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Glycoproteins isolated from *Atractylodes macrocephala* Koidz improve protective immune response induction in a mouse model

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Abstract This study examined the efficacy of *Atractylodes macrocephala* Koidz (AMK) protein and polysaccharide extracts as adjuvant or adjuvant booster when given together with porcine pleuropneumonia vaccine. Experimental mice (n = 5/group) were subcutaneously immunized with 25 μg ApxIIA #3 antigen, a target protein against *A. pleuropneumoniae*, together with alum and/or various concentrations (0–500 μg) of the AMK extracts, while the control group received PBS only. Immunization with ApxIIA #3 antigen increased the antigen-specific IgG titer and this increase was enhanced in the immunization together with AMK protein, but not polysaccharide extract. Supplementation of AMK protein extract exhibited dose-dependent increases in the antigen-induced protective immunity against *A. pleuropneumoniae* challenge and in the lymphocyte proliferation specific to the antigen. Glycoproteins present in the AMK extract were the active components responsible for immune response induction. Collectively, the present findings suggest that AMK glycoproteins are useful as immune stimulating adjuvant or adjuvant booster.

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Introduction

Vaccines offer various advantages, in which alum and MF59 are the common vaccine adjuvants (Ott et al., 1995; Podda and Del Giudice, 2003). However, most of adjuvants including the alum and MF59 require high dosages to achieve protective immunity and are to be potentially toxic to administer (Del Giudice et al., 2001). To improve a limited efficacy due to poor immunogenicity, many investigators have been paid their efforts to develop new adjuvants or to search efficient approaches for systemic and mucosal subunit vaccines (Del Giudice et al., 2001; Stevceva and Strober, 2004). The use of adjuvants together with active components that can increase synergistic activity of adjuvant is also considered as an attractive method in improving immune response induction to vaccination (Baudner et al., 2003). In addition, it is suggested that immunization in combination with immune stimulating components reduces the amount of antigens required and decreases the toxicity of adjuvants (Huber et al., 2003).

Traditional herbal plants are widely used for the enhancement of general wellbeing and the modulation of immune response induction. Among the bioactive materials present in traditional medicines, glycoproteins and polysaccharides are considered important macromolecules for stimulation of immune response induction (Lee et al., 2005). Specifically, *Atractylodes macrocephala* Koidz (AMK) has been widely used as one of traditional medicine prescriptions (Lee et al., 2005; Song et al., 2015). AMK glycoproteins and polysaccharides are known to exert beneficial effects on disordered intestinal flora (Wang

et al., 2014), macrophage activation (Ji et al., 2015), and Th2-type immune response-mediated allergic diarrhea (Kim et al., 2005). A previous report highlighted that immune stimulating activity of AMK is closely related to glycoproteins with molecular weights of around 30 kDa, especially with carbohydrate moiety rather than protein residues of the glycoproteins (Lee et al., 2007). Together, these reports suggest that AMK glycoproteins and polysaccharides may improve the efficacy of vaccination by acting as adjuvant or adjuvant booster.

In this study, AMK protein and polysaccharide extracts were prepared and their physiochemical properties were characterized. The potential of the AMK extracts to induce antigen-specific immune responses was also investigated. To this end, mice were subcutaneously immunized with the mixtures containing a partial fragment #3 of ApxIIA antigen together with alum and/or each of the AMK extracts. The antigen is one of the effective vaccine candidates against porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) (Seo et al., 2011; 2013). Subsequently, this study explored whether the immunization in combination with AMK protein or polysaccharide extract improves protective immunity of the antigen against challenge infection with *A. pleuropneumoniae*. Furthermore, the levels of the antigen-specific immunoglobulins (Igs) in the sera and feces, as well as of the antigen-specific splenocyte proliferation were determined after the first or boost immunization.

Materials and methods

Chemicals and plastics

All reagents used for cytokine production assay were obtained from PharMingen Inc. (San Diego, CA, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA) and [*methyl*-^{3}H] thymidine deoxyribose (TdR) and alum were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Unless otherwise specified, other chemicals were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA), and laboratory consumables were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA).

Experimental animals

Female BALB/c mice (4-weeks-old, total 95 heads) were purchased from Orient Co. (Kapyung, South Korea). Mice were randomly divided into cages (n = 5/cage) and housed in automatically controlled conditions with a 12-h light/-dark cycle at 22 ± 1 °C and 45–55% relative humidity. All mice had free access to standard rodent pellet food and water. All procedures for the animal care and use in this study followed the guidelines established by Institutional Animal Care and Use Committee of Chonbuk National University Laboratory Animal Center. The experimental protocol was approved by the University Committee on Ethics in the Care and Use of Laboratory Animals (Permit No.: CBU 2010-0007). All mice were acclimatized to new laboratory environment for 1 week before experimental use and monitored every 12 h per day before challenge infection with porcine pathogen.

Preparation of AMK extracts

AMK was provided by Dr. W.-H. Woo (College of Oriental Medicine, Wonkwang University, Iksan, South Korea) and authenticated by botanists at the Korea Institute of Oriental Medicine. A voucher specimen (# 2001-N3) has been deposited in the author's laboratory (J.-C. Lee). AMK protein and polysaccharide extracts were obtained from dried AMK (100 g) according to the methods described previously (Lee et al., 2005).

