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Expression and functional analysis of porcine aminopeptidase N produced in prokaryotic expression system

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ABSTRACT

Porcine aminopeptidase N (pAPN) is a cellular membrane protein and a functional receptor for porcine coronaviruses. Here, we describe the heterologous expression of pAPN without signal peptide in BL21(DE3)pLysS host cells. The *Escherichia coli* (*E. coli*) harboring the recombinant construct was efficiently induced to express the pAPN protein at a high level. The most optimal expression profile for pAPN expression was investigated. By inoculating a rabbit with the purified pAPN, a high tittered specific antibody was achieved. Biologically, the antibody reacted with either pAPN-expressing *E. coli* or native pAPN on the surface of swine testis cells. The pAPN and its specific antibody blocked transmissible gastroenteritis coronavirus infection *in vitro*. Furthermore, the localization of pAPN on the small intestine of swine was analyzed by immunohistochemistry.

1. Introduction

Aminopeptidase N (APN)/CD13 is a type 2 transmembrane ectopeptidase of 150 kDa that contains a zinc-binding motif (HEIAH) and forms a noncovalently bound homodimer on the cellular membrane. APN was extensively expressed on hematopoietic cells of myeloid origin, non-hematopoietic cells and tissues, such as fibroblasts, brain cells, and epithelial cells of the liver, kidney, and intestine as well as various solid tumors (Zhang et al., 2008; Tsukamoto et al., 2008). APN is a member of the metabolic complement of the blood–brain barrier that is involved in neuropeptide degradation (Mizutani et al., 1993).

It is known that porcine aminopeptidase N (pAPN) is a cellular receptor for most of group 1 coronaviruses (Delmas et al., 1992; Hansen et al., 1998; Schultze et al., 1995; Ren et al., 2008). The pAPN is highly expressed in small intestinal mucosa, where aminopeptidase represents about 8% of the protein content of the apical membrane of the differentiated enterocytes and catalyzes the removal of single amino acids from the amino terminus of peptides for the final steps of digestion (Delmas et al., 1992). Although APN as receptors for many coronaviruses are species-specific (Delmas et al., 1994; Kolb et al., 1996), the feline APN can also serve as a receptor for canine coronavirus, transmissible gastroenteritis and human coronavirus 229E in addition to feline infectious peritonitis virus (Tresnan et al., 1996).

Currently, there are some reports on the transient or stable expression of pAPN in eukaryotic systems for certain functional analysis (Kolb et al., 1996; Ortego et al., 2002). When the eukaryotic cells were transiently transfected with a recombinant plasmid, the gene will be replicated and the gene-encoded protein will be produced correspondingly. The transfected cells will eventually die due to the unlimited replication of the foreign DNA. The stably expressed proteins in mammalian cells are relatively expensive and not optimal for high-level production of foreign proteins.

Since somatostatin, a mammalian peptide hormone was firstly expressed *Escherichia coli* (*E. coli*), realizing the *in vitro* expression of a foreign gene in prokaryotic cells, the reformed *E. coli* has been used extensively as the cellular host for foreign protein expression due to its rapid growth rate, capacity for continuous fermentation, and relatively low cost (Wentworth and Holmes, 2001; Yin et al., 2007). In the current study, we got the high-level expression of pAPN in *E. coli* BL21 (DE3) pLysS [F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^R)], an efficient and often used host cells. The purified pAPN was used to elicit specific antibody in rabbit. In addition to the pAPN, the antibody also blocked TGEV infection *in vitro* efficiently and recognize native pAPN distributed in porcine small intestine. The protein, protein-producing system as well as the specific antibody are valuable biological reagents.

2. Materials and methods

2.1. Plasmids and bacterial strains

Recombinant plasmid, pcDNA3.1-apn containing full-length porcine aminopeptidase N (pAPN) was a gift from Dr. Georg Herrler

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of Institute for Virology, University of Veterinary Medicine Hannover, Germany. The sequence of pAPN gene has been deposited in the GenBank database of NCBI, and was assigned an accession no. NM214277. Expression vector pGEX-6P-1 contains the coding region for glutathione S-transferase (GST), and the expressed product will be in the form of a fusion protein with GST was purchased from Amersham Biosciences (USA). *E. coli* strain BL21(DE3) pLysS was purchased from Novagen Inc. Germany and used as host cells for recombinant protein expression.

