

## Original Article



# Expression and Purification of Recombinant Hepatitis Delta Virus (HDV) Antigen for Use in a Diagnostic ELISA for HDV Infection Using the High-Density Fermentation Strategy in *Escherichia coli*

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## Abstract

**Objective** Hepatitis Delt a Virus (HDV) antigen is widely used as a capture antigen in ELISAs for the identification of HDV infection; large amounts of recombinant HDV antigen with active antigenicity are required for this purpose.

**Methods** Reconstruct the gene of HDV antigen based on the bias code of *Escherichia coli*, the recombinant protein expresses by high-density fermentation with fed-batch feeding strategy, and purify by immobilized metal chromatography. The sensitivity and specificity of this antigen detect by ELISA method.

**Results** The expression of HDV antigen can reach 20% of the total cell mass in the soluble form. The recombinant HDV antigen can be conveniently purified (98%) by immobilized metal ion affinity chromatography (IMAC) using the interaction between a His-tag and nickel ions. Production of recombinant HDV antigen can reach 0.5 g/L under conditions of high-density cell fermentation. Applied to the diagnostic ELISA method, the recombinant HDV antigen shows excellent sensitivity (97% for IgM and 100% for IgG) and specificity (100% for IgG and IgM) for the detection of anti-HDV antibodies.

**Conclusion** Expression and purification the recombinant HDV antigen as a candidate protein for application in a diagnostic ELISA for HDV infection. Large-scale production of the protein can be achieved using the high-density fermentation strategy.

**Key words:** ELISA diagnosis; HDV; High-density cell fermentation; IMAC

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## INTRODUCTION

Although the hepatitis delta virus (HDV) was originally identified in 1977<sup>[1]</sup>, diagnosis of HDV by ELISA has not yet been standardized or characterized<sup>[2-4]</sup>. HDV antigen, which is a unique protein encoded by the HDV, can also exist in a form with an additional 19 amino acids at the C-terminal due to RNA editing during RNA

replication<sup>[5]</sup>. Both forms of antigen are capable of stimulating antibody production and specific IgG and IgM antibodies represent serum biomarkers of HDV infection, regardless of co-infection or super-infection<sup>[3-4]</sup>. Diagnosis of HDV infection based on ELISA detection of serum antibodies is a preferred method because it allows detection at an earlier stage than is possible using a method based on the appearance of the virion in serum<sup>[6]</sup>. The availability

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of antigenically active HDV antigen is critical for the ELISA method.

Undoubtedly, *Escherichia coli* (*E. coli*) is the most desirable option for the expression of recombinant proteins, due to the rapid growth and cost-efficiency, as well as the ease of manipulation and scale-up potential of this strain of bacteria. Currently, high-density fermentation technology using *E. coli* is widely applied for the large-scale production of recombinant proteins<sup>[7-11]</sup>. Although recombinant HDV antigen has been reported to be expressed *in vitro*, in this case, the antigen was produced as part of a fusion protein, rather than in the native form. Moreover, the low expression abundance as well as the inconvenient purification limited its large-scale industrial production<sup>[12-15]</sup>. High-density fermentation is critical for the large-scale production and efficient purification of native HDV antigen.

In the current study, the recombinant HDV antigen was efficiently expressed in *E. coli*. The purified protein retained the capacity to bind specific antibodies after codon optimization. Recombinant HDV antigen with high levels of purity was obtained by adjusting the concentration of NaCl in the buffers used for chromatographic purification. Large-scale and rapid production of recombinant HDV antigen with antigenic activity can be achieved using industrial high-density fermentation technology.

## MATERIALS AND METHODS

### Construction and Expression of Recombinant HDV Antigen

The gene sequence of the HDV antigen of the HDV strain isolate from Inner Mongolia, China (stored in our laboratory) was redesigned (codon-optimized) based on *E. coli* codon bias. The redesigned gene sequence was synthesized by TaKaRa Biotech (Dalian, China), and cloned into a pUC18 vector to generate the pUC10-HDV antigen vector. After digesting pUC18-HDV with *Nde*I and *Xho*I, the HDV gene fragment was inserted into a pET-43.1a (+) plasmid to generate the pET-43.1a+HDV antigen expression plasmid.

