

Profiling of hepatocellular proteins by 1D PAGE-MALDI/MS/MS in a rat heat stress model

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1. ABSTRACT

Heat induced complications cause an increase in a large number of proteins which play a role in diverse pathways during heat shock. A detailed characterization of these proteins is essential for understanding the molecular mechanisms involved in heat stroke. In this report, the proteins present in rat liver were compared at 37 °C (control) and at core temperature (Tc) 42 °C (heat stress) by 1D PAGE and MALDI/MS/MS. Among proteins identified in the sample after heat stress are dimethylglycine dehydrogenase, transketolase, carboxylic ester hydrolase, pyruvate kinase, L-type pyruvate kinase, arginosuccinate synthetase; fumarylacetoacetate hydrolase and peptidylpropyl isomerase A. These findings show that analysis of large scale proteins by MALDI/MS/MS provides a better understanding of the molecular mechanisms associated with heat shock. The resolution of proteins examined by 1D-PAGE was less than that obtained with 2D-PAGE. More specifically, 2D-PAGE allows better identification of low molecular weight proteins that can not be resolved by 1D-PAGE.

2. INTRODUCTION

Heat stress is induced by both exogenous and endogenous factors which are known to be involved in inflammatory and homeostatic responses (1). Exogenous factors include the lack of heat acclimatization, increase in ambient temperature and humidity, absence of breeze, and an increase in wet bulb reading. Endogenous factors include old age, obesity, exercise, dehydration, hypokalemia, alcoholism, midday overeating, cardiovascular disease, sweat gland dysfunction, sunburn, past history of heat stroke and certain drugs (1). Heat stress induced deaths are increasing in recent years (2-4). To prevent such deaths and develop treatment strategies, there is a need for better understanding of physiology and biochemistry of heat induced response (5, 6). However, the molecular mechanism of heat stress is not yet fully understood. Several reports have so far identified the proteins which are induced by heat shock. Among these are HSP (heat shock proteins) (7, 8), TNF (tumor necrosis factor) (9-12), IL (interleukins) (9-13), circulating endothelial markers (14), and adhesion molecules (15).

However, a full insight of the molecular mechanisms involved in heat shock requires a proteomic analysis. Profiling of proteins by conventional protein analytical techniques such as electrophoresis and Western blotting is not a practical approach (7). One approach which has recently been advocated is use of MALDI/MS/MS which allows simultaneous identification and analysis of a large number of proteins (16). To check the feasibility of this approach, we attempted to identify proteins induced by heat shock in liver by 1D PAGE and MALDI/MS/MS.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

CHCA (α -Cyano-4-hydroxycinnamic acid), ammonium bicarbonate, CHAPS and thiourea were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetic acid and trifluoroacetic acid (TFA) were from J. T. Baker (Griesheim, Germany). Sodium dodecyl sulphate, glycine, tris, acrylamide PAGE, methylenebisacrylamide, mercaptoethanol, bromophenol blue, glycerol, ammonium peroxosulphate, TEMED were purchased from Bio-Rad (Hercules, CA, USA). Mass spectrometry grade, Trypsin, was purchased from Promega Biosciences (San Luis Obispo, CA, USA).

3.2. Animal model

Adult male Wistar rats, weighing 400-450 grams, were used in this study. Rats were allowed to adapt to the environment for 1 week before the experiment and fed on laboratory chow. Water was provided *ad libitum*. Rats were subjected to heat stress at a core temperature of 42°C for 15 minutes. Subsequently, the rats were sacrificed and liver samples from individual rats were removed for further analysis. The liver samples from rats kept at 37°C were used as control.

