Expression and immunogenic analysis of recombinant polypeptides derived from capsid protein VP1 for developing subunit vaccine material against hepatitis A virus

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ABSTRACT

Three recombinant polypeptides, VP1-His, VP1-3N-His, and 3D2-His, were produced by Escherichia coli expression system. Recombinant VP1-His, VP1-3N-His, and 3D2-His were expressed as bands with molecular weights of 32, 38, and 30 kDa, respectively. These were purified by affinity chromatography using Ni-NTA Fast-flow resin and/or ion-exchange chromatography using DEAE-Sephacel Fast-flow resin. Intraperitoneal immunizations of recombinant polypeptides successfully elicited the productions of VP1-His, VP1-3N-His, and 3D2-His specific IgG antibodies (IgG subclass distribution of IgG1 > IgG2a > IgG2b > IgG3) in sera and induced the secretions of cytokines IFN-γ and IL-6 in spleen cells. Sera from recombinant VP1-His-, VP1-3N-His-, and 3D2-His-immunized mice neutralized the propagation of HAV. The highest neutralizing activity was shown in sera from recombinant VP1-3N-His-immunized mice. These results suggest that recombinant VP1-3N-His can be a useful source for developing hepatitis A virus (HAV) subunit vaccine candidates.

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Introduction

Hepatitis A virus (HAV) causes an acute viral hepatitis disease (hepatitis A) in human. It is classified within the genus hepatovirus of picornavirus family with a single-stranded, positive sense RNA genome coding a single polyprotein that subsequently is processed into structural and nonstructural proteins [1]. The replication of HAV in culture is slow and has poor yields, thus the production of inactivated vaccines is difficult and expensive. Recombinant subunit vaccines, eliciting an efficient immune response, offer the advanta-}

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**Abbreviations used:**
- BSA, bovine serum albumin
- EDTA, ethylenediaminetetraacetic acid
- ELISA, enzyme-linked immunosorbent assay
- FCA, Freund's complete adjuvant
- FIA, Freund's incomplete adjuvant
- FBS, fetal bovine serum
- FIA, Freund's incomplete adjuvant
- HAV, hepatitis A virus
- HRP, horseradish peroxidase
- IFN, interferon
- IgG, immunoglobulin G
- IL, interleukin
- IPTG, isopropyl β-D-thiogalactopyranoside
- LD, Luria–Bertani
- mAbs, monoclonal antibodies
- PBS, phosphate buffered saline
- RIPA, radioimmunoprecipitation assay
- RT-PCR, reverse transcription–polymerase chain reaction
- SDS, sodium dodecyl sulfate
- TMB, 3,3',5,5'-tetramethylbenzidine
- TBE, Tris borate EDTA
- TBE-urea, Tris borate EDTA-urea
- TCE, Tris-citrate-EDTA
block polyclonal human antibodies from binding HAV in competition immunoassays [3,4]. These suggest that recombinant polypeptides, containing a limited number of antigenic epitopes of HAV, can be much useful materials to develop recombinant subunit vaccine candidates.

Therefore, in this work, three recombinant polypeptides including full length VP1, N-terminal VP1 (VP1-N), and D2 domain of HAV were produced by E. coli expression system for developing subunit vaccine materials, containing a limited number of antigenic epitopes of HAV. An immunological assessment via intraperitoneal immunization of mice was performed with purified recombinant polypeptides. The effect of sera, obtained from recombinant polypeptides-immunized mice on the propagation of HAV, was further examined to evaluate neutralization activities of recombinant polypeptides-induced antibodies.