### Shake Flask Cultures

The resultant pET-43.1a+HDV antigen plasmid was then transformed into the *E. coli* BL21 (DE3) strain, which was cultured at 37 °C for 7-8 h in LB medium supplemented with 50 µg/mL ampicillin until OD<sub>600</sub> 0.8 was reached, when 1 mmol/L

isopropyl-β-thiogalactopyranoside (IPTG) was added to induce the expression of the recombinant protein. After 3 h, the cells were harvested by centrifugation at 4000 ×g and the pellets were suspended in a cold buffer containing 10 mmol/L Tris-HCl (pH 8.0) and 0.5% Triton X-100. The cells were then homogenized on ice by ultrasonic disruption, followed by centrifugation at 12,000 ×g to remove the insoluble material. All the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 13.5% gel).

### Fed-batch Fermentation

Cells in the mid-exponential growth phase were harvested, suspended in LB medium containing 50 mg/L ampicillin and 15% glycerol, and then store at -70 °C as stock. For fed-batch fermentation cultures, 50 µL of stored liquid was add to 200 mL LB medium containing 50 mg/L ampicillin and cultured in 500 mL flasks at 37 °C with agitation (240 rpm). When the OD<sub>600</sub> reached 0.8, the cells were harvested and suspended in LB medium containing 100 mg/L ampicillin. The resultant seed cultures were used to supplement the sterilized fermentation bioreactor of 6 L working volume (BCD-10 Baoxing, Shanghai). The temperature of the culture was adjusted to 37 °C, while dissolved oxygen levels were maintained above 40% for the growth phase, or 30% for induction, by automatically altering the agitation speed. The composition of the fed-batch fermentation medium was as follows: 15 g/L glucose, 3 g/L yeast extract, 10 g/L NaCl, 10 mmol/L MgSO<sub>4</sub>. The feed recipes were: Feed I (300 g/L glucose) and Feed II (300 g/L glucose, 10 g/L yeast extract). The flow rate was initially set at 6 L/min and gradually increased to 13 L/min. The pH of the media was maintained above pH 6.8 using concentrated ammonia water and 1 mol/L HCl. The agitation speed was decreased sharply before the addition of Feed I solution to control the specific growth rate close to 0.12. After 6 h, the feed was stopped, and 10 g/L lactose was added to initiate induction, which lasted for 1 h, followed by the addition of IPTG (1 mmol/L final concentration). Feed II was added subsequently. The volume of the feed solution added to the bioreactor per hour was calculated according to the following equation: (1)  $V(i)=27 \times X_1(t-t_0)$ , (2)  $V(a)=(27 \times X_1^6)X_2(t-t_0-7)$   $X_1=1.13$ ,  $X_2=1.1$ , where  $t$  represents the total time of the fermentation and  $t_0$  represents the start time of the fed-batch. Cells were collected after IPTG induction for 3 h and

centrifuged at 4000  $\times$ g for 10 min. Cell pellets were then stored at -20 °C.

### **Protein Purification**

The cells were suspended in lysis buffer (10 mmol/L Tris-HCl, 0.5% Triton X-100; pH 8.0) and lysed by ultrasonic disruption on ice. The disrupted cells were centrifuged at 12,000 rpm for 10 min to remove the insoluble matter. Subsequently, NaCl was added to the supernatant to a final concentration of 1 mol/L. The sample was loaded onto a nickel-nitriloacetic acid (Ni-NTA) affinity chromatography column and eluted using elution buffer 1 (10 mmol/L Tris-HCl, 1 mol/L NaCl, 60 mmol/L imidazole; pH 8.0) to remove the unbind proteins. Elution buffer 2 (10 mmol/L Tris-HCl, 1 mol/L NaCl, 300 mmol/L imidazole; pH 8.0) was used to elute the target recombinant HDV antigen. The recombinant proteins were dialyzed in reloading buffer (10 mmol/L Tris-HCl, 0.5 mol/L NaCl; pH 8.0) to remove imidazole. The process of purification by affinity chromatography was then repeated under conditions of 0.5 mol/L NaCl. All purified proteins were collected and analyzed by SDS-PAGE (13.5% gel).

