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Cloning, expression, and purification of recombinant bovine rotavirus hemagglutinin, VP8*, in *Escherichia coli*

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Abstract

Rotavirus VP8* subunit is the minor trypsin cleavage product of the spike protein VP4, which is the major determinant of the viral infectivity and neutralization. To study the structure–function relationship of this fragment and to obtain type-specific reagents, substantial amounts of this protein are needed. Thus, full-length VP8* cDNA, including the entire trypsin cleavage-encoding region in gene 4, was synthesized and amplified by RT-PCR from total RNA purified from bovine rotavirus strain C486 propagated in MA104 cell culture. The extended VP8* cDNA (VP8ext) was cloned into the pGEM-T Easy plasmid and subcloned into the *Escherichia coli* expression plasmid pET28a(+). The correspondent 30 kDa protein was overexpressed in *E. coli* BL21(DE3)pLysS cells under the control of the T7 promoter. The identity and the antigenicity of VP8ext were confirmed on Western blots using anti-His and anti-rotavirus antibodies. Immobilized Ni-ion affinity chromatography was used to purify the expressed protein resulting in a yield of 4 mg of VP8ext per liter of induced *E. coli* culture. Our results indicate that VP8ext maintained its native antigenicity and specificity, providing a good source of antigen for the production of P type-specific immune reagents. Detailed structural analysis of pure recombinant VP8 subunit should allow a better understanding of its role in cell attachment and rotavirus tropism. Application of similar procedure to distinct rotavirus P serotypes should provide valuable P serotype-specific immune reagents for rotavirus diagnostics and epidemiologic surveys.
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Rotaviruses are the leading cause of morbidity and mortality due to acute gastroenteritis in infants [1]. Rotavirus is also an important veterinary pathogen, causing disease in calves, sheep, swine, and poultry [2]. Because of the significant burden of rotavirus disease among children and animals worldwide, considerable efforts have been devoted towards the development of vaccines for disease prevention and control. Nevertheless, host susceptibility and immunity to severe rotavirus-induced diarrhea are not fully understood. The role of homotypic and heterotypic immune responses in protection against disease is still controversial despite its relevance to vaccine development. The production of individual rotavirus proteins or protein subunits to study their molecular structures and probable functions during virus infection is an alternative approach to address these questions.

Rotaviruses belong to the Reoviridae family and are composed of a genome of 11 segments of double-stranded RNA surrounded by three concentric layers of protein. Two proteins, VP7 and VP4, which independently, induce neutralizing antibodies to the virus, form the outermost layer [3]. VP7, a glycoprotein with a molecular mass of 34 kDa, forms the smooth external surface of the outer shell and determines the G serotype specificity of the virus. The other protein, VP4, is present as a series of 60 dimeric spikes, 10–12 nm in length, with a knob-like structure at the distal end, which projects outward from the VP7 shell [4].

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The viral spike, VP4, is nonglycosylated, has a molecular mass of 88 kDa, constitutes 1.5% of the virus protein, and determines the virus P serotype [5]. VP4 has been implicated in several important functions, including attachment to cellular receptors, cell penetration, hemagglutination, virulence, and neutralization [3,6].

Trypsin cleavage of VP4 produces two virion-associated fragments, VP8* (the 28 kDa N-terminal region), which has the hemagglutination domain [7] and VP5* (the 60 kDa C-terminal region), which permeabilizes membranes [8,9]. Proteolytic cleavage stabilizes the spike ensemble, confers icosahedral ordering on the VP4, and strongly enhances rotavirus infectivity, probably by enhancing virus penetration of the cell [10].