Materials and methods

Construction of expression plasmids

Gene fragment (about 2.95 kb) corresponding to the coding region of HAV P1-2A was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA extracts, isolated from African green monkey kidney B5C-1 cells (Korean Cell Line Bank) infected with HAVstrain HM175/18f (ATCC). The sense primer was 5'-GGATCCGAGAAGCTTATGAGCAGAATTTCCTAGG3' and the antisense primer was 5'-GGCAGGCGCATTTGCTTTAATTTCCTGAACCTCCAGAATC-3'. The amplified HAV P1-2A DNA fragment was cloned into a T/A cloning vector, pGEM-T (Promega). The identity of PCR fragment was confirmed by restriction enzyme mapping and DNA sequence analysis. The pGEM-T/HAV-P1-2A expression plasmid was cloned into E. coli expression system for developing recombinant subunit vaccine materials, containing a limited number of antigenic epitopes of HAV. An immunological assessment via intraperitoneal immunization of mice was performed with purified recombinant polypeptides. The effect of sera, obtained from recombinant polypeptides-immunized mice on the propagation of HAV, was further examined to evaluate neutralization activities of recombinant polypeptides-induced antibodies.

Expression of recombinant VP1-His, VP1-3N-His, and 3D2-His

E. coli BL21 (DE3) cells harboring the expression plasmids, pET-21a/HAV-His, pET-21a/VP1-3N-His or pET-21a/3D2-His, were cultured in Luria–Bertani (LB) broth medium containing 50 μg/ml ampicillin. The expressions of recombinant VP1-His, VP1-3N-His and 3D2-His were induced at an A600 of 0.6 through the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, a final concentration of 1 mM), and the cells were incubated for 3 h at 37°C. The cells were harvested by centrifugation at 3000g for 15 min, resuspended in PBS (pH 7.4) and mixed with 5 × sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) loading buffer. After boiling for 5 min, total protein extracts were collected by centrifugation at 30,000g for 20 min and resolved on SDS polyacrylamide gel. The expressions of recombinant VP1-His, VP1-3N-His and 3D2-His were verified by Coomassie brilliant blue staining and Western blot analysis.

Extraction of soluble proteins for purification experiments

To increase the production of soluble recombinant VP1-His, VP1-3N-His and 3D2-His, the expressions of recombinant VP1-His, VP1-3N-His and 3D2-His were induced at an A600 of 0.6 through the addition of IPTG (a final concentration of 0.1 mM), and the cells were incubated for 18 h at 20°C. The cells were harvested by centrifugation at 3000g for 15 min, resuspended in Tris buffer (50 mM, pH 8.0) for ion-exchange chromatography using DEAE-Sepharose Fast-flow resin (GE Healthcare Life Sciences) or Ni-NTA binding buffer (50 mM Na2HPO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) for affinity chromatography using Ni-NTA Fast-flow resin (Qiagen) and lysed by ultrasonication (Sonics & Materials Inc.). Soluble protein extracts were collected by centrifugation at 30,000g for 30 min and were used in ion-exchange or affinity chromatography.

Ion-exchange chromatography using DEAE-Sepharose Fast-flow resin

Soluble protein extract including recombinant VP1-His or 3D2-His was applied to a column containing DEAE-Sepharose Fast-flow resin previously equilibrated with Tris buffer (50 mM, pH 8.0). The bound proteins were eluted with 1 M NaCl solution. The presences of recombinant VP1-His and 3D2-His were determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) followed by silver staining and Western blot analysis.
Affinity chromatography using Ni-NTA Fast-flow resin

Soluble protein extract or protein mixture obtained from ion-exchange chromatography was applied to a column containing Ni-NTA Fast-flow resin previously equilibrated with Ni-NTA binding buffer. Weakly bound proteins were washed from the resin using the binding buffer, and contaminating proteins bound to the resin were removed by increasing the imidazole concentration to 100 mM. The polyhistidine-tagged recombinant VP1-His, VP1-3N-His, or 3D2-His was finally eluted in the elution buffer containing 300 mM imidazole. Fraction containing recombinant VP1-His, VP1-3N-His, or 3D2-His was dialyzed in PBS (pH 7.4) to remove the imidazole, concentrated with Vivaspin 20 (MW 10,000 Da; Vivascience), and stored at −80°C until use. Protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad) with bovine serum albumin (BSA) as a standard.