3.3. Sample extraction

Liver samples from control and treated animals were flushed through the hepatic vein with a cold NaCl solution to remove blood. For preparation of the total protein extract, liver tissue (1 gram) was suspended in 10 ml of sample buffer consisting of 20 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM 1, 4-dithioerythritol, 1 mM EDTA and a mixture of protease inhibitors (1 mM PMSF, one tablet complete™ (Boehringer Mannheim; Mannheim, Germany), phosphate inhibitors (0.2 mM Na₂PO₃ and 1 mM NaF) in 10 ml of suspension buffer. The suspension was homogenized with the use of a Polytron homogenizer (Kinematica, Luzern, Switzerland) for approximately 1 min, sonicated for 30 s and centrifuged at 15 000 g for 45 min and separated the total liver proteins from supernatant.

3.4. 1D SDS-PAGE analysis

SDS-PAGE was carried out in a Bio-Rad Protein system using 5%/12% stacking/separating polyacrylamide gels. The SDS sample buffer contained 4% SDS, 20% glycerol, 100 mM Tris-HCl and a trace amount of bromophenol blue. Prior to electrophoresis, the protein sample was mixed in the sample buffer and heated at 100 °C for 5 min. Gels were run at a constant voltage of 200V. After completion of electrophoresis, the gels were stained with Coomassie G-250 stain

buffer. Bio-Rad precision plus protein standards were used as standard molecular weight markers. Finally, the gel was washed for 10min in Milli Q water, scanned by a laser scanning densitometer (Image Scanner, Amersham Biosciences) and analyzed by Labscan (Amersham Biosciences).

3.5. In-gel tryptic digestion

The entire gel lanes were manually cut into 34 sections (17 gel pieces per lane per sample), and subjected to in-gel tryptic digestion (16). It should be noted that each lane in the gel was cut into 17 pieces according to their resolution due to molecular weight in 1D-PAGE by visual inspection of their Coomassie staining to protein bands. The gel pieces were washed with 100mM ammonium bicarbonate in 50% ACN for 20 min, followed by treatment with DTT for reduction of disulfide bonds and iodoacetamide for alkylation (17). After equilibration in 50% ACN, the gel bands were dehydrated in 100% ACN. Trypsin solution was added at a concentration of 20µg/ml and allowed to react with the gels overnight at 37°C. Peptide extraction solutions were concentrated and desalted using C18 ziptips (Agilent Technologies, Wilmington, DE, USA). Solution A (0.1% TFA in deionized water) was used for washing and solution B (0.1% TFA and 60% ACN) was used for elution.

3.6. Sample preparation for mass spectrometry

The extracted peptides (0.5 µL) were spotted on a 96X2 well target plate and crystallized with 0.5µL of matrix solution (5 mg/mL). The matrix solution was a saturated solution of α -cyano-4-hydroxycinnamic acid (CHCA) in 0.1% (v/v) trifluoroacetic acid (TFA) and 50% (v/v) ACN.

3.7. Mass spectrometry

The sample was analyzed on the 4700 Proteomics Analyzer (Applied Biosystems), Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight/Time-Of-Flight (MALDI/TOF-TOF). Instrument calibration was performed by using the 4700 calibration peptide mixture containing Des-Arg1-Bradykinin, Angiotensin I, Glu1-Fibrinopeptide B, ACTH (clip 1-7), ACTH (clip 18-39) and ACTH (clip 7-38). MS data were automatically acquired in the reflectron mode by using the Reflectron Method which consisted of the exclusion list for most common trypsin and keratin peaks. Consequently, 10 most intense ions from Peptide Mass Fingerprinting (PMF) data were automated selected for further MS/MS fragmentation and analysis. The collision energy of the MS system was set at 1KV and the collision gas used was nitrogen.

