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Expression and Evaluation of a Novel HAV-VP1 and HBS-Ag Fusion Protein for Potential Applications in Immunization and Diagnosis

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ABSTRACT

Introduction: Hepatitis A virus (HAV) is a causative agent of acute hepatitis in humans, infecting more than one million individuals every year, including both symptomatic and asymptomatic infections. The currently available preventive vaccines for HAV are based on either wild-type or live-attenuated virus strains, which can contribute to the costliness of the vaccination process. Therefore, it may be worthwhile to explore the potential of subunit vaccines that utilize immunogenic viral products. **Methods**: This study presents the results of a novel recombinant protein production study that employed the native structures of HAV-VP1 and HBs-Ag. The fusion protein underwent comprehensive characterization to evaluate its potential applications in diagnostics and immunization. The truncated recombinant protein, HAV-VP1 (position 99-259 aa) -HBs-Ag, was successfully expressed in the *Escherichia coli* BL21-DE3 system. **Results**: The recombinant protein, with a molecular weight of 46 kDa, was evaluated using SDS-PAGE gel electrophoresis and confirmed by western blotting. The fusion protein was successfully detected in serum samples positive for HBV or HAV using anti-HBs and anti-VP1 antibodies. Additionally, it elicited a potent humoral response in BALB/c mice. **Conclusion**: The novel recombinant protein described in this study has the potential to serve as a bivalent vaccine against HAV and HBV infections. The next step involves evaluating the immunogenicity and safety profile of the protein.

INTRODUCTION

Hepatitis is a liver disease that is often associated with documented outbreaks. It significantly impacts public health and can result in significant mortality in individuals with acute or chronic conditions [1, 2]. There are currently five recognized types of viral hepatitis. Hepatitis A and E are typically transmitted through the consumption of contaminated food or water. Meanwhile, hepatitis B, C, and D are primarily transmitted through exposure to infected body fluids or contact with infected blood [2-5].

Hepatitis A virus (HAV) is an RNA virus with a single-stranded genome that belongs to the *Picornaviridae* family. Hepatitis A virus is primarily transmitted through the fecal-oral route, including person-to-person contact. In response to the virus, the immune system can cause damage to hepatocytes [6, 7]. Hepatitis A poses a significant global health threat, with nearly 200 million people infected and 30,000 deaths caused by the virus each year [8-10].

Hepatitis B, caused by the double-stranded DNA virus HBV (hepatitis B virus) from the *Hepadnaviridae* family, is another form of viral hepatitis that often leads to chronic liver disease [11]. According to estimates, approximately 296 million people are living with chronic hepatitis B, and there were 820,000 related deaths recorded in 2019 [12].

Vaccination has consistently been considered the most effective method for preventing and treating infectious diseases [13-15]. Safely effective vaccines to prevent both hepatitis A and B were developed during the 1980s and 1990s [16]. The HAV vaccine was initially designed for individual prophylaxis and is now widely used to control HAV in endemic regions [17]. Both inactivated and live attenuated vaccines for hepatitis A have been convincingly effective in reducing HAV infections. However, in comparison to other childhood vaccinations, the coverage rates for the first and second doses of the vaccine are lower, at approximately 87% and 57%, respectively [18]. Hepatitis B vaccines primarily consist

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of various formulations of the recombinant viral surface antigen (HBsAg). Since 1996, a combined vaccine formulation has been gradually introduced to prevent both HAV and HBV infections, offering a single injection with excellent safety and immunogenicity profiles. This has been well-received in both the USA and Europe [16].

The vaccine design primarily targets the capsid of HAV, which is composed of 60 copies of capsid proteins VP1-VP4 [19]. The VP1 protein's immunodominant role in generating neutralizing antibodies has been recognized [20]. VP1 and VP3 contain sites that appear to interact with neutralizing antibodies, and specific amino acid stretches within these sites may contribute to this interaction [21, 22]. Many efforts have been made to express the HAV-VP1 protein in various host cells, such as plant cells, insect cells, and *E. coli*. Therefore, it is reasonable to harness the potential immunogenicity of VP1 in its recombinant form, which typically requires fewer resources compared to its natural form [23-25]. HAV vaccines are currently developed by generating infectious viruses, a process that is time-consuming [26].

