Muscle Proteome Analysis for the Effect of *Panax Ginseng* Extracts in Chicken: Identification of Proteins Using Peptide Mass Fingerprinting

K. C. Jung[1], S. L. Yu[1], Y. J. Lee[1], K. D. Choi[2], J. S. Choi[3], Y. H. Kim[3], B. G. Jang[4], S. H. Kim[5] D. H. Hahm[6] and J. H. Lee*

[1]Division of Animal Science and Resources, College of Agriculture and Life Sciences Chungnam National University, Daejeon 305-764, Korea

ABSTRACT : The present study was aimed to investigate proteome affected by *Panax ginseng* extracts in chicken muscles. The whole muscle proteins from chicken fed boiled extracts of 0% (control), 1%, 3%, and 5% *Panax ginseng* in water were separated by two-dimensional electrophoresis (2-DE) gels using immobilized non-linear gradient (pH 3-10) strips. More than 300 protein spots were detected on silver staining gels. Among them, four protein spots were distinctively up-regulated by *Panax ginseng* treatments and further investigated by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The obtained MS data were searched against SwissProt database using the Mascot search engine. The up-regulated proteins were finally identified as α-tropomyosin (2 spots), triosephosphate isomerase, and one unknown protein. Based on the known functions of the identified proteins, they are highly related to muscle development and enhanced immunity in chickens. These proteins can give valuable information of biochemical roles for *Panax ginseng* in chicken meats. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 7 : 922-926)***

Key Words : Chicken, Muscle, MALDI-TOF MS, 2DE, *Panax Ginseng*

INTRODUCTION

Proteomics is a relatively recently developed technique and covers the large-scale profiles of proteins expressed from a given cell or tissue. In multi-cellular organisms, all of the cells have the same set of genes. However, the proteomes from different cells or different tissues are differentially expressed, based on the circumstantial environment. Therefore, proteomic study includes the large-scale identification of expressed proteins and modified proteins, and protein-protein interactions in a given environment (Pandey and Mann, 2000). In other word, the proteomics provides a valuable link between DNA sequences and biological functions. Until recently, identified economic traits-related markers are mostly DNA markers. The association between *IGF1* gene polymorphism and growth traits in chicken is a good example (Wang et al., 2004). However there are some limitations for using DNA markers since the biological roles were determined by the final products: proteins. In cattle, new attempt was performed for identifying proteins affecting proteolysis in postmortem skeletal muscles using the proteomics techniques (Hwang, 2004).

The origin of proteomics research dates back to the 1970s when the two-dimensional electrophoresis (2-DE) was developed for the protein separation. 2-DE consists of the first separation of whole proteins based on the isoelectric point (p*I*) of proteins and the second separation by the molecular weight (M*r*) of each protein. However, there were some limitations for 2-DE analysis such as reproducibility, protein identification and separation. Recently, the development of analysis techniques and resources including MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) and animal genome sequences in the public database, opens up new era for the proteomics research.

Very recently, meat market requires secure and safe meat products as well as functional meat products. One good example of functional meat products is pork fed on diet containing ginseng leaf and stem extracts. When the pigs were fed on the diet comprising ginseng extracts, their meat showed higher levels of growth hormone to lead faster growth and high ability of water holding capacity, compared with their controls. In addition, ginsenoside Rg1 was detected from the loin of ginseng-fed animals. In cattle, the activities of neutrophils and lymphocytes in blood were dramatically increased, when they were fed on the diet containing ginseng roots (Hu et al., 1995; Concha et al., 1996). Also, ginseng in diet decreased the incidence of

* Corresponding Author: J. H. Lee. Tel: +82-42-821-5779, Fax: +82-42-825-9754, E-mail: junheon@cnu.ac.kr

[2] The Graduate School of Bio and Information Technology, Hankyong National University, Ansung 456-749, Korea.

[3] Proteome Analysis Team, Korea Basic Science Institute, Daejeon 305-333, Korea.

[4] National Livestock Research Institute, R.D.A., Daejeon 305-356, Korea.

[5] Department of Biological Science, Kyunghee University, Seoul 130-701, Korea.

[6] Department of Oriental Medical Science, Graduate School of East-West Medical Science, Kyung Hee University, Yongin 449-701, Korea.