### **IgM ELISA**

The purified recombinant proteins were dialyzed in 0.1 mol/L carbonate buffer (pH 9.6), and conjugated with horseradish peroxidases (HRP) by the periodate method according to a standard protocol. To determine the antigenicity of the recombinant HDV antigen in terms of IgM production, 100  $\mu$ L of serum samples from a total of 30 patients with HDV (preserved in our laboratory) was added to the anti-human IgM ( $\mu$ -chain specific) antibody (1:1000), Sigma- Aldrich, MO, USA-coated plates and incubated at 37 °C for 1 h. The wells were then washed and incubated for 30 min at 37 °C with 50  $\mu$ L of HRP-conjugated recombinant HDV antigen (1:2000). After a further three washes, 100  $\mu$ L of 3,3',5,5'-tetramethylbenzidine solution (TMB) was added to each well. The reaction was stopped after 20 min by the addition of 1.8 mol/L sulfuric acid (50  $\mu$ L per well), and the absorbance of all the samples was measured spectrophotometrically at two wavelengths (430 nm and 650 nm). BSA was used as the control and all samples with absorbance values above the average value measured for the control were regarded as IgM-positive. The sera from HDV-infected patients (30 cases), HCV-infected

patients (30 cases), healthy peers (30 cases) and patients with non-viral hepatitis (30 cases) were collected to measure the specificity and sensitivity of the ELISA for the detection of anti-HDV antigen-specific IgM antibodies. All serum samples were tested for the presence of HDV nucleic acids and the assay was repeated when the clinical information was considered.

### **Indirect IgG ELISA**

Microtiter plates (96-well) were coated with 10 ng of dialyzed recombinant HDV antigen at 4 °C overnight. Plates were then blocked with 1% BSA for 1 h. After drying, the diluted serum samples were added and incubated at 37 °C for 1 h. The wells were washed three times with PBS containing 0.2% Tween-20 and incubated at 37 °C for 30 min with HRP-conjugated mouse anti-human IgG antibody (1:10,000, Sigma- Aldrich, MO, USA). The wells were washed again and incubated with TMB (100  $\mu$ L per well) for 20 min, before the reaction was stopped by the addition of 1.8 mol/L sulfuric acid (50  $\mu$ L per well) and the absorbance of all the samples was measured spectrophotometrically at two wavelengths (430 nm and 650 nm). The specificity and sensitivity of the assay for the detection of recombinant HDV antigen-specific IgG antibodies was assessed against the control as described for the IgM ELISA.

## **RESULTS**

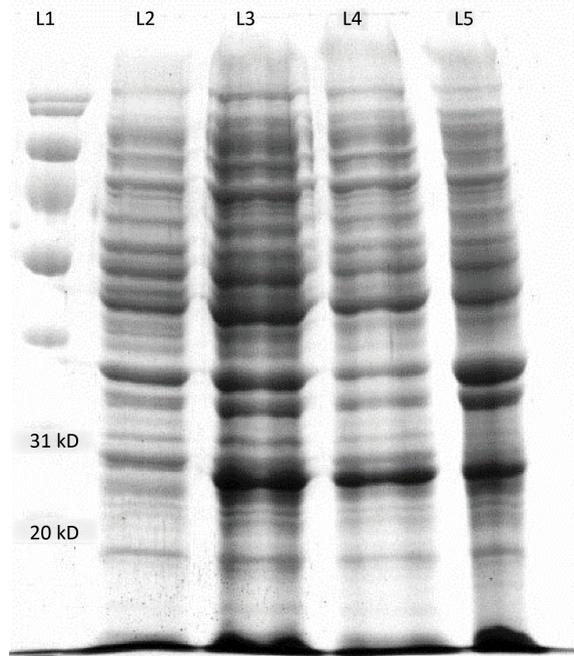
### **Generation of the Recombinant HDV Antigen**

Translation of the full length of the gene encoding the recombinant HDV antigen (612 bp) was initiated at the ATG created by *NdeI* restriction digestion, and ended at the termination codon after insertion of a 6 $\times$ His-tag (no other fusion proteins were included). The encoded recombinant consists of 203 amino acids with a theoretical molecular weight of 24 kD. SDS-PAGE analysis revealed that recombinant protein of the expected molecular weight was expressed predominantly in the soluble form. After 3 h of induction, the amount of recombinant protein comprised 20% of the total amount of endogenous bacterial proteins (Figure 1), indicating that the expression level was significantly improved by codon optimization.