Developing a vaccine based on a virus endemic to a particular region could offer a cost-effective and accessible solution for vaccinating the population. Recombinant protein vaccines are particularly effective in eliciting an immune response against conserved viral epitopes, especially in variable viruses [27, 28].

The latest bivalent vaccines for HAV and HBV combine recombinant HBV and live attenuated HAV vaccines. A bivalent vaccine against HAV and HBV, based on a recombinant subunit vaccine, has not yet been studied.

Therefore, this study aimed to express a novel recombinant fusion protein, which consists of a highly conserved region of HAV-VP1 and full-length HBs-Ag, as a bivalent vaccine candidate against both infections.

MATERIAL AND METHODS

Plasmids and bacterial strain. The Hepatitis, HIV, and Bloodborne Viruses Department of the Pasteur Institute of Iran provided the pET-24a plasmid containing the gene of interest. This gene encodes a truncated HAV-VP1-AAY-HBs-Ag linked to a proteasomal degradation sequence (GCAGCATAC). The truncated VP1gene sequence encodes amino acids 99 to 259, while the HBs-Ag gene sequence encodes the entire length of the protein. Cloning and protein expression were performed in *E. coli DH5α* and *E. coli BL21-DE3* strains.

Expression and purification of fusion protein. The fusion protein was expressed in the prokaryotic system by transforming *BL21-DE3* competent cells with recombinant pET-24a plasmids that contained the gene of interest. The protein expression was induced by adding 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside, Norgen, Canada) to the culture of bacterial mid-log phase at 37 °C. The cell lysis pellet was stored at -20 °C for further purification after incubation for 16 h.

The recombinant protein was purified under native conditions using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen, Germany), following the manufacturer's protocol. Briefly, the cell pellet was made soluble by incubating it in lysis buffer containing 5 mM imidazole for 30 min, followed by sonication (amplitude 60, 15 sec on, 30 sec off). The lysate was subsequently applied to the Ni-NTA column, followed by three washes using 20 mM imidazole. Next, protein purification was performed using an elution buffer that contained 250 mM imidazole. The protein was then purified and subjected to buffer exchange using Amicon Ultra centrifugal filters (Merck Millipore, Germany).

Confirmation of protein expression. To analyze the fusion protein of interest, a 12.5% SDS-PAGE gel was utilized. The gel was then transferred onto nitrocellulose membranes (Sigma, USA) following the manufacturer's protocol. A solution of Ponceau S red (0.3% w/v in trichloroacetic acid) was applied to confirm the transfer. After blocking the membrane with 3% (w/v) BSA in PBS, the membrane was incubated with a 1/2000 (v/v) dilution of an anti-His-tag polyclonal antibody (Abcam, USA) for one hour at room temperature, followed by three washes. In the final step, the membrane was incubated with an appropriate amount of diaminobenzidine tetrahydrochloride (DAB) (Roche, USA). Protein concentration was determined using a combination of methods, including absorbance measurement at 280 nm, a BCA protein assay kit (Pierce, USA), and optical assessment using a NanoDrop spectrophotometer [29].

