

## Effect of Astragalus and Astragalus Polysaccharide on Antimicrobial Peptides PR-39 and Protegrin-1 Gene Expression in Pigs

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Received: 07.10.2005

**Abstract:** This experiment was conducted to study the effects of micron Astragalus, 80 mesh Astragalus and Astragalus polysaccharide (APS) on antimicrobial peptides PR-39 and protegrin-1 gene expression in pigs using semi-quantitative RT-PCR analysis. One hundred twenty 60-day-old Duroc × Landrace × Yorkshire piglets ( $21.88 \pm 1.26$  kg of average initial body weight) were randomly allotted to 4 treatments. Each treatment had 3 replicates (i.e. pens) with 10 pigs per pen. The basal diets were supplemented with 0, 5 g kg<sup>-1</sup> APS, 5 g kg<sup>-1</sup> 80 mesh Astragalus, and 5 g kg<sup>-1</sup> micron Astragalus, respectively, and the feeding experiment lasted 30 days. After the feeding experiment, 12 pigs with 3 animals in each treatment were chosen to determine the effect of Astragalus or APS on PR-39 and protegrin-1 mRNA expression of pigs. The results showed that supplementation with 5 g kg<sup>-1</sup> micron Astragalus significantly ( $P < 0.05$ ) enhanced antimicrobial peptides PR-39 and protegrin-1 gene expression. Pigs fed the diet containing 5 g kg<sup>-1</sup> 80 mesh Astragalus also had higher PR-39 and protegrin-1 gene expression level than did the control ( $P < 0.05$ ). However, micron Astragalus was more effective than 80 mesh Astragalus in enhancing PR-39 and protegrin-1 gene expression. Supplementation with 5 g kg<sup>-1</sup> APS reduced PR-39 and protegrin-1 gene expression ( $P < 0.05$ ).

**Key Words:** Astragalus, PR-39, protegrin-1, antimicrobial peptide, pigs

### Introduction

Antimicrobial peptides are an important first line of defense against microbial invasion and play a prominent role in host defense mechanisms of innate immunity (1-4). Molecular cloning of mammalian bone marrow cDNA has revealed a family of antimicrobial peptides that share a highly conserved preprosequence followed by structurally variable mature peptide sequences (5). Because the prosequence is very similar to that of cathelin, a 96-amino-acid peptide originally isolated from porcine neutrophils, these antimicrobial peptides are called cathelicidins (5). Cathelicidins are the largest family of antimicrobial peptides in pigs and include PR-39, a proline-arginine-rich 39-amino-acid residue antimicrobial peptide (6-10); protegrins-1 to 5 (10,11); prophenins 1 and 2 (10,12); and porcine myeloid antimicrobial peptides 23, 36 and 37 (13).

In general, cathelicidins are synthesized by bone marrow progenitor cells (5), stored as proforms in

mature neutrophil granules (14), where few or no transcripts are expressed, and processed to mature peptides by enzyme cleavage (15). However, some cathelicidins are expressed in other tissues and are inducible, such as PR-39 and protegrin-1 in lymphoid tissues in young pigs (20). The 5'-promoter region of the porcine cathelicidin genes contains clusters of potential transcription regulatory elements, such as nuclear factors (NF)- $\kappa$ B, NF interleukin-6 (NF-IL-6), and IL-6 response elements (IL-6 RE) (7,10,16), suggesting that cathelicidin gene expression may be actively regulated.

Although in vitro and in vivo induction of antimicrobial peptides has been reported previously for cathelicidin (17), information on the in vivo modulation of cathelicidin gene expression is sparse. To date, the effect of a Chinese herbal medicine (CHM) or Chinese herbal medicinal ingredient (CHMI) on cathelicidin gene expression has not been reported. Studies showed that APS, a polysaccharide isolated from the roots of Astragalus, a medicinal herbal,

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could significantly activate NF- $\kappa$ B/Rel, whose binding sites were located in the promoter of PR-39 and protegrin-1 (18). In summary, these findings indicate that extrinsic modulation of PR-39 and protegrin-1 gene expression by Astragalus and Astragalus polysaccharide (APS) may be possible.

The purpose of this study was to determine the effect of Astragalus (prepared by superfine and conventional milling) and APS on PR-39 and protegrin-1 gene expression using semi-quantitative RT-PCR analysis, and further confirm the possibility of using Astragalus or APS as immune stimulators.

