The Journal of Poultry Science, 44: 182–188, 2007

Identification of Heat Stable Proteins in the Fatty Livers of Thyroidectomized Chickens

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Marked growth retardation has been well documented in thyroidectomized chickens. However, in these chickens, fatty liver and enlarged deposits of abdominal fatty tissue are also induced. The aim of the present study was to identify proteins that induce the fatty livers of thyroidectomized chickens. Heat stable proteins were selected for ease of analysis. Four protein bands were detected by SDS-PAGE with CBB staining after incubation at 70℃ for 10 min. The degree of CBB staining suggested that the expression of the 25-, 40- and 45-kDa proteins increased, whereas that of the 35-kDa protein decreased, in the fatty livers of thyroidectomized chickens. Partial N-terminal amino acid sequences were determined from the random peptides of these four proteins. Partial amino acid sequencing suggested that the 35-kDa protein was the lactate dehydrogenase B (LDHB, EC 1.1.1.27) subunit, which is primarily composed of LDH-1 isozyme, although this subunit is not abundant in mammal livers. Further analysis revealed that this 35-kDa protein acts as a dehydrogenase, with lactic acid as the substrate. It was thus identified as the LDHB subunit. The 40-kDa protein was identified as alcohol dehydrogenase 1 (ADH1, EC 1.1.1.1), and the 45-kDa protein as betaine homocysteine methyltransferase (BHMT, EC 2.1.1.5), an enzyme in the homocysteine cycle. The 25-kDa protein appeared to be a novel protein. These findings suggest that the metabolic pathway from pyruvic acid to ethanol is accelerated in the fatty livers of thyroidectomized chickens.

Key words: chicken, fatty liver, LDH, proteome, thyroidectomy

J. Poult. Sci., 44: 182–188, 2007

Introduction

The thyroid gland hormones profoundly influence metabolism, growth and development (Oppenheimer *et al.,* 1987), and it is well known that marked growth retardation occurs following thyroidectomy in chickens (Ringer, 1976). This retardation is caused mainly by inhibition of the growth of muscle, as well as inhibition of bone and intestinal organ growth (Raheja and Snedecor, 1970; King and King, 1976; Nikki and Nobukuni, 1994). On the other hand, the development of fatty liver and the accumulation of abdominal fat are marked in thyroidectomized chickens (Nobukuni *et al.,* 1989). In other words, hepatic carbohydrate and lipid metabolism are significantly influenced by thyroid hormones (Menahan and Wieland, 1969; Tata *et al.,* 1963). Therefore, if normal metabolism of the liver can be changed by removing the thyroid gland and artificially inducing a state of hypothyroidism, then the detection of this change in metabolism may help determine the mechanism by which thyroid hormones maintain normal metabolism at the molecular level. Moore *et al.* (1984) reported a change in metabolism and contractile activity of muscle in immature cockerels using this method. We aim to clarify the process by which thyroid hormones control metabolism in the liver of chickens using the fatty liver produced by thyroidectomy based on the concept of proteome analysis (Kahn, 1995; Swinbanks, 1995; Wilkins *et al.,* 1996).

Received: September 26, 2006, Accepted: December 19, 2006

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Shibata *et al.* (2003) reported that two proteins significantly increased in the fatty livers of surgically thyroidectomized chickens. One was betaine-homocysteine S-methyltransferase (BHMT; EC 2.1.1.5), which is an enzyme in the homocysteine cycle, while the other was a novel 29-kDa protein. However, analyzing all the proteins present in the liver by proteome analysis using electrophoresis and protein sequencing is difficult, as the small amounts present and crowding on the polyacrylamide gel hinder definitive identification.

In the present study, we focused on heat stable proteins, in order to clarify the proteins present in thyroidectomized fatty livers using advanced proteome analysis. Consequently, given that the electrophoretic pattern could be interpreted easily as heat unstable proteins had been removed, the dynamic state of each heat stable protein could be understood in detail. Here, we report protein changes detected in the fatty liver of thyroidectomized chicken.

