Journal of Korean Clinical Health Science. http://www.kschs.or.kr
Vol. 1, No. 1, June 2013, pp. 83-89

Proteomic Analysis of Serum Samples from Natural Herb and Immunoglobulin Yolksac (Ig Y) treated Porcine

Hyeon-Soo Park, Arulkumar Nagappan, Gyeong-Eun Hong, Silvia Yumnam, Ho-Jung Lee, Gon-Sup Kim*

Research Institute of Life science and College of Veterinary Medicine Gyeongsang National University

(Received March 17, 2013: Revised April 19, 2013: Accepted May 10, 2013)

Abstract

Purpose. Natural herb has been used for traditional medicine for thousands of years. For this reason, alternative medicine methods affect to domestic animal field. The main purpose of this study was to confirm the regulated proteins after feed additive treatment.

Methods. We used total thirty male pigs were used for this experiment. E. coli and Salmonella typhimurium, were used for promote enteritis. Animals were divided into a negative control group, positive control group and test group to determine the effect of an additive mixture on the changes in protein expression in serum. The pigs were given a food supplemented with a natural herbal additive containing immunoglobulin yolksac (IgY) at concentrations 1%. On the 1st week and after eight weeks of feeding, the serums were collected from each group. The changes in the serum proteome as a response to the herbal additive were examined using two-dimensional polyacrylamide gel electrophoresis.

Results. A total of 14 differentially expressed protein spots were identified by comparing the protein profiles of the control and additive treated porcine serums. Finally, 7 proteins were detected by MALDI-TOF/MS. Moreover, the proteins detected are involved in a cholesterol control factor, inflammation regulator,

Conclusion. These results support of the hypothesis that a natural herbal additive containing IgY can affect the immune regulation system and reduce the pathological process by microbial infections.

Key Words: Immunoglobulin Yolksac(Ig Y), Natural Herb, Porcine serum, Protemic analysis

* Corresponding Author: gosnkim@gnu.ac.kr

1. Introduction

Every year, 20% of neonatal pigs were died out by various reasons including microbial enteritis in Korea. For this reason, overdose of antibiotics have been raised as a controversial subject. Recently, natural herb feed additive focused on the substitute methods that instead of antibiotics. Previous studies shown that herbal components are positive effects in regulating immune response[1,2]. The main purpose of the immunomodulating in domestic animals is to control immune system for increased the production efficiency[3]. Previous time, we designed natural herb mixture with Ig Y for feed additive[4]. The egg yolks from laying hens are an excellent source of polyclonal antibodies. Specific egg yolk immunoglobulin (IgY) provides passive protection against ETEC infections in early post-weaning pigs[5, 6].

Two-dimensional gel electrophoresis (2-DE) is a powerful tool to separate proteins, as it separates them by their electric charge. Matrix assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF/MS) identifies proteins quickly and inexpensively. Our previous investigation clarified that natural herb feed additive affect to the spleen protein expression pattern4). However, the serum protein pattern after treatment of natural herb feed additive has not been studied. Thus, we analyzed the response to microbials, and the effect of the natural herb feed additive mixture with Ig Y using 2-DE and MALDI-TOF/MS.

2. Materials and Methods

2.1. Reagents

All the chemicals used in this study were purchased from AMRESCO Inc (Cochran, Solon, USA) and Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade. Specific IgY was prorided from animal Bio Resources Bank(ABRB)

2.2. Protein extraction

The protein was extracted from the porcine serum. Briefly, 15μl of the serum was dissolved in 500μl of the sample buffer containing 7M urea, 2M thiourea, and 4% (w/v) CHAPS. After sonication, the samples were centrifuged at 15000rpm and 4℃ for 1 hour and the supernatant was collected. For protein precipitation, 100μl of the supernatant was treated with 10% TCA (v/v) and incubated at -20℃ for 3 hours. The samples were then centrifuged at 15000rpm and 4℃ for 10min and the supernatant was discarded. The protein pellets were dried in a lyophilizer, dissolved in 500μl of the sample buffer and centrifuged at 15000rpm and 4℃ for 30min. The supernatant was transferred to another e-tube and stored -80℃ until needed. The protein concentration was estimated using a Non-interfering™ protein assay kit(Biosciences, st.Louis, MO, USA) according to the manufacturer's instructions.

2.3. Two-dimensional electrophoresis

In the first dimension, a total of 200 μg

of protein from each groups were applied to an IPG strip (Immobiline DryStrip™, pH 4-7L, 18cm, GE healthcare life sciences) for the IEF process, followed by 200 V for 1 h, 500 V for 30 min, 4000 V gradient step for 30 min, 4000 V for 1 h, 10000 V gradient step for 1 h, 10000 V for 12 h, and 50 V for 3 h. The protein samples were focused for a total of 140.2kVh. The strips were equilibrated the first time with 10mg/ml DTT in an equilibration buffer for 15 min and the second time with iodoacetamide for 15min with continuous shaking. In the second dimension, the equilibrated strips were placed onto 10% SDS-PAGE and run at a constant 30mA until the dye reached the bottom of the gel. The protein spots in the analytical gels were visualized by silver staining.