Western blot analysis

Protein samples were resolved on 12% (v/v) SDS–polyacrylamide gels and were transferred onto polyvinylidenedifluoride (PVDF) membranes (PALL Life Science). The membranes were pre-incubated with blocking solution [3% (w/v) non-fat dried milk in TBS-T (Tris-buffered saline with 0.1% (v/v) Tween-20)] for 1 h at room temperature, and then incubated overnight at 4°C with mouse anti-His (1:2000 dilution in blocking solution; Santa Cruz Biotech.), and incubated for 2 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:2000 dilution in blocking solution, Santa Cruz Biotech.). After washing with TBS-T buffer, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:2000 dilution in blocking solution, Santa Cruz Biotech.). After washing with TBS-T buffer, the proteins were detected using ECL Pico Western blotting detection reagents (Thermo Fisher Scientific Inc.).

Immunization experiments

Female Balb/c mice purchased from Orient Bio Inc. were provided with water and food ad libitum, and were quarantined in a specific pathogen-free environment with a 12 h light and 12 h dark photoperiod in an animal care facility, accredited by the Kyung Hee University Institutional Animal Care and Use Committee. Five-weeks-old female Balb/c mice were divided into four groups (8 mice per group) and were immunized intraperitoneally with 50 µg of purified recombinant VP1-His, VP1-3N-His, 3D2-His, or PBS (as a control) as a 1:1 emulsion with Freund's adjuvant (FA) 3 times at 2 weeks intervals. Freund's complete adjuvant (FCA) was used in the first immunization and Freund's incomplete adjuvant (FIA) was used for subsequent booster injections. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Kyung Hee University.

Detection of recombinant VP1-His, VP1-3N-His, and 3D2-His-specific antibodies

Blood was collected from the retro-orbital plexus at 1 week after the third immunization. Blood samples were allowed to clot at room temperature for 30 min, and sera were collected by centrifugation at 10,000g for 10 min and were stored at −80°C until use. Recombinant VP1-His, VP1-3N-His, and 3N-His-specific IgG antibodies in sera were analyzed by enzyme-linked immunosorbent assay (ELISA). A 96-well ELISA plate was coated overnight at 4°C with purified recombinant VP1-His, VP1-3N-His, or 3D2-His (0.2 µg/well) in a coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). The plate was washed three times with 200 µl of PBS-T [PBS supplemented with 0.05% (v/v) Tween-20]. Sera were diluted (1:100–1:1,000,000 dilutions in PBS-T), added to each well, and incubated for 1 h at room temperature. After washing with PBS-T, the plate was incubated with 100 µl of HRP-conjugated goat anti-mouse IgG (1:30,000 dilution in PBST; Sigma–Aldrich), IgG1, IgG2a, IgG2b, or IgG3 (all 1:30,000 dilution in PBS-T; Bethyl Laboratories) per well for 1 h at room temperature. The plate was washed and developed for 30 min with 100 µl of TMB One Component HRP Microwell Substrate (3’,5’-tetramethylbenzidine, SurModics) in phosphate-citrate buffer (pH 5.0) containing 0.002% (v/v) hydrogen peroxide. The reaction was stopped by addition of 50 µl of 2 M...
H₂SO₄ to each well. The absorbance was determined at 450 nm using an ELISA reader (Bio-Tek Inc.).

Cross-reactivity of recombinant VP1-His, VP1-3N-His, and 3D2-His-immunized sera to native hepatitis A viral antigen

A 96-well ELISA plate was coated with 100 μl of hepatitis A viral antigen (1 μg/ml of formalin-inactivated HAV; Meridian Life Science) per well in the coating buffer and left overnight at 4 °C. The plate was washed three times with 200 μl of PBS-T. Sera were diluted to 1:100 in PBS-T, added to each well, and incubated for 1 h at room temperature. After washing with PBS-T, the plate was incubated with 100 μl of HRP-conjugated goat anti-mouse IgG (1:30,000 dilution in PBS-T) per well for 1 h at room temperature. The plate was washed and developed according to the procedures described above. Hepatitis A viral antigens (100 ng per well) were resolved on 12% (v/v) SDS–polyacrylamide gels and transferred onto PVDF membranes. The membranes were pre-incubated with blocking solution for 1 h at room temperature, and then were incubated overnight at 4 °C with sera of recombinant VP1-His, VP1-3N-His, and 3D2-His-immunized mice (1:2,000 dilution in blocking solution). The membranes were washed three times with TBS-T and incubated for 2 h with HRP-conjugated anti-mouse IgG (1:10,000 dilution in blocking solution). After washing with TBS-T, protein bands were detected using ECL Pico Western blotting detection reagents.