Physicochemical characterization of AMK samples

Physiochemical properties of AMK protein and polysaccharide extracts were characterized according to the methods described previously (Lee et al., 2005). In brief, the protein and polysaccharide extracts were separated by SDS–PAGE (10–15% gel) and gels were then stained either with Coomassie brilliant blue (CBB) to detect proteins or with Schiff reagent to detect glycoproteins. To determine whether the immune stimulating activity of protein extract was due to glycoproteins, the sample was treated with pronase E to degrade the protein or with $NaIO_4$ to degrade the carbohydrate residues. The protein extracts treated with pronase E or $NaIO_4$ were dialyzed against PBS before use. In order to explore if the protein and polysaccharide extracts contained a lectin compound such as concanavalin A (Con A), these extracts (10 mg/sample) were dissolved in 1 mL distilled water in a tube and the tubes were soaked in a boiling water bath for 20 min. Alternatively, the protein and polysaccharide extracts (1 mg/sample) were dissolved in 1 mL of culture medium containing 1000 units of polymyxin B (PMB) and incubated at 37 °C for 3 h to determine if the extracts contained lipopolysaccharide (LPS)-like compounds. Each of the AMK extracts pretreated with pronase E, $NaIO_4$, PMB, or heating was added to splenocyte cultures at a concentration of 10 μg/mL. The mitogens such as Con A and LPS that were pretreated with PMB or heating were used as comparative positive controls. Finally, the effects of AMK extracts and mitogens on splenocyte proliferation and cytokine production were determined by TdR

incorporation assay and enzyme-linked immunosorbent assay (ELISA) methods.

In vitro assays on lymphocyte proliferation and cytokine production

A single-cell population of splenocytes was prepared from BALB/c mice and resuspended in RPMI-1640 medium supplemented with antibiotics and 10% FBS. The suspension of splenocytes (4×10^6 cells/mL) was divided into 24- (500 μL/well) or 96-well flat-bottomed plates (200 μL/well). After 24 h of incubation, culture media were replaced with fresh RPMI medium supplemented with 0.5% FBS and 10 μg/mL of AMK protein or polysaccharide extract, or 5 μg/mL of Con A or LPS that was pretreated or not with heating, PMB, pronase E, or $NaIO_4$. [*methyl*-^{3}H] TdR (0.5 μCi/mL) was added to each well of the 96-well plates for the last 16 h of a 48 h culture period and the incorporated tritium contents were determined using a liquid scintillation counter (Perkin-Elmer, Waltham, MA, USA). Alternatively, supernatants of the splenocyte cultures were collected from 24-well plates after 48 h of incubation, and the levels of cytokines such as interferon-γ (IFN-γ), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were determined by ELISA.

Production of recombinant ApxIIA #3 toxin protein

Recombinant ApxIIA #3 antigen protein was produced and purified according to the methods described previously (Seo et al., 2011). In brief, pCR2.1 vector containing full-length *apx*IIA gene (accession number: AF363362) was kindly provided by Prof. H.-S. Yoo (Seoul National University, South Korea). The cloning of ApxIIA #3 fragment from the full-length *apx*IIA gene was performed using an *Escherichia coli* (*E. coli*) expression system, pQE (Qiagen, Hilden, Germany) according to the manufacturer's instruction provided. Recombinant antigen protein was produced in the BL21 bacterial system and refined by Ni-NTA agarose (Qiagen). Authenticity of the antigen protein was confirmed through SDS–PAGE and Western blot analysis using both an anti-6 × His tag and polyclonal anti-ApxII antibodies.

Subcutaneous immunization

The efficacies of AMK protein and polysaccharide extracts as adjuvant or adjuvant booster were evaluated when immunized in combination with porcine pleuropneumonia vaccine. In this study, ApxIIA #3 toxin protein was used as a model antigen to the porcine pleuropneumonia, based on the previous findings (Seo et al., 2011; 2013). Briefly, mice were randomly divided into the control and experimental groups (n = 5/group). The experimental groups received subcutaneous injection of a mixture containing 25 μg/100 μL of ApxIIA #3 antigen, 100 μL of alum (Amersham Pharmacia Biotech Inc), and/or various concentrations (0–500 μg) of AMK protein or polysaccharide extract. The mice received a booster immunization with the same mixtures 2 weeks after the first immunization. In parallel, the control group received the same volume of PBS alone during the experimental period.

Collection of blood sera and feces

Blood samples were collected from the PBS control and experimental mice through the orbital vein 10 days after the first (1st sampling) and the boost immunization (2nd sampling), respectively. The sera were taken from the blood samples by centrifugation after 1 h clotting response and analyzed to monitor the presence of anti-ApxIIA #3 IgG. Feces were also harvested from the control and experimental groups at the same times to prepare fecal extracts according to the methods described elsewhere (Kim et al., 2006). Briefly, fecal pellet (0.1 g) was mixed with 1 mL PBS containing NaN_3 and vortexed for 10 min. The mixtures were centrifuged at 12,000×g for 10 min and the supernatants were collected into new tubes to measure the level of anti-ApxIIA #3 IgA.