2.2. Construction of expression plasmid

The gene encoding a truncated pAPN without the signal peptide sequence was amplified from pcDNA3.1-apn by polymerase chain reaction (PCR) using the sense primer, P1 (5′-GGGGGGATCCGAGAAGAACAAGAATGCC-3′) and antisense primer P2 (5′-CCCCCTCGAGTGCTGTGCTCTATGAACCA-3′). Both primers contained BamH I and Xho I restriction enzyme sites, respectively. The PCR profile included 95 °C 5 min, 30 cycles of 95 °C 5 min, 63.2 °C 30 s, 72 °C 1.5 min, and then a final extension of 72 °C 10 min. The PCR product was visualized by appropriate ethidium bromide-containing agarose gel electrophoresis (1% agarose, 80 V for 20 min) and subsequent UV transillumination. The PCR product was purified and digested with BamH I and Xho I, inserted into the pGEX-6P-1 expression vector, and then transformed into BL21(DE3) pLysS. A colony of positive cells was selected on Luria bertani (LB) agar plates containing Ampicillin (100 μg/ml) and screened by direct colony PCR. The extracted plasmid designated pGEX-apn was subjected to DNA sequencing by TaKaRa, Dalian, China.

2.3. Expression of the recombinant protein

To express the protein of interest, the pGEX-apn-transformed cells were cultured in either SOC (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) or LB medium containing Ampicillin (100 μg/ml) at 37 °C with shaking until the optical density of the culture at 600 nm reached 0.5. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 0.5 or 1 mM to induce the expression at 37, 30 or 25 °C for 4 h. The empty pGEX-6P-1 vector transformed culture was used as control. The bacteria were pelleted at 8000 × *g*, at 4 °C for 5 min. The pellets were re-suspended in buffer I (50 mM Tris and 1 mM EDTA, pH 8.0) and digested with lysozyme at a final concentration of 100 μg/l at room temperature (RT) for 30 min, after they were washed with PBS for twice. The cell suspension was sonicated on ice five times, each for 30 s with 30 s intervals. After sonication, the lysate was centrifuged at 5000 rpm for 10 min, and the supernatant was discarded and the pellet was softly re-suspended in buffer II (50 mM Tris, 0.5 mM EDTA, and 1% Triton-100, pH 8.0). The both samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. In parallel, the bacteria-containing culture was taken every 1 h for 7 h to analyze the optimal induction time.

2.4. Purification of inclusion bodies

As above-mentioned, the pellets re-suspended in buffer II were centrifugated at 10,000 rpm at 4 °C for 10 min. The precipitated inclusion bodies were washed with PBS for three times and then were re-suspended in SDS-PAGE loading buffer, boiled for 10 min and centrifugated at 12,000 rpm for 5 min. The supernatant was isolated by 8% SDS-PAGE. The sample-containing gel was washed with non-ionized water for three times, then washed with PBS for three times and then was stained with 0.25 mol/l cold KCl for 30 min. The protein of interest was collected by gel-cutting and homogenized gel was dissolved in PBS. After freezing and thawing with liquid nitrogen for three times, the samples were centrifugated at 5000 rpm for 10 min and the supernatant (purified protein) was collected.

