Screening and Antiviral Analysis of Phages That Display Peptides with an Affinity to Subunit C of Porcine Aminopeptidase

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The purified C subunit of the recombinant porcine aminopeptidase N (rpAPN-C) protein was used as an immobilized target to screen potential ligands against rpAPN-C from a 12-mer phage display random peptide library. After five rounds of biopanning, five phage clones showed specific binding affinities to rpAPN-C. In 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays, the phage clone PM1, which contained the HDAISWTHYHPW peptide sequence, had a protective effect against TGEV infection in swine testis cells. Therefore, the HDAISWTHYHPW peptide sequence has a potential use as a small molecular therapeutic agent against TGEV infection.

Introduction

TRANSMISSIBLE GASTROENTERITIS (TGE) is a highly contagious, enteric disease of swine caused by the transmissible gastroenteritis virus (TGEV) and characterized by severe diarrhea, vomiting, dehydration, and death, with a mortality rate of up to 100% in seronegative suckling piglets.⁽¹⁾ Presently, TGEV infection continues to cause frequent occurrences of diarrhea in piglets in many countries and markedly so in China.^(2,3) Porcine aminopeptidase N (pAPN) is reportedly a cellular receptor for TGEV entry⁽⁴⁾ and a type II transmembrane glycoprotein belonging to membrane-bound metalloproteinase family, composed of 963 amino acids with a molecular weight of 150 kDa.⁽⁵⁾ pAPN shows high expression levels on the surface of porcine intestinal epithelial cells, where it digests peptides at the N-terminus by the putative catalytic site HexxH (aa 383–387).^(6,7) Some pAPN molecules on the brush border membrane of enterocytes are cleaved into B subunits (aa 1–572, 95 kDa) and C subunits (aa 573–963, 50 kDa) by trypsin.⁽⁸⁾ The pAPN virus binding sites are mainly located in the C subunit (pAPN-C).^(5,9,10) Recent reports have described antiserum and phages that displayed peptides with potential antiviral effects against TGEV infection *in vitro*.^(9,11,12) Therefore, these properties suggest that pAPN may be a potential candidate for antiviral design and development. In the present study, phages displaying peptides against rpAPN-C protein were screened using a Ph.D.-12 phage library, and further antiviral effect of the identified phage clones was evaluated using the 3-(4, 5-dimethylthiazol-

2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The aim of this study was to explore small molecular therapeutic drugs based on the pAPN-C sequence to treat TGEV infections.

Materials and Methods

Viruses, bacteria, and phage library

TGEV was kindly provided by the Division of Swine Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. The recombinant bacteria expressing pAPN-C were stored at the Department of Veterinary Clinical Medicine, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University.⁽¹³⁾ The Ph.D.-12 Phage Display Peptide Library Kit was purchased from New England BioLabs (Beverly, MA).

Purification of recombinant pAPN-C protein

Purification of the recombinant pAPN-C protein (rpAPN-C) was performed according to the method described by Zhu and colleagues.⁽¹⁴⁾ Briefly, the recombinant pAPN-C-expressing bacteria were induced with 0.8 mM IPTG at 37°C for 4 h and then the rpAPN-C inclusion bodies were extracted. The extracted inclusion body proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was stained with RAPIDstainTM reagent (Merck, Darmstadt, Germany). The band of the rpAPN-C protein was cut from the stained gel, loaded into the

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FIG. 1. Purification of the rpAPN-C protein. Lane M, DNA marker DL2000; lane 1, purified rpAPN-C protein.

D-Tube™ Dialyzer Mini with a molecular weight cut-off of 12–14 kDa (EMD Millipore, Billerica, MA) containing 2.5 mL of Tris-glycine SDS, and then subjected to electroelution in a horizontal electrophoresis chamber. After electroelution, dialysis of the loaded tube was performed in 1 L phosphate-buffered saline (PBS, pH 7.4) for 3 h. The purified rpAPN-C proteins were collected from the dialysis tube and stored at -80°C .

