Differential Proteomics of Chick Serum Before and After Cold Stress [Postprint]

Authors: Yang Yuying, Liu Peng, He Jun, Yang Liu, Yao Ruizhi, Hongzhao Shi, Zhen Li, Tang Dejiang, Li Shize

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Abstract

This study aimed to comparatively analyze the differential expression of proteins in chick serum before and after cold stress and to identify key differentially expressed proteins. Thirty chicks were randomly divided into three groups: a cold stress group, a cold adaptation group, and a normal temperature control group. Blood was collected from chicks in each group to prepare serum, which was then subjected to two-dimensional gel electrophoresis (2-DE) to obtain 2-DE profiles of serum protein expression. Differential analysis of the 2-DE profiles was conducted using PDQuest 8.0 software. Differentially expressed proteins were subsequently identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and validated by Western blot. The results showed that 2-DE analysis of serum from the normal temperature control, cold stress, and cold adaptation groups yielded relatively complete differential protein data, with a total of 23 differential protein spots identified. MALDI-TOF-MS analysis of several reproducible and prominent protein spots successfully identified four differential proteins, two of which were fructose-bisphosphate aldolase C (ALDOC), proteins involved in energy supply through glucose and energy metabolism pathways. Western blot validation of the differential protein ALDOC subsequently yielded results consistent with those of 2-DE. The results indicated that protein expression in chick serum exhibited significant differences before and after cold stress, and these differential protein expressions may be associated with cold stress.

Full Text

A Proteomic Analysis of Differential Serum Protein Expression in Chicks Before and After Cold Stress

YANG Yuying, LIU Peng, HE Jun, LIU Yang, YAO Ruizhi, SHI Hongzhao, ZHEN Li, TANG Dejiang, LI Shize*

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing 163319, China

Abstract

This study aimed to compare and analyze differential protein expression in chick serum before and after cold stress and to identify key differential proteins. Thirty chicks were randomly divided into three groups: a cold stress group, a cold-adapted group, and a normal temperature control group. Serum samples were collected from each group and subjected to two-dimensional gel electrophoresis (2-DE) to obtain 2-DE maps of serum protein expression. Differential analysis of the 2-DE maps was performed using PDQuest 8.0 software. Differentially expressed proteins were then identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and verified by Western blot. The results revealed that 2-DE analysis of serum from the normal temperature control, cold stress, and cold-adapted groups yielded relatively complete differential protein data, with 23 differential protein spots detected. MALDI-TOF-MS analysis of several reproducible and prominent protein spots successfully identified four differential proteins, two of which were aldolase C fructose-bisphosphate (ALDOC), a protein involved in glucose and energy metabolism pathways. Subsequent Western blot verification of the differential protein ALDOC yielded results consistent with the 2-DE analysis. These findings indicate that protein expression in chick serum differs significantly before and after cold stress, and these differential expression patterns may be associated with cold stress responses.

Keywords: two-dimensional electrophoresis; cold stress; proteome; differential protein expression; differential protein spots

Introduction

Stress is a systemic nonspecific adaptive response that occurs when an animal' s homeostasis is threatened, representing a series of physiological reactions to various stressors. Cold climate conditions, particularly in northern regions during winter, constitute one of the most common factors causing stress responses in animals. Such stress severely impacts livestock production performance and disease resistance, significantly constraining the development of the animal husbandry industry.

The proteome refers to all proteins expressed by a genome, cell, or tissue, a term first introduced by Marc Wilkins and Keith Williams at a two-dimensional electrophoresis conference in 1994 [1]. Proteomics is the study of protein types, quantities, spatial structures, and interaction mechanisms at the protein level [2-3]. In recent years, despite the rapid development of proteomics and the widespread application of various proteomic technologies, two-dimensional gel

electrophoresis (2-DE) and mass spectrometry (MS) remain the most classical and fundamental methods in proteomics research. These techniques enable comparison of biological samples from the same organism under different states and monitor changes in protein expression, making them widely applicable for detecting differentially expressed proteins and disease diagnosis [4-9].