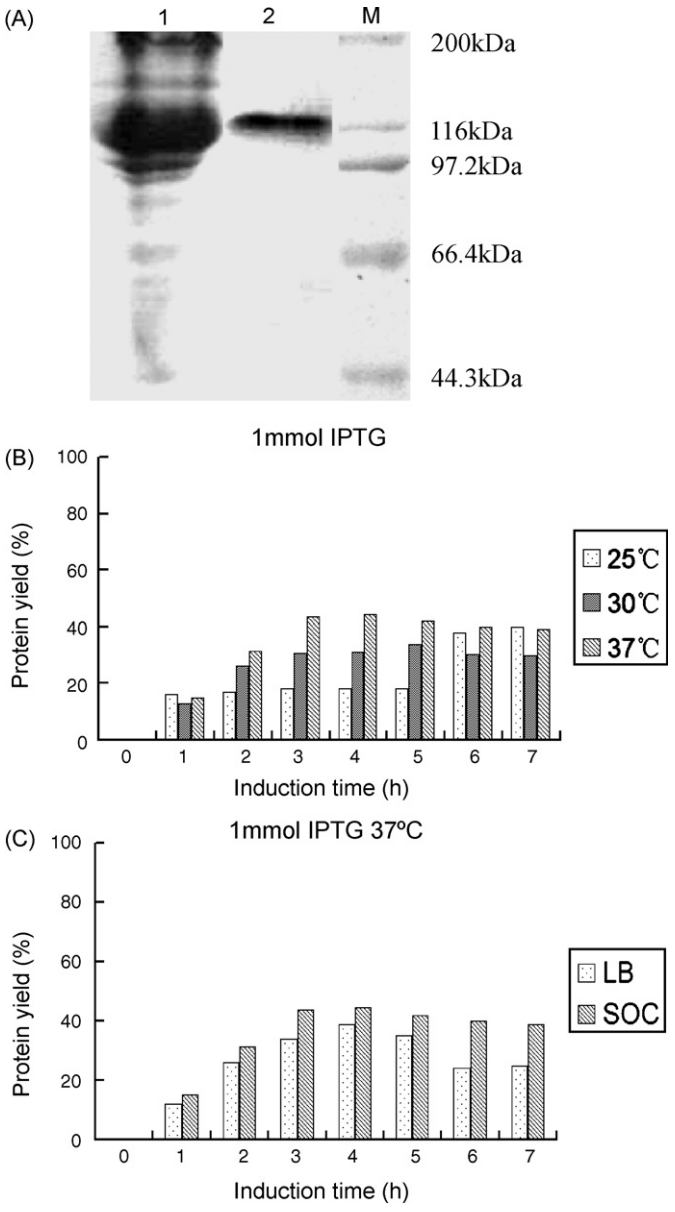

Fig. 1. SDS analysis of the expression of pAPN. The bacteria harboring pGEX-apn were induced with 1 mM IPTG for 4 h, and the bacterial protein were analyzed by SDS-PAGE. Panel A: The protein of interest in lane 1 is about 131 kDa and lane 2 is the gel-cutting purified protein with a very high purity; lane M is protein standard marker. The induction expression efficiency of pAPN at different temperatures using 1 mM IPTG is shown in Panel B. The discrepant expression efficiency of pAPN in SOC and LB medium is shown in Panel C. The protein expression level refers to percentage of pAPN content of the total cellular protein.

2.5. Renaturation of fusion protein by dialysis

The dialysis bag was boiled in 2% $NaHCO_3$ for 10 min, washed with distilled water, boiled in 1 mM EDTA for 10 min and then kept in 1 mM EDTA at 4 °C prior to renaturation. The purified protein-containing dialysis bag was put into 1 l renaturation solution (20 mM Tris–HCl pH 8.0, 0.1 mM glutathione of oxidized form, and 0.9 mM glutathione of reduced form) at 4 °C for 2 h, then put into 1 l new renaturation solution for another 4 h, and then put into

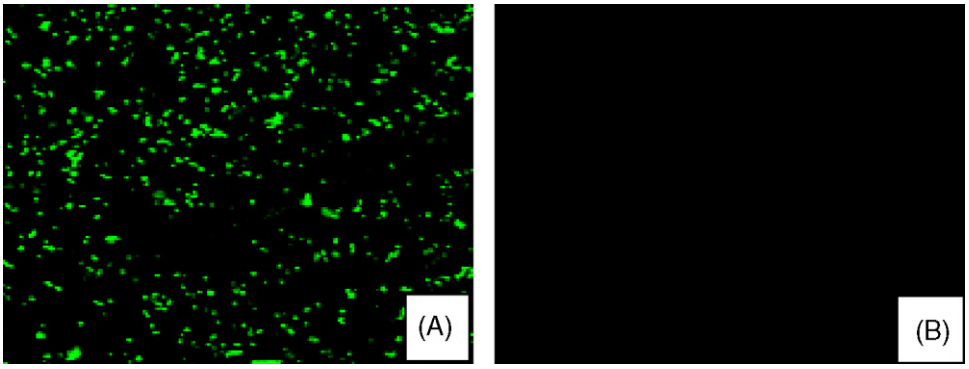

Fig. 2. Immunofluorescence analysis of pAPN-harboring bacteria. The bacteria harboring the pGEX-apn were subjected to indirect immunofluorescence. Panel A: The induced bacteria with the green fluorescence signals; Panel B: the non-induced bacteria control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1 l TE buffer (10 mM Tris-Cl, pH 7.5. 1 mM EDTA) for 4 h. The protein was concentrated using 50% PEG8000 at 4 °C.

2.6. Preparation of specific polyclonal antibodies

To prepare polyclonal antisera specific for pAPN protein, a New Zealand rabbit was immunized with purified pAPN (1.2 mg/ml) emulsified with equal amount of Freud's complete adjuvant via subcutaneous injection. Two weeks later, the equal volume of antigen mixed with Freud's incomplete adjuvant was injected at 10 days' interval for five times. Antisera were isolated from neck artery blood of the immunized rabbit.