## Material and Methods

### Chinese herbal medicine

Astragalus and Astragalus polysaccharide (APS) were chosen for the experiments. APS (purity: 50%) was a commercial product (Greensky Biological Tech. Co., Ltd, Hangzhou, China). Roots of *Astragalus membranaceus* were purchased from Huadong Pharmaceutical Company (Hangzhou, China), washed with water, then dried at 40 °C, and processed to ultramicro-powder and conventional fine powder respectively in our Institute of Feed Science. The conventional fine powder was ground through an 80-mesh sieve. Their particle sizes were determined by Mastersizer Laser Particle Size Analyzer (Malvern, UK). The particle sizes of 80 mesh Astragalus and micron Astragalus used in the current study were 180 and 6.32  $\mu$ m, respectively.

### Animals and experimental design

This experiment was approved by the Institutional Animal Care and Use Committee at Zhejiang University and was conducted in accordance with the National Institutes of Health guidelines for the care and use in experimental animals. The feeding trial was carried out in the Swine Research and Teaching Farm at Zhejiang University. One hundred twenty 60-day-old Duroc  $\times$  Landrace  $\times$  Yorkshire piglets, weighing an average of  $21.88 \pm 1.26$  kg, were randomly assigned to 4 treatments. The pigs had been weaned 36 days after birth. Each treatment had 3 replications (i.e. pens) with 10 pigs per pen. The treatments received the same basal diet and supplemented with 0, 5 g kg<sup>-1</sup> APS, 5 g kg<sup>-1</sup> 80 mesh Astragalus, and 5 g kg<sup>-1</sup> micron Astragalus,

respectively. Diets were formulated to meet or exceed requirements suggested by the NRC (1998) for 20-50 kg pigs. No antibiotic was included in the diets (Table 1). The feeding trial lasted 30 days after 7 days of adaptation. All pigs were housed in an open-front pig barn with a concrete floor and the size of the pens used was 350  $\times$  350 cm. Dry/wet feeders with 2 waterers were allocated in each pen for pigs. During the 30-day feeding trial, all pigs were given ad libitum access to feed and water.

Table 1. Ingredient and composition of experiment diets (as fed basis).

	composition, g kg <sup>-1</sup>
Maize	587.0
Soybean meal	250.0
Wheat middling	50.0
Yeast	10.0
Fish meal	20.0
Silkworm pupae meal	20.0
Limestone	10.0
Monocalcium phosphate	20.0
Sodium chloride	3.0
Premix <sup>a</sup>	30.0
DE <sup>b</sup> (MJ/kg)	14.46
Analyzed content	
Crude protein	195.0
Calcium	8.0
Phosphorus	6.6
Lysine	13.5
Methionine	3.3

<sup>a</sup> Premix provided the following per kg of complete diet: Fe, 100 mg; Cu, 6 mg; Mn, 4 mg; Zn, 100 mg; I, 0.14 mg; Se, 0.3 mg. Vit. A 2200 IU; Vit. D<sub>3</sub>, 500 IU; Vit. E 16 mg; Vit. K 0.5 mg; Vit. B<sub>1</sub> 1.5 mg; Vit. B<sub>2</sub> 4 mg; Vit. B<sub>6</sub> 2 mg; Vit. B<sub>12</sub> 0.02 mg; Niacin 22 mg; d-Pantothenic acid 12 mg; Biotin 0.08 mg; Folic acid 0.3 mg.

<sup>b</sup> DE based on calculated values

### Sample collection and assay

#### Sample Collection

A total of 12 pigs with 3 animals in each treatment were euthanized by electrocution, their femurs were dissected, and bone marrow cells were aspirated and immediately frozen in liquid nitrogen for RNA isolation.

#### Total RNA Extraction

Total RNA was isolated from the bone marrow cells by using TRIzol (Invitrogen Life Technologies, Carlsbad,

CA, USA) according to the manufacturer's manual. After pulverization and homogenization of the tissue, the homogenate was extracted with chloroform and then precipitated by isopropanol. The resulting pellets of total RNA were dissolved in ultra-pure water; the purity and concentration of total RNA were measured by a spectrophotometer at 260 and 280 nm.

