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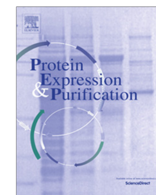

Preparation of the porcine secretory component and a monoclonal antibody against this protein

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ABSTRACT

Secretory component (SC) is a component of secretory IgA that is designated sIgA to distinguish it from IgA. The monoclonal antibody (MAb) against SC has been shown to be an excellent tool for the detection of the level of sIgA and for the evaluation of the efficacy of mucosal immunity. To prepare a monoclonal antibody against porcine SC, a recombinant porcine SC was expressed and purified. To develop this recombinant SC, the gene encoding the porcine SC was ligated into the pCold I vector. The recombinant vector was then transformed into *Escherichia coli* BL 21 (DE3), and gene expression was successfully induced by isopropyl-β-D-thiogalactoside (IPTG). After affinity purification with Ni–NTA resin and gel recovery, the recombinant SC protein was used to immunize BALB/c mice. Finally, three hybridoma cell lines showing specific recognitions of both recombinant SC and native SC were used as stable secretors of MAbs against porcine SC and were confirmed to have no reaction to porcine IgA or IgG. The successful preparations of recombinant SC protein and MAbs provide valuable materials that can be used in the mucosal infection diagnosis for porcine disease and mucosal immune evaluation for porcine vaccine, respectively.

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Introduction

The total surface area of the mucosal epithelia in the gastrointestinal, respiratory and urogenital tracts is 400 m^2, and this is the largest surface in contact with the external environment, in comparison to a total surface area of only 1.5 m^2 for the skin [1]. It has been reported that approximately 100 trillion microorganisms colonize mucosal surfaces [2]. Therefore, many foreign antigens can easily invade the bodies of animals through mucosal surfaces and cause damage. Secretory immunoglobulin A (sIgA)[2] is the major immunoglobulin of mucosal surfaces [3,4], and it binds to pathogens, preventing them from adhering to mucosal surfaces [5]. Thus, sIgA plays an important role in the protection and homeostatic regulation of intestinal, respiratory and urogenital mucosal epithelia separating the outside environment from the inside of the body [6]. sIgA is considered to be the first line of defense against mucosal infection, and secretory component (SC), which has also been called the transport component [6], is vital to the function of sIgA.

SC is the extracellular component of the polymeric immunoglobulin receptor (pIgR) that is responsible for the transcytosis of newly synthesized IgA (polymeric IgA, pIgA) [5]. To enter external secretions, locally synthesized IgA is transported across glandular and mucosal epithelial cells, and this process is mediated

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[2] *Abbreviations used:* sIgA, secretory immunoglobulin A; SC, secretory component; pIgR, polymeric immunoglobulin receptor; MAbs, monoclonal antibodies; DEPC, diethylpyrocarbonate; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PEG, polyethylene glycol; TMB, tetramethylbenzidine; BALF, bronchoalveolar lavage fluid; PVDF, polyvinylidene difluoride.

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by covalent binding of IgA to pIgR [7–9]. The pIgR–pIgA complex is transcytosed across the cell, and pIgR is then cleaved by a protease within a 42-amino acid region adjacent to the cell membrane at the luminal surface. This results in the release of sIgA into the lumen [10]. SC protects sIgA from degradation by host and bacterial proteases in the intestinal tract [11], promotes glycan-dependent adherence of sIgA to bacteria by nonspecific binding [12], and neutralizes inflammatory host factors, such as IL-8 [13,14]. SC improves the ability of the mouse respiratory tract to defend itself against bacterial affection [15], interacts with the surface proteins of *Salmonella typhimurium* to inhibit its invasion of and adherence to HeLa cells [16], and reduces *giardia* infection [17]. Soluble SC was isolated from human colostrum as a reference protein in tracheal aspirate fluid, using serial centrifugation, size-exclusion fractionation and ion-exchange chromatography [18]. The human SC gene was also successfully amplified for the construction of a prokaryotic expression plasmid, and monoclonal antibodies (MAbs) against SC were produced [19]. Although study on human SC has been developed rapidly, little is known about porcine SC. In the present study, the recombinant porcine SC protein and three MAbs against this protein were successfully prepared, which would open new opportunities for laboratory and basic research of porcine secretory immunity.

Materials and methods

Cell lines and culture

Mouse myeloma cells (SP2/0) were cultured in RPMI-1640 medium (Gibco, CA, USA) supplemented with 20% FBS. The cells were cultured at 37 °C in a humidified environment of 5% CO_2.

Preparation of porcine SC RNA and cDNA

Tracheal epithelial tissues of pigs were collected and cut into small pieces. Total RNA from the tissue was extracted using a Trizol reagent kit (Invitrogen, CA, USA). The prepared RNA was dissolved in 30 μl of diethylpyrocarbonate (DEPC) water.