2.7. Titration of the antibody by agar diffusion test and ELISA

The reaction between the pAPN and serially diluted antibodies (2–64-fold dilution in PBS) was analyzed by agar diffusion test at 37 °C for 24–72 h. In parallel, an indirect ELISA was performed. Briefly, ELISA plates were coated with 2-fold serially diluted pAPN (from 1:50 to 1:12800, 100 μl/well) at 4 °C overnight in carbonate–bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The wells were incubated with blocking buffer at 37 °C for 2 h. The wells were washed three times with PBS-0.1% Tween20 (PBST). The wells were incubated with serially diluted polyclonal antibodies at 37 °C for 1 h. After washing with PBST for four times, the plates were incubated with goat anti-rabbit IgG conjugated with peroxidase at 37 °C for 1 h. OPD substrate (100 μl/well) was added and incubated for 15 min after washing with PBST. 50 μl of stop buffer (2 M H_2SO_4) was added to each well and the optical density (OD) was read at 490 nm using an ELISA reader.

2.8. Immunofluorescence analysis for pAPN-expressing E. coli

The induced bacteria harboring either the pGEX-apn or empty vector were pelleted at 10,000 rpm for 2 min at RT. The pellets were re-suspended in appropriate volume of PBS and smeared on the poly-L-lysine treated microscope slides. The samples were air-dried and fixed in ice acetone for 10 min. Then they were incubated with the anti-pAPN sera (1:200 dilution in 1% BSA) at 37 °C for 1 h. After washing with PBS, The FITC labeled goat anti-rabbit IgG was added for another 1 h in dark. After three times washing with PBS and covered with cover glasses, the green fluorescence signals were analyzed by fluorescence microscope (Leica, Germany).

2.9. Immunofluorescence analysis for native pAPN on swine testis cells

The swine testis (ST) cell, a pAPN-containing cell line was cultured on coverslips in 24-well plates. The cell monolayers were fixed 4% (w/v) formalin in PBS for 20 min at RT. After quenching by 0.1 M glycine in PBS, the expression of pAPN on the surface of the cells was detected by using the specific polyclonal antisera against pAPN as the primary antibody (1:200 dilution in 1% BSA). The indirect immunofluorescence was performed as above.

2.10. Virus-binding blocking assay

Transmissible gastroenteritis virus (TGEV, Purdue 46-MAD strain, a gift from Prof. Luis Enjuanes, Centro Nacional de Biotecnología, CSIC, Department of Molecular and Cell Biology, Campus Universitario de Cantoblanco, Madrid, Spain) was used for virus infection or virus-binding blocking assay. To see whether the pAPN

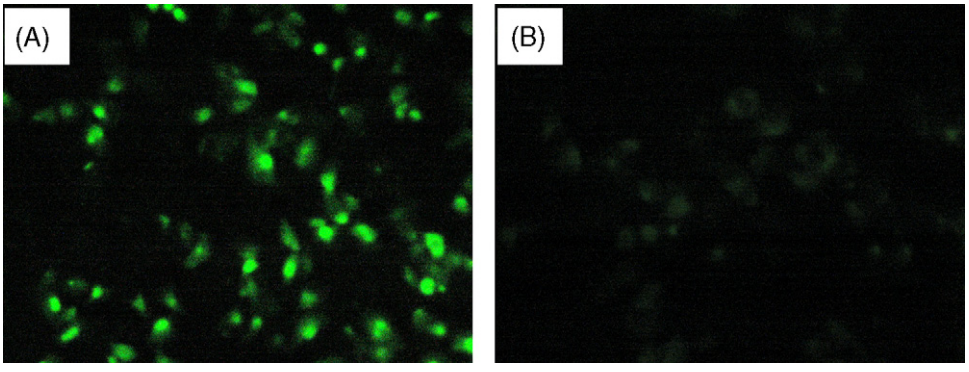

Fig. 3. Expression of pAPN on the surface of ST cells by immunofluorescence. The expression of pAPN on the surface of ST cells was analyzed by indirect immunofluorescence. Panel A: Expression of pAPN on ST cells detected by anti-pAPN antibody. Panel B: Cell control in which BSA was used as a primary antibody.