Measurement of antigen-specific immune responses

The levels of serum IgG and fecal IgA specific to ApxIIA #3 antigen were measured by direct ELISA according to the methods described elsewhere (Seo et al., 2011). Briefly, each well of ELISA plates was coated with 50 μL of 0.05 M carbonate–bicarbonate buffer containing 0.5 μg/mL of ApxIIA #3 protein and the plates were incubated overnight at 4 °C. The plates were incubated in PBS buffer containing 1% skim milk at 37 °C for 1 h and then treated with twofold serially diluted samples. After incubation overnight at 4 °C, alkaline phosphatase-labeled anti-mouse IgG (Fc specific) or IgA (α-chain specific) antibody was added to the plates followed by an additional-2 h incubation at 37 °C. The absorbance of each sample was measured at 405 nm using a SpectraCount™ ELISA reader (Packard Instrument Co., Downers Grove, IL, USA). The antibody titer was expressed as the reciprocal of the geometric $\log_2$ of the least concentrated sample that gave a basal level absorbance of 0.08, which was the value of the PBS control.

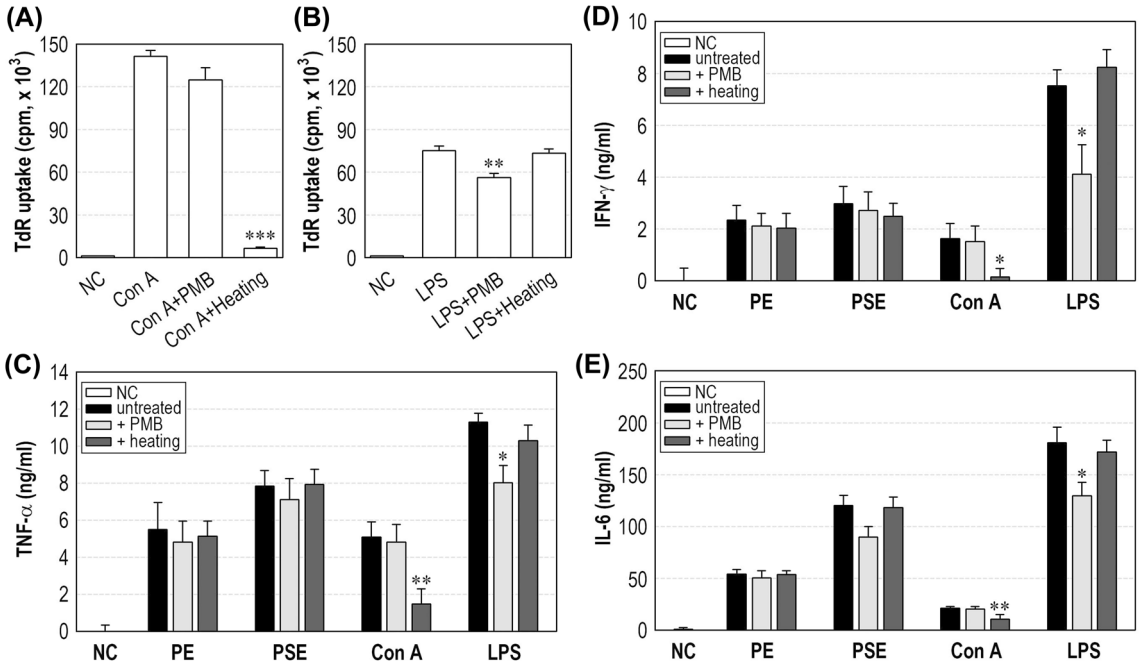

Fig. 1 Effects of AMK samples and mitogens treated with PMB or heating on lymphocyte proliferation and cytokine production. Splenocytes were incubated with 5 μg/mL of Con A (**A**) or LPS (**B**) that was pretreated with PMB or heating and the proliferation rates were determined by TdR incorporation assay. Splenocytes were also incubated with each of AMK extracts or mitogens that was pretreated with PMB or heating and the levels of TNF-α (**C**), IFN-γ (**D**), and IL-6 (**E**) were determined by ELISA 48 h after incubation. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared with the cells treated with the extract or mitogen alone (n = 5/group). NC, untreated negative control; PE, AMK protein extract; PSE, AMK polysaccharide extract

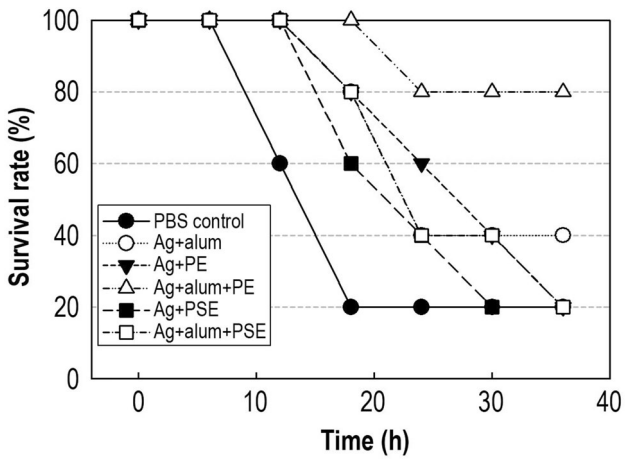

Fig. 2 Effects of AMK extracts on the induction of protective immunity against the challenge infection with *A. pleuropneumoniae*. Mice were subcutaneously immunized with the mixtures containing ApxIIA #3 antigen, alum, or both or in combination with each of AMK extracts. After 2 weeks of the boost immunization, mice (n = 5/group) were challenged intraperitoneally with 3×10^{10} CFU of the porcine pathogen and survival rate (%) was monitored every 6 h for 36 h. Ag, ApxIIA #3 antigen