Neutralizing monoclonal antibodies (NmAbs)[1] directed to either the amino or carboxyl tryptic fragments (VP8* or VP5*, respectively) of VP4 can inhibit virus attachment to cells and neutralize the virus in vitro [11,12]. Studies with animal and human rotavirus have identified eight neutralization epitopes on VP4, with five of them located on the VP8* region [13,14]. Analysis of the neutralization escape mutants selected with NmAbs suggested that rotavirus strain-specific epitopes, or perhaps limited heterotypic neutralization epitopes, are localized in the VP8* subunit of VP4 [13,15]. These findings indicated that VP8* is the main determinant of rotavirus P serotypes. However, lack of proper polyclonal or monoclonal antibodies owing to the extremely low abundance of VP4 in the virion hinder the characterization of rotavirus into P serotypes. Only the prototype strains, or a few unusual strains suspected to represent new P serotypes, have been characterized by the labor intensive plaque reduction neutralization (PRN) technique. The vast majority of human and animal rotavirus strains have been characterized by molecular methods such as nucleic acid sequencing, hybridization or amplification techniques, and in particular, by convenient PCR genotyping assays [16,17]. Although genotyping has been a reliable surrogate for serotyping, with proven full agreement for the VP7, or G, specificity [18,19], validation for the VP4, or P specificity awaits proper P-specific reagents. Meanwhile, two awkward and confusing classifications for P serotype and P [genotype] have been adopted [3,19]. Strains presenting <8-fold titer difference in PRN assay with polyclonal sera, or >89% amino acid homology of their VP4 proteins are classified into the same P serotype or P [genotype], respectively. The availability of purified VP8* protein would allow the production of serotype-specific polyclonal sera against spike proteins belonging to different P types. Those antisera would constitute the key ingredients for a reliable and universally acceptable ELISA for P serotyping of rotavirus field strains.

The limited amount of VP4 in the native viral particles or produced during replication also makes it difficult to obtain enough VP8* for structural and functional analysis by virus propagation and purification from infected cell cultures. Gene cloning and expression in heterologous hosts is one useful approach for obtaining large quantities of individual proteins for such studies. In this work, we attempted to produce high quantity of recombinant bovine rotavirus VP8* in *Escherichia coli* for such future studies.

Materials and methods

Strains, plasmids, and enzymes

The *E. coli* strains TOP10 and BL21(DE3)pLysS were used for cloning experiments and protein expression, respectively. Both strains were purchased from Invitrogen. The plasmid pGEM-T Easy vector was used for cloning and amplification of VP8ext cDNA and the plasmid pET28a(+) was used for protein expression. The plasmids were from Promega and Novagen, respectively. Restriction enzymes, *Taq* DNA polymerase, and T4 ligase were purchased from Promega and used according to supplier's recommendations.

Chemicals

Oligonucleotides were purchased from HWNN Research (IMPRINT/Brazil). The Super Script TM One-Step RT-PCR System with Platinum *Taq* DNA polymerase kit was from Invitrogen. Wizard SV Miniprep DNA Purification System was from Promega. Luria–Bertani (LB) broth media and NBT (Nitroblue Tetrazolium)-BCIP (5-bromo-4-chloro-3-indolyl phosphate) solutions were from Life Technologies. $NiSO_4$, Triton X-100, isopropyl-1-thio-β-D-galactoside (IPTG), alkaline phosphatase conjugated goat anti-mouse or anti-rabbit IgG, kanamycin, and ampicilin were from Sigma–Aldrich. GFX, PCR, DNA, and Gel band Purification and Chelating Sepharose Fast Flow resin were from Amersham Biotech. Monoclonal anti-His-Tag antibody was purchased from Novagen. Rabbit anti-rotavirus antibody was produced as previously described [20].

Virus and viral RNA

The G6P6[1] bovine rotavirus strain C486 was propagated in MA104, a fetal monkey kidney cell line, in the presence of 1 μg/ml trypsin, at 36 °C for 1 to 2 days. The cell debris was removed by low speed centrifugation and total virus RNA was purified from culture supernatant by adsorption to hydroxyapatite (HA) [21]. In brief, 600 μl of 6 M guanidine isothiocyanate (GITC) solution was added to 400 μl of the culture supernatant and homogenized. Then, 50 μl (two drops) of HA suspension was added and the tube was inverted or placed in a shaker for 20 min at

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[1] *Abbreviations used:* NmAbs, neutralizing monoclonal antibodies; PRN, plaque reduction neutralization; NBT, nitroblue tetrazolium; IPTG, isopropyl-1-thio-β-D-galactoside; HA, hydroxyapatite; GITC, guanidine isothiocyanate; PMSF, phenylmethylsulfonyl fluoride; IMAC, immobilized metal-ion affinity chromatography; MBP, maltose-binding protein; TMV, tobacco mosaic virus.

room temperature for nucleic acid adsorption to HA. The tube was centrifuged at 13,000*g* for 1 min, and the fluid was discarded. The complex RNA-HA crystals were washed twice with 1 ml of 10 mM potassium phosphate (KP), pH 6.8, and centrifuged at 13,000*g* for 1 min. The RNA was then eluted from the HA crystals with 40 µl of 200 mM KP, pH 6.8, and incubated at 56 °C for 5 min. The HA-free RNA solution was collected by centrifugation at 13,000*g* for 3 min and used immediately in the RT-PCR assay or stored at −20 °C.