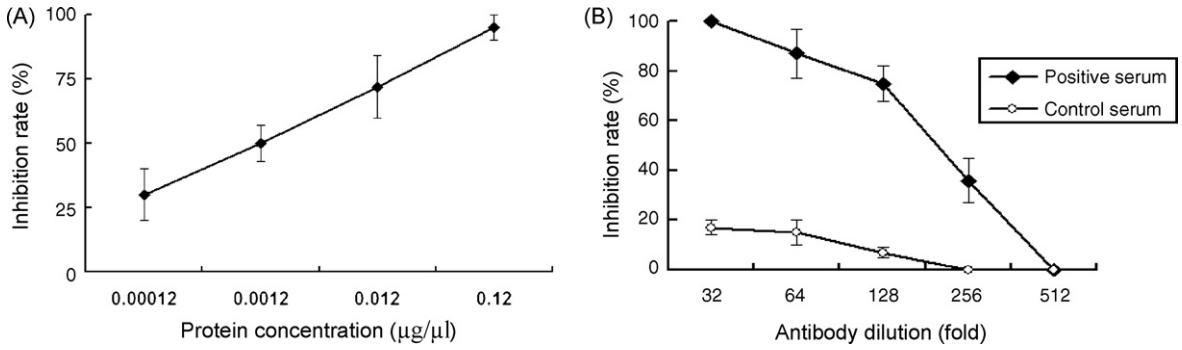

Fig. 4. Virus infection blocked by antisera against pAPN. Serially diluted pAPN was incubated with TGEV and then applied to ST cell monolayers (Panel A). In parallel, ST cell monolayers in 24-well plates were pre-treated with serially diluted polyclonal antibody against pAPN, then, TGEV was applied to the cells (Panel B). Infection efficiency was analyzed by plaque-reduction assays. Five wells were tested at dilution (values along the horizontal axis indicate protein concentration or antibody dilution) and the results are expressed as virus infection inhibition rate (vertical axis).

is able to block TGEV infection, pAPN protein serially diluted in serum-free medium at 37 °C for 1 h, and then the mixtures were infected swine testis (ST) cell monolayers seeded in 24-well plates at 37 °C for 48–72 h. To analyze the antiviral effect of the antibody, ST cell monolayers seeded in 24-well plates were pre-incubated with 200 μl serum-free medium containing either the test (anti-pAPN sera) or control (unvaccinated rabbit sera) at 37 °C for 1 h. Then, 200 μl virus dilution (1×10^5 pfu/ml) in medium was added to each well and incubated at 37 °C for 1 h. The monolayers were rinsed three times with PBS and fed with fresh medium followed by incubation for 48–72 h a humidified 5% CO_2 incubator at 37 °C. Positive (virus infection, without serum pre-incubation) and mock-infected controls were included. The wells were subjected to virus plaque-reduction assay using crystal violet staining.

2.11. Immunohistochemistry

Small intestine of ten-day piglets was used for preparation of acetone-fixed frozen sections. The sections were put in a humidified box, and then treated with 3% H_2O_2 20 min, trypsin for 5 min, 1% BSA for 20 min, respectively. Then they were incubated with anti-pAPN sera (1:200 dilution in PBS) at 37 °C for 1 h. After incubation with the biotin labeled goat anti-rabbit IgG (ZYMED, USA) at 37 °C for 1 h, the sections were reacted with the streptavidin-peroxidase complex (ZYMED, USA) at 37 °C for 40 min. Color developing was performed by diaminobenzidine (DAB) for 5 min at RT in dark using a commercially available Kit (blue, Usc-nlife, China). The nuclei were stained with hematoxylin for 10 min at RT.

3. Results

3.1. Construction and expression of recombinant plasmid

Using a pair of specific primers, the gene encoding a truncated pAPN was amplified and then subcloned into pGEX-6P-1 vector. The resulting recombinant plasmid, pGEX-apn was confirmed by DNA sequencing. After the pGEX-apn was transformed into host cells, IPTG was used to induce the expression of protein of interest. The GST-fused protein, pAPN was expressed as inclusion bodies in the bacterial cytosol. The SDS-PAGE showed that the protein was approximate 131 kDa as expected and the expression of the protein peaked at 4 h post induction. The protein was easily purified without significant loss by gel-cutting method (Fig. 1A). In contrast, the protein could not be detected in the empty vector transformed or non-induced bacterial culture (data not shown). The optimal expression condition was investigated by varying the induction time, temperature, IPTG concentration as well as media. Our results indicated that the maximum amount of the protein was achieved in SOC medium at 37 °C using 1 mM IPTG for induction (Fig. 1B and C). The expression amount of the protein accounted for about 45% of the total bacterial protein analyzed by the software BANDSCAN5.0. At different temperature and using 0.5 mM IPTG gave rise to similar results, however, the expression efficiency of the pAPN was lower than that induced by 1 mM IPTG (data not shown).