#### Semiquantitative RT-PCR

RT-PCR was performed in a thermocycler (Gene Amp PCR system 9600). Two micrograms of total RNA were converted in cDNA; 2 µg of total RNA and 2 µl of Random primers (500 µg/ml; Promega Corp., Madison, WI, USA) were denatured at 70 °C for 5 min, and cooled to 25 °C. The following components were added to give a total reaction volume of 25 µl: 5 µl M-MLV 5X reaction buffer (250 mM Tris HCl (pH 8.3), 375 mM KCl, 15 mM MgCl<sub>2</sub>, and 50 mM dithiothreitol), 2 µl dNTPs mix (10 mM each of dATP, dCTP, dGTP, and dTTP), and 1 µl M-MLV reverse transcriptase [200 units/µl; 0.5 µl rRNasin ribonuclease inhibitor and nuclease-free water (Promega Corp.)]. It was mixed gently by flicking the tube, and the reaction mixture was incubated at 37 °C for 60 min.

All PCRs were performed with 1 µl of each resulting cDNA in a 50-µl reaction volume containing 0.2 mM deoxynucleoside triphosphates, 2 mM Mg<sup>2+</sup> (PR-39) or 1.5 mM Mg<sup>2+</sup> (protegrin-1, β-actin), 0.4 µM each sense and antisense primer, and 1 U Taq DNA polymerase (Promega). The PCR primer sets used are shown in Table 2. Primer sequences for PR-39, protegrin-1 and β-actin were designed using the Prime program of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc., Madison, WI, USA) based on known sequences deposited in GenBank. The optimum PCR reaction cycle numbers, Mg<sup>2+</sup> concentration and annealing temperature

to give a linear amplification of each transcript were determined by a preliminary experiment (data not shown). The PCR profile for β-actin included denaturation at 94 °C for 2 min, followed by 29 cycles of denaturation at 94 °C for 50 s, annealing at 57.5 °C for 50 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR profile for PR-39 and protegrin-1 was similar to that for β-actin.

A 5-µl portion of each PCR product was subjected to electrophoresis on a 1% agarose gel with ethidium bromide. PCR products were normalized according to the amount of β-actin detected in the same cDNA sample, and PR-39/β-actin or protegrin-1/β-actin ratios were calculated. The expression level of PR-39 and protegrin-1 gene in bone marrow was compared on the basis of PR-39-to-β-actin, protegrin-1-to-β-actin ratio.

#### Data Analysis

Electrophoresis band intensities of the PCR products were quantified using Image Master VDS software (Amersham Pharmacia Biotech, Uppsala, Sweden). Mean PR-39 and protegrin-1 mRNA expression levels normalized against β-actin levels from each treatment group were presented in absolute integrated optical density. Each value was analyzed for statistical difference according to the Bonferroni/Dunn method.

#### Results

The effect of supplementation with 5 g kg<sup>-1</sup> micron Astragalus or 5 g kg<sup>-1</sup> 80 mesh Astragalus or 5 g kg<sup>-1</sup> APS on gene expression of antimicrobial PR-39 and protegrin-1 in bone marrow was investigated. The electrophoresis results of 3 pigs of each experimental group are shown in

Table 2. Primer sets for RT-PCR of porcine (*Sus scrofa*) PR-39, protegrin-1 and β-actin.

Gene	Gene special forward and reverse primers	Size of PCR product (bp)	Accession Number
PR-39	5'-CGCTGGTCACTGTGGCTTCT-3'(S) 5'-CTGCTTCACTCGCCATTCT-3'(AS)	285	X79868
protegrin-1	5'-CCGTGCTTGGTGCTGTGGA-3'(S) 5'-TGCCGTCGCAACCGTCAT-3'(AS)	355	L23825
β-actin	5'-CGGGACCTGACCGACTACCT-3'(S) 5'-GGCCGTGATCTCCTTCTGC-3'(AS)	411	CK408343

the Figure. Electrophoresis band intensities of PCR products were quantified and analyzed for statistical difference. The current results (see Figure) show that supplementation with micron Astragalus significantly ( $P < 0.05$ ) enhanced PR-39 mRNA expression level by 66.1%, 117% and 52.6%, and significantly ( $P < 0.05$ ) increased protegrin-1 mRNA expression level by 72.8%, 113% and 53.6% compared with control and other 2 treatment groups, respectively. The feeding diet containing 5 g kg<sup>-1</sup> 80 mesh Astragalus significantly ( $P < 0.05$ ) enhanced PR-39 mRNA expression level by 9.0% and significantly ( $P < 0.05$ ) enhanced (protegrin-1) mRNA expression level by 12.6% compared with the control. Supplementation with 5 g kg<sup>-1</sup> APS reduced PR-39 and protegrin-1 gene expression compared with the control ( $P < 0.05$ ).