Reverse transcriptase-polymerase chain reaction and cDNA cloning of VP8*

Primers were designed to copy and amplify an extended portion of the VP4 gene that codes for VP8* of strain C486 bovine rotavirus. The sense primer BpET8: 5′-TTA<u>CATA TGG</u>CTTCGCTCATTTATAG-3′ corresponds to nucleotides 10–29 of gene 4, starting at the initial codon ATG of the open-reading frame for VP4, and contains an *Nde*I restriction site (underlined) at the 5′-end to allow in-frame cloning into the expression vector pET28a(+) for *E. coli*. The anti-sense primer EpET8: 5′-CC<u>GGATCC</u>*CTA*GTC TTCATTAACTTGTGCTC-3′ was designed to include the trypsin cleavage-encoding region. It is complementary to nucleotides 768–749 on gene 4, and contains a *Bam*HI restriction site (underlined). A stop codon (italics) was added between the two sequences to terminate the template 18 nucleotides into the VP5*-encoding region. In this manner, reverse transcription of the fourth genomic segment of C486 with primer EpET8, followed by the polymerase chain reaction (PCR) with both primers would generate a cDNA that contains the coding region for full-length VP8* including the 16 amino acid residues that are removed upon trypsin cleavage, and the six first residues at the N-terminus of VP5* (Fig. 1).

The RT-PCR was performed as previously described [18] using Super Script TM One-Step RT-PCR System with Platinum *Taq* DNA polymerase kit. Briefly, purified RNA (8 µl) was added to a 0.2-ml tube containing 4 µl dimethyl sulfoxide (DMSO), 2 µl of a 20 mM solution of each primer BpET8 and EpET8, heated at 94 °C for 2 min and quenched in an ice-bath. Then, 86 µl of a reaction mixture containing buffer solution, 0.2 mM each dNTP, 1.5 mM MgCl$_2$, and 2 µl of the reverse transcriptase and *Taq* enzyme mixture

were added. The tube was placed in a thermocycler (Applied Biosystems 2400) programmed for a 60 min incubation at 42 °C, followed by 35 cycles of PCR (94 °C for 30 s, 42 °C for 50 s, and 72 °C for 70 s) and a final 7 min incubation at 72 °C.

The amplicon obtained was verified by agarose gel electrophoresis and purified on a GFX column. It was introduced into the pGEM-T Easy vector by using direct ligation of its 5′-T termination to the 3′A-hangover on the amplicon added by the *Taq* polymerase during template amplification. The resulting plasmid, named pGEM-T/ VP8ext, was used to transform competent *E. coli* TOP10 cells. The recombinant plasmid DNA was purified using the Wizard SV Miniprep DNA Purification System from a positive transformed colony. It was analyzed and quantified in agarose gel after electrophoresis and comparison with standard molecular weight marker.

Construction of VP8ext expression plasmid

The extended VP8* insert was isolated from 10 µg of the pGEM-T/VP8ext plasmid by digestion with *Nde*I and *Bam*HI restriction enzymes and ligated, overnight at 16 °C through a standard T4 DNA ligase procedure, into the *Nde*I/*Bam*HI-digested pET28a(+) vector, to construct the pET28a/VP8ext expression plasmid in *E. coli*. This construct has the hexa-histidine tag (6× His-Tag) and the thrombin recognition site in frame with the 5′ end of the extended VP8* insert. It was used to transform competent *E. coli* TOP10 cells. The clones were selected in LB plates supplemented with 50 µg/ml of kanamycin and were screened by digestion of Miniprep plasmids with *Nde*I and *Bam*HI. Cells were lysed by boiling for 2 min, and analyzed by PCR carried out with a plasmid primer (T7 promoter or T7 terminator) and a rotavirus specific primer (BpET8 or EpET8) to verify the presence and size of the viral cDNA insert. The PCR products from four clones were subjected to DNA sequence analysis in the automated 377 ABI Prism DNA sequencing system to confirm the construction sequences.