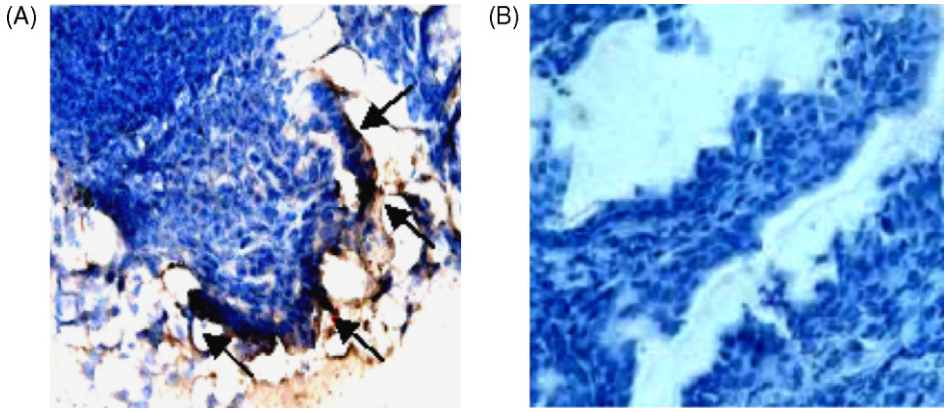

Fig. 5. Localization of pAPN in the small intestine. The small intestine of piglet was prepared as acetone-fixed frozen sections and then the sections were subjected to immunohistochemical analysis. The representative results taken from the jejunum are shown. Panel A: Positive expression of pAPN in the brush border (arrows). Panel B: The negative control. The cell nuclei were stained in blue. The images were taken at 400-fold magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Titration and specificity of the antisera

The purified pAPN was used as antigen for inoculating New Zealand rabbit, and ELISA indicated that the titer of the polyclonal antibody harvested was 1:2560. The agar diffusion test showed that there were clearly specific precipitation lines between antigen and serially dilution antibodies (up to 1:32 in dilution) (data not shown).

3.3. Immunoreaction analysis

Western blot showed that pAPN was recognized by the polyclonal antibody against pAPN (data not shown). The result was confirmed by indirect immunoflurescence. As shown in Fig. 2A, the positive signals on the surface of pAPN-expressing *E. coli* were detected using the polyclonal antibody, in contrast, there no green signals could be found in the control group, namely, empty vector transformed bacteria induced under the same induction condition as pAPN-expressing bacteria (Fig. 2B). Indirect immunofluorescence also showed that the specific polyclonal antibody against pAPN recognized native pAPN on the surface of ST cells (Fig. 3). There were no positive signals detected by immunofluorescence when the antibody was used to react with Vero, a monkey kidney cell line (data not shown).

3.4. Cell infection was inhibited by pAPN and its specific antibody

Using virus plaque-reduction assay, the effect of pAPN and its specific antibody on ST cell infection by TGEV was investigated. Our results indicated that cell infection was inhibited by incubation of pAPN with TGEV or by pre-treatment of cells with the specific antisera. The pAPN of 0.12 mg/ml inhibited TGEV infection *in vitro* efficiently (more than 95% inhibition rate) (Fig. 4A). When pAPN antibody was diluted below 1:32 in medium, the virus infection was totally inhibited (Fig. 4B). The inhibitory effect of the proteins to TGEV infection was in a dose-dependent manner.

3.5. Immunohistochemical analysis

The pAPN expression in the small intestine tissues was analyzed using the acetone-fixed frozen sections of small intestine tissue from ten-day piglets and the specific polyclonal antisera. The results showed that the pAPN distributed in the brush border of jejunum and ileum could be localized using the specific rabbit anti-pAPN antibody. A representative expression of the pAPN on the brush border of jejunum was given in Fig. 5.

4. Discussion

The pAPN plays important roles in many physical functions and serve as the cellular receptor for several coronaviruses (Delmas et al., 1992; Wentworth and Holmes, 2001; Ren et al., 2006). The obtaining of pAPN protein is useful for analyzing its biological functions. Selection of an optimal host cell systems for the expression of heterologous genes mainly bases on the productivity, bioactivity, purpose, and physicochemical characteristics of the interest protein (Yin et al., 2007). The pAPN has been expressed in eukaryotic system for specific functional analysis in several previous reports, however, the relatively high cost, complicated technology, and potential contamination with animal viruses of mammalian cell expression have been bottlenecks for its use in large-scale industrial production.