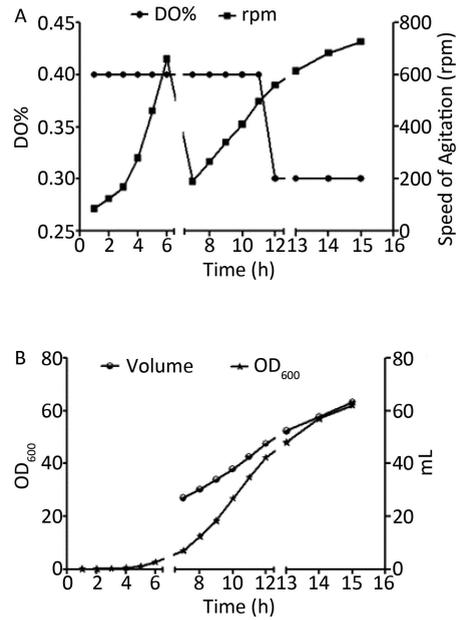
### **Fed-batch Fermentation**

To maintain the level of dissolved oxygen, the stirring speed was gradually elevated to 800 rpm

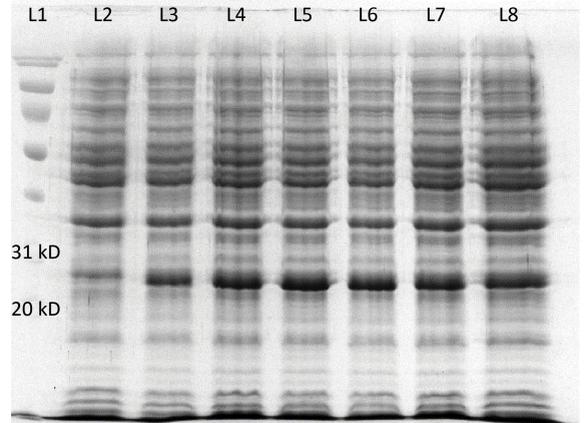
with the growth of the bacterium. The speed was slowed to 200 rpm after the carbon source was consumed, and the speed was adjusted once the feeding was initiated with fresh feed. The specific bacterial growth rate was controlled at 0.12 through addition of the carbon source (Figure 2A). The recombinant protein expression was started by the addition of the lactose inducer and IPTG was added to continue the induction. Peak expression was reached 3 h after the start of the induction, when the recombinant protein constituted approximately 23.6% of the total bacterial protein content (Figure 3). The production then decreased with prolonged induction time. Therefore, continuously increasing the fermentation cell density did not increase the recombinant protein production. The absorbance at OD<sub>600</sub> was monitored at different time-points, which were the OD<sub>600</sub> reached 62 at harvest (Figure 2B). The recombinant protein productivity was approximately 0.54 g/L if 0.382 g dry weight of bacterium per OD<sub>600</sub> absorbance unit was used for calculation.



**Figure 1.** SDS-PAGE showing recombinant HDV antigen expression in *Escherichia coli*. Lane 1: Low molecular weight marker. Lanes 2-5: The soluble and insoluble forms of the recombinant HDV antigen after induction.



**Figure 2.** Fed-batch fermentation process conditions. A, Dissolved oxygen levels. B, The number of bacteria (indicated by OD<sub>600</sub>) and feeding volume.



