J Food Sci Nutr
Vol 15, p 143~146 (2010)
DOI: 10.3746/jfn.2010.15.2.143

J Food Science and Nutrition

Effect of Organic Zinc on the Skin Characteristics of Broilers and the Expression Level of Skin Proteins

– Research Note –

Ju Youn Kim[1], Hyun Jin Kim[1], Hossan Md Salim[2], Bong Duk Lee[2], Hyun-Seok Chae[3], and Kyung Bin Song[1†]

Departments of [1]Food Science and Technology, [2]Animal Science and Biotechnology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Korea
[3]Poultry Science Division, National Institute of Animal Science, Chungnam 330-801, Korea

Abstract

Organic zinc was included in the diet of broiler chickens to examine its effect on the skin characteristics and the expression level of skin proteins. Broiler chicks (Ross×Ross) were fed a corn-wheat-soybean meal basal diet, either as control or containing an additional 80 ppm of zinc proteinate for 4 weeks, and then five broilers from each treatment were selected randomly, slaughtered, and their skin characteristics were examined. There were significant increases ($p<0.05$) in thigh skin epidermis and dermis thickness in the chicks fed organic zinc. Collagen content in the skin of broilers was also increased by the addition of organic zinc to the diet. 2D-gel electrophoresis patterns indicated that expression levels of the three proteins, glyoxylase 1, hypothetical protein, and dispersin B were affected by zinc feeding. These results suggest that adding organic zinc to the chicken's feed may contribute to decreased skin tearing.

Key words: broiler, organic zinc, skin tearing, collagen, 2D-gel electrophoresis

INTRODUCTION

Zinc is involved in various metabolic processes of animals and is an essential mineral for normal growth and carcass quality of broiler chickens (1,2). In poultry, zinc plays an important role in keratin and collagen biosynthesis (3). In particular, reduction in skin tearing was reported to be observed with the addition of organic zinc to the broiler's diet (4). The antioxidant capability of zinc provides a defense system to skin infection in broilers (5). Recently, organic mineral sources like zinc have been incorporated in the diet because of their high bioavailability, compared to inorganic source (6,7).

The objectives of this study were to examine the effect of organic zinc on the skin characteristics of broilers and to identify the skin proteins whose expression levels were affected by the addition of organic zinc.

MATERIALS AND METHODS

Preparation of samples

One-day-old broiler chicks (Ross×Ross 308) were allotted to 2 rice hull-littered floor pens with 1,500 birds per pen. The chicks were fed with corn-wheat-soybean meal basal diet or the diet containing 80 ppm zinc proteinate (Bioplex Zn, Alltech Inc., Nicholasville, KY, USA) for 4 weeks (Table 1). After 4 weeks, five birds from each treatment were selected randomly and slaughtered. Samples were then collected and stored at -10°C until the experiment proceeded.

Skin thickness measurement

For thickness measurement of skin layers, about 1 cm^2 of skin samples from the outer side of the thigh and pelvic back region of each broiler were collected. The samples were fixed in 10% formalin (pH 7.4), dehydrated in ethanol and embedded in paraffin, then stained with hematoxylin and eosin. The thickness of the skin layers was examined under a light microscope (Olympus Co., Ltd., BX 50, F-3, Tokyo, Japan) with a camera (Focus Light, Version 2.88). Significant differences ($p<0.05$) between mean values were determined using Duncan's multiple range test procedures.

