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Proteins Differentially Expressed in the Pancreas of Hepatic ADH-Deficient Deer Mice Fed Ethanol for Three Months

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Abstract

Objective—To identify differentially expressed proteins in the pancreatic tissue of hepatic alcohol dehydrogenase deficient (ADH⁻) deer mice fed ethanol to understand metabolic basis and mechanism of alcoholic chronic pancreatitis (ACP).

Methods—Mice were fed liquid diet containing 3.5g% ethanol daily for three months, and differentially expressed pancreatic proteins were identified by protein separation using 2-dimensional gel electrophoresis and identification by mass spectrometry.

Results—Nineteen differentially expressed proteins were identified by applying criteria established for protein identification in proteomics. An increased abundance was found for ribosome-binding protein 1, 60S ribosomal protein L31 like isoform 1, histone 4, calcium and ATP binding proteins and the proteins involved in anti-apoptotic processes, and endoplasmic reticulum (ER) function, stress and/or homeostasis. Low abundance was found for endoA' cytokeratin, 40S ribosomal protein SA, amylase 2b isoform precursor, serum albumin and ATP synthase subunit β , and the proteins involved in cell motility, structure and conformation.

Conclusions—Chronic ethanol feeding in ADH⁻ deer mice differentially expresses pancreatic functional and structural proteins, which can be used to develop biomarker(s) of ACP, particularly amylase 2b precursor, and 60 KDa heat shock protein and those involved in ATP synthesis, and blood osmotic pressure.

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Keywords

Ethanol; Deer mice; Proteomics; Pancreas

INTRODUCTION

Alcoholic chronic pancreatitis (ACP) is a major health problem causing significant morbidity and mortality among alcoholic patients. Chronic alcohol abuse costing ~\$250 billion to US economy and about 100,000 deaths each year is the major cause of ACP.^{1,2} Pathophysiology of ACP appears to be associated with dysregulation of lipids resulting in lipid accumulation (reversible stage), which can lead to necroinflammation of the gland with or without progressive fibrosis.^{3,4} Because advanced stages of the ACP are rarely reversible, identification of early markers of ethanol-induced pancreatic injury in chronic alcohol feeding model could be very critical for understanding the mechanism and metabolic basis of ACP, and also to develop preventive measures before the disease progresses to clinically overt stages.

About 90% of the ingested alcohol is metabolized in the liver by ADH, which is commonly inhibited in chronic alcoholic individuals, and in experimental animals after chronic ethanol feeding.^{5–10} Inhibition of hepatic ADH significantly impairs oxidative metabolism and increases body burden of ethanol, which together facilitate nonoxidative metabolism of ethanol to fatty acid ethyl esters (FAEEs) in the pancreas.^{4,11–13} Although hepatic microsomal cytochrome P-450 (CYP) 2E1, involved in ethanol oxidation, is induced during chronic alcohol abuse, but the Km of CYP2E1 is very high as compared to ADH 1 (key isoform responsible for ethanol oxidation). Therefore, inhibition of hepatic ADH 1 could be a key metabolic event resulting into an initiation of alternative mechanism of ethanol disposition *via* formation of FAEEs in the pancreas frequently damaged during chronic alcohol abuse.

Blood alcohol concentration (BAC) and lipid conjugates of ethanol such as FAEEs (nonoxidative metabolites of ethanol with endogenous fatty acids) together can diagnose acute vs chronic alcohol abuse.¹⁴ Earlier, we reported metabolic basis of ethanol-induced pancreatic and liver injury using hepatic alcohol dehydrogenase (ADH)-deficient (ADH⁻) vs hepatic normal (ADH⁺) deer mice.^{4,15} ADH⁻ deer mouse model mimics a metabolic condition (inhibition of hepatic ADH) similar to that observed in the chronic alcoholic individuals. However, impact of increased body burden of ethanol especially on pancreatic proteome and identifying the target proteins involved in maintaining the morphological and functional integrity of the pancreas under hepatic ADH inhibition are not well investigated. Therefore, differentially expressed proteins were identified in the pancreas of chronic ethanol feeding model of ADH⁻ deer mice, which could form a basis for identifying biomarker candidate(s) and therapeutic target(s) of ACP.

MATERIALS AND METHODS

Chemicals and Reagents

Unless indicated, all chemicals and reagents were obtained from Sigma-Aldrich Company (St. Louis, Mo).