2.4. Image and data analysis

The gel images were acquired using a BIO-RAD™ GS-800 scanner and imported into progenesis SameSpots™ 2D software (ver. 4.1, Nonlinear Dynamics, Newcastle, U.K.) for analysis. Protein spots showing more than a 2 fold ($p<0.05$) change in abundance or expression were considered to be differentially expressed proteins. All spots were also confirmed visually and edited manually.

2.5. Identification of differentially expressed proteins by mass spectrometry

The selected protein spots were excised from the 2-DE gel for identification of protein. In gel digestion of the selected protein spots on the gels was performed as described by[7] with moderate modification. The excised protein spots were proteolyzed in-gel with porcine trypsin. The tryptic fragment masses were detected by MALDI-TOF MS using a PerSeptive Biosystems Voyager-DE STR mass spectrometer. The proteins were identified by a Mascot-Peptide Mass Fingerprint (www.matrixscience.com) database search. The following parameters were used for the database searches: taxonomy, mammalians; cleavage specificity, trypsin with 1 missed cleavages allowed; Peptide tolerance of 100 ppm for fragment ions; allowed modifications, Cys Carbamidomethyl (fixed), oxidation of Met (variable). The MOWSE score and species were considered to identify the correct protein from the mascot results list.

3. Results and discussion

In this study, we investigated effects of feed additive on serum protein expression pattern using MALDI-TOF/MS. Total 14 differentially expressed protein spots were detected (more than two fold change was considered significant) in 1week and 8 weeks treatment groups (Figure 1 and Figure 2), and finally 7 protein spots were identified by maldi-TOF/MS. Table 1. lists the differentially expressed proteins that identified.

3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) convert HMG-CoA to mevalonate. This converting process is control step of the cholesterol synthesis. However, our results shown increased pattern of HMGCR. Previous study show that salmonella typhimurium infection decreased apolipoprotein A-I in cows[8]. Decrease of apolipoprotein A-I might be

influenced in HMGCR level but more detail mechanism study is demanded for this result.

Table 1. List of the proteins differentially expressed in porcine serum, as recognized by MALD-TOF MS analysis.

Spot#	protein	Accession #(a)	Nominal mass(Mr)	Matched peptide	Sequence Coverage (%)	Up/Down Regulation
1	3-hydroxy-3-methylglutaryl-coenzyme A reductase	172072667	98627	6	48	↑ ↑
2	albumin, partial	164318	71348	9	19	↓ ↓
3	albumin, partial	164318	71348	11	23	↑ ↓
4	hypothetical protein LOC100395808, partial	296201104	78812	6	9	↓ ↓
5	albumin	833798	71362	10	21	↓ ↓
6	complement C3 precursor	47522844	188229	31	17	↑ ↑
7	uncharacterized protein C5orf35-like	311273761	34596	6	21	↑ ↑

Note : a; Mascot search primary accession number

Complement C3 (C3) is critical protein in the immune system. C3 plays an important role in the innate immunity and complement system. Previous study shown that LPS originated from salmonella typhimurium induced overexpression of C3 in A/J mice[9]. Our study show that feed additive treatment decreased C3 precursor expression. This means that feed additive enhanced complement system and protect from microbial enterotoxin.

In conclusion, our study reveals that natural herb and Ig Y feed additive involved in cholesterol synthesis system and complement associated immune response. Further study for more mechanical finding will support our result and further study will focus on this results.

4. Acknowledgments

This study was supported by the National Research Foundation (NRF) of Korea grant funded by the Korean government (MEST) (No. 2012045015) and the National R&D Program for Cancer Control, Ministry for Health, Welfare, and Family Affairs, Republic of Korea (No. 0820050).

References

1. Mahajan SG, Mehta AA. Immunosuppressive activity of ethanolic extract of seeds of Moringa oleifera Lam. in experimental immune inflammation. Journal of ethnopharmacology, 2010;130(1):183-186.
2. Mrozikiewicz PM, Bogacz A, Karasiewicz M, at al. The effect of standardized Echinacea purpurea extract on rat cytochrome P450 expression level. Phytomedicine. International Journal of Phytotherapy and Phytopharmacology, 2010;17(10):830-833.
3. Blecha F. Immunomodulators for prevention

and treatment of infectious diseases in food-producing animals. The Veterinary clinics of North America. Food animal practice, 2001;17(3):621-33.