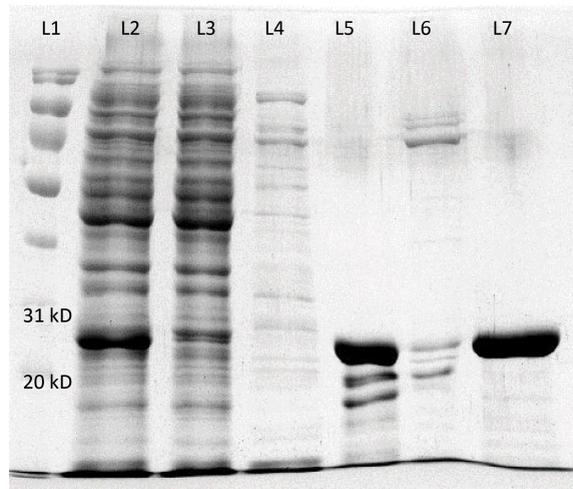
**Figure 3.** Expression of recombinant HDV antigen for fed-batch fermentation after IPTG induction. Lane 1: Low molecular weight marker. Lane 2: Lactose-induced expression. Lanes 3-8: Expression of recombinant HDV antigen at hourly intervals after induction. Peak expression peak was observed after induction for 3 h (Lane 6), with the recombinant protein accounting for 23.6% of the total bacterial proteins.

### Purification of the Recombinant HDV Antigen

The recombinant HDV antigen was purified via the His-tag at the C-terminus, which binds to Ni resin in the presence of 1 mol/L NaCl. Most of the impurities were removed by wash buffer 1, and the purity of targeted protein was not that satisfied followed with only wash buffer 2 washing. Following re-purification by the same procedure with the loading buffer containing 0.5 mol/L NaCl, the purity of the target protein reached 98% (show in Figure 4). In this study, purification of the protein by binding of the C-terminal His-tag to the Ni resin in the presence of 1 mol/L NaCl was an absolute requirement to achieve successful purification via the same procedure in the presence of 0.5 mol/L NaCl.

### Recombinant HDV Antigen for the Detection of Serum anti-HDV Antibodies by ELISA

Serums amples with absorbance values above the average value measured for the BSA control were regarded as positive. Using the recombinant HDV antigen as a capture antigen, all 30 HDV patients



**Figure 4.** Purification of recombinant HDV antigen using immobilized metal ion affinity chromatography (IMAC). Lane 1: Low molecular weight marker. Lane 2: soluble form of rHDV antigen after *E. coli* lysis (loading sample). Lane 3: Flow-through solution with the recombinant protein bound to Ni resin. Lane 4: Impurities eluted by the washing buffer 1. Lane 5: The eluted target protein after the first purification. Lanes 6 and 7: The purified target protein after the first (1 mol/L NaCl) and second (0.5 mol/L NaCl) purification. The final purity of the recombinant protein was 98%.

tested positive for IgG by ELISA, while the remaining 90 non-HDV patients tested negative. Similarly, 29 of 30 HDV patients tested positive for IgM by ELISA, with no false positive results among the 90 non-HDV patients (Table 1).

### DISCUSSION

*E. coli* is the strain of bacteria predominantly exploited in large-scale industrial recombinant protein production, owing to its high yield of protein products. *E. coli* is compatible for high-density cultivation, thereby substantially lessening the costs of large-scale protein production.

In this study, by optimizing the codon bias, the yield of HDV antigen expression was dramatically increased and accounted for 20% of the total bacterial proteins. In this case, the G+C content was reduced from 70% to 48% by codon optimization, which is beneficial for protein expression. The small HDV antigen consists of amino acids, with a molecular weight of approximately 24 kD. The relatively short length seems to confer the ability to fold rapidly into the correct and appropriate conformation throughout the translation process, with the HDV antigen synthesized in soluble form. The HDV antigen is a basic protein that contains a number of alkaline amino acids, with a predicted isoelectric point (pI) of 9.8. Consequently, the HDV antigen not only binds negatively charged nucleic acids, but also interacts with water molecules through hydrogen bond formation to generate the soluble protein. Most viral nucleoproteins are synthesized in soluble form, rather than in inclusion bodies that subsequently require problematic dialysis and renaturation procedures.