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mastitis and the number of somatic cells in milk in Holstein cattle (Hu, 2001). Moon (1999) was indicated that the dietary ginseng gave lots of pharmaceutical effects in the body containing elevation of basic metabolism and protein synthesis, resistance in anemia, diabetes, high blood pressure, and response against stress. In this present study, we aimed to investigate differentially expressed proteins in chicken muscles affecting edible *Panax ginseng* extracts as well as address the possibility for functional chicken meat products.

MATERIALS AND METHODS

Sample preparations

For making edible ginseng water, 1 kg of dried ginseng leaves and stems were put into 1,000 ml water and boiled. As a result, total amounts of obtained saponins (Rh_2: 6.32, Rg_2: 53.119, Rg_1: 48.197, Rf: 6.2, Re: 1.7, Rd: 17.55, Rc: 36.25, Rb_2: 1.65) were 170.98 μg/ml. This extract was diluted to 0% (control), 1%, 3%, 5% and gave to three-breed-cross chickens (Korean Native Chicken×Rhode Island Red×Cornish) for two weeks before slaughter. Muscles from chicken breast were immediately removed just after slaughter and quenched in liquid nitrogen for subsequent 2-DE gel analysis.

Frozen muscle samples (1 g) were crushed in a mortar containing liquid nitrogen and mixed with 10 ml of solution containing 0.3% SDS, 50 mM Tris-HCl pH 8.0, 1 mM PMSF (phenylmethylsulfonyl fluoride, Roche, Germany) and 200 mM DTT. The mixtures were incubated at room temperature for 30 min and boiled at 100°C for 10 min. The mixtures were then transferred to ice and incubated with solution containing 40 U DNase I, 14 U RNase A, 50 mM Tris-HCl pH 8.0, 0.1 mM $MgCl_2$ for 10 min. After centrifugation at 15,000 g at 4°C for 30 min, the supernatant was precipitated with 50% TCA solution for 1 hr at -20°C. The protein pellet was washed with ice-cold acetone at least four times to remove contaminants and lyophilized. The washed pellet was dried using vacuum centrifuge prior to 2-DE analysis.

Electrophoresis

Before rehydration, muscle supernatants containing proteins were solubilized in rehydration solution containing 8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, Amersham Pharmacia, Uppsala, Sweden), 60 mM DTT, 0.5% IPG buffer (Amersham Biosciences, Uppsala, Sweden) with a trace amount of bromophenol blue. Protein concentration was determined according to Bradford (Bradford, 1976) using BSA as a standard. The protein solution containing 250 μg of protein was applied on immobilized pH 3-10, 18 cm nonlinear gradient strips and rehydrated for 12 h. Isoelectric focusing (IEF) was performed using an IPGphor system (Amersham Biosciences, Uppsala, Sweden). Focusing was performed in 4 steps (200 V 1 h, 500 V for 1 h, 1,000 V for 1 h and final focusing step of 8,000 V for 8 h) at 20°C. Proteins resolved in the first dimensional strip were reduced and alkylated prior to second dimensional electrophoresis. Strips were incubated for 15 min in a solution containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT, followed by 15 min in the same solvent containing 2.5% (w/v) iodoacetamide in place of the DTT. The second dimension was run on a 12% polyacrylamide SDS gel using a PROTEAN II xi electrophoresis kit (Bio-Rad, Hercules, USA). After laying the strip on top of a 12% polyacrylamide SDS gel and sealing it with agarose, electrophoresis was carried out at 15 mA per gel until the dye front reached the lower end of the gel. Staining was carried out according to the method of Heukeshoven and Dernick (Heukeshoven and Dernick, 1985) using a Silver Staining Kit (Amersham Biosciences, Uppsala, Sweden).

Image analysis

Gels were scanned using a Powerlook III image scanner (UMAX data system, Taiwan) and gel images were saved as TIFF file formats. The gels were compared using the 2D Image Master software (Amersham Biosciences, Uppsala, Sweden). Protein spots whose levels had changed by 2-fold or more were excised. Stained protein spots were excised from the gel and digested with trypsin (Promega, Madison, WI, USA). After washing with 10 mM ammonium bicarbonate and 50% acetonitrile, gel pieces were swollen in digestion solution (50 mM ammonium bicarbonate, 5 mM $CaCl_2$ and 12.5 ng/μl trypsin) and incubated at 37°C for 15 h. The peptides were recovered by stage extraction using 50 mM ammonium bicarbonate and 100% acetonitrile. The resulting peptide extracts were pooled, lyophilized in a vacuum centrifuge and stored at -20°C.