Diagnostic utility of the recombinant protein. To evaluate the binding ability of the recombinant antigen with associated antibodies, an ELISA assay was performed. Six serum samples positive for HBV and HAV were obtained from commercial kits (Dia. Pro Diagnostic Bioprobes, Italy). In addition, to control for cross-reactivity, confirmed HIV and HCV serum samples were also examined. These samples were obtained from the serum archives of the Department of Hepatitis, HIV, and Bloodborne Viruses at the Pasteur Institute of Iran. Flat-bottomed 96-well polyvinyl chloride plates (Nunc, Denmark) were coated with 100 µl of truncated VP1-HBs-Ag protein (at a concentration of 3 µg/ml) and incubated overnight at 4 °C. Subsequently, 100 µl of each serum sample was added to wells. The plate was then subjected to an hour of incubation at 37 °C, followed by washing and blocking steps. Next, 100 µl of a 1:10,000 dilution of goat anti-human total IgG-HRP antibody (Sigma, USA) was added to each well, and the plates were incubated for one hour. In the final step, 100 µl of tetramethylbenzidine (TMB) (Sigma-Aldrich) was added, and the optical absorbance was then measured at 450 nm.

Immunization of mice with the potential bivalent vaccine. Female BALB/c mice, aged 6-8 weeks, were obtained from the Pasteur Institute of Iran and treated in

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accordance with international animal care ethics standards. The mice were immunized either with or without alum adjuvant via subcutaneous injection into the loose skin over the neck three times at 3-week intervals (at 0, 3, and 6 weeks). Blood samples were collected from the retroorbital sinus of mice at 0, 5, and 8 weeks. The sera were then isolated and stored at -70°C prior to analysis.

Humoral response to HBS and VP1 in mice via indirect ELISA. An indirect ELISA assay was performed to evaluate the antibody responses in the immunized mice. Serial dilutions of serum samples were prepared for each group. Briefly, 100 µl of truncated VP1-HBs-Ag protein (3 µg/ml) was coated onto flat-bottomed 96-well polyvinyl chloride plates (Nunc, Denmark) and incubated overnight at 4 °C. Next, 100 µl of each sample's serum, diluted 1:800, was added to its designated well. The plates were incubated at 37 °C for one hour, followed by washing and blocking. Next, 100 µl of a 1:1000 dilution of goat anti-mouse total IgG-HRP antibody (Sigma, USA) was added to each well, and the plates were further incubated for one hour. Finally, 100 µl of tetramethyl benzidine (TMB) solution (Sigma-Aldrich) was added. The optical absorbance was measured at a wavelength of 450 nm.

Statistical analysis. Statistical analysis was performed to compare the control and test groups using one-way ANOVA (GraphPad Prism, USA). Each sample was analyzed in triplicate, and the error bars on the graph represent the standard deviation (±SD) of each group. *P*-values less than 0.05 were considered statistically significant.

Ethical statement. This study was approved by the Ethics Committee of the Pasteur Institute of Iran under reference number IR.PII.REC.1398.053.

RESULTS

Protein expression and purification. The expression of the truncated VP1-AAY-HBs-Ag fusion protein in the prokaryotic system resulted in the production of a C-terminally His-tagged protein, as depicted in Figure 1. The VP1-AAY-HBs-Ag fusion protein, corresponding to the selected sequence, was observed as a protein of approximately 46 kDa on a 12.5% SDS-PAGE gel.

