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

Alexsandra R.M. Favacho [a], Eleonora Kurtenbach [b], Silvia I. Sardi [c], Vera S. Gouvea [a,*]

[a] *Departamento de Virologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil*
[b] *Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil*
[c] *Departamento de Imunologia, Universidade Federal da Bahia, Salvador, BA, Brazil*

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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.

Keywords: Rotavirus spike; VP8* subunit; Expression in *Escherichia coli*; His-Tag purification

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].

* Corresponding author. Fax: +55 21 2560 8028/8344.
E-mail address: vera.gouvea@micro.ufrj.br (V.S. Gouvea).

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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

[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.