3.8. Protein identification

MASCOT (version 2, Matrix Science Ltd., London, UK) was used for peptide mass mapping by searching the GenBank annotation using the parameter values for mass error tolerance to MS data (150ppm), mass error tolerance to MS/MS data (0.2Da), fixed modification (carbamidomethylation of cysteine) and variable modification (methionine oxidation). The MASCOT annotation produced protein names in each

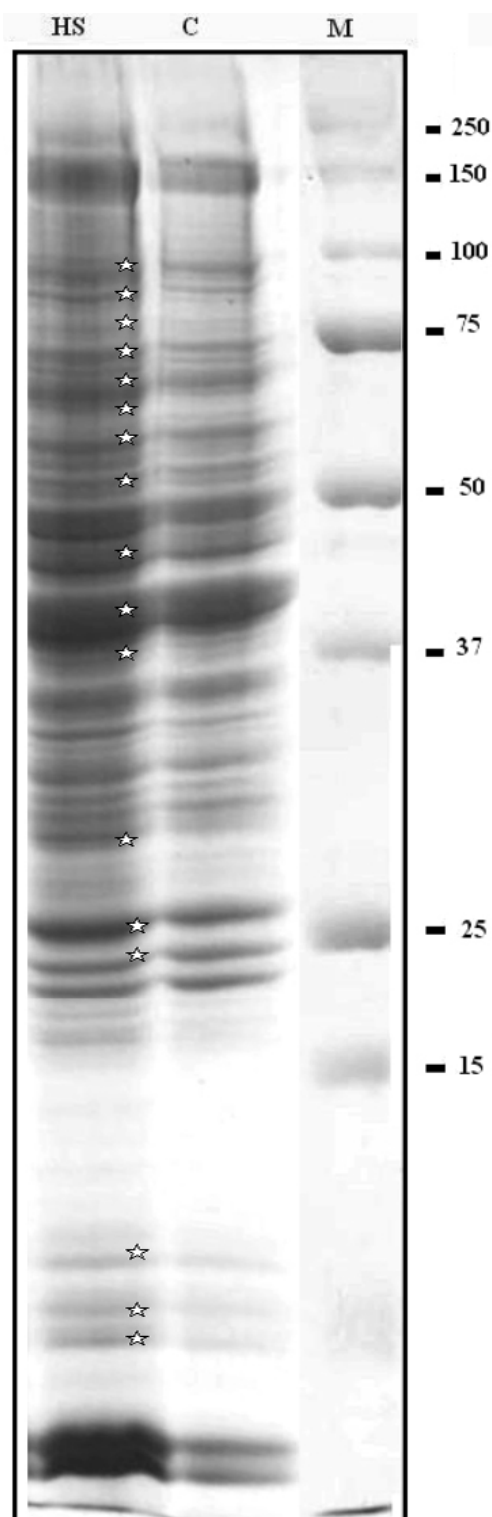


Figure 1. One dimensional-Poly-Acrylamide Gel Electrophoresis (1D PAGE) of control and heat stress induced proteins in rat liver. 17 distinct bands were removed and protein mixture from each band was eluted for MALDI/MS/MS analysis. HS = heat stress (42 °C); C = control (37 °C); M = Marker Proteins.

protein band from GenBank description. The protein annotation was manually curated (eliminated by reading through the protein names using domain expert individuals) by restricting to those of the genus “*Rattus*” for reducing inter-kingdom contamination in naming. This approach is simple, less sensitive and high specific due to manual curation.

4. RESULTS AND DISCUSSION

Identification of molecules expressed by heat induced inflammatory and homeostatic responses can provide an insight on the mechanisms involved in heat stress. Similarly, an array of proteins acts in a synchronized manner to induce heat tolerance. Among the proteins identified during heat stress are HSP (heat shock proteins) (7, 8), TNF (tumor necrosis factor) (9 - 12), IL (interleukins) (9-13), circulating endothelial markers (14), and adhesion molecules (15). However, the available information is yet insufficient to draw a concerted conclusion in understanding the molecular mechanisms involved in heat stress. Such an insight may only be gleaned from identification of all proteins involved in these processes. Identification of the full array of molecules that participate in heat stress or tolerance is only feasible by protein profiling (16, 17). To identify proteins involved in heat stress, we have initiated using MALDI/MS/MS in a rat liver model. The results from 1D PAGE of rat liver samples at Tc 37 °C (control) and 42 °C (heat stress) are shown in Figure 1. The 1D PAGE in Figure 1 shows 17 distinct bands identified in each condition. Elution of proteins corresponding to each of these bands and subsequent analysis of eluted proteins using MALDI/MS/MS produced the m/z frequency distribution of proteins in these samples. Further analysis of MALDI/MS/MS data using MASCOT identified a number of GenBank annotated proteins under each condition for different band eluates.

