Asian-Aust. J. Anim. Sci.
Vol. 21, No. 10 : 1383 - 1388
October 2008

www.ajas.info

Identification of Differentially Expressed Proteins at Four Growing Stages in Chicken Liver

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ABSTRACT : Because of high growth rate and large deposition of fat in the abdomen, the chicken has been used as a model organism for understanding lipid metabolism, fattening and growing. In this study, differentially expression of proteins in chicken liver, one of the important organs for lipid metabolism, has been investigated at four different growing stages. After separation of proteins using two-dimensional electrophoresis (2-DE), more than 700 protein spots were detected. Among them, 13 growing stage specific proteins in chicken liver were selected and further investigated by matrix-assisted laser adsorptions ionization-time of flight mass spectrometry (MALDI-TOF MS). Of these, 12 proteins were matched to existing proteins based on a database search. The identified fat-related proteins in this study were fatty acid synthase (FASN) and malic enzyme (ME1). These proteins were more highly expressed at week 32 than at other weeks. In order to confirm the differential expression, one of the proteins, FASN, was confirmed by western blotting. The identified proteins will give valuable information on biochemical roles in chicken liver, especially for lipid metabolism. (**Key Words :** Chicken Liver, Different Growing Stages, MALDI-TOF MS, 2-DE)

INTRODUCTION

Chicken (*Gallus gallus*) has been regarded as a valuable model organism, especially for studies of vertebrate development, since the success of embryo manipulations *in vitro* (Stern, 2004 and 2005) and facilitated from the success in chicken genome sequencing (Hillier et al., 2004). Besides, 70 million tons of poultry meat and 47 million tons of eggs were consumed per year in the world. Among the poultry meat, 85% was the chicken meat and 96% was the chicken eggs (Arthur and Albers, 2003). Therefore, the chicken meat and eggs are the valuable protein sources for human.

Along with the recent development of molecular genetic techniques, proteomics is one of the powerful technologies, mainly because this technique allows us to investigate the final products of the metabolism, proteins. Previous results indicated that there were very less correlations between the mRNA expression levels and the amount of target proteins (Futcher et al., 1999; Gygi et al., 1999). In order to understand the expressed proteins from genes, protein-protein interactions and modified proteins in a given environment, proteomics becomes the essential part of many researches (Pandey and Mann, 2000).

Until recently, a number of proteomics research has been carried out in livestock species, including cattle, pig and chicken (Kelly et al., 2006; Jung et al., 2007; Kim et al., 2007). Proteomics can also be widely applied in order to find proteins that are controlling endocrine system mechanism, embryo development and organism developmental changes (Naaby-Hansen et al., 2001). People can anticipate the phenotypic changes by investigation of the diverse changes in metabolites (Fiehn, 2002; Weckwerth, 2003; Dettmer and Hammock, 2004). In case of chicken, Doherty et al. (2004) investigated chicken skeletal muscle proteomes at the specified time points after hatching and identified a number of biologically important proteins. Also, the chicken muscles between layer and broiler breeds were compared by proteomics approach in order to identify economically important growth related proteins (Jung et al., 2007). However, only the comparison of chicken muscles

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Received November 8, 2007; Accepted April 23, 2008

was not enough for the understanding the complex biological process in the muscle. There are also big problems for abdominal fat in adult chicken and the breeding strategy has been aimed to decrease the abdominal fat contents and increase the feed efficiency. In the chicken organs, liver is the main lipogenic tissue, where adipose tissue is also primarily responsible for lipogenesis in mammals (Kanai et al., 1997). After few days of hatching, the majority of the accumulated lipids in the liver of birds is esterified cholesterol, which is stored within lipolysosomes in the hepatocytes (Kanai, 1989; Kanai et al., 1994). Previously, differentially expressed genes in chicken liver from two divergent chicken lines were investigated (Ding et al., 2008). In the present study, we investigated the proteomes in chicken liver at different growing stages in order to identify biologically important fat-related proteins.