Characterization of protein spots by MALDI-TOF MS

Dried samples were dissolved in 0.5% TFA (trifluoroacetic acid). To improve the ionization efficiency of MALDI-TOF MS, sample peptides were purified with ZIP-tip C18 (Millipore, Bedford, MA, USA) before MS analysis. Equal volumes of peptide and matrix solution were mixed and crystallized on the sample plate. The matrix solution consisted of 10 mg α-cyano-4-hydroxycinnamic acid dissolved in 1 ml of a solution containing 50% acetonitrile and 0.5% TFA. The peptide solution was analyzed using a modified Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Desorption and ionization of samples was performed using a nitrogen laser (λ = 337 nm). The proteins were identified by searching against NCBI non-redundant

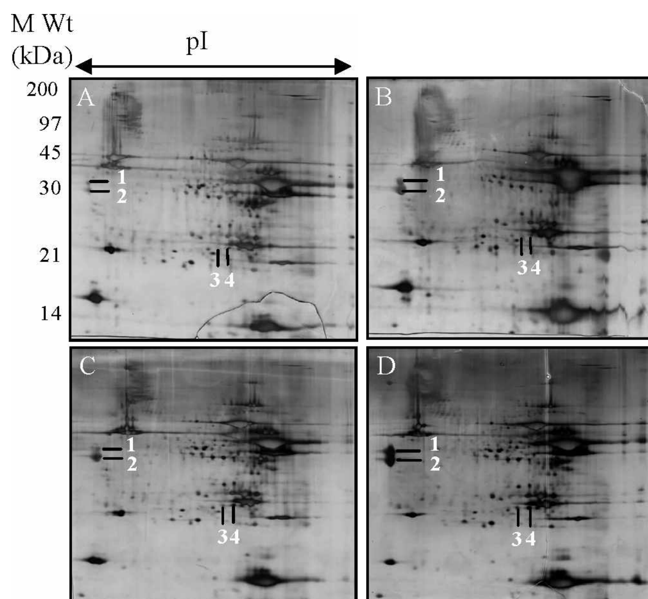


Figure 1. 2-DE gel images of chicken muscles. Muscles from chickens fed on 0% (A), 1% (B), 3% (C) and 5% (D) boiled ginseng extracts in edible water. Four protein spots in muscle affected by ginseng treatment are indicated by numbers. Molecular size markers and isoelectric point (p*I*) are also shown.

database using MASCOT peptide mass fingerprint software (http://www.matrixscience.com).

RESULTS AND DISCUSSION

Whole muscle proteins were extracted from chickens fed on 0% (control), 1%, 3%, and 5% *Panax ginseng* extracts in water. More than 300 protein spots on silver stained 2-DE gels were identified with the pH range between 3 to 10 and molecular weight range between 15 to 100 kDa (Figure 1). With replicated experiments, four differentially expressed protein spots induced by boiled ginseng extracts in water were identified on 2-DE gels. These proteins were further analyzes by MALDI-TOF MS and searched against protein database using Mascot search engine. These up-regulated proteins for the ginseng treatments were identified as α-tropomyosin (2 spots), triosephosphate isomerase and one unknown protein (Table 1). The actual protein spots were illustrated in Figure 2. All four protein spots identified were significantly increased based on the dose-dependent manner when supplemented with *Panax ginseng* extracts. For example, spot number 4

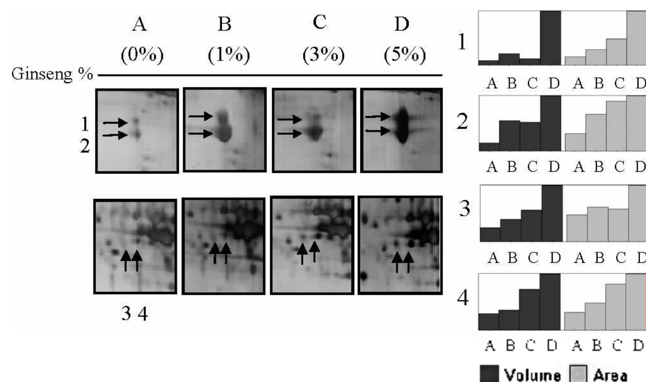


Figure 2. Comparative analysis of four identified proteins listed in Table 1. All four protein spots identified are up-regulated when the ginseng concentration in water is increased. Spot volumes and areas are indicated on the right.