Expression of VP8 in E. coli

The expression plasmid, pET28a/VP8ext, containing the expected sequence was used to transform competent *E. coli* BL21(DE3)pLysS cells by electroporation. The bacterial cells were cultured in LB broth (1% Bacto-tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.5) containing 50 µg/ml kanamycin and shaken overnight at 37 °C. Then culture was transferred into 50 ml fresh LB broth flask and grown at 37 °C with vigorous shaking. When absorbance at 600 nm reached 0.8, an aliquot (t_0) was taken and expression of the His-Tag-VP8ext fused protein was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG). One milliliter samples were taken every hour, up to 4 h after induction, and the induced bacterial cells were pelleted by centrifugation at 10,000*g* for 2 min, resuspended in
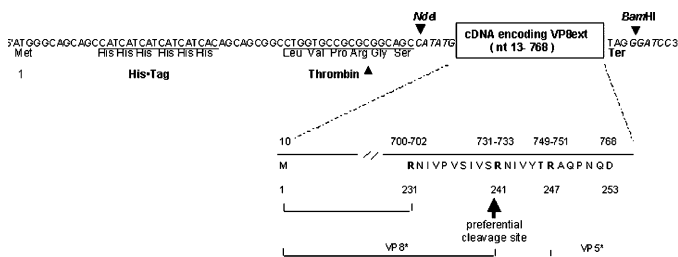

Fig. 1. Schematic illustration of the construct showing the cDNA/VP8ext insert in pET28a (+) vector.

100 µl of 1× SDS loading buffer, boiled for 5 min, and analyzed by SDS–PAGE.

Preparation and isolation of VP8ext

Four hours after induction, 50-ml cultures were centrifuged at 10,000g for 15 min at 4 °C and the supernatant was saved for SDS–PAGE. The pelleted cells were thawed on ice and resuspended in 5 ml buffer EL (50 mM Tris–HCl, and 0.5 M NaCl, pH 8.0) supplemented with 1% Triton X-100, 40 µg/ml phenylmethylsulfonyl fluoride (PMSF), and 25 U of DNAse and incubated at room temperature for 20 min. Lysozyme was added to a concentration of 100 µg/ml for 1 h on ice. The suspension was sonicated for 30-s pulses, at least five times, at 1 min intervals, using a microtip (Branson Ultrasonic Corporation). The lysate was clarified at 10,000g for 10 min at 4 °C and both the soluble fraction and the pellet containing the insoluble fraction (inclusion bodies) were analyzed by SDS–PAGE.

Solubilization of inclusion bodies

The inclusion bodies pellet was washed with 5 ml buffer A (0.01 M Tris–HCl, 0.1 M sodium phosphate buffer, and 2 M urea, pH 8.0) and incubated at room temperature for 30 min. After washing, the inclusion bodies were recovered by centrifugation at 10,000g for 10 min. The washing was performed three times to release the trapped protein. The purified inclusion bodies were solubilized in denaturing buffer B (0.01 M Tris–HCl, 0.1 M sodium phosphate buffer, and 8 M urea, pH 8.0), and incubated for 1 h on ice. After centrifugation at 16,000g for 30 min, the supernatant was collected and the protein concentration was determined. The protein solution was stored at 4 °C. All pellets and supernatants were analyzed by SDS–PAGE.

Purification of VP8ext

The His-tagged recombinant protein was purified using immobilized metal-ion affinity chromatography (IMAC). The chelating Sepharose Fast Flow resin was pre-treated as follows. The Sepharose resin was washed with 10 ml of sterile water charged with Ni^{2+} ions by treatment with 0.1 M $NiSO_4$ for 10 min at room temperature with occasional shaking. The charged resin was then washed with 10 ml of sterile water again. The washing was performed twice to remove unbounded Ni^{2+}, and resuspended in buffer B.

The soluble fraction containing the denatured recombinant protein was loaded onto the IMAC column previously equilibrated in the same buffer B, and let for 30 min at room temperature to promote adsorption of the His-Tag to the nickel ions. The column was then washed twice with 10 ml of buffer B containing 10 mM imidazole and a third washing with buffer C (0.01 M Tris–HCl, 0.1 M sodium phosphate buffer, and 8 M urea, pH 6.3). The recombinant protein was then eluted using buffer B incorporating 100 mM EDTA and 2 mM β-mercaptoethanol. The dena-

tured protein eluted was dialyzed against Tris buffer saline (TBS) pH 4.0, containing 20 mM β-mercaptoethanol and decreasing amounts of urea (8, 6, 4, 2, and 1 M) at 4 °C. Then, two more dialysis steps were done against TBS, pH 5.5, containing 2 mM β-mercaptoethanol, and a last dialysis against TBS. The dialysate solution was concentrated by speed vacuum, and VP8 recombinant was further identified by SDS–PAGE analysis.

