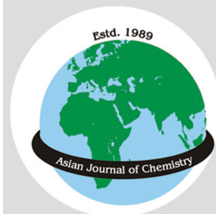

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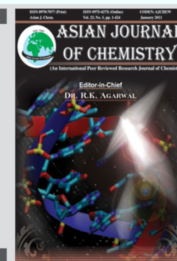

Identification of Protein Peptides by HPLC-Mass Spectrometry

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Fluid albumin as one important structure in egg was not focused on protein changes by scientists in the past years, especially effects by different breeding modes on laying hens. Through HPLC-mass spectrometry method, protein peptides in fluid albumin could be identified. Free-cage (FC) and in-cage (IC) mode were performed in this study to breed the laying hens and the eggs were collected only after laying on one day. Through a rapid 1-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS PAGE), fluid albumin were separated and concentrated quikly. The uper-gel on SDS PAGE were cut and digested by trypsin. By nano-liquid chromatography coupled with mass spectrometry identification, 9 proteins were found, including 5 were in in-cage breeding mode and 4 were in free-cage breeding mode. However, 1 protein, lysozyme C, was celected as a new poteitial marker to distinguish the in-cage and free-cage eggs.

Key Words: Fluid albumin, HPLC, Lysozyme C, Mass spectrometry.

INTRODUCTION

In general, mass spectrometry (MS) was useful not only for organic compound but also for biological macromolecule. The identification of mass spectrometry could be draw into poultry industry because poultry industry has an important role to supply food production in developing countries including China. The major sections in poultry production in China consists of meals and eggs. Hens eggs always provied high qualitiy proteins in human diet as nutritious source. Egg white of avian occupied immportant places not only for stablilizing the york place in the center of egg but also for providing nutrition for the development of chicken embryo. In particular, fluid albumin in the egg white were also used as the resource of nutrition for the cultivation of chicken embryo *in vitro*[1,2].

In general, different environment caused different influence on the same species, especialy for protein expression level[3-6]. Enviroment influence also affected the quality of egg[7]. For example, temperature affected the vitamin volume of egg[8]. In China, native chicken breeding in free-cage (FC) mode always produced free-cage eggs which displayed similarity and significant difference in many aspects with eggs produced by chicken breeding in in-cage mode by feeds, such as the size of egg, the weight rate between yolk and the whole egg, the weight of fluid albumin and so on. Free-cage hens was not only bred by feeds but also by native worm on the ground of mountain and what they had to eat was added less addiction comparing to in-cage hens. Due to the ecological reasons, people have a fancy for buying the free-cage eggs than in-cage eggs. What's more, few research was reported about the different of nutrition between free-cage and in-cage egg, especially for egg white. However, the difference between free-cage and in-cage egg about fluid albumin were not clear at present. In this paper, the fluid albumins of free-cage and in-cage eggs were extracted, respectively. According to shotgun proteome strategy, proteins dissolved in SDS PAGE gel were diggested by trypsin enzyme and dilivered to identified by nano-LC MS/MS. To compare the two kinds fluid albumin, five proteins were found in each type of egg. While only one differential proteins were found between them including lysozyme C in in-cage egg and keratin in free-cage egg, which indicated lysozume C could be a potential marker to distingush eggs betweem free-cage and in-cage eggs.

EXPERIMENTAL

12 In-cage eggs and free-cage eggs were collected, respectively from laying hens in aged around 35 weeks, after which produced by hens only in one day. In this study, three parameters were choose to evaluate the egg properties, such as shape index, yolk weight rate and fluid albumin weight

rate. The length and the width were messured by a vernier caliper. Shape index was calculated by the length and the width of egg through the following evaluation[9]. Shape index = (width/ length) × 100 %. At the same time the weight of each egg was calculated by eclectronic balance and weighted within 0.1 g. To collect the fluid albumin component from the whole egg, the fluid albumin was passed through the sieve after egg was slightly broken and the thick albumin and york were excluded. Yolk weight rate and fluid albumin weight rate were also calculated by the following evaluation.

$$\text{Yolk weight rate} = \left(\frac{\text{Weight of yolk}}{\text{Weight of egg}}\right) \times 100\ \%$$

The fluid albumin weight rate

$$= \left(\frac{\text{Weight of fluid albumin}}{\text{Weight of eggs}}\right) \times 100\ \%$$

General procedure: The fluid albumin components were pericipated by 10 fold volume of ice-cold acetone overnight, washed three times by acetone and resuspended in lysis buffer (7 M urea, 2 % SDS) to produce the concentrated fulid albumin proteins. These sample were quantified by modified Bradford method[10] and then stored at -80 ºC before using. Gel electrophoresis were performed by Bio-rad Mini-PROTEAN Tetra Electrophoresis System and 50 µg protein was loaded for 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To run the electrophoresis under 120 V until coommassie brilliant blue line entered into the 12 % SDS concentrated gel around 1 cm. The part of gel upon the coommassie brilliant blue line were cut, washed by double distilled water twice and stored at -20 ºC, respectively.