MATERIALS AND METHODS

Experimental animals

Liver samples from two female birds of White Leghorn breed were obtained at 0, 10, 21 and 32 weeks of age. These samples were collected from National Institute of Animal Science (NIAS), Seong-hwan, Korea and were immediately stored at liquid nitrogen until use.

Sample preparation

Two hundred mg of frozen liver samples were mixed with 200 μl of solution containing 0.3% sodium dodecyl sulfate (SDS) (GE Healthcare, Sweden), 50 mM Tris-HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Roche, Germany), 50×Protease inhibitor (Roche, Germany), 200 mM dithiothreitol (DTT) (GE Healthcare, Sweden). These mixtures were homogenized by sonication (Hielscher, Germany) and then centrifuged at 15,000 g for 10 min at 4°C. Obtained supernatants were incubated with solution containing 40 U DNase I (Roche, Germany), 14 U RNase A (Roche, Germany), 50 mM Tris-HCl pH 8.0, 0.1 mM $MgCl_2$ in ice for 50 min. After centrifugation at 4°C with 15,000 g for 15 min, the supernatants were used for further analysis. Protein concentration of each sample was determined according to Bradford assay (Bradford, 1976).

Two-dimensional electrophoresis (2-DE)

For isoelectric focusing (IEF), immobilized pH gradient (IPG) strips (pH 3-10 NL, 18 cm: GE Healthcare, Sweden) were rehydrated in solution containing 8 M urea (GE Healthcare, Sweden), 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (GE Healthcare, Sweden), 60 mM DTT, 0.5% IPG buffer (GE Healthcare, Sweden) and 0.01% bromophenol blue (BPB) (GE Healthcare, Sweden) for 12 h. Total 110 μl solution containing 1 mg protein of each sample was loaded onto rehydrated IPG strips and focusing was performed in 50 V for 10 h, 100 V for 1 h 40 min, 300 V for 1 h 40 min, 600 V for 1 h 40 min, 1,000 V for 1 h 40 min, 3,000 V for 1 h, 5,000 V for 1 h and final focusing step of 8,000 V for 12 h. All focusing steps were performed at 20°C using Multiphor II apparatus (GE Healthcare, Sweden). The focused IPG strips were equilibrated in 10 ml of 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-dimensional electrophoresis was performed on 8-16% linear gradient SDS-polyacrylamide gels. The gels were placed into an Ettan DALT (GE Healthcare, Sweden). After the second dimensional separation, the gels were fixed for 1 h in solution containing 40% methyl alcohol, 5% phosphoric acid and stained for 24 h in Colloidal Coomassie Blue G-250 solution (CCB) (Fluka, Switzerland). Then the gels were destained in solution containing 1% acetic acid until the appropriate background was obtained.

Image analysis

The stained gels were scanned using a GS-710 imaging calibrated densitometer (Bio-Rad, USA) and analyzed using an ImageMaster 2D Platinum software version 5.0 (GE Healthcare, Sweden). The gel images from different growing stages were compared and differentially expressed spots were further investigated.

MALDI-TOF MS

In-gel digestion was performed as described by Park et al. (2004). Protein spots were excised from stained gels and the proteins were subjected to in-gel trypsin digestion and peptide extraction using a Mass-Prep™ digestion robot (Micromass, UK). Peptides were analyzed using a modified Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, USA). The proteins were identified using MASCOT peptide mass fingerprint software (http://www.matrixscience.com/cgi/search_form.pl) and profound program (http://www.unb.br/cbsp/paginiciais/profound.htm).

Western blot analysis

For western blotting, approximately 25 μg proteins was electrophoresed on 12% SDS-PAGE gel and the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) using Trans-Blot apparatus (Bio-Rad, USA). After incubation of the membranes in blocking buffer (5% non-fat dry milk) for 1 h, the membranes were subjected washing three times for 10 min with washing buffer (TBS-T: 10 mM Tris-HCl pH, 7.4, 0.1 M NaCl, 0.1%