Splenic cytology and cytokine detection

Spleens were surgically removed and gently crushed using the plunger of a disposable syringe on a Falcon cell strainer (BD Biosciences). The splenic stroma was washed with 200 μl of PBS and incubated for 2 min at 37 °C. After washing with PBS-T, the plate was incubated with 100 μl of HRP-conjugated anti-mouse IgG (1:1,000,000 dilution in PBST; Mediagnost) for 1 h at room temperature. The plate was washed and developed according to the procedures described above. Hepatitis A viral antigens (100 ng per well) were resolved on 12% (v/v) SDS–polyacrylamide gels and transferred onto PVDF membranes. The membranes were pre-incubated with blocking solution for 1 h at room temperature, and then were incubated overnight at 4 °C with sera of recombinant VP1-His, VP1-3N-His, and 3D2-His-immunized mice (1:2,000 dilution in blocking solution). The membranes were washed three times with TBS-T and incubated for 2 h with HRP-conjugated anti-mouse IgG (1:10,000 dilution in blocking solution). After washing with TBS-T, protein bands were detected using ECL Pico Western blotting detection reagents.

Neutralization assay

BS-C-1 cells were seeded in 6 well culture plate at a density of 5 × 10⁶ cells per well, and were cultured to 70–80% confluence. All sera in a group were pooled, and 5 μl of pooled sera or 1 μg of anti-HAV IgG (M40; Mediagnost) were mixed with 500 μl of HAV (HM175/18F strain). The mixtures of sera (or anti-HAV IgG) and HAV were incubated for 1 h at room temperature and were added to BS-C-1 cells after removal of culture medium. The cells were incubated for 1 h at 37 °C in a humidified incubator with 5% CO₂. The media was replaced with fresh RPMI-1640 containing 2% FBS. The HAV-infected cells were incubated for 5 days longer. After three freeze-thaw cycles of the cultures, the medium fraction containing HAV was separated by centrifugation at 10,000g for 10 min. The contents of HAV were determined by ELISA. A 96-well ELISA plates were coated with 100 μl of anti-HAV IgG (M40) (1 μg/ml) per well in the coating buffer and were left overnight at 4 °C. After washing with PBS-T, the plate was incubated with 100 μl of HAV-cultured media (or normal BS-C-1 cultured media as a control) per well for 1 h at room temperature. The plates were washed with PBS-T and incubated with HRP-conjugated anti-HAV IgG (1:1,000,000 dilution in PBST; Mediagnost) for 1 h at room temperature. The plates were washed and developed according to the procedures described above.

Statistical analysis

All data are presented as mean ± SE. Student’s t test was used to compare recombinant VP1-His, VP1-3N-His, or 3D2-His-immunized groups and PBS-immunized control groups (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Expression of recombinant VP1-His, VP1-3N-His, and 3D2-His

Full length VP1, VP1-3N (three copies of VP1-N connected by GGGGS linker), and 3D2 (three copies of D2 connected by GGGGS linker) were respectively cloned into E. coli expression vector, pET-28a (Fig. 1). VP1-N corresponds to N-terminal 100 amino acid residues of VP1, whereas D2 domain corresponds to C-terminal 25 amino acid residues of VP1 and N-terminal 51 amino acid residues of 2A. Three copies of VP1-N and D2 were chosen to produce recombinant VP1-3N and 3D2 with similar molecular mass of full length VP1. Polymidine epitope was C-terminally fused to detect and purify recombinant polypeptides. Total protein extracts were collected from E. coli BL21 (DE3) cells harboring pET-21a/VP1-His, pET-21a/VP1-3N-His, and pET-21a/3D2-His and the expression of recombinant VP1-His, VP1-3N-His, and 3D2-His were determined by SDS–PAGE followed by Coomassie brilliant blue staining and Western blot analysis using anti-His (Fig. 2A and B). Recombinant VP1-His, VP1-3N-His, and 3D2-His were expressed as single bands in total protein extracts of E. coli BL21 (DE3) cells harboring pET-21a/VP1-His, pET-21a/VP1-3N-His, and pET-21a/3D2-His. Molecular masses of recombinant VP1-His, VP1-3N-His, and 3D2-His were 32, 38, and 30 kDa, respectively.

