™; Lane 1, 2: pET32a(+)/ChIFNα recombinant plasmid cut by restriction enzymes EcoRI and HindIII

To further confirm that the designed ChIFNα gene was ligated into the pET32a(+) vector, the pET32a(+)/ChIFNα plasmid was sequenced using T7 promoter and terminator primers. The sequence of the ChIFNα gene in the expression vector is 100% identical to the gene synthesized by GeneScript, showing that the designed ChIFNα gene was successfully inserted into the expression vector.

Expression of recombinant pET32a(+)/ChIFNα in *E. coli* Rosetta

The pET32a(+)/ChIFNα recombinant plasmid was transformed into *E. coli* Rosetta by the heat-shock method. The transformant colonies were cultured in LB broth containing ampicillin until OD_{600} reached 0.6. First, to optimize the expression temperature, induction was performed with 1 mM IPTG at 15, 25, 30 and 37 ^{o}C. In the design of the pET32a(+)/ChIFNα expression vector, ChIFNα was fused with a Trx tag to improve the solubilility and level of expression of this protein, and a His tag for purification purposes. The protein expression was observed on 12.5% SDS-PAGE electrophoresis (Fig. 2). The theoretical molecular mass of the recombinant ChIFNα protein is approximately 40 kDa, including 21.9 kDa ChIFNα, a Trx-tag and 2 N- and C-terminal His-tags. At 1 mM IPTG induction, the recombinant ChIFNα protein was not expressed after 16 hours of incubation at 15 ^{o}C. At 25, 30 and 37 ^{o}C, the 40 kDa band appeared in the insoluble protein lanes. The largest band was detected when the bacterial strain was cultured at 37 ^{o}C and collected 4 hours after induction.

As low temperatures and low concentrations of inducers have been described to improve the solubility of recombinant proteins expressed in *E. Coli* (Costa et al., 2014; Sørensen & Mortensen, 2005), the expression level of recombinant ChIFNα was further examined at 0.5 mM IPTG in 15 ^{o}C up to 72 hours post-induction (Fig. 3). The results showed that at 15 ^{o}C ChIFNα was weakly expressed even at 72 hours after IPTG induction, and the protein was mostly expressed as inclusion body.

Following trials were carried out with 0.2 mM and 0.5 mM IPTG, at 37 ^{o}C in 4 hours of cultivation (Fig. 4). At lower concentrations of IPTG, the protein appeared to express both in soluble and insoluble forms, however, the majority of the expressed recombinant ChIFNα remained insoluble. Besides, the amount of protein expressed with 0.2 mM and 0.5 mM IPTG induction at 37 ^{o}C appeared to be lower than that induced with 1 mM IPTG.

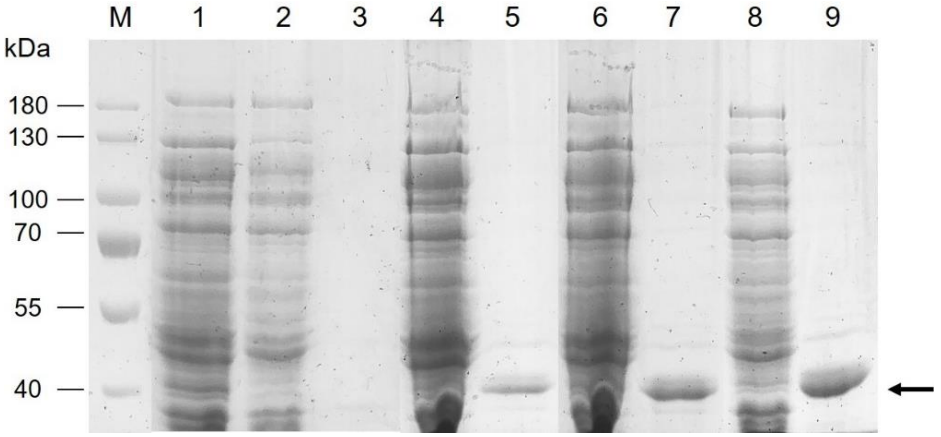

Figure 2. Induced expression of recombinant ChIFNα in *E. coli* Rosetta with 1 mM IPTG at different temperatures**.** M: Marker PageRuler^{TM} Prestained ThermoScienific; Lane 1: Total cellular proteins from *E. coli* Rosetta without IPTG induction; Lane 2-9: soluble and insoluble proteins, respectively, expressed post-IPTG induction at 15 ^{o}C in 16 hours (Lane 2-3); at 25 ^{o}C in 10 hours (Lane 4-5); at 30 ^{o}C in 10 hours (Lane 6-7); and at 37 ^{o}C in 4 hours (Lane 8-9). The recombinant ChIFNα bands were indicated in an arrow