The *E. coli* based system is a typical prokaryotic expression system and has the highest expression potential as it can afford inexpensive scale-up (Yin et al., 2007; Jaiswal et al., 2004). Therefore, we wanted to express the pAPN in such system for the first time. In designing strategies for the expression of the protein, we used BL21 (DE3) pLysS as host cells for expression based on the following reasons. Firstly, BL21 strains are the most widely used hosts for protein expression and have the advantage of being deficient in both *lon* and *ompT* proteases. The DE3 indicates that the host is a lysogen of DE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter. Such strains are suitable for production of protein by induction with IPTG. The types of pLysS strains express T7 lysozyme, which further suppresses basal expression of T7 RNA polymerase prior to induction, thus stabilizing recombinants encoding target proteins that affect cell growth and viability. This BL21 system is rather matured and therefore is extensively used (Stabile et al., 2000; Rina et al., 2000; Böck et al., 2007; Volontè et al., 2008). At the same time, the pGEX-6P-1 is a good prokaryotic expression vector with a tac promoter for chemically inducible, high-level expression as well as an internal lac I^q gene for use in any *E. coli* host. Several foreign proteins have been successfully using pGEX vector (Mikami et al., 2006; Capparelli et al., 2007; Chuan et al., 2008).

In the current study, the combined usage of BL21 (DE3) pLysS and pGEX-6P-1 were very optimal for pAPN expression. We found that *E. coli* harboring the recombinant construct was efficiently and stably induced to express high levels of the pAPN protein. The highest yield of pAPN in the *E. coli* was achieved in SOC media at 37 °C using 1 mM IPTG as inducer. In addition to IPTG concentration and induction time, the media also was a vital condition for pAPN expression. The SOC media was superior to LB media in terms of the ability of inducing protein expression, and we putatively believe that the high nutrition components of media should be one of the crucial factors for a maximal production of foreign protein in prokaryotic system. The expressed protein was found in the insoluble phase of the cell lysates. We also found that the truncated pAPN gene without signal peptide sequence was inducible in the prokaryotic system, and the full-length pAPN gene was not (data not shown).

To achieve the matured pAPN with good biological activity, the inclusion bodies were solubilized using a strong denaturant and the purified protein was renaturated using a cheap and effective dialysis method in regeneration solution (20 mM Tris–HCl pH 8.0, 0.1 mM glutathione of oxidized form, and 0.9 mM glutathione of reduced form). Because the GST tag cannot influence the application of pAPN protein, therefore, we used a very simple gel-cutting method instead of using Factor Xa to cleave the GST tag to purify the fusion protein. The former method is very cheap and effective. Subsequent biological experiments indicated that the purified pAPN was a good immunogen for eliciting highly titered and specific polyclonal antibodies in the inoculated rabbit. The pAPN could be recognized not only by the corresponding polyclonal antibody, but also by anti-GST-tag monoclonal antibody in a Western blot analysis (data not shown). Interestingly, in our immunofluorescence assay, we also found that the protein was detected on the surface of the bacteria harboring the recombinant plasmid. We speculate that the transport of pAPN to the cell surface may be due to either the overexpression of the protein or some unidentified secreting signals, which needs to be investigated in the future. The specific antibody induced by the pAPN also reacted with the natural pAPN on the surface of ST cells, the swine testis cells by indirect immunofluorescence assay, demonstrating that the antibody recognize aminopeptidase N in a species-specific way. However, the pre-incubation of anti-pAPN sera with the ST cells blocked the cell infection by TGEV efficiently, confirming the utility and specificity of the pAPN and its antibody. The expression of pAPN on the brush border of small intestine was detected using the specific antibody, which is consistent with clinical infection sites of TGEV in vivo. The antibody is also a good diagnosis reagent for localizing pAPN in other tissues.

In conclusion, we have expressed and purified biologically functional recombinant pAPN protein and provided the expression

profile for achieving high yield of the recombinant protein in inexpensive media. The pAPN and prepared specific polyclonal antibody are valuable for analyzing the role of pAPN physiologically and virologically.

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References

Böck, R.A., Soulages, J.L., Barrow, W.W., 2007. Substrate and inhibitor specificity of *Mycobacterium avium* dihydrofolate reductase. FEBS J. 274, 3286–3298.

Capparelli, R., Ventimiglia, I., Palumbo, D., Nicodemo, D., Salvatore, P., Amoroso, M.G., Iannaccone, M., 2007. Expression of recombinant puroindolines for the treatment of staphylococcal skin infections (acne vulgaris). J. Biotechnol. 128, 606–614.

Chuan, Y.P., Lua, L.H., Middelberg, A.P., 2008. High-level expression of soluble viral structural protein in *Escherichia coli*. J. Biotechnol. 134, 64–71.