### Discussion

Components of the gut mucosal barrier or non-specific immune factors (e.g., antimicrobial peptides) are important for piglet growth and immunity in the growing phase (19). In the current experiment, we investigated the effect of Astragalus with different particle sizes and Astragalus polysaccharide derived from Astragalus species on gene expression of antimicrobial peptides PR-39 and protegrin-1 in vivo by semi-quantitative RT-PCR. The major findings of this study are that Astragalus (CHM) can upregulate PR-39 and protegrin-1 mRNA expression in the bone marrow cells of pigs, and micron Astragalus is more effective than 80 mesh Astragalus in enhancing PR-39 and protegrin-1 mRNA expression in pigs. These results showed the possible roles of neutrophils in

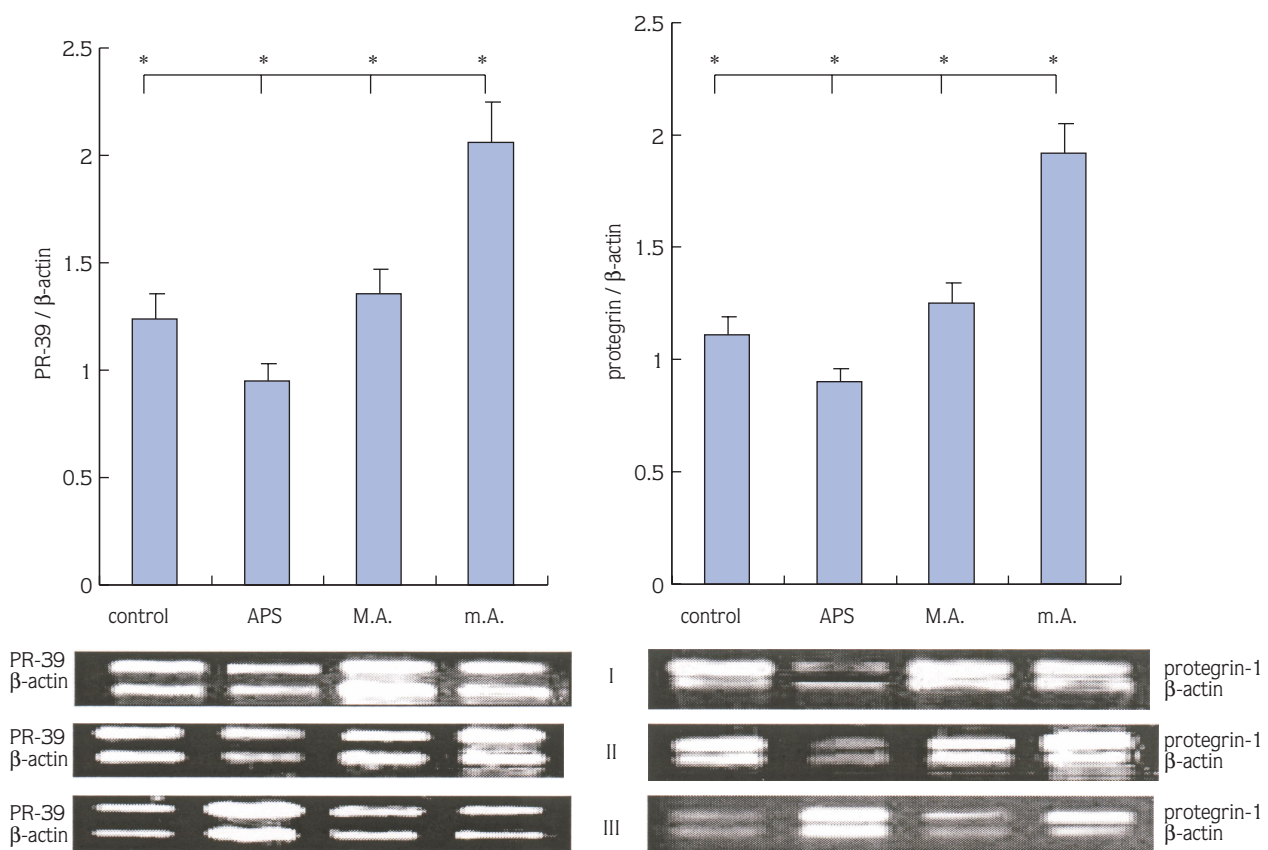


Figure. Micron Astragalus increased PR-39 (A) and protegrin-1 (B) mRNA expression in bone marrow cells. Semiquantitative RT-PCR was used to quantify mRNA. Total RNA (0.08 μg) was used as templates using 29 cycles for PR-39 or protegrin-1 or β-actin. PR-39/β-actin or protegrin-1/β-actin ratios were determined. I, II and III: The results from the first, second and third piglets in each experimental group, respectively. APS, M.A. and m.A. represent the results from piglets in the Astragalus polysaccharide, 80 mesh Astragalus and micron Astragalus groups, respectively. Values are means ± SD from 3 pigs in each treatment. \* $P < 0.05$ .