Collagen content measurement

Hydroxyproline (Hyp) content of skin samples was analyzed to estimate the collagen content. Hyp content was determined as described by Ignateva et al. (8) with some modifications. Samples (12 mg) were hydrolyzed by concentrated hydrochloric and acetic acids (2:1, v/v, 200 μL) at 166°C (oil bath) for 25 min. The hydrolyzed samples were then mixed with a buffered chloramine B reagent, and oxidation was allowed to proceed for 20 min at room temperature. Excess of chloramine B was de-

†Corresponding author. E-mail: kbsong@cnu.ac.kr
Phone: +82-42-821-6723, Fax: +82-42-825-2664

Table 1. Composition of experimental diets

Ingredients (%)	Treatment	
	Control[1)]	Zn
Corn	25.78	25.78
Wheat	30.00	30.00
Soybean meal	22.99	22.99
Rape seed meal	3.00	3.00
DDGS	10.00	10.00
Yellow grease	4.26	4.26
DL-Methionine	0.30	0.30
L-Lysine (98%)	0.38	0.38
L-Threonine (98%)	0.08	0.08
Salt	0.23	0.23
Limestone	1.79	1.74
Monocalcium phosphate	0.66	0.66
Phyzyme	0.05	0.05
Mineral mixture[2)]	0.20	0.20
Vitamin mixture[3)]	0.05	0.05
Choline chloride	0.12	0.12
Salinomysine	0.10	0.10
Avylamycine	0.03	0.03
Total	100.00	100.00
Zinc proteinate	−	80 ppm
Calculated composition		
Crude protein (%)	20.11	20.11
ME (kcal/kg)	3070	3070
Ca (%)	0.99	0.97
Total P (%)	0.55	0.55
Available P (%)	0.48	0.48
Lysine (%)	1.24	1.24
Methionine (%)	0.61	0.61
Total Zn (ppm)[4)]	33.57	133.66

[1)]Control, without zinc supplement.
[2)]Mineral mixture supplied per kilogram of complete feed: iron, 146 mg; copper, 72 mg; iodine, 0.95 mg; selenium, 0.4 mg; manganese, 89 mg.
[3)]Vitamin mixture supplied per kilogram of complete feed: vitamin A, 12001 IU; vitamin D_3, 3,000 IU; vitamin E, 32 mg; vitamin K_3, 2.06 mg; choline, 1608 mg.
[4)]Analyzed values.

composed by the addition of 3.15 M $HClO_4$ solution, and Erlich's reagent was added. The absorbance was measured at 557 nm and Hyp content was determined, based on the standard curve. The total collagen content was calculated by multiplication of Hyp content by 7.5 (9). Significant differences ($p<0.05$) between mean values were determined by using Duncan's multiple range test procedures.

2D-gel electrophoresis (2D-GE)

Skin samples from the slaughtered chickens were frozen in liquid nitrogen and ground using a pestle and mortar. The ground powder was dissolved in the phosphate-buffered saline soultion (137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate dibasic, 1.8 mM potassium phosphate monobasic) containing 1 mM phenylmethylsulfonyl fluoride and 2% NP40 and centrifuged at 4,000×*g* for 20 min. To the supernatant, trichloroacetic acid/acetone solution was added and proteins were precipitated. After centrifugation, the precipitated proteins were washed more than 3 times with cold acetone containing 0.007% β-mercaptoethanol. The pellets were then freeze-dried and used as a sample for 2D-GE.

For the first dimension isoelectric focusing (IEF), Ettan IPGphor system (Amersham Biosciences, Uppsala, Sweden) and non-linear immobiline IPG strips (18 cm, pH range 4～7) were used (10). The lyophilized protein pellets were dissolved in rehydration buffer (8 M urea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer, 0.002% (w/v) bromophenol blue). After centrifugation, the supernatant (340 μL) was placed into a strip holder, taking care to ensure there were no trapped air bubbles, and covered with Drystrip cover fluid (Amersham Biosciences). The rehydration step was performed at 50 μA for 18 hr at room temperature and the isoelectric focusing (500 V for 1 hr, 1000 V for 1 hr, 4000 V for 4 hr, and 8000 V for 4 hr) proceeded.