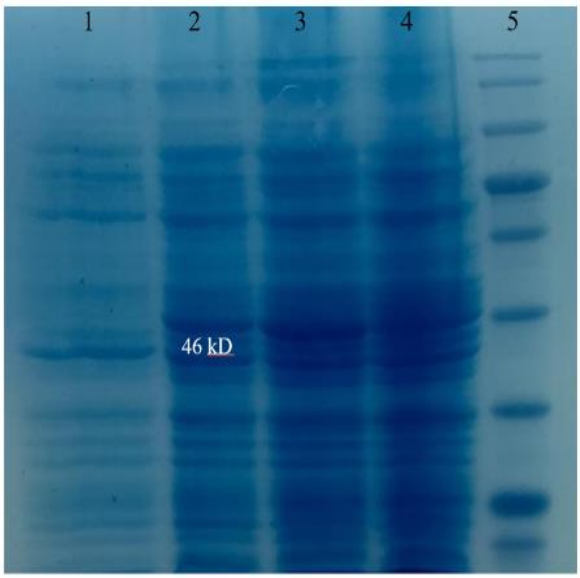


Fig. 1. Analysis of the HAV-1-AAY-HBsAg fusion protein in bacterial samples before and after induction was performed using SDS-PAGE. Lane 1: uninduced bacterial lysate; Lane 2: fusion protein purified from bacterial lysate 3 h after induction; Lane 3: fusion protein purified from bacterial lysate 4 h after induction; Lane 4: fusion protein purified from bacterial lysate 16 h after induction; Lane 5: pre-stained protein ladder (Sinaclon, Iran).

Successful protein purification was achieved under native conditions, resulting in the presence of a distinct 46 kDa protein band, as depicted in Figure 2, corresponding to the SDS-PAGE analysis.

Western blot analysis was conducted using an anti-His-tag polyclonal antibody (Alexis Biochemicals, USA), revealing a distinct protein interaction with the crude lysate (Fig. 3). Using the 280 nm absorbance method and the BCA protein measurement kit, we achieved a purified protein yield of 10 µg/µL.

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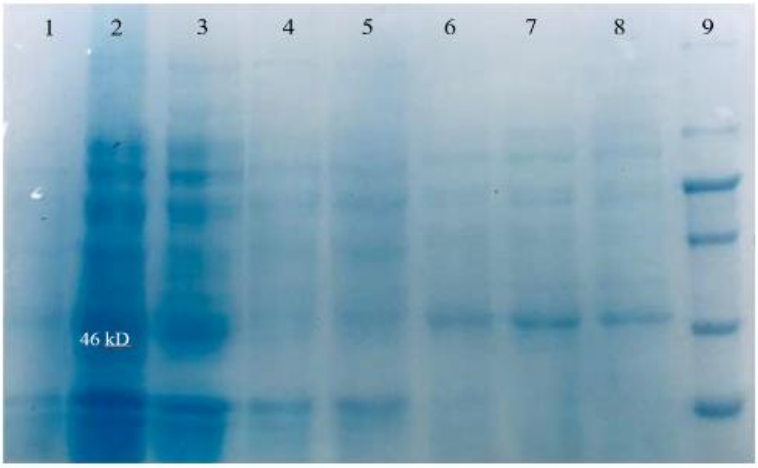


Fig. 2. SDS-PAGE analysis of the purified truncated HAV-1-AAY-HBsAg fusion protein. Lane 1: uninduced bacterial lysate; Lane 2: fusion protein purified from bacterial lysate 4 h after induction; Lane 3: crude lysis sample; Lane 4: flow-through sample; Lane 5: Sample from the wash step; Lanes 6-8: eluted samples; Lane 9: pre-stained protein ladder (Sinaclon, Iran).

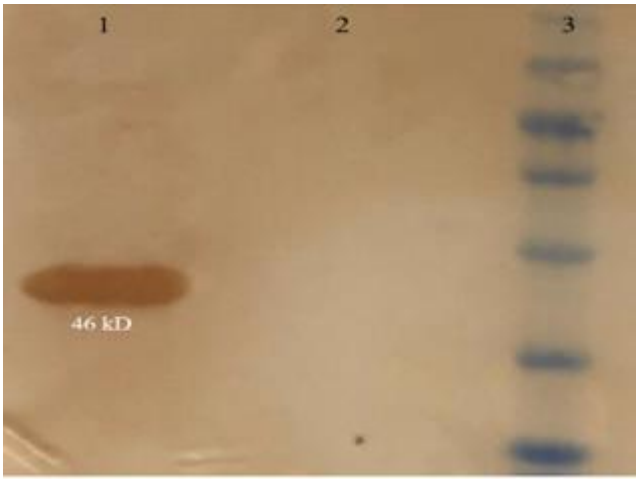


Fig. 3. Western blot analysis of the truncated VP1-AAY-HBs-Ag protein. Lane 1: eluted protein sample; Lane 2: uninduced bacterial sample; Lane 3: pre-stained protein ladder (Sinaclon, Iran).