Materials and Methods

Twenty male Single Comb White Leghorn (SCWL) chickens were divided into thyroidectomized and control groups. The thyroidectomized group was surgically thyroidectomized at 10 to 11 days of age by the method of Marvin and Smith (1943) with slight modifications.

All chicks were kept in a brooder at 30–33℃. At 28 days of age, the room temperature was decreased to 24℃. Chicks had *ad libitum* access to feed, which was supplied at 0900. Two types of commercial feed for layer chickens were used: starter feed (22% CP) from 1 to 21 days of age and grower feed (17% CP), from 22 days of age until the end of the experiment. The photoperiod was 14L:10D.

Five birds in each group were decapitated at 30 and 50 days of age, and the livers were weighed and stored at −20℃ until analysis. The livers were homogenized in 10-fold lysis buffer (8 M Urea, 2% Nonidet-P40, 5% 2-mercaptethanol), and extracts were obtained by centrifugation at 105,000×g for 30 minutes. Extracts were stored at −20℃ until electrophoresis. Blood plasma T_3 levels were determined by ELISA using a clinical kit (Coenzyme Test T_3, Boehringer Mannheim) at 50 days of age. The extracts were incubated at 70℃ for 10 min using a heating block, after which they were centrifuged at 20,000×g. The proteins in the supernatants were then analyzed by SDS-PAGE and two-dimensional electrophoresis (2-DE). SDS-PAGE was performed in accordance with the method of Laemmli (1970) with slight modifications. A separate SDS gel (160×160×1 mm) was prepared using 14% polyacrylamide and 50 μg of protein from each liver extract was then loaded. Conventional native PAGE was performed to detect enzymes in the liver.

2-DE was used as described by Hirano *et al.* (2000). Immobilized pH gradient isoelectric focusing (IPG) in a glass tube (150 mm long, 3.0 mm ID) was used for the first dimension electrophoresis, with 150 μg of protein loaded into each column. Development of 2-DE was performed as described above for SDS-PAGE. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R250 (CBB R 250) in 50% methanol and 5% acetic acid and then destained with 5% methanol and 7% acetic acid.

Amino acid sequences were determined for the proteins of interest. The protein bands on the SDS-PAGE gel were excised with a knife and these segments destained with acetonitrile. After reduction of the proteins with 1 g of guanidine hydrochloride, 5 μl of 4-vinylpridine and 10 μg of tri-n-butylphosphine in 0.5 M Tris-Cl (pH 8.5), they were digested with *Achromobactor* Protease I (lysyl endopeptidase) in 0.1 M Tris-Cl (pH 9.0) containing 0.1 M EDTA and 10% SDS at 35℃ overnight.

Digested peptides were separated on a reversed-phase HPLC column (Inertsil HPLC Column Peptides C-18, 2.1×150 mm, GL Science Co. Ltd). Peptides were eluted using a linear gradient system composed of 0.1% trifluoroacetic acid (solvent A) and 90% acetonitrile in solvent A (solvent B). The solvent B gradient of 0 to 50% was used for 60 min. Single peaks of peptides were selected for amino acid sequence analysis of the N-terminal regions by the Edman method using a protein sequencer (PPSQ-21, Shimadzu Co. Ltd).

The basic local alignment search tool (BLAST) (Altschul *et al.,* 1990) using the ExPASy Molecular Biology Server (www.expasy.ch/) was used to identify the proteins.

Results

Measurements and Determination

The body weights of the thyroidectomized chickens were approximately 66% at 30 days and 60% at 50 days of age of the control chickens at the corre-

sponding age (Table 1). There was no significant difference in liver weights between the control and thyroidectomized groups at any given age. However, the relative liver weight (weight per 100 g body weight) of the thyroidectomized group at 50 days of age was significantly greater than that of the control group, whereas that at 30 days of age was only slightly greater. The supernatant of the first thyroidectomized liver extract contained considerable yellow fat. The levels of plasma T_3 at 50 days of age was markedly lower in the thyroidectomized group compared to the control group (Table 1).