Screening and identification of phages displaying peptides against rpAPN-C

The Ph.D.-12 phage display library was subjected to biopanning using rpAPN-C as an immobilized target and performed according to the manufacturer's instructions. Briefly, 96-well plates were coated with purified rpAPN-C at a concentration of $10\text{ }\mu\text{g}/\text{well}$ in 0.1 M NaHCO_3 (pH 8.6) buffer at 4°C for 12 h. Then the plates were blocked with 5% (w/v) nonfat dried milk in TBST (50 mM Tris-HCl [pH 7.5], 150 mM NaCl , 0.1% Tween-20) at 37°C for 1 h. After washing six times

with TBST, $100\text{ }\mu\text{L}$ of diluted Ph.D.-12 phage display library (2×10^{11} plaque-forming units (pfu)/mL) was added to the coated plate and incubated at room temperature for 30 min with gentle rocking. Unbound phages were removed by washing the plates 10 times with TBST. Bound phages were eluted using $100\text{ }\mu\text{L}$ of elution buffer (0.2 M glycine-HCl , pH 2.2). After neutralization with $15\text{ }\mu\text{L}$ of 1 M Tris-HCl (pH 9.1), the eluates were amplified in *Escherichia coli* ER2738 cells. The amplified phages were purified using the polyethylene glycol precipitation method and phage concentrations were tittered according to the manufacturer's instructions. Next, four rounds of biopanning were performed according to the above-described procedure.

Enzyme-linked immunosorbent assay (ELISA) plates were coated with the purified rpAPN-C proteins ($10\text{ mg}/\text{well}$) with His-tagged proteins being used as a control. Then, the plates were blocked with 5% (w/v) nonfat dried milk in TBST at 37°C for 1 h. After washing five times with TBST, the plates were incubated with the selected phages at a concentration of 2×10^{11} pfu/well for 1 h at 37°C . Horseradish peroxidase-conjugated monoclonal mouse anti-M13 antibody (Pharmacia, Uppsala, Sweden) diluted 1:5000 in TBST was added to the wells and incubated for 1 h at 37°C . After washing six times with TBST, $100\text{ }\mu\text{L}$ of substrate 3,3',5,5'-tetramethylbenzidine solution was added to the wells and incubated at room temperature for 10 min. The reaction was stopped by the addition of $50\text{ }\mu\text{L}$ of $2\text{ M H}_2\text{SO}_4$ to each well and then the absorbance at 450 nm was measured. OD₄₅₀ value of the rpAPN-C/OD₄₅₀ value of the His tag > 2 was determined as positive standard for the phage ELISA. Furthermore, five positive phage clones were sequenced according to the manufacturer's instructions.

Antivirus analysis of phages displayed peptides against rpAPN-C

The antiviral activity of the PM1 and PM4 phage clones was analyzed using the MTT assay as described by Ren and colleagues.⁽¹²⁾ Briefly, swine testis (ST) cells were incubated with the PM1 and PM4 phages (2×10^{11} pfu/well, respectively) at 37°C for 1 h in 96-well plates. TGEV ($10^3\text{ TCID}_{50}/0.1\text{ mL}$) was used to infect the cells at 37°C for 24–48 h. After the wells were

FIG. 2. Phage ELISA using purified rpAPN-C as antigen.

TABLE 1. DEDUCED AMINO ACID SEQUENCES OF PHAGE CLONES PM1, PM2, PM3, PM4, AND PM5

Phage clones	Sequence of deduced amino acids
PM1	HDAISWTHYHPW
PM2	LWCITQDCSHRM
PM3	LWCITQDCSHRM
PM4	LWCITQDCSHRM
PM5	LWCITQDCSHRM

washed three times with PBS, 20 μ L of MTT buffer (5 mg/mL) were added to each well and the plates were then incubated at 37°C for 4 h. The supernatant was discarded and 150 μ L dimethyl sulfoxide were added to each well and incubated at room temperature for 10 min with gentle shaking. The optical density at 490 nm of the wells was determined using a plate reader. The TGEV, phage clones, and ST cells were individually included as controls. Each experiment was repeated eight times. Statistical analysis was performed by one-way ANOVA, using STATVIEW software (Abacus Concepts, Berkeley, CA). For $p < 0.05$ in the ANOVA, Bonferroni/Dunn's HSD test was used to locate differences.

Results

Purification of rpAPN-C

After IPTG induction, the inclusion bodies expressing rpAPN-C were extracted from the induced recombinant bacteria. The rpAPN-C protein was purified from the extracted inclusion bodies using a simple gel-cutting purification procedure followed by electroelution and dialysis. Our SDS-PAGE results indicated that a high-purity rpAPN-C protein was obtained (Fig. 1).