immune activity of Astragalus, and the mechanism was explained in 2 ways. The first mechanism was that the Astragalus preparations increased the expression of protegrin-1 and PR-39 per cell. The other was related to the increase in the number or percentage of PR-39/protegrin-1-producing cells in the bone marrow. These possible regulation mechanisms need further investigation. As in previous research (17,20,21), in the current study we only determined the total mRNA levels of protegrin-1 and PR-39.

Wu et al.'s (20) results suggested the existence of regulatory elements controlling PR-39 expression and raised the possibility of altering these control elements to modulated PR-39 expression. Wu et al. (17) reported that lipopolysaccharide (LPS), interleukin-6 (IL-6) and retinoic acid (RA) upregulate PR-39 and protegrin-1 gene expression. Many studies revealed that the 5' flanking region of the PR-39 and protegrin-1 gene contained several potential regulatory motifs, nuclear factor (NF)- $\kappa$ B, NF interleukin-6 (NF-IL-6) and IL-6 response elements (IL-6 RE). These reports implied the possibility of extrinsic modulation of PR-39 and protegrin-1 gene expression. Lee and Jeon (18) showed that NF- $\kappa$ B/Rel is positively regulated by APS. NF- $\kappa$ B/Rel exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, I $\kappa$ B. Macrophage activation by certain external stimuli results in the phosphorylation of I $\kappa$ B, thus releasing the active DNA-binding form of NF- $\kappa$ B/Rel. The active NF- $\kappa$ B/Rel translocates to the nucleus and binds to  $\kappa$ B motifs in the regulatory region of a variety of genes. Their immunohistochemical staining, EMSA, and reporter gene assay verified APS-induced activation of NF- $\kappa$ B/Rel DNA binding, nuclear translocation, and transcriptional activation, respectively. Scientific investigations have indicated that the immunopotentiating effects of the roots of Astragalus species are associated with their polysaccharide fractions (Astragalus polysaccharides, APS) (22-25). The results of this study confirmed that Astragalus could upregulate PR-39 and protegrin-1 mRNA expression in pigs. The results also demonstrated that micron Astragalus was more effective than 80 mesh Astragalus in enhancing PR-39 and

protegrin-1 mRNA expression. Many studies confirmed the efficacy of superfine comminution-treated CHM (26,27). The possible mechanisms were that the cell walls of CHM prepared by super-fine comminution were highly disrupted, which allows access to the released intracellular contents by digestive enzymes of animal; on the other hand, due to fine particle and large surface area, its adhesive function was enhanced, and time of settling and releasing of medicine in the body were prolonged. Those all benefit bioavailability. Other studies showed that an additional dosage of APS was associated with its efficacy (28,29). Every CHM contains many complicated ingredients. The efficacy of each depends on complicated combinatory effects of many ingredients although the effects of some ingredients have not been determined. On the other hand, APS has a bi-directional modulation function. Therefore, in the current study, supplementation with 5 g kg<sup>-1</sup> APS reduced PR-39 and protegrin-1 gene expression.

Our findings suggested that cathelicidin gene expression could be regulated by Astragalus (CHM). In short, micron Astragalus was more effective than 80 mesh Astragalus in enhancing antimicrobial peptides PR-39 and protegrin-1 mRNA expression. Supplementation with 5 g kg<sup>-1</sup> Astragalus polysaccharide failed to upregulate the mRNA expression of PR-39 and protegrin-1. Further research needs to be conducted to better understand the effects and mode of action of Astragalus with different particle sizes and APS at different additional dosages, and to explore the feasibility of extrinsic modulation of this important innate immune mechanism. If further studies confirm their immunostimulatory effects, this potential may stimulate the development of new-type immunopotentiators and they may be used in the pig industry.

### Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (30571348) and Program for New Century Excellent Talents in University (NCET-04-0543).

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