Electrophoresis Patterns

The SDS-PAGE patterns of the liver protein extracts from the thyroidectomized and control chickens obtained by incubation at 70℃ for 10 min are shown in Fig. 1. As most proteins had been removed as sediment following thermal denaturation, differences between proteins from the thyroidectomized and control groups were easily identifiable. Four proteins (25-, 35-, 40- and 45-kDa) were observed on the SDS-PAGE gel after this treatment. In addition, two or three ambiguous bands were detected, but these were not analyzed in this study. Based on the intensity of CBB R250 staining, the proteins in bands HR-1 (45-kDa), HR-2 (40-kDa) and HR-4 (25-kDa) appeared to be more abundant in the thyroidectomized group than the control group, whereas the staining intensity of HR-3 (35-kDa) in the thyroidectomized group was markedly lower.

The 2-DE patterns of the extracts after incubation are shown in Fig. 2. Each protein without HR-1 was detected as a single spot on the two-dimensional gel. Only HR-1 consisted of a few spots.

Amino Acid Sequence and Identification

Protein bands were excised from the SDS-PAGE gel after CBB R250 staining, as each protein existed as a simple spot in the two-dimensional gel. The protein bands were then digested with lysyl endopeptidase and analyzed by peptide mapping and amino acid sequencing.

The protein sequencer was also used to analyze two or three additional peptides randomly selected from peptide maps. The sequence of each peptide, starting from the N-terminal end, as well as those of the candidate proteins, is shown in Table 2. An amino acid sequence homology search revealed that the HR-3 band, which was markedly less abundant in the thyroidectomized chickens, was most likely the chicken lactate dehydrogenase B (LDHB, EC 1.1.1.27) subunit, since the sequences of the two peptides showed

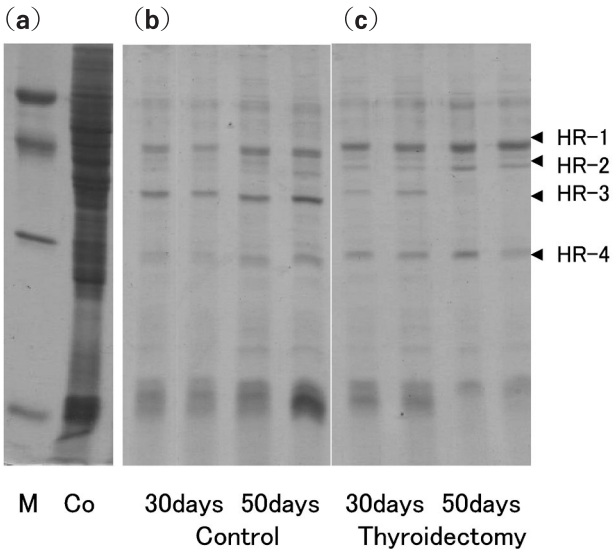

Fig. 1. **SDS-PAGE of protein using the coomassie brilliant blue R250 staining procedure from the liver of thyroidectomized chickens (c) and control chickens (b) at 30 and 50 days of age.** HR-1, -2, -3 and -4 indicates the protein pattern after treatment at 70℃ for 10 min. M indicates molecular marker of 14.2, 29, 45, 66-kDa protein from the anode (under). Co indicates the protein pattern in the crude liver extract of control chicken at 50 days of age (a).

Table 1. **Body and liver weights, and concentration of thyroid hormone T_3 of sham-operated and thyroidectomized SCWL chickens at 30 and 50 days of age**

Item	30 days of age		50 days of age	
	Control	Thyroidectomy	Control	Thyroidectomy
Body weight (g)	357±9	237±17*	687±38	409±82*
Liver weight (g)	9.3±1.6	9.2±1.1	19.8±1.7	15.0±3.0*
Liver weight/100 g BW (g)	3.1±0.4	3.9±0.7	2.9±0.3	3.8±1.0*
T_3 (ng/m*l*)	—	—	4.0±0.4	0.4±0.3*

Means and SD were calculated from five birds. Statistical analysis was carried out using Student's *t*-test (*$P<0.05$).
SCWL; Single Comb White Leghorn.