Purification of recombinant VP1-His, VP1-3N-His, and 3D2-His

Polymidine-tagged recombinant VP1-His, VP1-3N-His, and 3D2-His were purified from soluble protein extracts collected from E. coli BL21 (DE3) cells, harboring expression plasmids, by a simple one-step Ni-NTA affinity chromatography. Recombinant VP1-3N-His was successfully purified after elution with buffer containing 300 mM imidazole. However, the elution fractions of recombinant VP1-His and 3D2-His contained a few contaminated proteins (data not shown). To increase the purity of the purified recombinant VP1-His and 3D2-His, the two step purification method was employed. The first step was a partial purification of recombinant VP1-His and 3D2-His, by ion-exchange chromatography, using an anion-exchanger DEAE-Sepharose Fast-flow resin. It was followed by the second step of affinity chromatography using Ni-NTA Fast-flow resin. In the first step of purification, recombinant VP1-His and 3D2-His did not bind to DEAE-Sepharose Fast-flow resin, equilibrated with 50 mM Tris buffer (pH 8), and passed through the column (data not shown). Recombinant VP1-His and 3D2-His with negligible contaminants could be successfully purified by Ni-NTA affinity chromatography of the flow-through fractions, containing partially purified recombinant VP1-His and 3D2-His. The purities and identities of the purified proteins were verified with SDS–PAGE followed by silver staining and Western blot analysis using anti-His (Fig. 3A and B).
Antigenicity analysis of recombinant VP1-His, VP1-3N-His, and 3D2-His

To evaluate the antigenic activities of recombinant VP1-His, VP1-3N-His, and 3D2-His, Balb/c mice were intraperitoneally immunized with purified recombinant VP1-His, VP1-3N-His, 3D2-His, or PBS (as a control). The presences of recombinant VP1-His, VP1-3N-His, and 3D2-His-specific IgG antibodies in sera were determined by ELISA. Absorbance values of recombinant VP1-His, VP1-3N-His, and 3D2-His-immunized sera were not increased in PBS-coated ELISA plates (Fig. 4A). When sera were applied to the recombinant VP1-His-coated ELISA plates, absorbance values of recombinant VP1-His-, VP1-3N-His-, and 3D2-His-immunized sera were significantly greater than those of the PBS-immunized control (Fig. 4B). These are almost constant in sera of recombinant VP1-His-, VP1-3N-His-, and 3D2-His-immunized mice. Recombinant VP1-His, VP1-3N-His, and 3D2-His-specific IgG antibodies in sera were also determined in recombinant VP1-3N-His- and 3D2-His-immunized sera (Fig. 4C and D). Sera of recombinant VP1-His and VP1-3N-His-immunized mice resulted in higher absorbance values in recombinant VP1-3N-His-coated ELISA plates, compared to sera of recombinant 3D2-His- or PBS-immunized mice (Fig. 4C). In ELISA plates coated with recombinant 3D2-His, sera of recombinant VP1-His-immunized mice showed higher absorbance values, compared to sera of recombinant VP1-His- and VP1-3N-His-immunized mice (Fig. 4D). The IgG subclass distributions of recombinant VP1-His-, VP1-3N-His-, and 3D2-His-specific antibodies were also investigated by ELISA. Predominant IgG subclass elicited by the intraperitoneal immunizations of recombinant VP1-His-, VP1-3N-His-, and 3D2-His was IgG1 (Fig. 5). The IgG subclass distribution of recombinant VP1-His-, VP1-3N-His-, and 3D2-His-specific antibodies was IgG1 > IgG2a > IgG2b > IgG3.