4. Park HS, Park KI, Nagappan A, et al. Proteomic analysis of effects on natural herb additive containing immunoglobulin Yolksac (IgY) in pigs. The American Journal of Chinese Medicine, 2011; 39(3):477-488.
5. Marquardt RR, Jin LZ, Kim JW, et al. Passive protective effect of egg-yolk antibodies against enterotoxigenic Escherichia coli K88+ infection in neonatal and early-weaned piglets. FEMS immunology and medical microbiology, 1999;23(4): 283-288.
6. Yokoyama H, Peralta RC, Diaz R, et al. Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic Escherichia coli infection in neonatal piglets. Infection and immunity, 1992;60(3):998-1007.
7. Shevchenko A, Wilm M, Vorm O, et al. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Analytical Chemistry, 1996;68(5): 850-858.
8. Oikawa S, Katoh N, Itoh H. et al. Decreased serum apolipoprotein A-I concentrations in cows infected with Salmonella typhimurium. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire, 1997;61(3):182-186.
9. Nakano A, Kita E, Kashiba S. Different sensitivity of complement to Salmonella typhimurium accounts for the difference in natural resistance to murine typhoid between A/J and C57BL/6 mice. Microbiology and immunology, 1995;39(2):95-103.

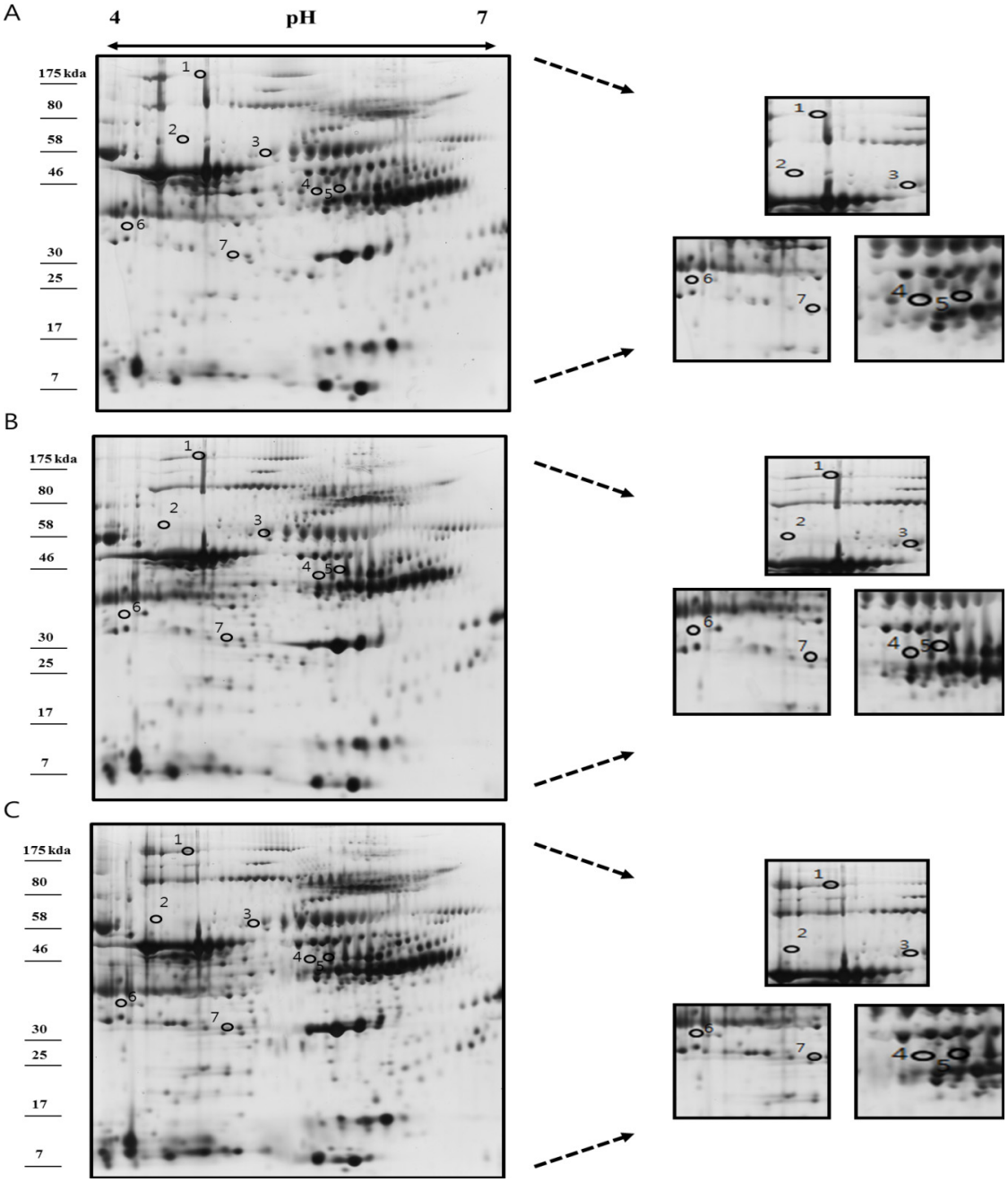

Fig 1. Representative 2-DE pattern of the negative control group (A), 1week positive control group (B) and 1week test group (C). The sample was resolved by 2-D on nonlinear pH 4-7 IPG strips followed by separation on a 10% SDS-PAGE gel in the second dimension. The proteins were visualized by silver nitrate staining.