Detection of anti-HBs-Ag and anti-VP1 antibodies using the fusion protein. Figure 4 illustrates the binding affinity of the recombinant fusion protein with human serum samples from confirmed cases (cut-off:0.27). ELISA testing detected specific binding of the positive HBV/HAV samples (S1-S6). In contrast, the parallel control group comprised HIV- or HCV-positive samples (S11-S14) that showed no reactivity to the coated antigen. Furthermore, the HBV- or HAV-negative controls showed no binding to the antigen, indicating the VP1-AAY-HBs-Ag protein's capability ability to detect specific associated antibodies without cross-reactivity. This feature enables the candidate proteins to be used in immunoassay tests for the concurrent detection of HAV and HBV antigens.

Specific IgG response to the fusion protein in mice. The results for total IgG are presented in Figure 5. Compared to the controls, all mice immunized with the bivalent antigen exhibited a significant IgG response against the truncated VP1+HBs-Ag protein (*P*-value < 0.0001). As depicted in Figure 5A, the mice immunized with the antigen and Alum regimen exhibited the highest IgG titer level. Moreover, the specific antibody titer level in all immunized mice exhibited a rapid increase after the third injection. Furthermore, the same indirect ELISA assay was used to test serial dilutions of the serum from the main group (Truncated VP1+HBs+Ag+Alum Adj.). The results indicated that dilution rates of approximately 1/6400 remained detectable (above the cut-off value) when using 3 µg/ml of the fusion antigen (Fig. 5B).

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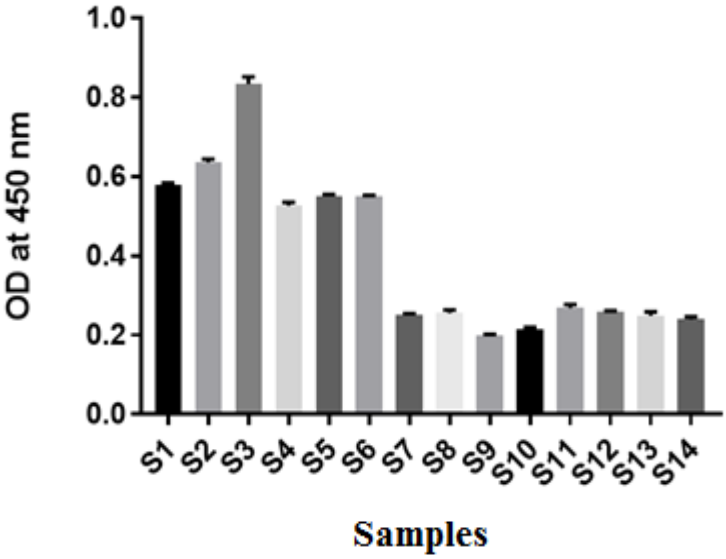


Fig. 4. Assessment of antibody detection potency. S1-S3: HBV-positive samples, S4-S6: HAV-positive samples, S7-S8: HBV-negative samples, S9-S10: HAV-negative samples, S11-S12: HIV-positive samples, S13-S14: HCV-positive samples. All sera were confirmed using appropriate commercial kits.