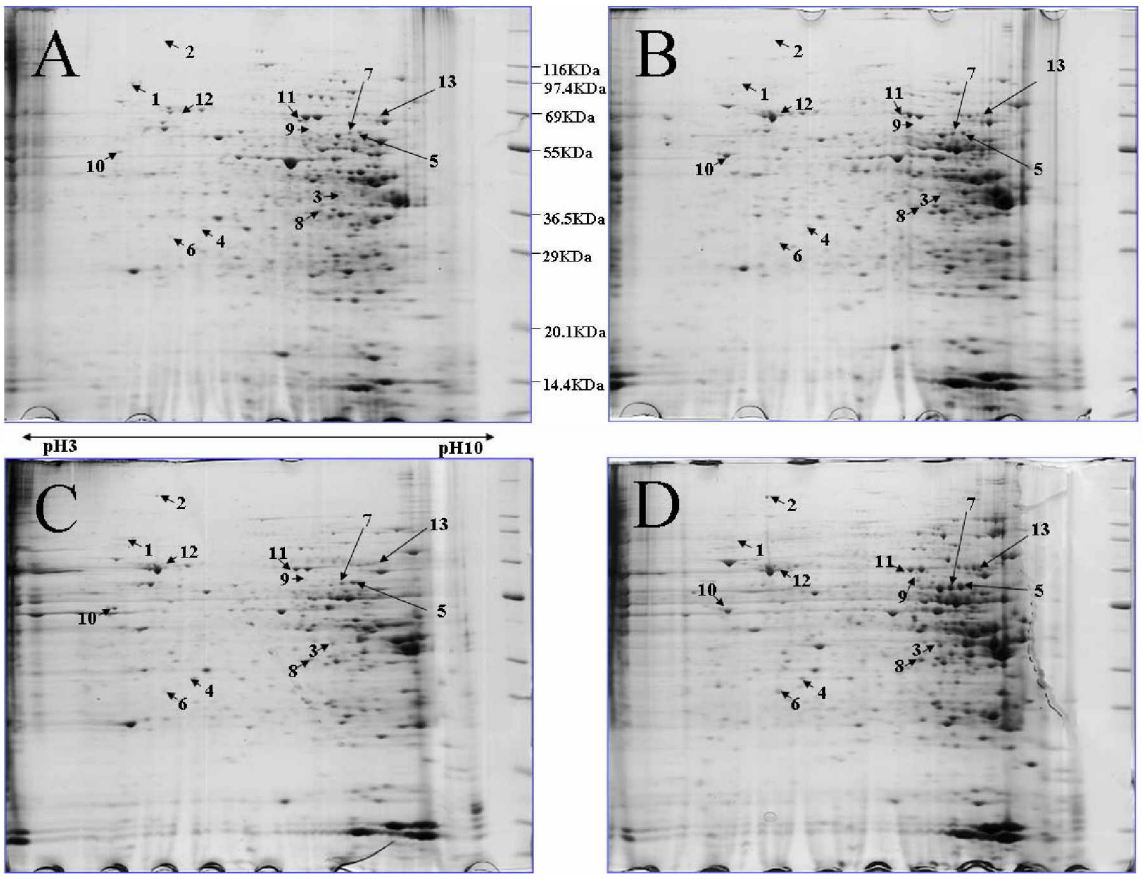

Figure 1. 2-DE Gel images of chicken liver at 0 (A), 10 (B), 21 (C) and 32 (D) weeks of age. The locations of 13 differentially expressed proteins were indicated by arrows.

Tween 20). Then, the membrane was incubated in 1:1,000 diluted FASN specific antibody (Abcam Co. Ltd., UK) along with 1:2,000 diluted control β-action antibody (Genway, USA) for 1 h. After that, the membrane was incubated in 1:10,000 diluted secondary antibody (Goat polyclonal to chicken IgY H & L; Abcam Co. Ltd., UK) for 1 h. The detection procedure was carried out in dark room using an ECL kit (GE Healthcare, UK). The detection reagent was poured onto the membrane and the membrane was exposed with a sheet of X-ray film (Agfa, Belgium) in a film cassette. The molecular weights of the fragments were determined using the AlphaEase™ Stand Alone Software (Alpha Innotech Corp., USA).