Numerous studies have demonstrated that long-term exposure to cold environments alters internal homeostasis, triggering a series of abnormal physiological responses that increase heat production to cope with cold stimulation [10]. The abundant proteins in serum can reflect an animal' s physiological environment, and analyzing their differential expression can help determine physiological status and identify diagnostic biomarkers. This study utilized high-throughput proteomic technology to analyze and detect differential protein expression in chick serum before and after cold stress, aiming to identify biomarker proteins whose expression changes under cold stress conditions. These findings hold significant importance and value for investigating molecular mechanisms and enabling early disease diagnosis and prevention.

Materials and Methods

Experimental Animals

The experimental animals consisted of 30 one-day-old Roman cockerels housed in an artificial intelligence climate chamber with adequate ventilation and free access to water and feed throughout the experimental period.

Animal Model and Grouping

The one-day-old Roman cockerels were randomly divided into three groups (n=10 each). Group 1 served as the normal temperature control group, maintained at 33-35°C (this control group was processed in two batches to serve as a baseline for both the cold stress and cold-adapted groups). Group 2 was the cold stress group, which at 3 days of age was placed at 25°C for 2 hours, after which chicks from this group and five chicks from the control group were decapitated for blood collection. Group 3 was the cold-adapted group, maintained at 4-6°C lower than the control group; at 14 days of age, chicks from this group and the remaining five control group chicks were decapitated for blood collection. Two milliliters of blood were collected from each chick and slowly injected into a sterile 5 mL centrifuge tube. After standing at room temperature for 1 hour, the samples were centrifuged at 3,500×g for 5 minutes at 4°C, and serum was carefully collected.

Protein Sample Preparation

Fifty microliters of serum sample were taken from each group. High-abundance proteins (albumin and immunoglobulin G) were removed using an Aurum Serum

Protein Mini Kit. Serum protein concentration was determined following the instructions of a bicinchoninic acid (BCA) quantification kit.

Two-Dimensional Gel Electrophoresis (2-DE)

2-DE was performed according to the Bio-Rad 2-DE manual. Isoelectric focusing (IEF) employed 17 cm IPG strips with a pH range of 4-7. Samples were loaded in rehydration solution at 1 mg per strip. Following IEF, the immobilized pH gradient (IPG) strips were equilibrated in Equilibration Buffer I [50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 1% sodium dodecyl sulfate (SDS), 0.2% dithiothreitol (DTT), trace bromophenol blue] on a horizontal shaker for 15 minutes. Excess buffer was removed with filter paper, and the strips were transferred to Equilibration Buffer II [50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 1% SDS, 3% iodoacetamide (IAA), trace bromophenol blue] for an additional 15 minutes. After equilibration, the strips were rinsed with deionized water, blotted dry, and subjected to second-dimension electrophoresis on 12.5% SDS-polyacrylamide gels.

Following electrophoresis, gels were immediately immersed in fixation solution (40% ethanol, 10% acetic acid) for 2 hours. The gels were stained with Coomassie brilliant blue overnight at 30°C with shaking, then repeatedly washed with 10% acetic acid until the background was clear. Stained gels were scanned using a PowerLook 2100XL scanner and analyzed with PDQuest 8.0 software.

Protein Identification and Verification

Gel Excision and Destaining Selected differential protein spots and non-protein gel regions of appropriate size were excised and placed in 1 mL EP tubes, washed twice with ultrapure water, aspirated dry, and stored at -20°C. For destaining, 50 L of destaining solution (30 mmol/L potassium ferricyanide and 100 mmol/L sodium thiosulfate mixed 1:1) was added to each tube and vortexed for 5 minutes; this step was repeated once. After three washes with ultrapure water, destaining was performed with 50% acetonitrile for 20 minutes, repeated once, followed by incubation in 50 mmol/L ammonium bicarbonate solution for 1 hour. The gel pieces were then washed with 180 L ultrapure water for 5 minutes and lyophilized for 20 minutes.