The resolution of proteins by 1D PAGE was less than that obtained from MALDI/MS/MS. If these initial findings can be validated, they provide a persuasive support for carrying out large scale analysis by 1D PAGE – MALDI/MS/MS to provide a full insight of the molecular repertoire involved in heat stress and tolerance. The only drawback of this approach is the inability of annotation software to identify and annotate novel proteins. The MASCOT identified GenBank annotated proteins were then manually curated. The list of proteins induced by heat stress at Tc 42 °C and at control Tc 37 °C is provided in Table 1. From this data, the differential expression of proteins can be further inferred. Proteins identified in the sample after heat stress included dimethylglycine dehydrogenase, transketolase, carboxylic ester hydrolase, pyruvate kinase, L-type pyruvate kinase, arginosuccinate synthetase; fumarylacetoacetate hydrolase and peptidylpropyl isomerase A and these proteins have not been reported earlier. Among these, carboxylic ester hydrolase, peptidyl prolyl isomerase A, pyruvate kinase, transketolase, dimethyl glycine dehydrogenase and fructose 1, 6- biphosphatase 1 were confirmed for differential expression using real time PCR (Figure 2).

Table 1. Proteomics analysis of proteins in rat liver after heat stress

No	MW kDa	Control (37°C)	Heat Stress (42°C)
1	93- 110	Formyltetrahydrofolate dehydrogenase; Liver glycogen phosphorylase	Formyltetrahydrofolate dehydrogenase; Liver glycogen phosphorylase
2	87- 93	Heat shock protein, 90 kDa	<i>Eukaryotic translation elongation factor 2</i>
3	71- 75	Albumin	Heat shock protein, 90 kDa; <i>Dimethylglycine dehydrogenase</i>
4	68- 71	Catalase	Albumin
5	64- 68	Catalase	<i>Transketolase</i>
6	62- 64	Glucose regulated protein, 58 kD	Catalase
7	57- 62	Carboxylesterase	Glucose regulated protein, 58 kDa; Carboxylesterase; <i>Carboxylic ester hydrolase</i>
8	52- 57	Methylmalonate semialdehyde dehydrogenase; Prolyl 4-hydroxylase, beta polypeptide	<i>Pyruvate kinase, L-type pyruvate kinase</i>
9	40- 43	Mitochondrial aldehyde dehydrogenase precursor, Tubulin beta 5	Methylmalonate semialdehyde dehydrogenase; Prolyl 4-hydroxylase, beta polypeptide
10	37- 40	Actin	<i>ATP synthase, H⁺ transporting, mitochondrial F1 complex, alpha subunit, isoform 1; H(+)-transporting ATP synthase</i>
11	36- 37	Fructose 1,6 biphosphatase 1	<i>Arginosuccinate synthetase; Fumarylacetoacetate hydrolase</i>
12	27- 28	Aldolase B, Aldob protein	<i>Laminin receptor 1</i>
13	25- 26	Carbonic anhydrase 3	Aldolase B; Aldob protein
14	23- 25	Glutathione S-transferase, mu 1; Glutathione S-transferase RN) mu 2; Glutathione S-transferase, mu type3	Carbonic anhydrase 3, <i>Enoyl Coenzyme A hydratase</i>
15	10.5- 11.5	Glutathione S-transferase, alpha type2 ; Glutathione S-transferase alpha; Glutathione S-transferase A5	Glutathione S-transferase, mu 1 ; Glutathione S-transferase, mu type 3
16	10- 10.5	Ribosomal protein S19	Glutathione-S-transferase, alpha type2; Glutathione S-transferase alpha; Glutathione S-transferase A5
17	8- 10	Hist2h2aa1 protein	<i>Peptidylprolyl isomerase A</i>
		Beta-globin; Hemoglobin beta chain complex	Hist2h2aa1 protein
		III beta-3 globin	Beta-globin; Hemoglobin beta chain complex ; III beta-3 globin; <i>III beta-2 globin</i>