Cross-reactivity of recombinant VP1-His, VP1-3N-His, and 3D2-His-immunized sera to native hepatitis A viral antigen

Cross-reactivity of the antibodies, after intraperitoneal immunization with recombinant VP1-His, VP1-3N-His, and 3D2-His in mice against hepatitis A viral antigen, was investigated by ELISA. When sera at 1:100 dilution were applied to wells coated with 0.1 μg of hepatitis A viral antigens (Meridian Life Science), the mean ELISA values were 0.35, 0.32, 0.14, and 0.14, respectively, for recombinant VP1-His-, VP1-3N-His-, and 3D2-His-immunized mouse groups (Fig. 6A). Cross-reactivity of the antibodies against hepatitis A viral antigen was further determined by Western blot analysis. As shown in Fig. 6B, a 33 kDa band corresponding to a band for HAV VP1 was detected in Western blot analysis using sera of recombinant VP1-His- and VP1-3N-His-immunized mice. Under the same conditions of Western blot analysis, sera of PBS- and recombinant 3D2-His-immunized mice did not detect the hepatitis A viral antigens.
Cytokine IFN-γ and IL-6 analysis in splenocyte culture media

Splenocytes were prepared from recombinant VP1-His-, VP1-3N-His-, and 3D2-His-immunized mice and were cultured in the presence of each antigen (recombinant VP1-His, VP1-3N-His, and 3D2-His). The synthesis of IFN-γ and IL-6 was examined by ELISA using splenocyte culture media. As shown in Fig. 7A, splenocytes that were isolated from mice immunized with recombinant VP1-His, VP1-3N-His, and 3D2-His and that were cultured in the presence of the each antigen, secreted large amounts of IFN-γ, compared to splenocytes isolated from PBS-immunized mice and cultured in the presence of PBS. When splenocytes from mice immunized with recombinant VP1-His, VP1-3N-His, and 3D2-His were cultured with the each antigen, the productions of IL-6 were also increased (Fig. 7B).

Neutralization activity of recombinant VP1-His, VP1-3N-His, and 3D2-His-immunized sera

To determine the neutralizing activities of recombinant VP1-His-, VP1-3N-His-, and 3D2-His-immunized sera, HAVs pre-incubated with sera were infected to BS-C-1 cells. The propagated HAV was determined by ELISA as described in Materials and methods. Pre-incubations with sera of recombinant VP1-His-, VP1-3N-His-, and 3D2-His-immunized mice reduced the propagations of HAV by 17.9, 36.7, and 22.5%, respectively, compared to PBS-immunized sera (Fig. 8). This means that intraperitoneal immunizations of recombinant VP1-His, VP1-3N-His, and 3D2-His induced the production of specific antibodies to neutralize the propagation of HAV.

Discussion

In the previous experiments to produce recombinant polypeptides containing a limited number of antigenic epitopes that induce HAV neutralizing antibodies, the N-terminal, middle, and C-terminal regions of immunodominant capsid protein VP1 were separately expressed by E. coli expression system. N-terminal region of VP1 was successfully expressed in E. coli cells. However, the expressions of middle and C-terminal regions of VP1 were not observed (data not shown). HAV D2 domain, corresponding to C-terminal 25 amino acid residues of VP1 and N-terminal 51 amino acid residues of 2A, has been suggested to induce a virus-neutralizing antibody response [13]. Peptides derived from the N-terminus of VP1 induced HAV neutralizing antibodies [6,7]. The N-terminus of VP1 has been suggested to be the secondary neutralization site [14]. Here, we produced three recombinant polypeptides (VP1-His, VP1-3N-His, and 3D2-His) derived from HAV VP1 via E. coli expression system. Generally, there is a correlation between the size of antigen and its immunogenicity. The antigen of molecular mass greater than 100 kDa is the potent immunogen, whereas the antigen of molecular mass less than 10 kDa can be a poor immunogen [15]. As produced, the molecular masses of antigenic peptides VP1-N and D2 were in the range of 10 kDa. In an
attempt to address its immunogenicity, we have constructed a larger antigenic peptide using tandem repeats of antigenic peptides VP1-N and D2. The tandem repeat constructs consisting of two or three copies of an antigenic peptide originated from the antigen of *Streptococcus* species have been reported to successfully enhance the immunogenicity, compared to a single copy of peptide [16,17]. We previously reported a strategy for the production of VP1 vaccine antigens against HAV using insect cell and plant system [9,10]. In this study, we attempted to examine the efficiencies of VP1-N and D2, compared to the control immunogen VP1. Thus, in the construction of a larger antigenic peptide using tandem repeats of antigenic peptides VP1-N and D2, the molecular mass of the constructed peptides needs to be in the range of the size of VP1. Therefore, a triplet repeat construct for VP1-N and D2 was chosen to produce the optimal peptide size among the 2–4 repeat constructs.