After IEF, the IPG strips were equilibrated for 30 min in SDS equilibrium buffer (50 mM Tris, 8 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) containing 65 mM dithiothreitol (DTT), and re-equilibrated in SDS equilibrium buffer containing 135 mM iodoacetamide. The equilibrated strips were loaded otol (Dtop of a vertical SDS-PAGE (1g 5%) gel for the second dimension separation (20 cm×20 cm× thm, PROTEAN II 2D Cell system, Bio-Rad laboratories Inc., Hercules, CA, USA). The strips otol (Dgel were fixed with 0 5% lowt heDS ng agarose dissolved in running buffer. Electrophoresis was performed at tW per gel for the initial 30 min and then at 250 W. After running, the gels were visualized by silver staining kit (No. 17-1150-01; Amersham Biosciences) and scanned for image analysis.

Protein spots which were differently expressed on the gel between the samples were excised, and the gel pieces were destained with 100 mL of destaining solution (30 mM potassium ferricyanide, 100 mM sodium thiosulfate) with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated with 100 mL of acetonitrile and dried in a vacuum centrifuge. The dried gel pieces were then rehydrated with 20 mL of 50 mM ammonium bicarbonate containing 0.2 mg modified trypsin (Promega, Madison, WI, USA) for 45 min on ice. After removal of solution, 30 mL of 50 mM ammonium bicarbonate was added. The digestion was performed at 37°C overnight. The peptide solution was then desalted using a custom-made C_{18} nano column. A

Table 2. Skin layer thickness (μm) of broilers fed with 80 ppm organic zinc supplementation

Treatment	Thigh skin		Back skin	
	Epidermis	Dermis	Epidermis	Dermis
Control	35.52±4.24[b]	159.97±12.58[b]	35.90±4.27[a]	244.27±63.82[a]
Zn	45.82±5.25[a]	277.50±5.44[a]	40.11±6.20[a]	252.09±77.34[a]

[a,b]Means in the same column followed by different letters are significantly different ($p<0.05$).

column of Poros reverse phase R2 material (PerSeptive Biosystems) was packed in a GELoader tip (Eppendorf, Hamburg, Germany). Thirty microliters of the peptide mixture from the digestion supernatant was diluted in 5% formic acid (final volume, 45 μL), loaded onto the column, and washed with 30 mL of 5% formic acid. For the mass spectrometric analysis, peptides were eluted with 1.5 mL of 50% methanol/49% H_2O/1% formic acid into a precoated borosilicate nanoelectrospray needle (Micromass, Manchester, UK).

Mass spectrometry analysis

MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (AB Sciex Instruments, CA, USA). The source temperature was 80°C A potential of 1 kV was applied to the precoated borosilicate nano-electrospray needles (Econo-Tip™, New Objective, USA) in the ion source combined with a nitrogen back-pressure of 0～5 psi to produce a stable flow rate (10～30 nL/min). The cone voltage was 40 V. The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The collision gas was Ar at a pressure of $6～7\times 10^{-5}$ mbar and the collision energy was 25～40 V. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a micro-channel plate detector and a time-to-digital converter. The data were processed using a peptide sequence system.

Protein identification and sequence processing

To identify the protein, peptide masses from the mass spectrometer were matched with the theoretical peptides of proteins in the NCBI database using MASCOT software. Also, all MS/MS spectra recorded on tryptic peptides derived from spot were searched against protein sequences from NCBI and EST databases using the MASCOT search program (www.matrixscience.com).