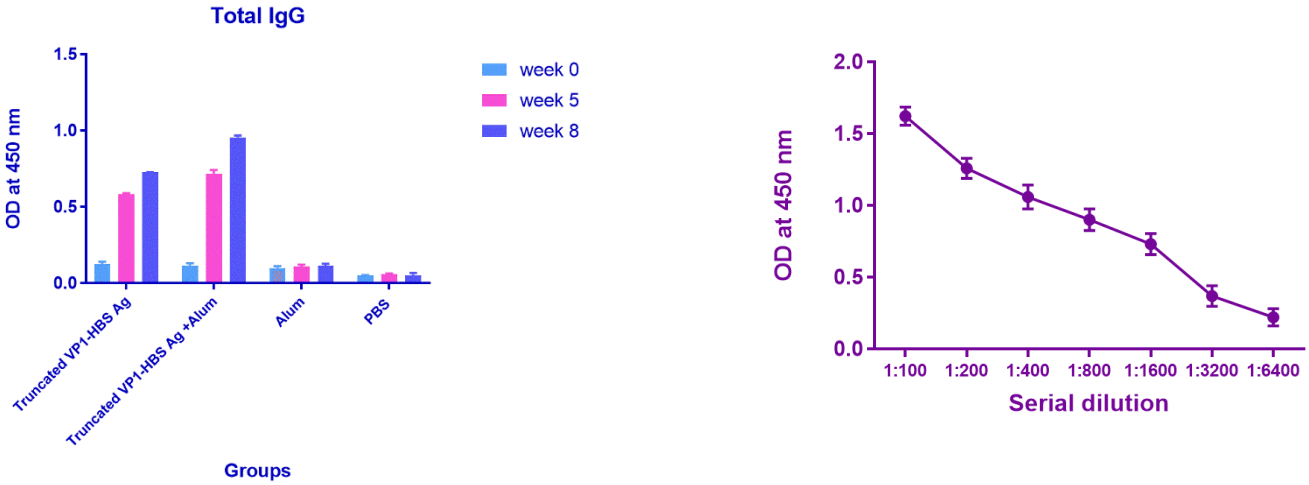


Fig. 5. Analysis of humoral responses. (A) Total IgG levels were determined using a 1:800 dilution of mouse sera. (B) Evaluation of specific antibody titer in different serial serum dilutions of mice immunized with the truncated VP1-HBsAg + Alum regimen. All analyses were performed in triplicate for each sample, and error bars represent the ± standard deviation (SD) within each group. The data are presented as the mean of triplicate measurements ± SD

DISCUSSION

Hepatitis A virus, which causes acute liver inflammation, is typically self-limiting. However, in rare cases, it can progress to liver failure. In many South Asian regions, HAV-related hepatitis was predominantly an asymptomatic childhood illness [30, 31]. However, with the rapid improvement in socio-economic conditions, the disease has transitioned into an epidemic of symptomatic

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infection epidemic among young people. In recent years, there has been a growing risk of outbreaks in China and Europe [32, 33].

Multiple agencies have recommended routine childhood immunization against HAV as a preventive measure to mitigate future outbreaks. Consequently, the number of countries that have included HAV vaccination as part of routine immunization has increased to 34 since 2019. The cost of vaccines remains unaffordable in many developing countries, hindering accessibility. Furthermore, the currently available inactivated viral vaccines (Havrix or Twinrix) depend on the slow-growing HAV culture, which incurs high costs for large-scale purification [25, 33, 34]. Therefore, given these factual limitations, the development of subunit vaccines against hepatitis A is a high priority. This approach offers a fast and cost-effective method, similar to other extensive endeavors against viral infections [35, 36].

In this study, we utilized a prokaryotic system to express a recombinant fusion protein consisting of a highly immunogenic portion of HAV-VP1 (amino acid position 99 to 259) and the full length of HBs-Ag. The fusion protein was connected by a flexible linker (AAY). Our aim was to develop a bivalent subunit vaccine targeting hepatitis A and B. The protein was successfully expressed in *BL21(DE3)* cells, purified, and confirmed to have the predicted size of 46 kDa. The one-step purification method enables us to obtain sufficient amounts of the protein in its native conformation, rendering it suitable for various purposes and potential applications.

The main components in commercial kits used for the diagnosis of HBV and HAV are the Ag protein and VP1 protein, respectively. The ELISA test results demonstrated the detectability of this protein by specific antibodies against HBV and HAV from confirmed cases, without any cross-reactivity (Fig. 4).