Total RNA was then reverse transcribed to cDNA. Briefly, 10.5 μl of the RNA extract was added into a reaction mixture with a final volume of 20 μl, containing 4 μl of 5 × M-MLV buffer, 1 μl of M-MLV reverse transcriptase (TaKaRa, Dalian, China), 0.5 μl of RNase inhibitor, 2 μl of dNTP and 2 μl of Oligo (dt) 15 primer (Promega, WI, USA). The samples were incubated at 45 °C for 1 h and then at 95 °C for 5 min for enzyme deactivation.

For the polymerase chain reaction (PCR), the primers were designed according to the pIgR genome of pig from Genbank (accession No. NM 214159.1): SC F: 5′-GCGGAATTCAAGAGTCCCA TATTCG-3′; SC R: 5′-TTAAGCTTTTTGGAGCCCCC-3′. The thermal cycling parameters were as follows: one initial denaturation step at 95 °C for 5 min; 30 cycles of (i) 95 °C for 90 s, (ii) 50 °C for 90 s, and (iii) 72 °C for 120 s; and a final extension step at 72 °C for 10 min. PCR products were analyzed on a 1% agarose gel.

Construction of recombinant plasmid

The SC porcine gene was T/A cloned into the cloning vector pMD18-T (Takara, Dalian, China) to generate the recombinant vector pMD18-T-SC for sequencing. Then the SC gene was cloned into the prokaryotic expression vector pCold I (Takara, Dalian, China) from pMD18-T-SC, and the resultant plasmid was named pCold I-SC. The cloning of SC into these plasmids was confirmed by enzymatic digestion with *EcoR I* and *Hind III* (pCold I) and DNA sequencing. The transformants were cultured on Luria–Bertani (LB) agar plates supplemented with 100 μg/ml ampicillin.

Expression and purification of SC protein

The recombinant plasmid pCold I-SC was transformed into *Escherichia coli* BL21 cells (Transgen Biotech, Beijing, China) and cultured at 15 °C until reaching an optical density of 0.4–0.5 at 600 nm (OD600). Protein expression was induced by isopropyl-β-D-thiogalactoside (IPTG) at 15 °C with a final concentration of 1.0 mM for 24 h.

Every gram of cell pellet was resuspended in 5 ml of lysis-equilibration-washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Lysozyme was added at a final concentration of 1 mg/ml, and then the suspension was stirred on ice for 30 min. Subsequently, the suspension was sonicated for 10 min. Inclusion bodies were collected after centrifugation and 100 mg of them were resuspended in 10.0 ml of denaturing solubilization buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8.0).

The SC protein was purified using a Protino® Ni-TED 2000 packed column (Macherey–nagel, Neuman, Germany). Briefly, the column containing 1 ml of Ni-TED resin was equilibrated with four bed volumes of denaturing solubilization buffer. The inclusion bodies were added into the column, followed by washing with eight bed volumes of denaturing solubilization buffer and eluting with three bed volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 250 mM imidazole, pH 8.0) for three times. The primarily purified SC protein in the elution buffer was dialyzed in PBS (pH 7.4) and concentrated by PEG-6000. After that, the SC protein was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for further purification using PAGE gel protein recovery kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instruction. Briefly, the gels were immersed into chilled 250 mM KCl for 1 min, and then the white bands were excised, cut into pieces and dipped into the recovery buffer A for 18 h. Transferred the supernatant into another new Eppendorf tube after centrifuging and the recovery buffer B was added. Centrifuged again and the sediment was the recombinant SC protein. The purified SC protein was confirmed by SDS–PAGE and Western blotting assay. The concentration of the purified protein was determined by the Bradford assay, using bovine serum albumin (BSA) as a protein standard.

Preparation of MAbs

Five 8-week-old female BALB/c mice (purchased from Comparative Medicine Centre of Yangzhou University) were immunized subcutaneously with 100 μg recombinant SC protein emulsified in Freund's complete adjuvant (Sigma–Aldrich, Saint Louis, USA) at a ratio of 1:1. Two booster immunizations were given with 100 μg recombinant protein emulsified in Freund's incomplete adjuvant (Sigma–Aldrich, Saint Louis, USA) at 2-week intervals. Afterwards, blood samples were collected from the tail vein to detect the antibody titer by an indirect enzyme-linked immunosorbent assay (ELISA) using plates coated with the 100 ng purified recombinant SC protein every well (ELISA-SC). The last injection was performed intraperitoneally with 200 μg of antigen. Three days later, the spleen of the immunized mouse was eviscerated for fusion with the pre-prepared myeloma cells (SP2/0).

Selection of positive hybridoma cells

The splenocytes were fused with SP2/0 while under activation by polyethylene glycol (PEG) (Sigma–Aldrich, Saint Louis, USA). Hybridoma cells were cultured in HAT and HT medium (Sigma–Aldrich, Saint Louis, USA). Secreting hybrids were identified by detection of the hybridoma culture supernatants. Positive hybridomas, those secreting antibodies against recombinant SC protein,

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