RESULTS AND DISCUSSION

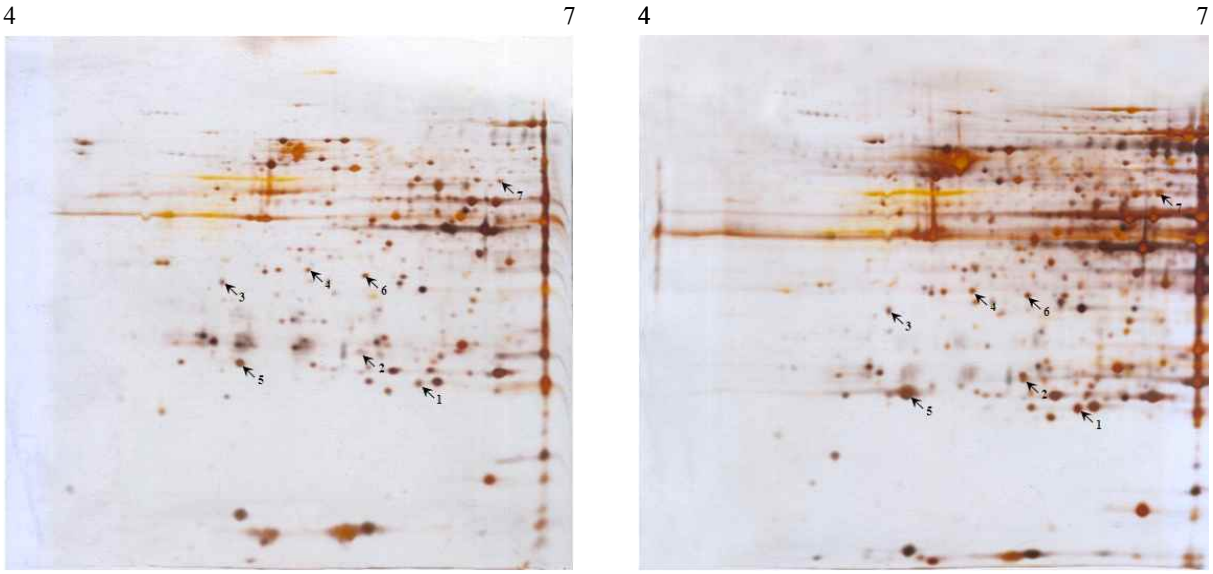
Epidermis and dermis thickness of back skin were not influenced by zinc supplementation, but significant increases ($p<0.05$) in thickness of thigh skin epidermis and dermis were observed (Table 2). Epidermis thickness of thigh skin increased from 35.5 μm for the control to 45.8 μm for the broilers treated with 80 ppm organic zinc, and dermis thickness also increased from 159.9 to 277.5 μm. The thicker the skin layer was, the stronger the resistance to infection by pathogens was (11). The results were also supported by collagen content data. Skin layers are mostly composed of collagen, which is responsible for the skin strength of broilers. Collagen contents, as determined by Hyp content, were significantly different between the control group and the broilers fed with 80 ppm of organic zinc (Table 3). Collagen content of thigh skin increased from 9.3 mg/g for the control broiler to 24.9 mg/g for the broiler fed with zinc. In addition, collagen content of back skin increased from 13.4 to 29.6 mg/g. Rossi et al. (4) also reported that higher levels of organic zinc supplementation increased collagen content in the skin of broilers, which is consistent with our results. Therefore, the results indicate that feeding with organic zinc can increase skin thickness and collagen content of broilers, resulting in decrease of skin tearing.

Skin tearing results in downgrading and economic losses to the broiler industry (2). The degree of skin tearing is affected by growing condition, dietary factors, and skin collagen content. Downgrades of broilers ranging from 5 to 7% have been reported due to skin tearing (12). Therefore, to examine the relationship between zinc in the diet and the degree of skin tearing, the expression level of skin proteins affected by organic zinc feeding was evaluated on 2D-GE. Protein spots were separated using pH 4～7 IPG strip and the expression level of each protein was compared on the silver stained gel between the control and the broilers fed with 80 ppm organic zinc (Fig. 1). Among the protein spots, difference in the expression level of proteins by an image analyzer was examined carefully and repeatedly, and the 7 spots showing distinct and consistent pattern in terms of difference in density were selected. The protein spots were analyzed using MALDI-Q-TOF-MS after trypsin digestion. The peptide mixtures of the protein spots were analyzed

Table 3. Collagen content (mg/g) in skin of broilers fed with 80 ppm organic zinc supplementation

Treatment	Thigh skin	Back skin
Control	9.28±0.22[b]	13.43±3.5[b]
Zn	24.88±4.29[a]	29.60±3.97[a]

[a,b]Means in the same column followed by different letters are significantly different ($p<0.05$).