Detection method: All samples from gel were digested by trypsin. The gel particles were cut into small cubes of 1 mm^3 that were washed twice with 50 µL of 50 % acetonitrile. The supernatant were discarded after each washing step. After adding 50 µL acetonitrile, the gel particals were dehydrated. Disulfide bonds were destroied by incubating the gels for 1 h at 56 ºC with 50 µL of 10 mM DTT in 50 mM ammonium bicarbonate buffer. Alkylation of cysteines was performed by the addition of 50 µL of 10 mM iodoacetamide in 50 mM ammonium bicarbonate buffer and incubation of the samples for 45 min at room temperature in darkness. Gel particles were washed once with H_2O and 50 % acetonitrile and dehydrated with 50 µL of acetonitrile. Gel pieces were covered with trypsin solution (10 ng/µL in 50 mM ammonium bicarbonate buffer). After a 5 min incubation of ice, the remaining trypsin solution was removed and 10 µL of 50 mM ammonium bicarbonate were added. Proteolysis was performed overnight at 37 ºC and stopped by adjusting the samples to 0.1 % formic acid. The supernatant was directly used for analysis by mass spectrometry. Nanoscale liquid chromatography tandem mass spectrometry (nano LC-MS/MS) experiments were performed on an ultra 3000 nanoflow system (Dionex Corporation, USA) connected to a 7-tesla Bruker apex-ultra FT mass spectrometry (Bruker Daltonics, Germany) equipped with a nanoelectrospray ion source (Bruker Daltonics, Germany). Briefly the mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS. Survey full scan MS spectra (from m/z 300-2300) were acquired by FT-ICR with resolution r = 25,000 at m/z 400. The three most intense ions were sequentially isolated for MS/MS acquisition by auto MS/MS mode.

Each MS/MS spectra file of auto mode was send to MASCOT search engine (Matrix Science) against the publicly available NCBIr data base with carbamidomethyl cysteine as fixed modification. Protein N-acetylation, oxidized methionine were searched as variable modifications. Searches were done with tryptic specificity allowing 1 missed cleavages and an initial tolerance on mass measurement of 5 ppm in MS mode and 0.5 Da for MS/MS ions.

RESULTS AND DISCUSSION

Phenotypes in eggs caused by different breeding environment: Data including shape index, yolk weight rate and fluid albumin weight rate were summarized by descriptive statistics and were presented in Fig. 1. Although many methods to measure the shap index of egg, method to calculate the width and the length was used in this paper because of the simplicity[11]. Shape index of the two breeding mode were closed to 70 %, which suggested that chickens bred by free-cage mode were no significant difference in shape index comparing to chickens bred by in-cage mode. No change in shape index indicated that the genetic properties were no change between different environments. In egg enterprise, albumin and yolk as well as their rate directly effected the price of products. Then the rate of fluid albumin and yolk were also detected. However, yolk weight rate and fluid albumin weight ratio were not similar in the two kinds of breeding mode. Free-cage breeding chickens produced the higher values around 5 % for youlk weight rate than in-cage breeding chickens. On the contrary, the in-cage breeding chickens had the higher value over 8 % for fluid albumin weight rate. The above two parameters probably suggested the fulid albumin in egg white were significant different.

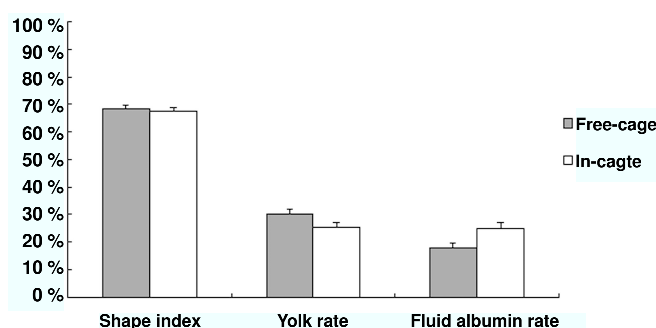

Fig. 1. Comparison of phenotypes between free-cage and in-cage eggs. The shape index were closed to 70 % in the two kinds of eggs, but yolk weight rate were 31.1 ± 1.85 % in free-cage and 25.5 ± 1.59 % in in-cage egg. The fluid albumin weight rate were 17.7 ± 1.69 % in free-cage and 25.1 ± 2.18 % and in-cage eggs