Delmas, B., Gelfi, J., Kut, E., Sjostrom, H., Noren, O., Laude, H., 1994. Determinants essential for the transmissible gastroenteritis virus-receptor interaction reside within a domain of aminopeptidase-N that is distinct from the enzymatic site. J. Virol. 68, 5216–5224.

Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjostrom, H., Noren, O., Laude, H., 1992. Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. Nature 357, 417–420.

Hansen, G.H., Delmas, B., Besnardeau, L., Vogel, L.K., Laude, H., Sjostrom, H., Noren, O., 1998. The coronavirus transmissible gastroenteritis virus causes infection after receptor-mediated endocytosis and acid-dependent fusion with an intracellular compartment. J. Virol. 72, 527–534.

Jaiswal, S., Khanna, N., Swaminathan, S., 2004. High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in *Escherichia coli*. Protein Expr. Purif. 33, 80–91.

Kolb, A.F., Maile, J., Heister, A., Siddell, S.G., 1996. Characterization of functional domains in the human coronavirus HCV 229E receptor. J. Gen. Virol. 77, 2515–2521.

Mikami, S., Kobayashi, T., Yokoyama, S., Imataka, H., 2006. A hybridoma-based in vitro translation system that efficiently synthesizes glycoproteins. J. Biotechnol. 127, 65–78.

Mizutani, S., Goto, K., Nomur, S., Ino, K., Kikkawa, F., Kurauchi, O., Goldstein, G., Tomoda, Y., 1993. Possible action of human placental aminopeptidase N in feto-placental unit. Res. Commun. Chem. Pathol. Pharmacol. 82, 65–80.

Ortego, J., Escors, D., Laude, H., Enjuanes, L., 2002. Generation of a replication-competent, propagation-deficient virus vector based on the transmissible gastroenteritis coronavirus genome. J. Virol. 76, 11518–11529.

Ren, X., Glende, J., Al-Falah, M., de Vries, V., Schwegmann-Wessels, C., Qu, X., Tan, L., Tschernig, T., Deng, H., Naim, H.Y., Herrler, G., 2006. Analysis of ACE2 in polarized epithelial cells: surface expression and function as receptor for severe acute respiratory syndrome-associated coronavirus. J. Gen. Virol. 87, 1691–1695.

Ren, X., Glende, J., Yin, J., Schwegmann-Wessels, C., Herrler, G., 2008. Importance of cholesterol for infection of cells by transmissible gastroenteritis virus. Virus Res. 137, 220–224.

Rina, M., Pozidis, C., Mavromatis, K., Tzanodaskalaki, M., Kokkinidis, M., Bouriotis, V., 2000. Alkaline phosphatase from the Antarctic strain TAB5. Properties and psychrophilic adaptations. Eur. J. Biochem. 267, 1230–1238.

Schultze, B., Enjuanes, L., Herrler, G., 1995. Analysis of the sialic acid-binding activity of the transmissible gastroenteritis virus. Adv. Exp. Med. Biol. 380, 367–370.

Stabile, H., Curti, B., Vanoni, M.A., 2000. Functional properties of recombinant *Azospirillum brasilense* glutamate synthase, a complex iron-sulfur flavoprotein. Eur. J. Biochem. 267, 2720–2730.

Tresnan, D.B., Levis, R., Holmes, K.V., 1996. Feline aminopeptidase N serves as a receptor for feline, canine, porcine, and human coronaviruses in serogroup I. J. Virol. 70, 8669–8674.

Tsukamoto, H., Shibata, K., Kajiyama, H., Terauchi, M., Nawa, A., Kikkawa, F., 2008. Aminopeptidase N (APN)/CD13 inhibitor, Ubenimex, enhances radiation sensitivity in human cervical cancer. BMC Cancer 8, 74.

Volontè, F., Marinelli, F., Gastaldo, L., Sacchi, S., Pilone, M.S., Pollegioni, L., Molla, G., 2008. Optimization of glutaryl-7-aminocephalosporanic acid acylase expression in *E. coli*. Protein Expr. Purif. 61, 131–137.

Wentworth, D.E., Holmes, K.V., 2001. Molecular determinants of species specificity in the coronavirus receptor aminopeptidase N (CD13): influence of N-linked glycosylation. J. Virol. 75, 9741–9752.

Yin, J., Li, G., Ren, X., Herrler, G., 2007. Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. J. Biotechnol. 127, 335–347.

Zhang, R., Hua, G., Andacht, T.M., Adang, M.J., 2008. A 106-kDa aminopeptidase is a putative receptor for *Bacillus thuringiensis* Cry11Ba toxin in the mosquito *Anopheles gambiae*. Biochemistry 47, 11263–11272.