(A) (B)

Fig. 1. Comparison on the 2D-GE pattern of skin proteins between the control broiler (A) and the broiler fed with 80 ppm of organic zinc (B).

based on the mass spectrum (data not shown). The proteins that were affected by zinc in the diet were finally identified using the MASCOT search engine, the NCBI database, and an MS BLAST homology search. However, it should be noted that 4 of the spots (spot 3, 4, 5, 6) were not identified due to lack of information regarding chicken proteins in the database. However, in this study, we were able to identify three proteins. They are glyoxylase 1 (spot 1), hypothetical protein (spot 2), and dispersin B (spot 7). Glyoxylase is related to detoxification of metabolic byproducts, and dispersin B is involved in detachment of bacterial cells. Thus, these two proteins may contribute to a higher immunity to foreign microorganisms, resulting in resistance to skin infection by pathogens. Therefore, our results suggest that organic zinc feeding affects the expression level of skin proteins of broilers, which may be involved in immune response or inhibition of bacterial infection, resulting in decrease of skin tearing.

ACKNOWLEDGEMENTS

This work was supported by a grant from RDA, Republic of Korea.

REFERENCES

1. Salim HM, Jo C, Lee BD. 2008. Zinc in broiler feeding and nutrition. *Avian Biol Res* 1: 5-18.
2. Salim HM, Lee HR, Jo C, Lee SK, Lee BD. 2010. Effect of sources and levels of zinc on the tissue mineral concentration and carcass quality of broilers. *Avian Biol Res* 3: 23-29.
3. McDowell LR. 2003. Zinc. In *Minerals in animal and human nutrition.* Cunha TJ, ed. Elsevier Science, Amsterdam, Netherlands. p 357-396.
4. Rossi P, Rutz F, Anciuti MA, Rech JL, Zauk NHF. 2007. Influence of graded levels of organic zinc on growth performance and carcass traits of broilers. *J Appl Poult Res* 16: 219-225.
5. Elizabeth FR, Holly VD, Doren LM, Sheldon RP. 2002. Evidence supporting zinc as an important antioxidant for skin. *Int J Derm* 41: 606-611.
6. Ao T, Pierce JI, Power R, Dawson KA, Pescatore AJ, Cantor AH, Ford MJ. 2006. Evaluation of bioplex Zn as organic zinc source for chicks. *Int J Poult Sci* 5: 808-811.
7. Nollet L, Van der Klis JD, Lensing M, Spring P. 2007. The effect of replacing inorganic with organic trace minerals in broiler diets on productive performance and mineral excretion. *J Appl Poult Res* 16: 592-597.
8. Ignateva NY, Danolv NA, Averkiev SV, Obrezkova MV, Lunin VV, Sobol EN. 2007. Determination of hydroxyproline in tissue and the evaluation of the collagen content of the tissues. *J Anal Chem* 62: 51-57.
9. Bonifer LJ, Froning GW. 1996. Chicken skin composition as affected by aqueous washings. *J Food Sci* 61: 895-898.
10. Sun NK, Jang JW, Lee SH, Kim SK, Lee SH, Hoe KR, Chung KS, Kim DW, Yu HS, Won M, Song KB. 2005. The first two-dimensional reference map of the fission yeast, *Schizosaccharomyces* pombe proteins. *Proteomics* 5: 1574-1579.
11. Jonhson RW, Escobar J, Webel DM. 2001. Nutrition and immunology of swine. In *Swine Nutrition*. Lewis AJ, Southern LL, eds. CRC Press, Boca Raton, FL, USA. p 545-562.
12. Leeson S, Summers JD. 2005. Feeding programs for broiler chickens. In *Commercial poultry nutrition*. Leeson S, Summers JD, eds. University Books, Guelph, Ontario, Canada. p 281-284.

(Received March 11, 2010; Accepted April 8, 2010)