RESULTS AND DISCUSSION

In order to detect differentially expressed proteins, liver proteins were obtained from White Leghorn breeds at 0 (at birth), 10 (growing period), 21 (early laying period) and 32 (late laying period) weeks of age. Figure 1 shows the 2-DE patterns of chicken liver proteins at four different growing stages using pH 3-10 non-linear (NL) strips. Approximately 700 protein spots were detected on the coomassie stained gels and most of them were appeared in the pH range between 5 to 9 and molecular weight between 20 to 100 kDa. Thirteen identified protein spots were selected as distinctly differentially expressed proteins in chicken liver at four different growing stages. We found that the protein spot 1 was highly expressed at hatching (week 0) and the protein spot 2 was expressed only in late laying period (week 32). Protein spots from 3 to 9 were gradually increasing the protein expression when the bird aged. On the other hand, protein spots from 10 to 13 were gradually decreasing the protein expression as the bird aged (Figure 1). These proteins were further analyzed by MALDI-TOF MS and searched against the protein database. The identified proteins were listed in Table 1. Twelve spots were identified as previously known proteins and one spot (spot number 1) was unable to match with database. Based on the database search, 11 of the proteins were actually matched with chicken proteins, indicating the high reliability of the identified proteins that we generated.

Since the lipid metabolism-related proteins were the major interest in our research, differentially expressed proteins such as FASN (No. 6 in Table 1) and ME1 (No. 9 in Table 1) were more carefully investigated. When the

Table 1. The identification results for the differentially expressed proteins in four growing stages in chicken liver

Spot ID	Accession No	Protein information	%	pI	kDa	Highest expression[1]
1	-	-	-	-	-	0
2	gi|45383974	albumin	39	5.51	69.872	32
3	gi|50752703	similar to Sorbitol dehydrogenase	52	7.09	38.12	32
4	gi|62738642	Chicken Annexin V	39	5.61	36.045	32
5	XP_421496.1	similar to catalase	31	7.3	55.77	32
6	gi|1345958	Fatty acid synthase	7	5.94	274.607	32
7	XP_421496.1	similar to catalase	30	7.3	55.77	32
8	gi|57530355	malate dehydrogenase 1	34	6.92	36.52	32
9	gi|15420977	malic enzyme	30	6.45	61.961	32
10	gi|57524986	heat shock 70 kDa protein 9B	17	6.09	73.141	0
11	XP_421496.1	similar to catalase	26	7.3	55.77	0
12	gi|230360	Triose phosphate isomerase	38	7.26	26.527	0
13	gi|46048768	Enolase 1	24	6.17	47.275	0

Note that two proteins (spot No. 6, 9) were identified as lipid related proteins.
[1] Observed highest expression among four growing stages (0, 10, 21, 32 weeks of age).

expression levels were compared, FASN expression level was continually increased as the birds aged. Especially, the FASN expression level in week 32 was 4.5 times higher than week 0. FASN is a complex multifunctional enzyme (Wakil, 1989; Witkowski et al., 1991; Smith, 1994; Smith et al., 2003) and plays important roles in the essential components of all biological membrane lipids and energy metabolism. In fact, FASN in typical cells is responsible for energy storage from excess carbohydrate (Kuhajda, 2000). Therefore we can expect that the high expression of FASN protein in later period of growth is highly related with the fat storage.

The ME1 was not expressed exponentially in the growing stage at 0 and 21 weeks. But its expression level was roughly 10 times higher in week 32 comparing to week 0 (Figure 2). ME1 is a lipogenic and widely distributed in metabolic pathways. ME1 catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO_2 and NADPH. Therefore we also postulated that the ME1 is very important protein in lipid metabolism and biological pathways. Previous research indicated that FASN and ME1 in liver usually change drastically when animals were subjected to different development, nutritional and hormonal states. In chicken, FASN and were very low in the embryonic liver. FASN began to significantly increase the day just before hatching but, there was no increase in ME1 activity. In addition, FASN and ME1 were increased when neonatal birds were fed, and decrease when food is withheld (Goodridge, 1968 and 1973). In order to confirm the differential expressions that were observed from 2DE analysis, western blot was performed for the FASN protein (Figure 3). We found the consistent results from the Western blot analysis indicating the high confidence of our 2-DE results obtained in this study.