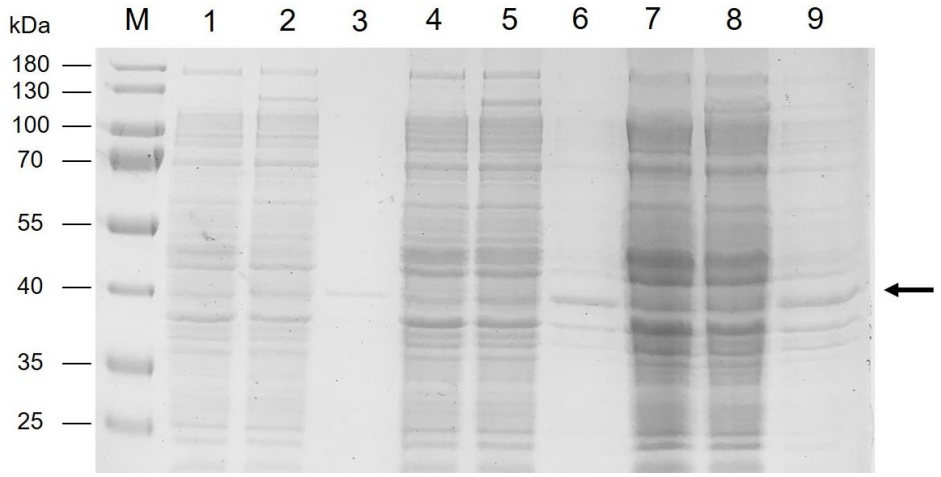

Figure 3. Weak expression of recombinant ChIFNα in *E. coli* Rosetta at 15 ^{o}C up to 72 hours post-IPTG induction. M: Marker; Lane 1-3: Total cellular protein without IPTG induction (Lane 1), soluble proteins (Lane 2) and insoluble proteins (Lane 3) 24 hours post-IPTG induction; Lane 4-6: Total cellular protein without IPTG induction (Lane 4), soluble proteins (Lane 5) and insoluble proteins (Lane 6) 48 hours post-IPTG induction; Lane 7-9: Total cellular protein without IPTG induction (Lane 7), soluble proteins (Lane 8) and insoluble proteins (Lane 9) 72 hours post-IPTG induction. The recombinant ChIFNα bands were indicated in an arrow

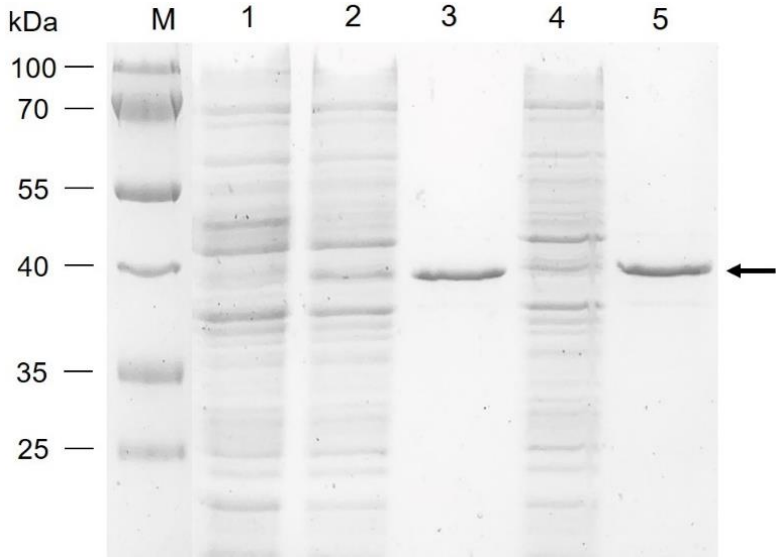

Figure 4. Recombinant ChIFNα remained mostly insoluble after 4-hour induction with 0.2 mM and 0.5 mM IPTG, 37 ^{o}C in *E. coli* Rosetta. M: Marker; Lane 1: Total cellular protein without IPTG induction; Lane 2: soluble, and Lane 3: insoluble proteins expressed with 0.2 mM IPTG induction; Lane 4: soluble, and Lane 3: insoluble proteins expressed with 0.5 mM IPTG induction. The recombinant ChIFNα bands were indicated in an arrow

Rapid purification of the recombinant ChIFNα protein

The Ni^{2+} chelate affinity chromatography was used to purify the recombinant His-tagged ChIFNα protein. After being eluted with the elution buffer containing 400 mM imidazole, 8 fractions were collected. The results on 12.5% SDS-PAGE gel electrophoresis revealed that the purified recombinant protein was present in fractions 3–8, with the largest bands at 40 kDa observed in fractions 5 and 6 (Fig. 5). The purity of recombinant ChIFNα was 94% as estimated by ImageJ software. This value was consistent with the molecular mass of the recombinant ChIFNα protein fused with the Trx and His tags. The expression efficiency was approximately 30 mg/L biomass culture volume. There were traces of contaminated proteins until fraction 6, indicating that the purification procedure needs further optimization. To identify the protein, the purified product was subsequently subjected to Western blot analysis, using a Trx-specific antibody. The back dot on the PVDF membrane showed a consistent molecular mass value as in the SDS-PAGE analysis (Fig. 5).