SDS–PAGE and Western blotting analysis

SDS–PAGE was carried out on 12.5% acrylamide gel in the discontinuous Laemmli system [22]. Proteins were visualized by Coomassie Blue R250 staining. For Western blotting, the proteins were separated by 12.5% SDS–PAGE under reducing conditions and then electroblotted onto a nitrocellulose membrane (Millipore) using a Semi-Dry Transfer System (Bio-Rad). Non-specific protein binding was blocked by incubating the membrane in TBS containing 0.05% Tween 20 and 5% slim milk at room temperature overnight. After washing with TBS–Tween 20, the membrane was then incubated with either monoclonal mouse anti-His-Tag (1:10,000) or rabbit anti-rotavirus C486 strain antiserum (1:500) [20] for 2 h at 37 °C under constant shaking. The membrane was washed five times for 10 min with TBS–Tween 20 and incubated at 37 °C with the appropriate alkaline phosphatase conjugated anti-mouse immunoglobulin or anti-rabbit IgG for 1 h, and subsequently washed five times for 10 min with TBS–Tween 20. Bound antibody was revealed by the addition of the NBT-BCIP substrate.

Measurement of protein concentration

The purified protein solution was concentrated under vacuum and quantified by the Lowry method [23] standardized with bovine serum albumin, and estimated by its molar extinction coefficient at 280 nm.

Results and discussion

The expected 776 bp cDNA encoding a subunit in VP4 that includes VP8*, the trypsin cleavage region and the first six amino acid residues of VP5* of the bovine rotavirus C486, was amplified by RT-PCR from viral dsRNA. This cDNA was purified and ligated directly into the pGEM-T Easy cloning vector, which was used to transform competent *E. coli* TOP10 cells. The resulting recombinant plasmid pGEM-T/VP8extC486 was digested with *Nde*I/*Bam*HI and the VP8extC486 fragment was subcloned into the *Nde*I/*Bam*HI-digested pET28a(+) expression vector. The ligation product was stabilized in transformed *E. coli* TOP10 cells selected on kanamycin plates. The new construct, pET28a/VP8ext, was verified by nucleotide sequencing, and showed the expected in-frame presence of the viral insert. Once the presence of an open-reading frame that encoded a translation product of 273 amino acids, consisting of a six-His-Tag and thrombin cleavage peptide (first 20 amino acids) and

the extended VP8* (last 253 residues M1-D253) of rotavirus C486 fourth gene was confirmed, the construct was used to transform electrocompetent *E. coli* BL21(DE3)pLysS cells. Single colonies were picked from kanamycin plates, grown overnight, and the presence of the VP8extC486 expression cassette in the transformed clones was further confirmed by PCR using specific primers for pET plasmid and rotavirus VP4 gene (Fig. 2).

Colonies presenting the strongest amplicon of the expected size were chosen for small-scale expression trials to determine the best harvesting time. Fig. 3A shows one

such experiment, in which maximum expression was attained at 3–4 h after IPTG induction. By 1 h after induction, an additional faint band of approximately 30 kDa could be detected among the endogenous bacterial proteins in Coomassie blue stained gel, becoming increasingly evident during the following 3 h.