Previous studies have identified numerous antigenic domains in HAV. Khudyakov *et al*. (1999) utilized a set of 237 overlapping synthetic peptides, each spanning 20 amino acids, to cover the HAV polyprotein. They then examined serum samples from individuals with acute HAV infection. Among the capsid proteins, the VP1 protein, along with others, was identified as immunodominant in the C-terminal region, encompassing amino acids 767 to 842. The obtained data demonstrated that both structural and nonstructural proteins of HAV contain immunogenic regions that can be effectively represented by short synthetic peptides [37]. In this study, our investigation focused on a truncated form of HAV-VP1, which includes immunodominant human epitopes. The aim was to eliminate highly variable regions of VP1 and develop a chimeric vaccine candidate against HAV and HBV. The results of our study demonstrated that the novel protein, when combined with alum adjuvant, can induce a strong humoral response (Fig. 5).

Junior HC *et al*. (2017) assessed the utility of recombinant VP1 protein as a diagnostic marker for HAV infection. Similar to our study, *E. coli* was used for the expression and purification of the protein of interest. The purified rVP1 was successfully employed in the detection of IgM antibodies in sera obtained from HAV-infected patients via ELISA. The results highlight rVP1 as a reliable antigen for detecting antibodies against HAV [38]. Lee HH *et al*. (2009) used *Drosophila melanogaster* S2 cells to express recombinant VP1. Subsequent Southern blot analysis confirmed the presence of multiple copies of the HAV-VP1 gene in the genome of the transformed cells. The recombinant HAV-VP1 protein, with a molecular weight ranging from 42 to 49 kDa, was secreted into the culture medium at a maximum density of 6.24 mg/liter during the T-flask culture of Drosophila cells. Furthermore, this protein elicited a specific IgG response when administered via the intraperitoneal route and induced IgA secretion in the small intestine through oral immunization. The authors concluded that the secretory form of recombinant VP1, generated by transformed S2 cells, could serve as an effective immunogenic agent for developing a vaccine platform against HAV [23]. In our study, the recombinant truncated VP1-AAY-HBs-Ag, with a molecular weight of 46 kDa, was expressed and purified from *E. coli Bl21-DE3* cells using an Amicon filtration system, resulting in a yield of 10 µg/µl of purified protein.

Chung HY *et al*. (2011) reported on the expression of a chimeric protein, HAV-VP1, fused to an Fc antibody fragment (HAV VP1-Fc) using a replicating vector and infiltrating tobacco plants. The chimeric HAV VP1-Fc protein, with a molecular mass of 68 kDa, was expressed and subsequently purified. The authors demonstrated that the fusion protein induced the production of specific IgG antibodies in the sera through intraperitoneal immunization. Moreover, the secretion of IFN-γ and IL-4 was increased in spleen cells [24].

In another study, recombinant polypeptides, VP1-3N-His (38 kDa), VP1-His (32 kDa), and 3D2-His (30 kDa), were expressed in *E. coli* cells. The proteins were purified using Ni-NTA chromatography. Subsequently, intraperitoneal immunization successfully induced the production of specific IgG antibodies against the polypeptides, while also inducing the secretion of IFN-γ and IL-6 in spleen cells. The sera obtained from mice immunized with the recombinant protein showed neutralization activity against HAV [39].

Developing a recombinant subunit vaccine could offer a more accessible and effective approach to prevent hepatitis A. The HAV capsid protein VP1 has shown promise as an antigen in numerous studies. In this study, we successfully expressed and purified an immunodominant segment of VP1 in conjunction with HBs-Ag using the *E. coli BL21-DE3* system. Furthermore, our initial findings, which demonstrate the detection of this novel fusion protein by anti-HBs and

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HAV antibodies, suggest its significant potential for use in diagnostic assays and as a bivalent vaccine candidate. However, additional biological and immunological assays are underway to confirm its viability.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest are associated with this manuscript.

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