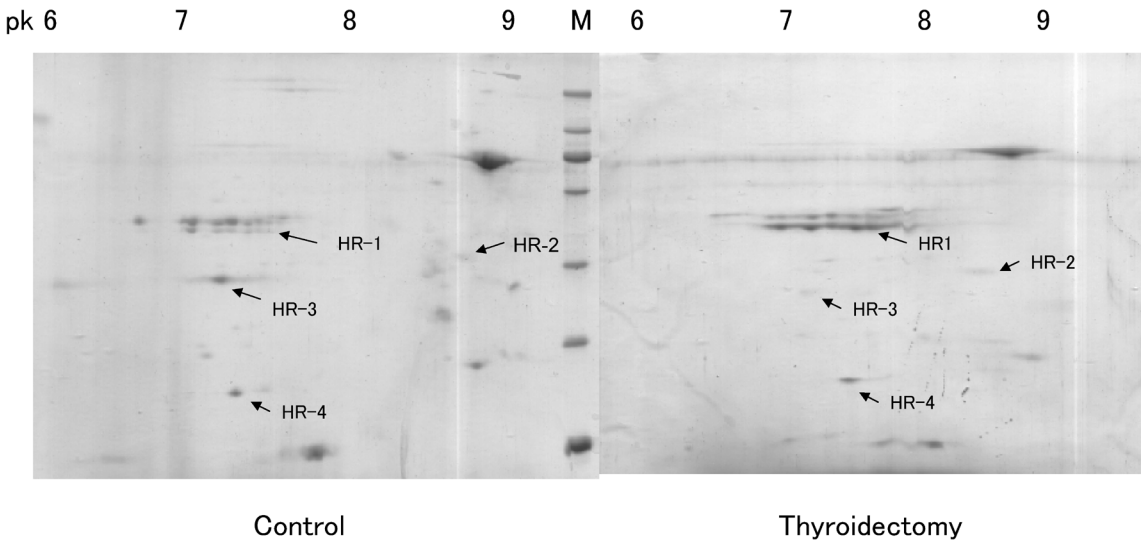

Fig. 2. **Two-dimensional gel electrophoresis of proteins treated at 70℃ for 10 min from the liver of thyroidectomized chickens (right) and sham-operated controls (left) at 50 days of age using the coomassie brilliant blue R250 staining procedure.** HR-1, -2, -3 and -4 indicate protein spots detected. M indicates molecular markers of 20, 30, 40, 60, 80, 100 and 150-kDa proteins from the anode (under).

Table 2. **Sequenced partial amino acid sequences and estimated proteins identified in thermally stable liver proteins in chicken**

Symbol for Protein band	MW (kDa)[1]	Amino acid sequence of a peptide	Estimated protein	MW (Da) (Origin)	Accession[2] number
HR-1	45	AGSNVLQTFT… LENRGNYVAE…	betaine homocysteine S-methyltransferase (EC 2.1.1.5)	44969 (human)	Q93088
HR-2	40	ELGATECIN… GNLCIK…	alcohol dehydrogenase 1 (EC 1.1.1.1)	39676 (chicken)	P23991
HR-3	35	DYAVTANSK… AYEVL…	lactate dehydrogenase B subunit (EC 1.1.1.27)	36187 (chicken)	P00337
HR-4	25	AVGDKPPLDV… DPNLVIVPLE…	unknown unknown		

[1] Molecular weights were estimated using molecular markers on electrophoresis gel.
[2] Accession number is the UniprotKB/Swiss-Prot entry code on the Expasy server.
All of the peptide sequences for HR-1, -2 and -3 showed completely homology to estimated proteins.

100% homology with the partial amino acid sequences for the chicken LDHB subunit (AC: P00337). The characters and numerals in parentheses indicate the accession number of the UniProtKB/Swiss-Prot entry code.