Screening and identification of phages displaying peptides against rpAPN-C

To obtain the phages displaying peptides against rpAPN-C, biopanning was performed using rpAPN-C as the coating antigen. A total of five phage clones (PM1, PM2, PM3, PM4, and PM5) showed high binding activity with the rpAPN-C using a phage ELISA (Fig. 2). The nucleotide sequences of the five phages were determined and the deduced amino acid

sequences are shown in Table 1. The amino acid sequence of phage clone PM1 was HDAISWTHYHPW and those of PM2, PM3, PM4, and PM5 were LWCITQDCSHRM.

Antiviral analysis of phages displaying peptides against pAPN-C

Effects of the phage clones PM1 and PM4 on TGEV infection of ST cells was evaluated using the MTT assay. Our results indicated that the PM1 phage clone had a protective effect against TGEV infection in ST cells, whereas the PM1 and PM4 phage clones had no effect on ST cell growth (Fig. 3).

Discussion

TGEV is a member of the genus *Coronavirus*, family Coronaviridae, order Nidovirales, and its S protein is a major structural protein involved in viral entry into cells. Interaction of the S protein with pAPN plays a significant role in the process of viral infection. The small molecules binding to pAPN, which have potential use for blocking TGEV infection *in vitro*, have become interesting targets for TGEV prevention and therapy.^(9-13,15-17) In the current study, five phage clones with a binding affinity to the rpAPN-C protein were obtained from the Ph.D.-12 phage display library by biopanning. The phage clone PM1 (HDAISWTHYHPW) showed a protective effect against TGEV infection in ST cells. Bioscreening of the phage display library revealed that the purity and activity of the coating antigen was crucial for identification of the ligands of interest. In a previous study, the rpAPN-C protein was successfully expressed in *E. coli* cells using a His-tagged pET-32a vector. The rpAPN-C protein was purified using a simple gel-cutting assay. To improve the purification process, the D-Tube™ Dialyzer was used for electroelution and dialysis of the rpAPN-C protein isolated from the polyacrylamide gels. The procedure efficiently recovered the proteins and simultaneously removed ampholytes. The purified rpAPN-C protein was deemed suitable for downstream bioscreening of the phage library.

Phage display is a powerful tool to screen and identify ligands binding to the targets of interest. The Ph.D.-12 phage display library is widely used to identify small molecules for diagnosis and therapy.^(12,18,19) In the present study, five high-affinity phage clones against the rpAPN-C protein were

FIG. 3. MTT assay of the phage clones PM1 and PM4. Small letters represent statistically significant differences ($p < 0.05$) among six groups.

identified using the Ph.D.-12 phage display library. The results demonstrated that the Ph.D.-12 phage display library was useful to screen the ligands binding to the targets of interest. However, the five identified phage clones only displayed two different peptides and attempts to obtain more phage clones displaying different peptides were unsuccessful in our study. Our results regarding the numbers of identified phage clones were similar with previous observations.^(12,18,19) The MTT assay indicated that the HDAISWTHYHPW sequence of the PM1 phage clone provided a protective effect to ST cells against TGEV infection. The specific peptide sequence HDAISWTHYHPW has a potential use for treatment of TGEV infection, especially in combination with other special small molecules, such as the SVVPSKATWGFA, HVTTFAPPPPR, and FKPSPPSITLW peptides described by Ren and colleagues.⁽¹²⁾ Compared to normal ST control cells, the PM1 and PM4 phage clones had no effect on ST cell growth. This result suggested that the recombinant phages displaying specific peptides could be directly used for therapeutic purposes, in place of expensive peptide synthesis.

In conclusion, we identified five phage clones that bound to the rpAPN-C protein from the Ph.D.-12 phage display library. Of these five identified phage clones, PM1, which contained the HDAISWTHYHPW sequence, showed a protective effect in ST cells against TGEV infection. To the best of our knowledge, this preliminary study is the first to describe phage-displayed peptides against rpAPN-C. However, further antiviral research of the identified peptides is necessary to formulate therapeutic applications.

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Author Disclosure Statement

The authors have no financial conflicts to disclose.

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