Measurement of antigen-specific lymphocyte proliferation

The level of antigen-specific lymphocyte proliferation was measured by TdR incorporation assay according to the methods described previously (Seo et al., 2013). Briefly, spleen was incised from the control and experimental groups 10 days after the first immunization and dissociated with a needle. The cell suspension was filtered with a 40 μm fine cell strainer and the filtrates were collected into a tube. The cell pellets were resuspended in 6 mL of 40% Percoll (Amersham Pharmacia Biotech Inc.) and floated on 75% Percoll followed by centrifugation at $1500 \times g$ for 20 min. Mononuclear cells were collected from the interface between the 75 and 40% Percoll and washed with serum-free media. The suspension of cells (2×10^6 cells/mL) was divided into each well of 96-well flat-bottomed plates (200 μL/well) together with 2 μg of ApxIIA #3 antigen. The culture plates were incubated for 72 h at 37 °C and then [*methyl*-^{3}H]-TdR (0.5 μCi/well) was added to the plates. After incubation for an additional 12 h, the cells were collected with a cell harvester (Inotech, Dottikon, Switzerland) and the incorporated tritium contents were counted using a liquid scintillation counter (Perkin-Elmer). Stimulation indices were calculated by dividing the

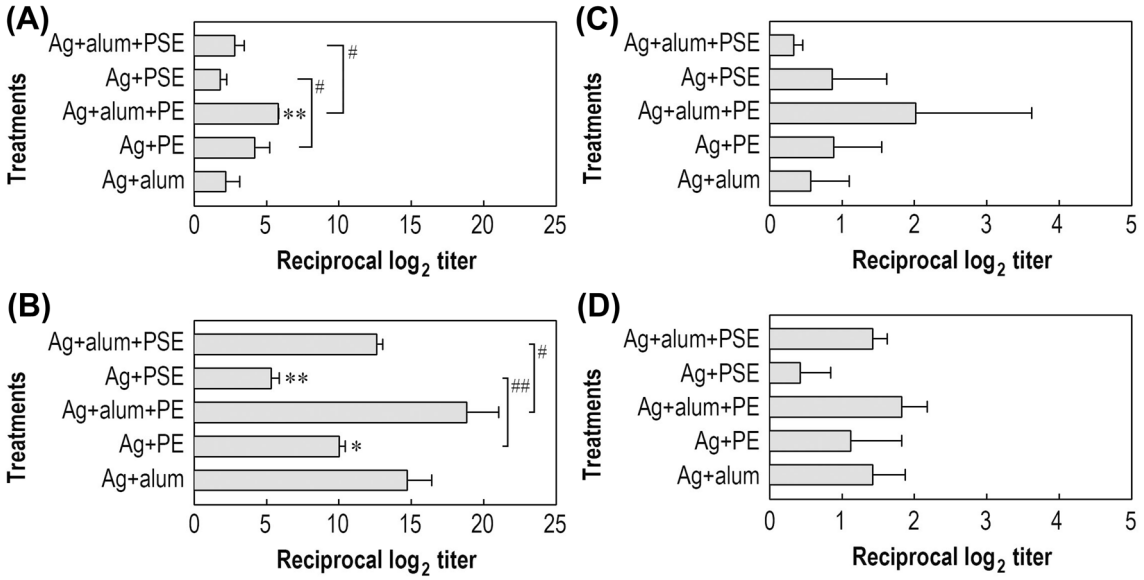

Fig. 3 Stimulating effect of AMK extracts on the induction of antigen-specific systemic or mucosal immune response to ApxIIA #3 antigen. Mice were subcutaneously immunized with the mixtures containing ApxIIA #3 antigen, alum, and/or each of AMK extracts. Ten days after the first (**A**, **C**) and the boost immunization (**B**, **D**), antigen-specific IgG titer in the sera (**A**, **B**) or antigen-specific IgA titer in the fecal extracts (**C**, **D**) was measured by direct ELISA. The results are represented as the reciprocal of the geometric mean log2 titer (n = 5/group). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the mice immunized with the antigen and alum. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ indicate significant differences of the titers between the two groups

tritium incorporation level (cpm) in cells treated with antigen with that in the control cells treated with PBS.