Analysis of the proteins that became more abundant after thyroidectomy revealed that the N-terminal amino acid sequences for HR-1 protein had a high degree of homology with the betaine homocysteine S-methyltransferase (BHMT, EC 2.1.1.5) subunit (AC: Q93088) reported by Shibata *et al.* (2003). The sequences of the peptides for HR-2 protein showed high homology with the alcohol dehydrogenase 1 (ADH1, EC 1.1.1.1) subunit (AC: P23991). All six and nine residues of the peptides for HR-2 agreed with the partial amino acid sequences for chicken alcohol dehydrogenase. Conversely, HR-4 protein was not identified by the amino acid homology search with the two random peptides.

Enzymatic Determination

HR-3, which decreased markedly after thyroidectomy, was isolated and purified by incubation at 70℃ for 10 min, anion exchange chromatography and gel filtration (unpublished data). Purified HR-3 and crude extract from liver were separated by conventional PAGE and detected by enzymoelectrophoresis with the formazan reaction using lactic acid as the substrate for detecting of enzyme activity.

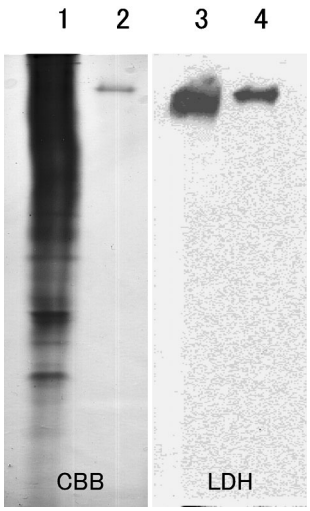

Fig. 3. **The coomassie brilliant blue R250 (1 and 2; CBB) and formazan (3 and 4; LDH) staining patterns using lactic acid as a substrate in conventional PAGE.** 1 and 3 indicate crude extracts, 2 and 4 indicate purified HR-3 protein, which decreased in abundance in thyroidectomized chicken liver. Purified HR-3 protein was shown to have LDH activity.

Enzymatic activity of purified HR-3 and crude extract were detected at the same position on conventional PAGE gels (Fig. 3). Thus, it was confirmed that the HR-3 protein was the LDHB subunit.

Discussion

Usually, plasma T_3 levels decrease markedly after a thyroidectomy. The plasma T_3 level at 50 days of age decreased to approximately 0.4 ng/m*l* from approximately 4 ng/m*l* of plasma. In the present study, although the plasma T_3 level at 30 days of age was not measured, Shibata *et al.* (2003) reported that the T_3 level in thyroidectomized chickens at 20 days of age was lower than normal levels. We therefore believe that the T_3 levels in our thyroidectomized chickens were lower at 30 days of age. Body weight, liver weight and liver weight relative to body weight at 30 and 50 days in our control and thyroidectomized chickens were similar to those observed in other studies (Nikki and Nobukuni, 1994; Shibata *et al.,* 2003).

Shibata *et al.* (2003) reported an increase in the 29-kDa and 45-kDa proteins in the crude extract of thyroidectomized chicken liver, and identified a 45-kDa protein as betaine homocysteine S-methyltransferase (BHMT, EC 2.1.1.5). In the present study, incubation at 70℃ for 10 min was performed to examine heat stable liver proteins that may have changed due to thyroidectomy. The HR-1 protein we obtained was identified as the same 45-kDa protein identified as BHMT by Shibata *et al.* (2003). On the other hand, the HR-3 protein, which was markedly less abundant following thyroidectomy, was identified as chicken LDHB by amino acid sequencing of peptides. Chicken LDHB has a molecular weight of 36187 Da, consists of 332 amino acid residues (AC: P00337), and has an isoelectric point of 7.22 estimated from amino acid residues. The molecular weight of HR-3 was estimated to be approximately 35-kDa, and its isoelectric point was between 7 and 7.5 by 2-DE. However, LDH has two subunits; LDHA and LDHB. It is well known that LDHA is the skeletal muscle type (M type) while LDHB is the heart muscle type (H type) (Fondy and Kaplan, 1965; Markert, 1968). The molecular weight of the LDHB subunit is the same as the LDHA subunit, and LDHB has 80.6% amino acid homology with LDHA. Moreover, there are five native LDHs (LDH1 to 5), which form tetramers using combinations of the LDHA and LDHB subunits. Therefore, we compared the sequences of each peptide between HR-3 and the same position in chicken LDHA (AC: P00340). One of the analyzed peptides from HR-3 was located between amino acids 80 and 89 from the N-terminal of the reported chicken LDHB sequence (AC: P00337). Another was located between amino acid 237 and 242. However, the former HR-3 peptide and the same position on LDHA differed by two amino acids, whereas HR-3 and LDHB were in complete agreement at the same position and had the same sequence as each other. Therefore, the peptides from HR-3 were considered to be chicken LDHB.