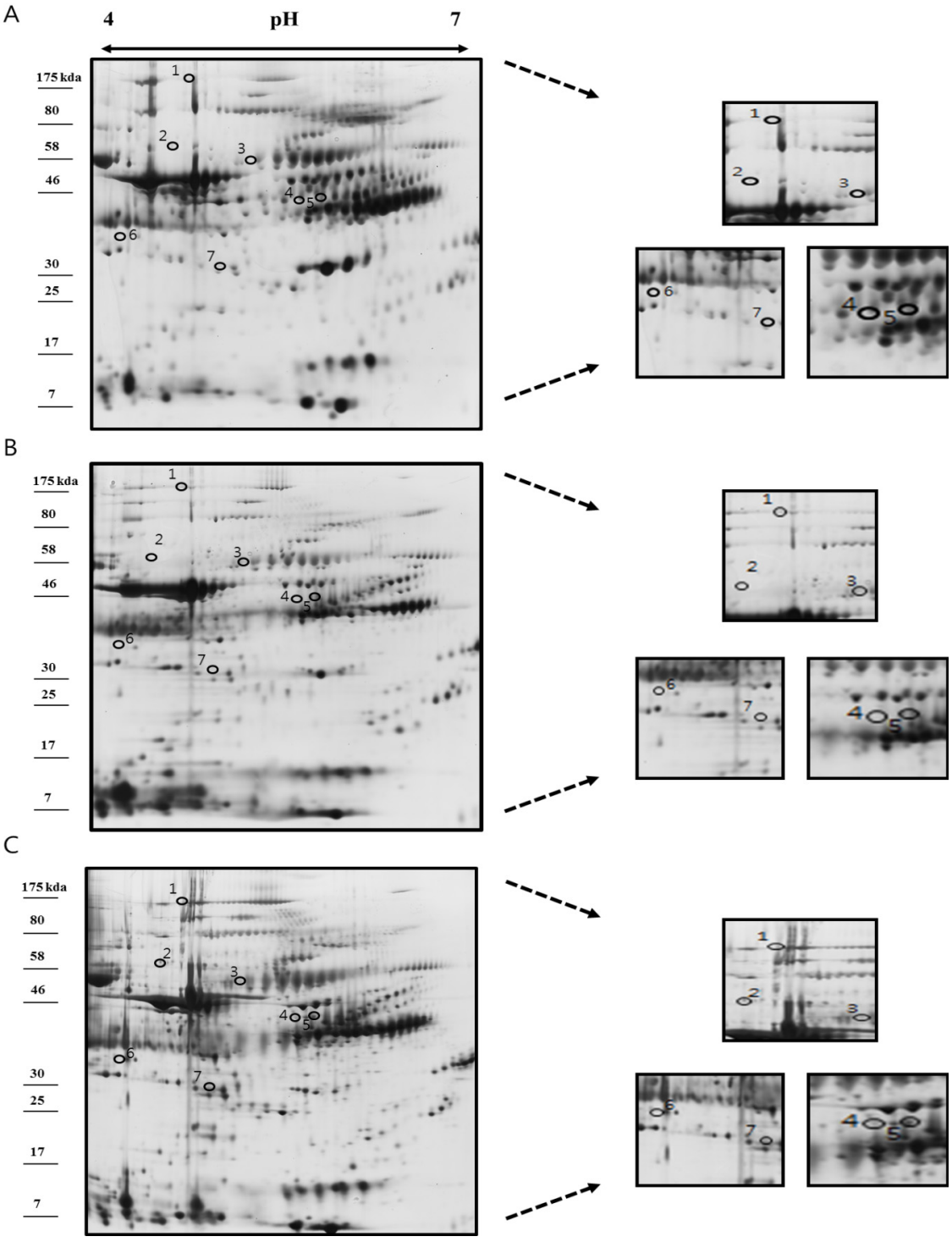

Fig 2. Representative 2-DE pattern of the negative control group (A), 8weeks positive control group (B) and 8week test group (C). The sample was resolved by 2-D on nonlinear pH 4-7 IPG strips followed by separation on a 10% SDS-PAGE gel in the second dimension. The proteins were visualized by silver nitrate staining.