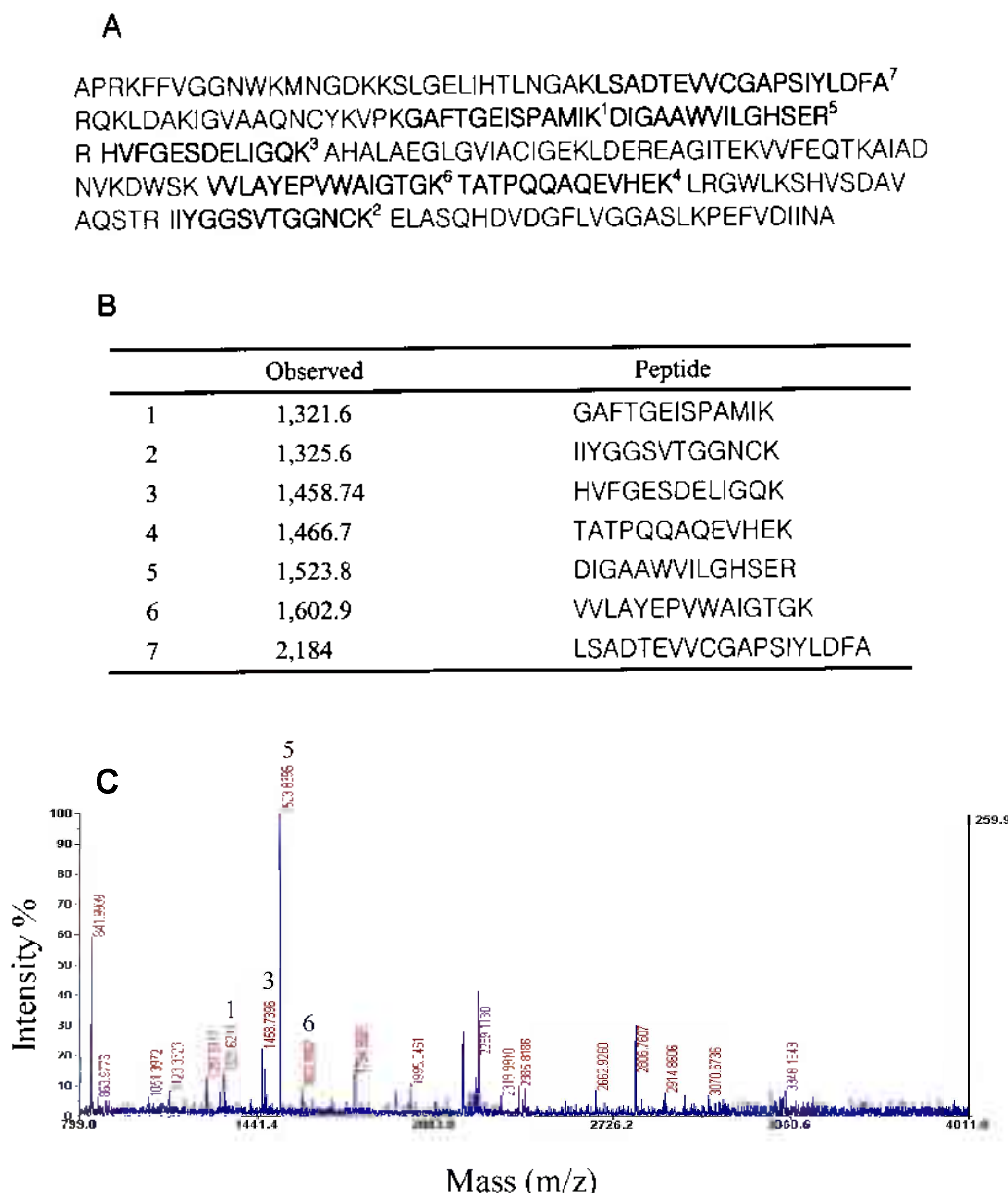
was identified as triosephosphate isomerase and the spot volume and area were increased when the ginseng concentration was increased. The example of whole process for identifying proteins followed by MALDI-TOF and database search was illustrated in Fig. 3. Of these proteins identified, spot number 3 in Table 1 and Figure 2 was estimated as protease of human immunodeficiency virus type 1. However, the molecular weight and pH from the 2-DE map were different compared with results obtained from MALDI-TOF MS data. Therefore we concluded that the spot number 3 was accidentally matched as protease protein, possibly due to the lack of information in the database.