To positively identify HR-3 as LDHB, we needed to determine whether HR-3 had any enzymatic activity. We therefore purified HR-3 by heat-treatment, anion exchange chromatography and gel filtration, and then analyzed whether it acted as LDH using lactic acid as the substrate. HR-3 did indeed exhibit LDH activity and was subsequently identified as the chicken LDHB subunit, which has a high thermal stability.

The LDH-5 isozyme, which is primarily composed of subunit A (M type), has previously been considered to be the most abundant isozyme in mammalian livers (Fine *et al.,* 1963). However, our findings in

chickens do not support this hypothesis. Previous studies using Titan-Gel electrophoresis, which is applied to most animal tissues, have shown that the technique does not produce good separation of LDH in chicken tissue and provides only one isozymatic pattern (Kaplan *et al.,* 1960; Heinova *et al.,* 1996). Recently, Heinova *et al.* (1999) demonstrated that visualization of LDH isozyme distribution in chickens could be improved using isoelectric focusing. They reported that five isozymatic patterns exist for chicken LDH, which is the same number reported for mammals (Michalek and Marcanik, 1975). However, several differences in LDH isozyme profiles did appear among tissues; LDH-1 was dominant in the heart and LDH-5 was dominant in breast muscle in both mammals and chickens. However, LDH-1 was dominant in chicken liver and LDH-5 was dominant in chicken serum, which was opposite to that in mammals. These findings support the results obtained in the present study.

Cachexia exophthalmica in humans is associated with increased carbohydrate metabolism, promotion of glycogenolysis and accentuation of glyconeogenesis, and a lowering of glycogen synthesis (Holness and Sugden, 1987). Consequently, the production of lactic acid is promoted, and it is known that both the blood lactate concentration and the activity of the Cori cycle increase (Kuzuya, 2002). Since the T_3 concentration in the blood of thyroidectomized chickens has been demonstrated to decrease, the present results are thought to represent the reverse condition to cachexia exophthalmica in humans. Therefore, the production of lactic acid in the livers of thyroidectomized chickens is suppressed and the chickens experience acidosis due to pyruvic acid accumulation in their livers. Conversely, the abundance of HR-1 and HR-2 proteins increased in our thyroidectomized chickens. HR-4 is an as yet unidentified novel protein and HR-2 was identified as the ADH1 subunit by partial amino acid sequences. In addition, the molecular weight and isoelectric point for HR-2 were very close to those of ADH (MW, 39.8-kDa; pK, 8.69). ADH catalyzes the process that forms ethanol from acetaldehyde, which is composed of pyruvic acid. Shibata *et al.* (2003) reported that BHMT increases in the liver of thyroidectomized chickens while Barak *et al.* (1987) reported that ethanol induces the activity of BHMT in rat liver. Our findings suggest that the process of obtaining ethanol from pyruvic acid through the glycolytic pathway is accelerated in the fatty liver of thyroidectomized chicken in order to avoid acidosis due to the accumulation of pyruvic acid. However, further analysis of the changes in enzymatic activities is required. In addition, the function of the novel protein observed in the fatty livers of our thyroidectomized chickens needs to be determined.

Acknowledgments

We greatly appreciate the assistance of Mr. Hideo Tsukamoto of the Support Center for Research and Education at Tokai University for the determination of amino acid sequences in this study. Part of this work was supported by a grant from the Tokai University Educational System.

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