Challenge assay

The *A. pleuropneumoniae* used for this study were kindly gifted from Prof. H.-S. Yoo. The bacteria were cultivated at 37 °C in brain heart infusion broth with β-NAD (10 μg/mL) according to the methods described elsewhere (Seo et al., 2013). The PBS control and immunized mice (n = 5/group) received a challenge infection by single dose intraperitoneal injection with 200 μL of *A. pleuropneumoniae* preparation (3×10^{10} colony-forming units; CFU) 2 weeks after the boost immunization. Survival rates of mice were recorded every 6 h after the challenge infection for 36 h.

Statistical analyses

All results are expressed as the mean ± standard deviation (SD) of five different samples. One-way ANOVA was used to determine the significance of differences among or over three groups using Statistical Package for the Social Sciences (SPSS) (version 12.0). When one-way ANOVA was significant ($p < 0.05$), Post-hoc Tukey test was used to determine significance differences among groups. Student's t test was used only when the significance of differences between two sets of data was determined using the SPSS program. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

Physicochemical properties of AMK samples

Similar to our previous findings (Lee et al., 2005; 2007), the stimulating activity of AMK protein or polysaccharide extract on the proliferation of splenocytes was not reduced by treatment with PMB or heating (data not shown). In contrast, the Con A- or LPS-stimulated proliferation of splenocytes was inhibited by the heating or PMB treatment [Fig. 1(A, B)]. Similarly, PMB or heating treatment did not affect AMK samples' activity to stimulate the production of TNF-α [Fig. 1(C)], IFN-γ [Fig. 1(D)], and IL-6 [Fig. 1(E)] by splenocytes, whereas ConA or LPS treated with heating or PMB exhibited significantly lower activity for the production of these cytokines compared with the untreated mitogen. These results suggest that AMK protein and polysaccharide extracts did not contain lectin compounds and were not contaminated with LPS-like mitogen.

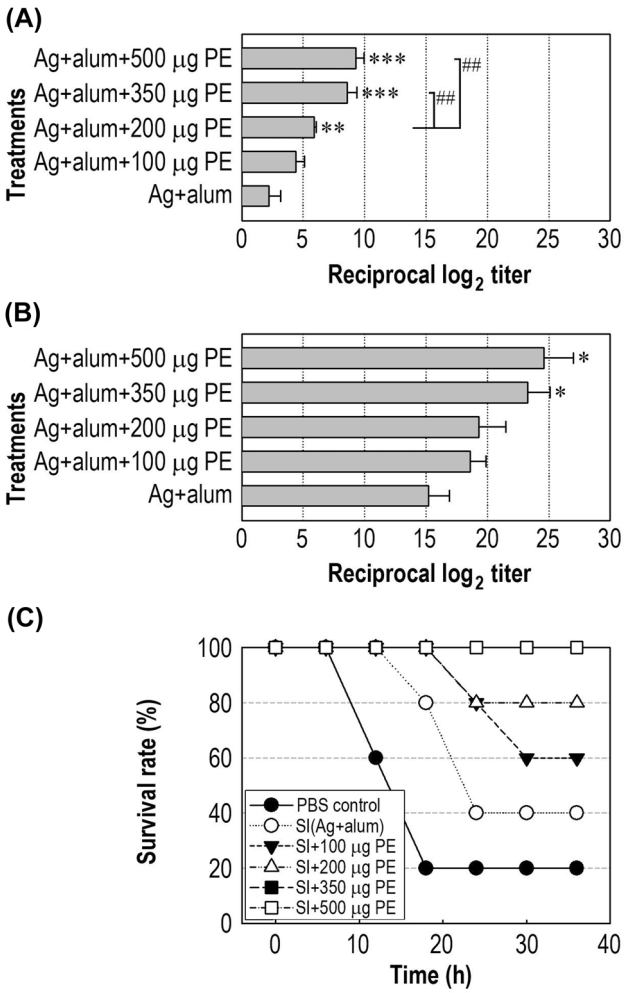

Fig. 4 Combined immunization with AMK protein extract enhances the ApxIIA #3 antigen-specific systemic immune response and the protective immunity in a dose-dependent manner. Mice were subcutaneously immunized with the mixtures containing ApxIIA #3 antigen and alum, or in combination with the increasing amounts (0–500 μg) of AMK protein extract. After 10 days of the first (**A**) and the boost immunization (**B**), antigen-specific IgG titer in the sera was measured by ELISA and the results are represented as the reciprocal of the geometric mean log2 titer (n = 5/group). $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the mice received a subcutaneous immunization only. $^{\#\#}p < 0.01$ indicates a significant difference of the titers between the groups. (**C**) Two weeks after the boost immunization, mice (n = 5/group) was also challenged intraperitoneally with 3×10^{10} CFU of the porcine pathogen and survival rate (%) was monitored for 36 h. SI, subcutaneous immunization

AMK samples improves the protective immunity against *A. pleuropneumoniae* infection by stimulating systemic antigen-specific immune responses

Subcutaneous immunization with ApxIIA #3 antigen and alum induced the protective immune response, in which four and two out of five mice of the immunized mice were still alive after 18 and 36 h of the pathogen challenge infection, respectively (Fig. 2). The immunization together with AMK protein or polysaccharide extract in the absence of alum also induced the protective immunity, although the survival rates of these groups were reduced similar to that of the PBS control after 36 h of the bacterial injection. However, when mice were immunized in combination with alum and AMK protein, only one mouse was dead 24 h after the challenge injection and the remaining four mice were alive until 36 h after the injection. These results indicate that AMK samples act as adjuvant or adjuvant booster, where the protein extract enhances protective immune response greater than the polysaccharide extract.