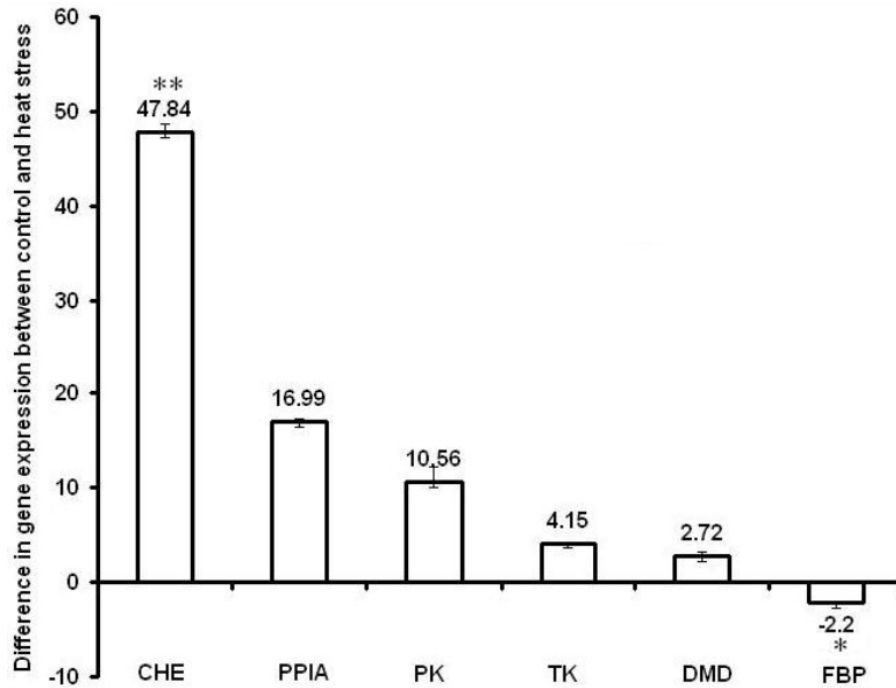


Figure 2: Differential gene expression as assessed by quantitative real time PCR in rat liver after heat stress treatment compared to control at 37°C. The levels of gene expression were normalized to the housekeeping gene (β -actin). Values are expressed as \pm SE. (* $p < 0.05$, ** $p < 0.01$ as compared to control). CEH = carboxylicester hydrolase; PPIA = peptidylprolyl isomerase A; PK = pyruvate kinase; TK = transketolase; DMD = dimethylglycine dehydrogenase; FBP = Fructose-1,6- biphosphatase 1. RNA extractions were performed using RNeasy® mini kit (QIAGEN, CA, USA). Quantitative real-time PCR was carried out according to the manufacturer's instructions using the Light Cycler system (Roche Diagnostics, Basel, Switzerland) as an independent method for assessing relative gene expression. Reverse Transcription (RT) of RNA and QRT-PCR were performed as follows: single stranded cDNA was generated from 4 μ g of total RNA. 200 μ M nucleotides, 500 units Superscript II reverse transcriptase and 1.5 μ M oligo(dT)₁₅ primers in 50 μ l reactions. Reverse transcription was stopped after 1 h by heating to 95°C for 5 min. All primers were synthesized by 1st BASE Pvt. Ltd. Singapore. The expression of β -actin was used as an internal calibrator for equal RNA loading and to normalize relative expression data. The real-time quantitative RT-PCR data were quantified using relative quantification as described elsewhere (18). Y-axis: Difference in gene expression between 37°C and 42°C in rat liver.

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