Polyhistidine-tagged recombinant VP1-His was successfully expressed as a band with a molecular mass of ~32 kDa (Fig. 2). Three copies of N-terminal region of VP1 (corresponding to N-terminal 100 amino acid residues of VP1) and D2 domain were respectively connected by GGGS linker (composed of four glycines and one serine) to facilitate the proper folding of the inserted polypeptides as independent domains [18]. Polyhistidine-tagged recombinant VP1-3N-His and 3D2-His were expressed as bands with molecular mass of 38 and 30 kDa, respectively. These molecular masses agree with the predicted molecular masses of recombinant polypeptides (http://web.expasy.org).

Polyhistidine-tagged recombinant VP1-3N-His was successfully purified from the soluble protein extract of *E. coli* BL21 (DE3) cells, harboring pET-21a/VP1-3N-His, by one-step affinity chromatography using Ni-NTA Fast-flow resin. However, recombinant VP1-His and 3D2-His with high purities could not be obtained in this one-step affinity chromatography. To purify recombinant VP1-His and 3D2-His, we performed two step purification methods, which are ion-exchange chromatography followed by Ni-NTA affinity chromatography. Recombinant VP1-His and 3D2-His with negligible contaminants could be purified by these two step purification methods. Approximately, 1.8, 23.8, and 5.5 mg of recombinant VP1-His, VP1-3N-His, and 3D2-His were respectively purified from the soluble proteins of 1 liter culture (data not shown).

The immunogenicity of recombinant VP1-His, VP1-3N-His, and 3D2-His derived from *E. coli* cells was examined in animal experiments. The intraperitoneal immunization of recombinant VP1-His, VP1-3N-His, and 3D2-His induced the productions of IgG antibodies. The intraperitoneal immunization of recombinant VP1-His, VP1-3N-His, and 3D2-His induced the productions of IgG antibodies to detect VP1-His antigen with similar efficiencies. However, these showed different binding efficiencies against VP1-3N-His and 3D2-His antigen, due to differences in antigenic epitopes. The IgG subclass distribution was IgG1 > IgG2b > IgG2a > IgG3. Taken together, these results indicate that intraperitoneal immunizations of recombinant VP1-His, VP1-3N-His, and 3D2-His induced the generation of specific IgG antibodies in sera of mice showing different efficiencies. The cross-activities of the

![Fig. 5. IgG subclass analysis in sera from mice intraperitoneally immunized with recombinant VP1-His, VP1-3N-His, and VP1-3D2-His. Sera collected at 1 week after the third immunization were diluted 1:100 and incubated in purified recombinant VP1-His-coated plate. After probing with peroxidase-conjugated goat anti-mouse IgG1 (A), IgG2a (B), IgG2b (C) or IgG3 (D), the absorbance was determined at 450 nm using an ELISA reader. The results from triplicate assays for all 8 mice per group are presented as mean ± SE (**p < 0.01, ***p < 0.001).](http://www.koang.net/proteinexpressionandpurification.com/100/1-9)
antibodies, developed in intraperitoneally immunized mice against hepatitis A viral antigen (Meridian Life Science), were further examined. In ELISA and Western blot analysis, the antibody contents recognizing hepatitis A viral antigen, especially VP1, were higher in serum samples of recombinant VP1-His- and VP1-3N-His-immunized mice, compared to serum samples of recombinant 3D2-His-immunized mice. This strongly indicates that antibodies, produced by the intraperitoneal immunizations of recombinant VP1-His and VP1-3N-His, efficiently recognize epitopes presented in target hepatitis A viral proteins, compared to the recombinant 3D2-His-induced antibodies.