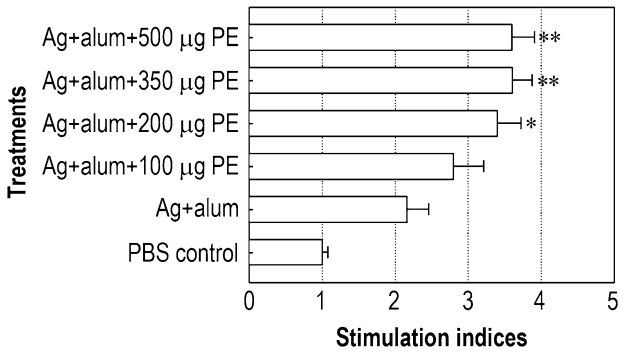

Fig. 5 Combined immunization with AMK protein extract enhances ApxIIA #3 antigen-specific lymphocyte proliferation. Lymphocytes were isolated from spleen tissues of the PBS control and immunized mice 10 days after the first immunization. The level of ApxIIA #3 antigen-specific lymphocyte proliferation was measured by TdR incorporation assay after in vitro stimulation with the antigen and the results are expressed as stimulation indices. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the mice that received immunization without the extract (n = 5/group)

To evaluate the efficacy of AMK samples on the induction of systemic immune responses to ApxIIA #3 antigen, serum level of the antigen-specific IgG was measured by ELISA. When the sera collected 10 days after the first immunization were analyzed, the titer of ApxIIA #3-specific IgG in the mice immunized together with AMK protein, but not polysaccharide extract, was significantly higher than that in the mice immunized without the extract [Fig. 3(A)]. After the boost immunization, serum IgG titer specific to the antigen was further increased in all experimental groups compared with that after the first immunization [Fig. 3(B)]. The immunized mice in combination with AMK protein extract also showed higher titer of the antigen-specific serum IgG compared with the mice immunized together with polysaccharide extract. However, there was no a significant difference in the fecal IgA titer specific to the antigen among the experimental mice groups after both the first [Fig. 3(C)] and the boost immunization [Fig. 3(D)]. These results indicate that subcutaneous immunization together with AMK protein extract