The identity of this band as the recombinant protein was demonstrated by Western blot assay using monoclonal antibody to the His-Tag (Fig. 3B). Further, the results showed that the same recombinant protein detected by the anti-6× His mAb, also reacted positively to a polyclonal rabbit serum produced against purified bovine rotavirus NCDV strain (Fig. 4). Bovine strains NCDV and C486 are antigenically homologous, sharing both G6 and P6 serotype specificities as determined by PRN assays [17,19]. However, strain to strain diversity probably accounted for the faint band of antigenic recognition observed on the Western blot. Although amino acid sequence comparison
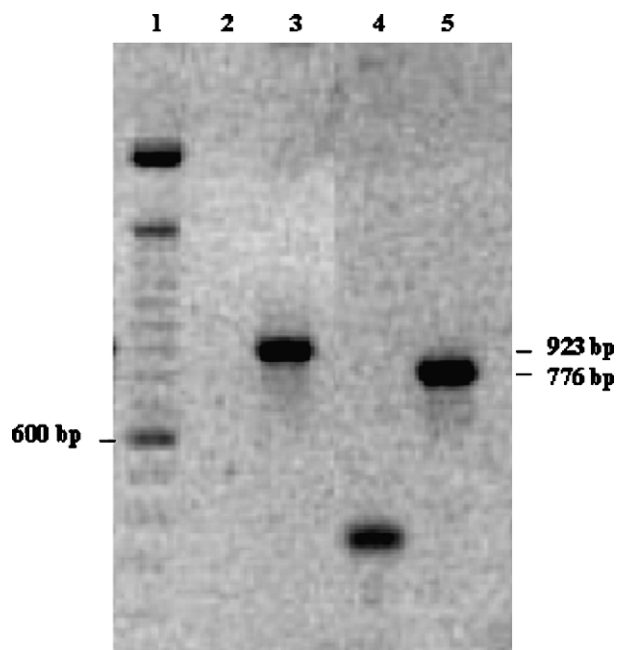

Fig. 2. PCR analysis of a transformed BL21(DE3)pLysS cells. Agarose gel electrophoresis of PCR products show the 923 bp fragment amplified with the pET-specific sense primer T7 pro and the rotavirus-specific anti-sense primer EpET8 (lane 3), and the 776 bp fragment amplified with the rotavirus-specific set of primers (lane 5). Controls without primers (lane 2) and of pET vector without insert (lane 4) are shown. Lane 1, 100 bp DNA ladder.
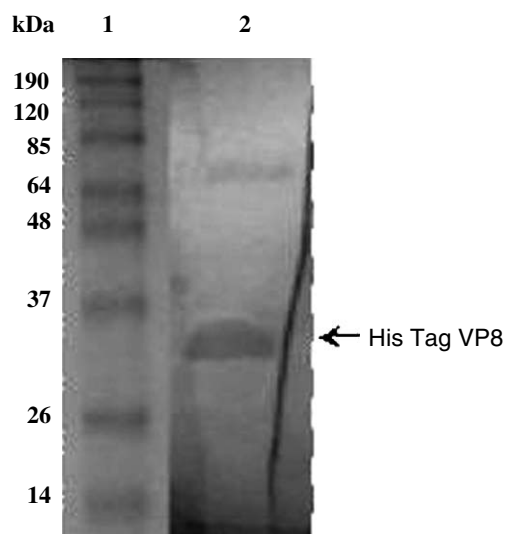

Fig. 4. Western blot of His-Tag/VP8ext fused protein in whole cell lysate after 4 h induction is revealed (arrow) with anti-bovine rotavirus hyperimmune serum (lane 2). Lane 1, protein molecular weight markers.
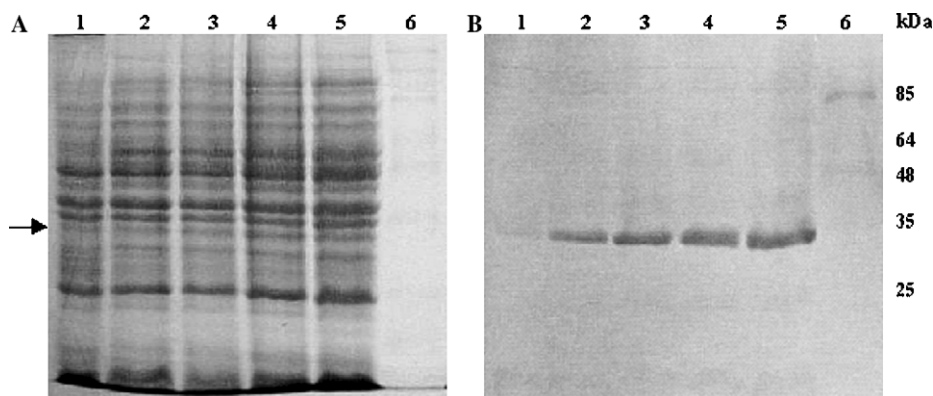

Fig. 3. Expression and localization of the His-Tag/VP8ext fused protein. (A) 12.5% SDS–PAGE and (B) Western blot using monoclonal anti-His antibody of the whole cell lysate before induction (lane 1) and after 1, 2, 3, and 4 h induction (lanes 2–5, respectively). Lane 6, benchmark prestained protein ladder (Invitrogen). The arrow indicates the recombinant VP8ext protein.

showed >98% homology between the VP8 peptides of those two viruses, four non-conserved substitutions (residues at positions 93, 94, 109, and 157) within the major region of antigenic determinant of P specificity were observed. The recombinant protein however, was not recognized by polyclonal serum raised against the human P1A[8] Wa or P1B[4] DS1 strains (results not shown), demonstrating the specificity of the antigenic determinants on the VP8extC486 recombinant peptide, as expected for the VP8 portion of the spike protein.