To produce a large amount of ChIFNα, there have been various challenges including efficient expression, insolubility, and purification. ChIFNα was expressed in different systems including *Pichia pastoris* (yeast) (Tam et al., 2014) and *Bacillus subtilis* (bacteria) (Thu, 2012), yet the preventive effect of the expressed products against different chicken pathogens was not high and constant. Moreover, in comparison with the yeast system, the *E. coli* bacterial system has certain advantages such as easier workflow and more cost-effective. The main purpose of this study is to design a recombinant ChIFNα gene to be fused with the Trx tag in the pET32a(+) vector and expressed in the *E. coli* Rosetta system. The Rosetta strain is one of the BL21 derivatives that contains tRNA codons rarely used in *E. coli*, which may promote the expression of eukaryotic proteins (Kaur et al., 2018; Rosano et al., 2019). Besides, a Trx tag placed at the N-terminal of the target protein can theoretically act as a solubility enhancer and avoid the chance of

producing an inclusion body when expressing recombinant protein (LaVallie et al., 1993; Sun et al., 2010; Tomala et al., 2010). Different IPTG induction concentrations, temperatures and cultivation times for the best yield and solubility of the protein were examined. Under the control of the T7 promoter, the recombinant ChIFNα was efficiently expressed, with the largest amount obtained from the conditions of either 0.5 or 1 mM IPTG final concentration, and cultivation at 37 ^{o}C in 4 hours. Recombinant ChIFNα was also successfully expressed in different *E. coli* system in previous studies. For example, Zhao et al. also constructed pET32a/ChIFNα vector and succeeded in soluble expression in *E. coli* Rosetta (DE3) system (Zhao et al., 2019). This is not consistent with our results. Although the vector design, selection of host strains and expression conditions were optimized in our study to obtain the soluble protein, it is not clear why our recombinant protein was expressed as an inclusion body. The Trx tag was intentionally used to improve the solubility, but it was found to not fulfill this aim for any recombinant protein. In an attempt to express fibroblast growth factor (FGF) 15/19 by Kong and Guo, the Trx tag improved the soluble expression of FGF19 protein in the Rosetta-gami strain but failed to increase the yield of soluble FGF15 protein in both Rosetta-gami strain and BL21 (DE3) *E. coli* systems (Kong & Guo, 2014). In the case of our target protein, soluble expression of ChIFNα was also obtained in the study by Ma et al. using a pHis-NusA expression vector in BL21 (RILP) strain, however, the purification of this protein appeared challenging as there were many bands of unrelated proteins (Ma et al., 2014). Our recombinant ChIFNα protein, despite being expressed insolubly, was easy to perform using affinity column chromatography, with about 95% purity at the last fractions. In addition, Western blot analysis revealed its immunoreactivity with the specific antibody, suggesting that this protein is likely to retain its biological activity. Further experiments should be conducted to try refolding the protein, and test its activity.

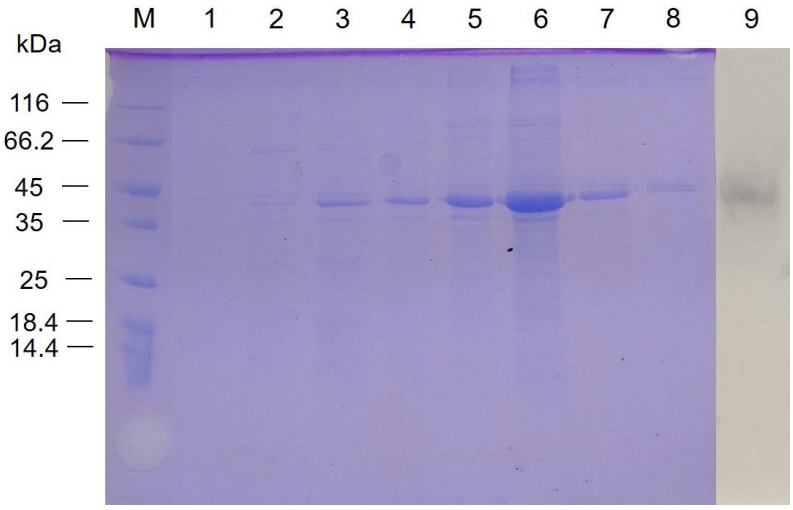

Figure 5. Purification and Western blot analysis of recombinant ChIFNα. M: Marker Thermo^{TM};
Lane 2-8: Recombinant ChIFNα fractions eluted with 400 mM imidazole;
Lane 9: Immunoreactivity of the recombinant protein to the specific antibody

CONCLUSION

In this study, the gene encoding Chicken interferon-alpha was optimized according to the codon usage in *E. coli*. The best yield of the recombinant protein was obtained (about 30 mg/L culture) in the expression condition at 0.5 mM IPTG and cultivation at 37 ^{o}C in

4 hours. The recombinant ChIFNα was successfully purified by Ni- affinity column chromatography with a purity of 94% and can be subjected to functional assays.

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