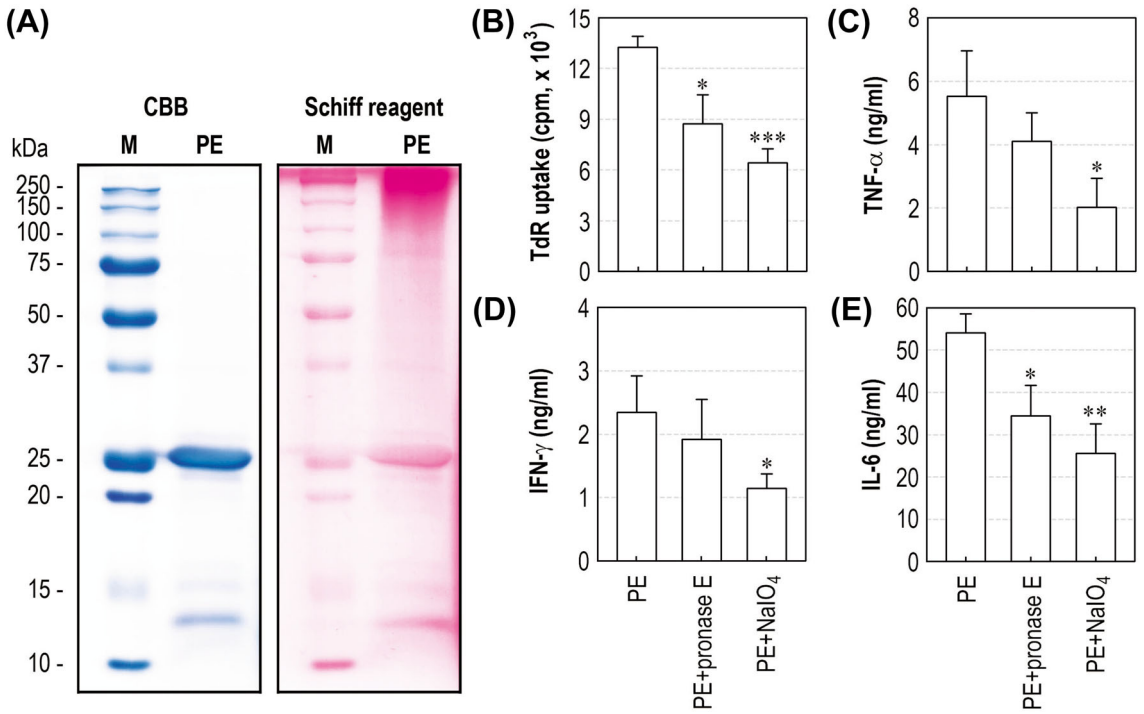

Fig. 6 Effects of AMK protein extract treated with pronase E or $NaIO_4$ on in vitro lymphocyte proliferation and cytokine production. (**A**) AMK protein extract (200 μg/lane) was electrophoresed in a 12% SDS–PAGE and then stained with CBB (left panel) or Schiff reagent (right panel). M, molecular weight marker; PE, AMK protein extract. Splenocytes were incubated with 10 μg/mL of AMK protein extract pretreated with pronase E or $NaIO_4$ and the lymphocyte proliferation rate (**B**) along with the levels of TNF-α (**C**), IFN-γ (**D**), and IL-6 (**E**) in culture supernatants were determined by TdR incorporation assay and ELISA 48 h after incubation, respectively. $*p < 0.05$; $**p < 0.01$, and $***p < 0.001$ compared with the untreated PE values (n = 5/group)

stimulates systemic immune response rather than mucosal immunity. Similarly, there are several reports demonstrating that systemic immunization through intramuscular or subcutaneous injection does not stimulate a mucosal IgA response (Chen and Cerutti, 2010; Holmgren and Czerkinsky, 2005; Seo et al., 2013).

Supplementation with 100, 200, 350, and 500 μg of AMK protein extract augmented the antigen-specific serum IgG titer up to 2-, 2.6-, 3.9-, and 4.2-folds, respectively, compared with the mice that received immunization only (2.2 ± 0.94) [Fig. 4(A)]. When the serum IgG titer was determined after the boost immunization, the groups immunized together with more than 200 μg of the AMK protein extract significantly increased the titer compared with that in the group immunized with the antigen and alum [Fig. 4(B)]. In parallel, combined treatment with AMK protein extract showed a dose-dependent protection against the infection with *A. pleuropneumoniae* [Fig. 4(C)]. Subcutaneous immunization with ApxIIA #3 antigen and alum also increased the priming efficiency on the antigen compared to that of the PBS control mice, such that the immunized group augmented stimulation index up to 2.1-fold compared with the control mice (Fig. 5). Further, immunization together with the protein extract enhanced the antigen-specific lymphocyte proliferation in a dose-dependent manner, where stimulation indices in the immunized mice were augmented to 1.62-, 1.71-, and 1.71-folds in combination with 200, 350, and 500 μg of the protein extract, respectively. All of these findings support that subcutaneous immunization together with AMK protein extract elevates serum IgG titer specific to the antigen in a dose-dependent manner. It is also suggested that AMK protein extract increases antigen-specific lymphocyte proliferation.

Following the underlying mechanisms by which AMK samples improve the antigen-stimulated protective immunity against *A. pleuropneumoniae* infection, it is considered that (1) the antigen-stimulated protective immunity against the porcine pathogen and its improvement by AMK protein extract is closely associated with the antigen-specific increases in serum IgG rather than fecal IgA titer, (2) the increase in systemic immune response induction after the first immunization is more linked with the improved protective immunity than that after the boost immunization, and (3) the supplementation with AMK protein extract stimulates both the humoral and cellular immune responses specific to the antigen.

The balance between the Th1 and Th2 cell subpopulations plays an important role in modulating immune response induction. Th1 cells that produce IL-2, IFN-γ, and lymphotoxin are involved mostly in cell-mediated immune reactions, while Th2 cells that mainly produce IL-4, IL-5, IL-6, IL-10, and IL-13 are associated with strong antibody and allergic immune responses (Mosmann et al., 1986). The current data along with the previous findings (Ji et al., 2015; Kim et al., 2005; Lee et al., 2005) suggest that AMK samples potentially enhance Th2-mediated and/or macrophage-mediated (or B cells) immune responses. It is also likely that subcutaneous immunization stimulates prominently Th2- more than Th1-type cytokine expression. Collectively, it is postulated that supplementation of AMK protein extract improves protective immunity against the porcine pathogen and this is at least in part associated with the potential of the extract to stimulate host immune responses specific to Th2-type T lymphocytes. Overall, AMK protein extract is to be useful as adjuvant or adjuvant booster for human, because the AMK has long been used in the traditional medicines.

Glycoproteins are the active components of AMK protein extract and its immune stimulating activity is due to the carbohydrate, rather than protein residues

AMK extract showed two major groups of bands around 27 kDa and smaller than 15 kDa together with a broad spectrum of molecular weights by CBB staining and mainly of approximately 200, 27, and 13 kDa by Schiff reagent staining [Fig. 6(A)]. Treatment of the extract with either pronase E or $NaIO_4$ decreased the potential for the sample to stimulate TdR incorporation in splenocytes [Fig. 6(B)]. The AMK extract-stimulated production of TNF-α [Fig. 6(C)] and IFN-γ [Fig. 6(D)] in the cells was also significantly ($p < 0.05$) inhibited when the extract was treated with $NaIO_4$. In addition, treatment with pronase E ($p < 0.05$) or $NaIO_4$ ($p < 0.01$) led the protein extract to exhibit significantly attenuated production of IL-6 [Fig. 6(E)]. These results confirm that glycoproteins are the main components of AMK protein extract responsible for its immune stimulating activity.