Mass identification of proteins in rapid gel: To survey breeding mode-ralationship profie in egg fluid albumin, eggs used in this study were only collected in one day after which were layed. To exclude other environmental elements such as storage time and temperature[12], sample preparations were executed as soon as possible. Due to the shortcoming of other

shot-gun proteome methods, the rapid seperation were choosed in this paper with a few modification[13]. The mixtrue of fluid albumin in 6 eggs from each breeding mode were seperated by the rapid 1D-SDS PAGE. To overcome potential disadvantage of this seperated method, nano-LC MS/MS spectrometry were performed to decrease the system error in the process of identification and to remedy the insuficient in gel seperation. As described in Table-1, totally 9 proteins were found in the fluid albumin of the two breeding mode, in which five proteins were identified in in-cage eggs and four proteins were identified in free-cage eggs. Ovotransferrin and egg ovalumin were identified in both kinds of eggs. However, three proteins, lysozyme C, keratin 9 and mucin 5, were only identified in in-cage egg. And type II keratin subunit protein were also only identified in free-cage egg. As keratin probablely could be the contamination in the process of trypsin digestion, lysozyme C were collected as the unique potential marker to distinguish the in-cage egg and free-cage egg. Fig. 2 shown the mass identified result of lysozyme C.

Lysozyme from egg attracted the sight of many scientists and deep studies of lysozyme in egg were achieved including analysis of amino acid, crystal structrue and enzymic activity. Due to the genotype and age of hens and storge time and temperature of egg also could affected the egg qulities, lysozyme C should be a potential marker for research in the future.

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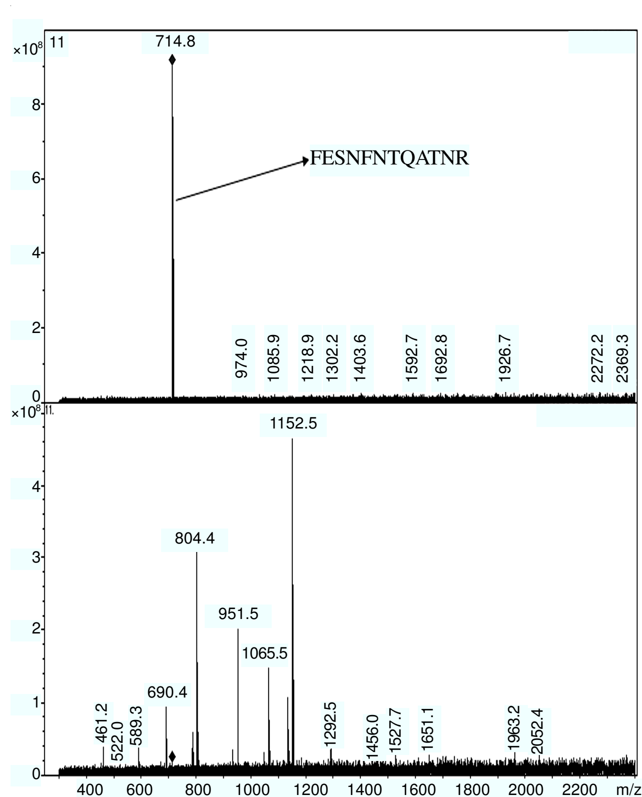

Fig. 2. Identification of lysozyme C by mass spectromety. The up-image shown the parent ion of 714.8^{2+} in the fist scan MS which corresponded the peptide of FESNFNTQATNR and the down-image shown the daughter MS/MS peaks of 714.8^{2+}

TABLE-1
IDENTIFICATION BY MASS SPECTROMETRY IN IC FLUID ALBUMIN

Accesion No.	Proteins	Protein scores	No. of unique peptides	Total of accepted peptides	Sequence coverage	Theoretical molecular weight	Unique peptides
Gi|1351295	Ovotransferrin	171	6	12	7	79	DSAIMLK SCHTAVGR VAAHAVVAR DQLTPSPR KDQLTPSPR TCNPSDILQMCSFLEGK
Gi|129293	Ovalbumin	147	3	8	10	43	VYLPR ISQAVHAAHAEINEAGR EVVGSAEAGVDAASVSEEFR
Gi|126608	Lysozyme C	135	2	4	18	16	FESNFNTQATNR IVSDGNGMNAWVAWR
Gi|55956899	Keratin 9	98	2	3	4	62	FSSSGGGGGGGR SGGGGGGGLGSGGSIR
Gi|45382809	Mucin 5, subtype B	38	1	2	< 1	24	IQEIATDPGAEK
Gi|1351295	Ovotransferrin	94	5	12	8	79	FGVNGSEK VAAHAVVAR DQLTPSPR VEDIWSFLSK TDERPASYFAVAVAR
Gi|129293	Ovalbumin	75	2	4	6	43	HIATNAVLFFGR LTEWTSSNVMEER
Gi|82190473	Ovalbumin	66	1	1	4	52	GGLESINFQTAADQAR
Gi|386854	Type II keratin subunit protein	63	1	1	2	52	WELLQQVDTSTR

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