Optimization assays revealed small differences in the level of expression of the recombinant protein between cultures induced with 0.5–1 mM IPTG incubated at 28 °C, but an overall higher level of expression at 37 °C. Therefore, a clone with the highest expression level of the recombinant protein was selected to upscale production in culture containing 1 mM IPTG and harvesting after 4 h of incubation at 37 °C (Fig. 5). Cells from the induced culture were suspended in EL buffer, sonicated, clarified, and analyzed by SDS–PAGE (Fig. 6). This lysate should contain cytoplasmic proteins including the heterologous His-Tag/VP8extC486 expressed protein. However, the recombinant protein remained associated to the insoluble fraction after cellular disruption, most likely as inclusion bodies as demonstrated in Fig. 6 (lane 1). Reduction of the IPTG concentration (0.1–0.2 mM) did not prevent intracellular segregation of the recombinant protein and its accumulation as inclusion bodies.

To recuperate the recombinant protein, the pellet was washed with a mildly denaturing buffer (buffer A, 2 M urea), to remove contaminating soluble cytoplasmic proteins, and then with highly denaturing (buffer B, 8 M urea) to disrupt the inclusion bodies. This treatment resulted in dissolution of the aggregated recombinant protein, which could then be applied to the IMAC column (Fig. 7, lane 1).
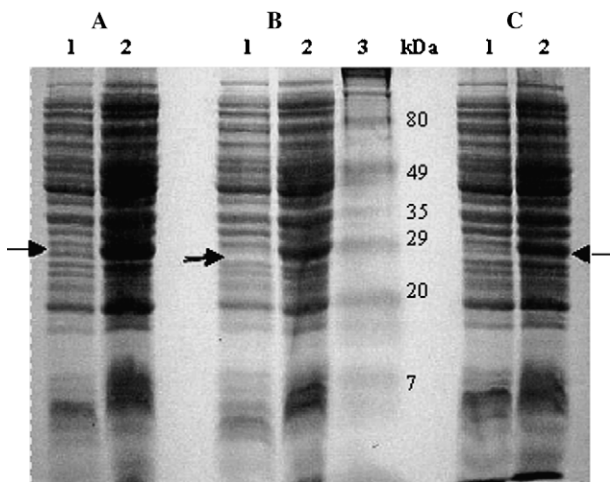

Fig. 6. 12.5% SDS–PAGE analysis of the purification steps: (lane 1) the insoluble pellet of the cell lysate after sonication; (lane 2) pooled three washings of the pellet; (lane 3) soluble fraction of the sonicated cell lysate; (lane 4) protein molecular weight markers.

Fig. 7. Purification of the recombinant VP8ext after suspension and dissolution of the inclusion bodies. 12.5% SDS–PAGE corresponds to fractions obtained by Ni-ion affinity chromatography of: lane 1, crude supernatant; lane 2, flow-through; lanes 3–5, sequential column washings; lane 6, protein molecular weight marker; lanes 7 and 8, elution of recombinant VP8ext protein from the column. The arrow shows whole recombinant VP8ext protein eluted in a first elution volume and was of apparent good purity.

Three washings, with the denaturing buffer and very low concentration of imidazole, were sufficient to remove all cellular contaminants, and a last wash to dilute out the imidazole (Fig. 7, lanes 2–5). The recombinant protein was then eluted from the column still in denatured form by discharging with EDTA (Fig. 7, lanes 7 and 8). It was then allowed refolding upon dialysis against decreasing concentrations of urea in the presence of a reducing agent. SDS–PAGE showed that the final recombinant protein obtained was of apparent good purity (Fig. 7, lane 7), as Coomassie blue, or the more sensitive, AgNO$_3$ stained-gels showed no other protein band besides the approximately 30 kDa band identified as the recombinant rotavirus VP8 protein.