In summary, glycoproteins and polysaccharides derived from traditional medicines could be potentially used as efficacious vaccine adjuvant candidates or adjuvant boosters (Bao et al., 2015; Pi et al., 2014). The current findings highlight that AMK extracts, especially protein extract, are useful as adjuvant and/or adjuvant booster to improve the efficacy of vaccination by enhancing systemic immune response. This study also supports that glycoproteins are the important immune active components present in AMK protein extract and the extract did not contain lectin compounds and were not contaminated with LPS-like mitogens.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Human and animal rights This research proposal was approved by the Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals, which is considered as our institutional animal care and use committee in scientific research.

References

Bao LD, Ren XH, Ma RL, Wang Y, Yuan HW, Lv HJ. Efficacy of *Artemisia annua* polysaccharides as an adjuvant to hepatitis C vaccination. Genet. Mol. Res. 14: 4957–4965 (2015)

Baudner BC, Giuliani MM, Verhoef JC, Rappuoli R, Junginger HE, Giudice GD. The concomitant use of the LTK63 mucosal adjuvant and of chitosan-based delivery system enhances the immunogenicity and efficacy of intranasally administered vaccines. Vaccine 21: 3837–3844 (2003)

Chen K, Cerutti A. Vaccination strategies to promote mucosal antibody responses. Immunity 33: 479–491 (2010)

Del Giudice G, Podda A, Rappuoli R. What are the limits of adjuvanticity? Vaccine 20 (Suppl 1): S38–S41 (2001)

Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat. Med. 11 (4 Suppl): S45–S53 (2005)

Huber VC, Arulanandam BP, Arnaboldi PM, Elmore MK, Sheehan CE, Kallakury BV, Metzger DW. Delivery of IL-12 intranasally leads to reduced IL-12-mediated toxicity. Int. Immunopharmacol. 3: 801–809 (2003)

Ji GQ, Chen RQ, Zheng JX. Macrophage activation by polysaccharides from *Atractylodes macrocephala* Koidz through the nuclear factor-κB pathway. Pharm. Biol. 53: 512–517 (2015)

Kim SH, Jung HN, Lee KY, Kim J, Lee JC, Jang YS. Suppression of Th2-type immune response-mediated allergic diarrhea following oral administration of traditional Korean medicine: *Atractylodes macrocephala* Koidz. Immunopharmacol. Immunotoxicol. 27: 331–343 (2005)

Kim SH, Lee KY, Kim J, Park SM, Park BK, Jang YS. Identification of a peptide motif enhancing mucosal and systemic immune responses against the peptide conjugated antigen after oral administration in mice. Mol. Cells 21: 244–250 (2006)

Lee JC, Lee KY, Jung HN, Kim JG, Jang YS. Glycoproteins contained within Soamsan, a traditional Oriental medicine, are the main class of active ingredients responsible for the medicine-induced immune stimulation. J. Biochem. Mol. Biol. 38: 253–257 (2005)

Lee JC, Lee KY, Son YO, Choi KC, Kim J, Kim SH, Chung GH, Jang YS. Stimulating effects on mouse splenocytes of glycoproteins from the herbal medicine *Atractylodes macrocephala* Koidz. Phytomedicine 14: 390–395 (2007)

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348–2357 (1986)

Ott G, Barchfeld GL, Van Nest G. Enhancement of humoral response against human influenza vaccine with the simple submicron oil/water emulsion adjuvant MF59. Vaccine 13: 1557–1562 (1995)

Pi CC, Chu CL, Lu CY, Zhuang YJ, Wang CL, Yu YH, Wang HY, Lin CC, Chen CJ. Polysaccharides from *Ganoderma formosanum* function as a Th1 adjuvant and stimulate cytotoxic T cell response in vivo. Vaccine 32: 401–408 (2014)

Podda A, Del Giudice G. MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. Expert Rev. Vaccines 2: 197–203 (2003)

Seo KW, Kim DH, Kim AH, Yoo HS, Lee KY, Jang YS. Characterization of antigenic determinants in ApxIIA exotoxin capable of inducing protective immunity to *Actinobacillus pleuropneumoniae* challenge. Immunol. Invest. 40: 465–480 (2011)

Seo KW, Kim SH, Park J, Son Y, Yoo HS, Lee KY, Jang YS. Nasal immunization with major epitope-containing ApxIIA toxin fragment induces protective immunity against challenge infection with *Actinobacillus pleuropneumoniae* in a murine model. Vet. Immunol. Immunopathol. 151: 102–112 (2013)

Song HP, Li RL, Zhou C, Cai X, Huang HY. *Atractylodes macrocephala* Koidz stimulates intestinal epithelial cell migration through a polyamine dependent mechanism. J. Ethnopharmacol. 159: 23–35 (2015)

Stevceva L, Strober W. Mucosal HIV vaccines: where are we now? Curr. HIV Res. 2 (1): 1–10 (2004)

Wang R, Zhou G, Wang M, Peng Y, Li X. The metabolism of polysaccharide from *Atractylodes macrocephala* Koidz and its effect on intestinal microflora. Evid. Based Complement. Alternat. Med. 2014: 926381 (2014)