Enzymatic Digestion Ten microliters of dithiothreitol solution (sufficient to cover the gel pieces) was added and incubated at 60°C for 30 minutes, followed by addition of 10 L iodoacetamide. The supernatant was discarded, and the gel pieces were washed with ammonium bicarbonate. The gel was cut into 2-4 mm^3 pieces and transferred to washed EP tubes. After dehydration with acetonitrile, the gel pieces were digested with trypsin for 15 hours while maintaining moisture. Peptides were extracted and lyophilized below -20°C.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis Sample preparation is a critical step in MALDI, as the analyte must be incorporated into the matrix crystal, and contamination with salts or buffers can significantly affect this process. One microliter of the lyophilized peptide digest was dissolved and mixed 1:1 with matrix; 1 L of this mixture was spotted onto a stainless steel plate, dried at room temperature until crystallized, and then analyzed by MALDI-TOF-MS in positive ion reflectron mode using a 337 nm nitrogen laser at an accelerating voltage of 2 kV.

Database Searching Peptide mass fingerprint (PMF) data obtained from mass spectrometry were processed using FlexAnalysis software and searched against the NCBInr or Swissprot databases using Mascot. Search parameters included: fixed modification of carbamidomethylation (C), variable oxidation of methionine, mass tolerance of ±0.5 Da or within 1×10 , minimum sequence coverage of 15%, and species restricted to chicken. Proteins with a MOWSE score 75 ($P<0.05$) were considered successfully identified.

Western Blot Verification Samples were loaded at 80 g per well. Electrophoresis was performed on 12% separating gels at 80 V for 45 minutes, followed by 120 V for approximately 90 minutes. Polyvinylidene fluoride (PVDF) membranes were activated in methanol, and proteins were transferred at 80 V for 2 hours. After transfer, membranes were blocked in 5% skim milk dissolved in Tris-buffered saline with 1% Tween (TBST), then incubated with primary antibodies [rabbit anti-aldolase C fructose-bisphosphate (ALDOC) polyclonal antibody and glyceraldehyde-phosphate dehydrogenase (GAPDH)] followed by fluorescently labeled secondary antibodies. Membranes were washed three times with phosphate-buffered saline containing 1% Tween (PBST) for 10 minutes each. Images were scanned and saved using a PowerLook 2100XL scanner.

Results and Analysis

2-DE Analysis Results

PDQuest 8.0 image analysis software was used to compare and analyze gel images. Each serum protein 2-DE map contained 500-600 detectable protein spots [Figure 1: see original paper]. Comparative analysis of the experimental groups with the normal temperature control group revealed 14 differential proteins in the cold stress group (8 up-regulated, 6 down-regulated) and 13 differential proteins in the cold-adapted group (7 up-regulated, 6 down-regulated).

Differential Protein Identification Results

Analysis of the 2-DE maps identified 23 differential protein spots. MALDI-TOF-MS analysis of eight reproducible and prominent spots successfully identified four differential proteins, two of which were ALDOC; the remaining two were

unidentified proteins. ALDOC content was elevated in the cold stress group serum. The peptide mass fingerprint for one ALDOC protein is shown in [Figure 2: see original paper], and the protein identification information obtained by Mascot searching of the NCBInr database is presented in .

Western Blot Verification

Western blot analysis using GAPDH as an internal control confirmed ALDOC expression levels in chick serum across groups, with results consistent with the 2-DE analysis [Figure 3: see original paper] and [Figure 4: see original paper]. ALDOC expression levels were higher in both the cold stress and cold-adapted groups compared to the normal temperature control group, with the difference between the cold stress and control groups reaching statistical significance ($P<0.05$).