Recombinant VP1-His, VP1-3N-His, and 3D2-His promotes the expression of Th1-type cytokine, IFN-γ. The Th1 response characterized by the production of IFN-γ leads to a cytotoxic T cell response and virus clearance. IFN-γ activates the bactericidal activities of macrophages, induces B cells to make coating and complement-fixing antibodies, and leads to the cell-mediated immunity [19]. The Th2 response is characterized by the release of IL-6 and other cytokines, which is associated with IgG and IgE responses. In this study, IFN-γ and IL-6 cytokine analysis in the culture media of splenocytes collected from mice intraperitoneally immunized with recombinant VP1-His, VP1-3N-His, and VP1-3D2-His. Splenocytes were collected from spleen and cultured in the presence of the recombinant VP1-His, VP1-3N-His, and 3D2-His (or PBS as a control). After 48 or 72 h, the culture media were collected and the levels of IFN-γ (A) and IL-6 (B) were measured using mice cytokine ELISA kits. Data are shown as mean ± SE of triplicate assays with splenocytes polled from all eight mice per group (\( ^\star p < 0.05, ^\star\star p < 0.01, ^\star\star\star p < 0.001 \)).
of IL-4 and IL-6, which results in the activation of B cells to make neutralizing non-cytolytic antibodies, leading to humoral immunity. Generally, Th1 responses are more effective against intracellular pathogens (viruses and bacteria that are inside host cells), while Th2 responses are more effective against the extracellular bacteria, parasites, and toxins. Our results also show that recombinant VP1-His, VP1-3N-his, and 3D2-His induce a typical Th2 response in mice. Although cell-mediated immunity (Th1 response) is known to be responsible for controlling intracellular infections such as HAV infection, the humoral immunity (Th2 response) is essential for controlling the extracellular infections. Therefore, co-stimulation of both cellular and humoral immunity has been proposed as necessary for the host to control infections [20]. Hence, the induction of both cellular and humoral immunity of our recombinant polypeptides can be a useful feature for vaccine candidate targeted against HAV, as suggested in the immunological analysis of recombinant VP1 produced from plant expression system [10].

Previously, 42 antigenic peptides were identified across the HAV polyprotein, by using a total of 237 synthetic peptides spanning the entire polyprotein and a panel of serum samples from acutely HAV-infected patients [13]. Also, D2 domain fused to rotavirus VP7 (D2/VP7) has been proved to be immunogenic and to elicit neutralizing antibody response against HAV [21]. Our results showed that recombinant VP1-His, VP1-3N-His, and 3D2-His can induce the neutralizing antibody response to inhibit the propagation of HAV. Interestingly, the neutralizing antibody response was the highest in sera of recombinant VP1-3N-His-immunized mice. This agrees with other suggestion that the antigenic sites responsible for inducing neutralizing antibodies are closer to the N-terminal end of VP1 [7].

In summary, we report a strategy for the production of more efficient recombinant subunit vaccine materials against HAV. The three recombinant polypeptides, VP1-His, VP1-3N-His, and 3D2-His, were produced by E. coli expression system. Intraperitoneal immunizations of recombinant VP1-His, VP1-3N-His, and 3D2-His successfully elicited the productions of VP1-His, VP1-3N-His, and 3D2-His specific IgG in sera. The recombinant VP1-3N-His, and 3D2-His induced both the cellular and humoral immunities. Sera of recombinant VP1-His, VP1-3N-His, and 3D2-His-immunized mice contained the neutralizing activity to inhibit the propagation of HAV. The highest neutralizing activity was observed in sera of recombinant VP1-3N-His-immunized mice. Our results suggest that recombinant VP1-3N-His can be a useful source for developing HAV subunit vaccine candidate, which contains a limited number of antigenic epitopes that induce the generation of HAV-neutralizing antibodies.

Acknowledgments

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ009526), Rural Development Administration, Republic of Korea.

References