In conclusion, 13 growing stage specific proteins in chicken liver were investigated and two proteins that were related to lipid metabolism in chicken liver have been identified in this study. The detailed investigation of the identified proteins can give valuable information for chicken liver in relation to the different growing stages.

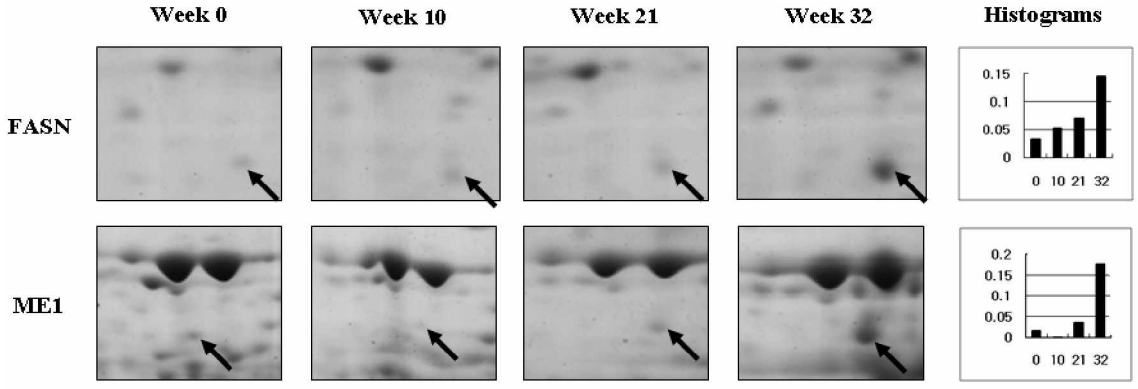

Figure 2. The gel images of two differentially expressed proteins that were related lipid metabolism at four growing stages in chicken liver. In the histogram, X- and Y- axes indicate the weeks of age in four growing stage and the relative expression level of the spot, respectively.

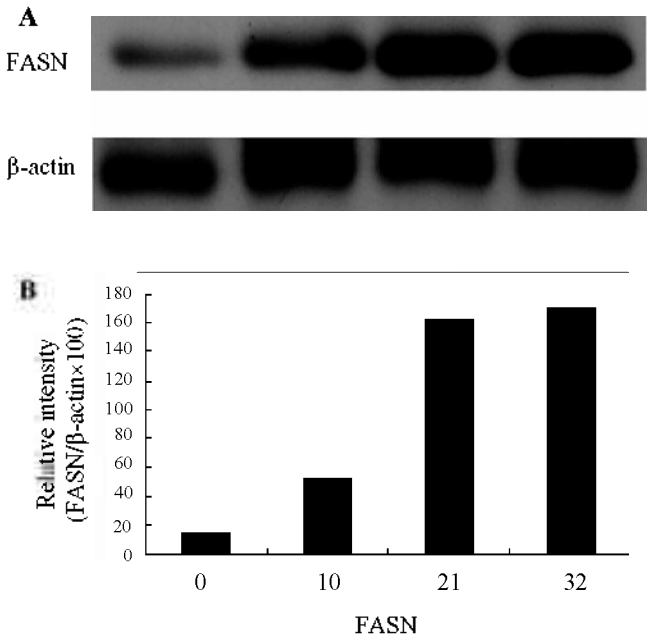

Figure 3. Western blot results of FASN protein. (A) Representative bands by anti-FASN and β-actin at four growing stages. (B) Densitometric quantification of protein levels at four growing stages. X axis shows 0, 10, 21, 32 weeks of age, respectively. Y axis shows relative intensity of the FASN protein. The FASN protein was quantified in comparison with β-actin expression level in each sample.

ACKNOWLEDGMENTS

This work was supported by a grant (code #20050301034359) from Biogreen21 Program and by a grant of FTA research project (Project No. 20080101-081-386-001-02-00), Rural Development Administration, The Republic of Korea.

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