Discussion

Blood components change with physiological conditions, and numerous diseases including cancer, cardiovascular disease, and metabolic disorders alter blood protein content. In modern proteomics research, serum and plasma are frequently used for early disease diagnosis. Analyzing relationships between diseases and key proteins facilitates understanding of early pathological mechanisms and holds broad prospects for disease prognosis and treatment [11-21]. In this study, 2-DE analysis of serum proteins from cold stress, cold-adapted, and normal temperature control groups compared protein expression profiles across the three groups. The results revealed three differential proteins that were up-regulated in both cold stress and cold-adapted groups, one differential protein that was down-regulated in both groups, 12 differential proteins expressed only in the cold stress group, and nine differential proteins expressed only in the cold-adapted group. Clear, reproducible, and significantly differential protein spots on the 2-DE maps were selected as breakthrough points for animal cold stress proteomics research and subjected to subsequent analysis. Other detected differential proteins remain to be identified and verified using peptide mass fingerprinting.

MALDI-TOF-MS analysis of differentially expressed proteins successfully identified four proteins. Notably, two were unidentified proteins requiring further investigation, while the other two were both identified as ALDOC—a phenomenon likely caused by post-translational modifications in 2-DE. Changes in blood protein content during cold stress likely play important roles in its onset and progression. In-depth protein research not only lays the foundation for investigating the pathogenesis of cold stress-related diseases but also provides scientific references for preventing and treating cold stress-related complications.

Aldolase (ALD) is a glycolytic enzyme widely present in nature that catalyzes aldol reactions (producing aldols) or the reverse reaction (cleaving aldols). Three aldolase isoenzymes exist in mammals: aldolase A (ALDOA), aldolase B (AL-

DOB), and aldolase C (ALDOC). ALDOC, located in mitochondria, participates in glucose metabolism pathways as a key enzyme in glycolysis [22] and has also been identified as a protein related to lipid metabolism. Previous studies on biochemical indicators related to glucose and energy metabolism in chick serum after cold stress, along with other biochemical markers and immune factors, demonstrated significant changes in concentrations of glucose (GLU), insulin (INS), and free fatty acids (FFA), with increasing trends as stress duration extended [23-24]. When animals experience cold stress, increased secretion of growth hormone and glucagon promotes glycogenolysis and gluconeogenesis, leading to elevated serum glucose concentrations. Insulin promotes glycogen, fatty acid, and protein synthesis, while free fatty acids serve as direct heat sources, with concentrations increasing significantly in cold environments. These biochemical changes suggest that ALDOC likely plays an important role in energy metabolism following cold stress. Ren et al. [25] reported that cold stimulation affects chickens' physiological environment through increased basal metabolic rate, enhanced energy metabolism, deeper respiration, increased oxygen consumption, elevated blood flow, and reduced glycogen storage in liver and muscle—all of which enhance thermogenic capacity. During cold exposure, animal thermogenesis is primarily mediated by adenylate cyclase (AC) 分解 ATP, which increases cyclic adenosine monophosphate (cAMP) concentrations, enhances protein kinase A activation, and promotes glucose decomposition, elevating both fructose-6-phosphate and fructose-1,6-bisphosphate concentrations. Aldolase is the enzyme that catalyzes the cleavage of fructose-1,6-bisphosphate. In this study, ALDOC content in both cold stress and cold-adapted groups was higher than in the normal temperature control group, with significantly up-regulated expression. We therefore hypothesize that under cold stress conditions, expression of proteins related to glucose and energy metabolism pathways increases, promoting the decomposition of energy substances like glucose, enhancing metabolism and heat production to cope with cold environments. Elevated ALDOC expression in chick serum under cold conditions indicates that cold stress enhances energy metabolism and suggests that ALDOC may be a sensitive biomarker for cold stress responses. However, the mechanism underlying this specific expression pattern remains unclear, and whether ALDOC can be applied to cold stress diagnosis, monitoring, and treatment requires further investigation.

This study employed 2-DE combined with MALDI-TOF-MS to investigate differential serum proteomic changes in chicks after cold stress. The identified differential proteins were associated with glucose and energy metabolism, with particular emphasis on the potential importance of ALDOC in cold stress responses, which likely correlates with physiological functional changes induced by cold stress.

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Note: Figure translations are in progress. See original paper for figures.