All four proteins were up-regulated for increasing the ginseng concentration in water. Also many protein spots showed variations in ginseng treatments but they were excluded for the further analysis because of lacks of consistency among treatments. The identified α-tropomyosin (2 spots), triosephosphate isomerase, and one unknown protein expressions in the 5% ginseng treatment were 11.2, 6.5, 3.4 and 4.0 times higher than controls.

To estimate biological roles in chicken muscles, the proteins identified were carefully investigated. Tropomyosins are ubiquitous proteins of 35 to 45 kD associated with the actin filaments of myofibrils and stress fibers (Lees-Miller and Helfman, 1991). Interestingly, we identified two differentially expressed protein spots for α-tropomyosin with different expression levels. These two α-tropomyosins were assumed to be different isoforms. In vertebrates, four known tropomyosin genes code for diverse isoforms that are expressed in a tissue-specific manner and

Table 1. Identification of four proteins in chicken muscles affected by ginseng treatments

Spot no.	p*I*	M*r*	Sequence coverage (%)	Protein identification	Accession	Species
1	4.8	30,045	39	Alpha-tropomyosin (partial)	gi/212815	Gallus gallus
2	4.8	30,045	39	Alpha-tropomyosin (partial)	gi/212815	Gallus gallus
3	7.0	25,000		Unknown	-	-
4	6.9	26,487	5	Triosephosphate isomerase	gi/136056	Gallus gallus



A

APRKFFVGGNWKMNGDKKSLGELIHTLNGAKLSADTEVVCGAPSIYLDFA[7]
RQKLDAKIGVAAQNCYKVPKGAFTGEISPAMIK[1]DIGAAWVILGHSER[5]
R HVFGESDELIGQK[3] AHALAEGLGVIACIGEKLDEREAGITEKVVFEQTKAIAD
NVKDWSK VVLAYEPVWAIGTGK[6] TATPQQAQEVHEK[4] LRGWLKSHVSDAV
AQSTR IIYGGSVTGGNCK[2] ELASQHDVDGFLVGGASLKPEFVDIINA

B

	Observed	Peptide
1	1,321.6	GAFTGEISPAMIK
2	1,325.6	IIYGGSVTGGNCK
3	1,458.74	HVFGESDELIGQK
4	1,466.7	TATPQQAQEVHEK
5	1,523.8	DIGAAWVILGHSER
6	1,602.9	VVLAYEPVWAIGTGK
7	2,184	LSADTEVVCGAPSIYLDFA

C

Figure 3. MALDI-TOF MS peptide map of spot 4. A: Amino acid sequence of Triosephosphate isomerase (Q54714). B: observed peak data. C: seven peptides identified by peptide mass fingerprinting are indicated in the protein sequence.

regulated by an alternative splicing mechanism (Lees-Miller and Helfman, 1991). This evidence supports that two protein spots are isoforms of α-tropomyosin. Based on the gene ontology search results, this protein affects muscle development and agreed that the ginseng treatments can cause muscle cell developments (Park et al., 1990).

Another up-regulated protein, triosephosphate isomerase acts as important role in glycolytic pathway and mutation of this gene can cause degeneration of brain and muscle distonia (Casal et al., 1987; Ationu et al., 1999). Interestingly, this gene causes eye tumors and $CD4^+$ T cells were activated for suppression of the eye tumors. Therefore, this protein is highly related with immune responses and supports the results that ginseng treatments elevate immune ability (Moon, 1999).

Recently, proteomics researches are dramatically growing. In case of human and mouse, protein databases for liver, red blood cells, and kidney protein databases have been widely used (http://us.expasy.org/). However, the proteomics in livestock animals is launched recently and the protein databases are ultimately needed. Recently, high-resolution protein 2-DE reference maps for bovine tissues and biological fluids have been determined (Talamo et al., 2003). Very recently, proteome of chicken skeletal (*pectoralis*) muscles have been investigated and 90 proteins spots were characterized by MALDI-TOF MS. The chicken

genome sequences released on March 2004 will give good opportunity for the improvement in chicken genomics and proteomics research.

In conclusion, three proteins (four protein spots) have been identified in this study affecting ginseng treatments. Based on the known functions of the identified proteins, they are related to the muscle development and enhanced immunity in chicken. Upon tracing exact biological roles for the identified proteins, they can be used as valuable biomarkers for meat quality, productivity and disease in chicken.

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