Purification and proper folding of the recombinant protein is required for detailed structural analysis to allow a better understanding of its role in cell attachment and

Fig. 5. Optimization of expression. 12.5% SDS–PAGE of cultures non induced (lane 1) or induced (lane 2) under distinct conditions of temperature and of IPTG concentration: (A) 1 mM IPTG at 37 °C, (B) 0.5 mM IPTG at 28 °C, and (C) 1 mM IPTG at 28 °C. Arrow indicates the induced 30 kDa VP8ext protein. Lane B3, protein molecular weight markers.
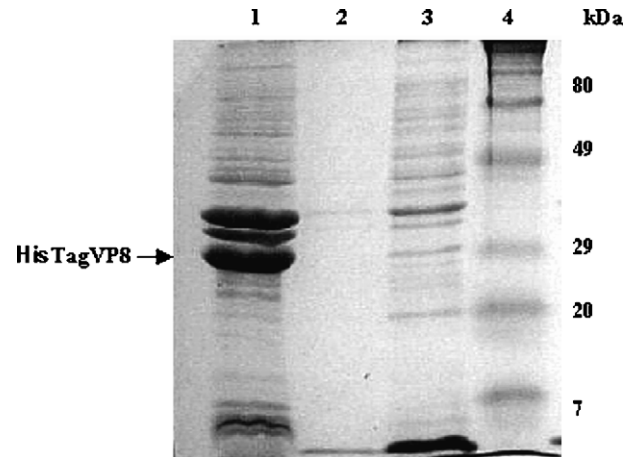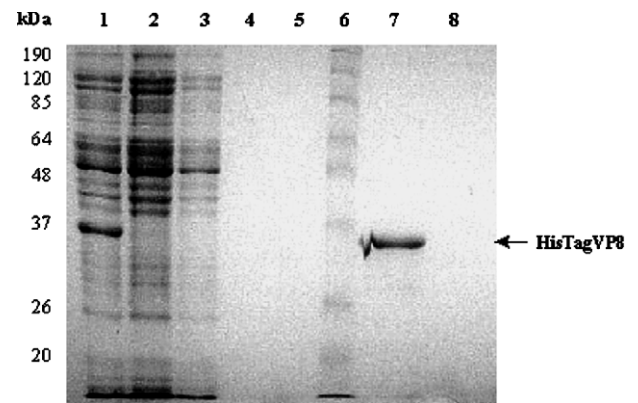
rotavirus tropism. Nevertheless, purified recombinant VP8 protein, still in its aggregated form or solublized in 8 M urea, has been shown to be a good immunogen for the production of neutralizing antibodies in immunized animals, and to prime the immune system of mice [24,25]. Protein bands excised from SDS–PAGE after Coomassie blue staining have been often used as immunogens. In one such study, soluble VP4 and VP8 proteins of human rotavirus IS2 were expressed in *E. coli* as fusion proteins of maltose-binding protein (MBP) for the purpose of producing immune reagents for P serotyping of rotaviruses in epidemiological studies [26].

The yield of His-Tag/VP8extC486 was determined to be 4 mg/L culture. This seems to be a good yield when compared to previously reported results for the VP8* recombinant protein obtained by others. Expression of human rotavirus Wa strain VP8* as a fusion protein with glutathione *S*-transferase (GST/VP8*) using the pGEX-4T-2 vector in *E. coli* resulted in 1.8 mg/L culture of pure VP8* [27]. In another study, a tobacco mosaic virus (TMV) construction with a $7\times$ His-Tag was used as vector to express bovine rotavirus C486 VP8* in tobacco leaves [28]. In this system, 5 µg of His/VP8* per 50 g of fresh leave tissue was obtained after IMAC purification. Interestingly, in both studies the recombinant VP8* produced a duplex band on acrylamide gels, not found in our study. A single band of ∼30 kDa was produced, although obtaining double or triple bands due to digestion by bacterial proteases would not be surprising. Our unique construct was designed to produce a translation product that included the entire trypsin cleavage region and six amino acid residues on VP5* so that the influence of this region, or parts of it, may be investigated in future functional studies of the expressed protein. Determination of the precise protein mass and amino acid terminal sequencing should further characterize the recombinant protein obtained in this study. High yields of recombinant VP8* should allow production of much needed P type-specific immune reagents, subsequent detailed studies on the three-dimensional structure of rotavirus VP8* and its relationship to the many important functions of this subunit.

Acknowledgments

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