It has been reported that during high-density fermentation, the expression yield is dependent on the phase of growth at which the bacteria cells were harvested and used for protein expression. Typically,

**Table 1.** Detection of HDV Infection Using the Recombinant HDV Antigen for Detection of Serum HDV-specific Antibodies by ELISA

Item	IgG (P/T)	IgM (P/T)
HDV (+)	30/30	29/30
HCV(+)	0/30	0/30
Healthy peers	0/30	0/30
Non-viral hepatitis	0/30	0/30

**Note.** P: positive cases, T: total cases.

*E. coli* cells in the mid-logarithmic phase are optimal for protein expression. In our study, bacteria were maintained in the mid-logarithmic phase by continuous addition of the carbon source to the fermentation broth. Collectively, our results demonstrate that when the specific growth rate increased to a level exceeding 0.17, the protein expression is negatively affected as a result of acetic acid accumulation, while a specific growth rate of 0.12 is optimal for a high yield<sup>[16]</sup>. The ratio of carbon to nitrogen in the early stages has a crucial influence on the ultimate protein yield; that is, when bacterial cells divide too actively due to the availability of high levels of the carbon source in the medium, the phase of declining growth is reached before the carbon source is depleted, leading to a reduction in the protein yield. On the other hand, excessive nitrogen sources lead to slow bacterial growth, thereby prolonging the duration of the fermentation process and increasing the risk of contamination and plasmid loss, and leading to inferior yield and production efficiency. To stabilize the plasmids, we introduced divalent cations such as Mg<sup>2+</sup> into the medium, which proved to be effective in increasing the expression yield. Lactose, which is non-toxic, widely available, and inexpensive<sup>[16]</sup>, was initially used as the carbon source to induce protein expression instead of IPTG, which is relatively expensive. This approach is regarded as beneficial for therapeutic application in view of its advantages in terms of drug safety and cost reduction. In addition, we added yeast extract to the feed solution during induction to boost the ratio of the nitrogen source in the medium, with beneficial effects on protein expression. In our study, no increase in protein abundance was observed after induction for 3 h. Instead, the data showed a reduction in the yield, possibly due to resulting the declining bacterial growth phase or plasmid loss.

The wide application of IMAC is attributed to its low-cost, high efficiency, and the potential to achieve a high degree of purity (>95%) in a one-step purification process. Characterized by its short length (only 6 amino acids), the His-tag is preferred for protein purification because of its lack of immunogenicity making it a perfect purification tag for the production of antigens for clinical diagnosis. The introduction of NaCl into the binding buffer not only substantially weakened the interference in ion exchange, but also boosted the chelate- interaction of the His-tag with metal ion. The arginine-rich domain (ARD) at the C- terminal of the HDV antigen

is highly charged, causing interference with the interaction of the His-tag and metal ion. Therefore, the fusion protein could not bind to the chromatography column at the routine NaCl concentration of 0.5 mol/L, although good binding was achieved with the NaCl concentration was elevated to 1 mol/L. We speculate that this was because the high levels of NaCl interacted with the charged amino acid residues on the protein surface, and consequently reduced the negative effect on the chelation of the His-tag by the nickel ions.

The HDV genome encodes a unique protein designated the HDV antigen (HDV antigen), which has exists in distinct small (S-HDV antigen) and large (L-HDV antigen) forms. The S-HDV antigen is crucial for HDV RNA replication, while the L-HDV antigen, which suppresses HDV replication, appears at the later stage and is sustained at relatively high levels during the chronic infection period. The 19 amino acids at the C-terminal of the L-HDV antigen are related to the patient serotypes, and extremely divergent among different serotypes. The original C-terminal epitope (9E4) can be shielded against recognition by the immune system by prenylation<sup>[17]</sup>. In addition, the C-terminal 19 amino acids can also have a negative effect on protein expression, irrespective of a higher yield achieved by means of codon bias optimization. Collectively, our data indicate that the S-HDV antigen is suitable for use as a capture antigen in an ELISA for the diagnosis and determination of HDV infection. Although there are many problems in the clinical diagnosis HDV infection, expression and purification of the recombinant HDV antigen has important significance in serological diagnosis.

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