Animal Studies

Hepatic ADH 1 deficient (ADH⁻) deer mice, genetic variant of *Peromyscus maniculatus* (male, ~one year old, ~19g body weight), were purchased from Peromyscus Stock Center, University of South Carolina, Columbia).⁴ Animals were divided into two groups, experimental and pair fed control. After one week of acclimatization in animal quarters, each mouse was individually housed in a cage and fed regular Lieber-DeCarli liquid diet (Dyets Cat No.710260, Dyets, Inc, Bethlehem, Pa) for three days. Ethanol concentration in the liquid diet was incrementally increased from 0 to 3.5g% in two weeks and then fed 3.5g % ethanol in the liquid diet daily for additional three months. Control group was pair-fed with an isocaloric liquid diet (Dyets Cat. No. 402851) in which ethanol calories were substituted with Maltose-Dextrin. At the end of three months, all the animals (5 in each group) were anaesthetized by pentobarbital sodium Solution, USP (NEMBUTAL®, 100 mg/kg body weight *via* intra peritoneal injection). Blood was collected from the heart, and 50 µl of the whole blood transferred to glass vial and sealed with Teflon lined cap for the analysis of blood alcohol by head space gas chromatography.⁴ The pancreas from each animal was harvested, cut into pieces and immediately frozen in liquid nitrogen for proteomic study and a portion was fixed in 10% buffered formalin, thin sections cut and stained with Hematoxylin & Eosin (H&E) for the morphological evaluation.^{4,15}

Proteomic Studies

For the proteomic studies, four animals per group were used. Frozen pancreatic tissues were thawed, 15 mg tissue was homogenized in DeStreak rehydration buffer (GE Healthcare, Life sciences, Pittsburgh, Pa) with protease inhibitor cocktail (Sigma-Aldrich, Milwaukee, Wis). The homogenate was treated with benzonase (E1014, Sigma-Aldrich) at 150 U/ml for 30 min at room temperature to remove nucleic acids followed by centrifugation at 13,000 g for 15 min. The supernatant was collected and protein concentration was measured using RC DC protein assay kit (Bio-Rad, Hercules, Calif Cat # 500-0122). Two-dimensional gel electrophoresis (2-DE), fluorescent staining, imaging, gel analysis, mass spectrometry (MS) and protein identification were done essentially according to methodologies established in our Proteomic Center.¹⁶⁻¹⁹

2-DE for Pancreatic Proteins

A total of 200 µg protein equivalent supernatant of pancreatic homogenate was loaded on 11-cm long precast immobilized pH gradient (IPG) strips of pH range 3–5.4, 5.4–7.0 and 7–10. The strips were rehydrated overnight and focused at 20°C in six steps, 50 V for 11h, 250 V for 1h, 500 V for 1h, 1000 V for 1h, 8000 V for 2h, and held at 8000 V for 6h for a total of 480000Vh. After isoelectric focusing (IEF), the IPG strips were stored at -80°C till the next step. Prior to the 2-DE, the IPG strips were incubated at 22°C in 4 ml of equilibration

buffer (6M urea, 2%SDS, 50mM Tris-HCl, pH8.8, 20% glycerol) containing 10µl of 0.5M tris (2-carboxyethyl) phosphine with shaking for 15 minutes. In the subsequent step, IPG strips were incubated in 4 ml of equilibration buffer with 25 mg/ml of iodoacetamide for 15min at 22°C with shaking. After completion of equilibration, 2-DE was performed at 150 V for 2.25h at 4°C with precast 8 to 16% polyacrylamide gels in tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS; pH 8.3)^{16–19}.

Gel Staining

Gels were fixed in the fixing buffer containing 10% methanol and 7%acetic acid for 2h at room temperature and then stained with SyproRuby stain (Invitrogen, Carlsbad, Calif) over night at room temperature. The gels were destained with 10% ethanol for 1h before imaging.

Image Analysis

The destained gels were imaged using ProExpress 2D Proteomic Imaging System (PerkinElmer Life and Analytical Sciences, Waltham, Mass) using 480 nm excitation and 620 nm emission filters. For obtaining the most intense protein spots on the gels, the exposure time was adjusted to achieve 55,000 to 63,000-pixel intensity value and the gel images were analyzed using Progenesis SameSpots software version 4.0 (TotalLab, Newcastle Upon Tyne, UK). The program selected one of the Images as the reference gel and all the other gel images were aligned to it based on a selected subset of the aligned gels, and background subtraction and spot volume intensity normalization to those of the reference gel were done. Data were analyzed by ANOVA and fold changes 1.5 with p values 0.05 considered significant.

Spot Excision and In-gel Digestion, Mass Spectrometry, and Protein Identification

Gel spots of interest (differentially expressed proteins) were excised and incubated at 37°C for 4h with trypsin (20 µg/ml in 25 mM ammonium bicarbonate, pH 8.0; Promega Corp., Madison, Wis) and analyzed by MALDI-TOF/TOF mass spectrometry using Proteomics Analyzer 4700 (Applied Biosystems, Foster City, Calif) for peptide mass finger-printing and sequencing (Settings and other parameters of the MALDI-TOF/MS-MS are provided in Supplemental Digital Content 1, Supplementary Material). Protein identification was performed by recording both MS/MS data and the MASCOT search engine (Matrix Science, London, UK) with NCBI mouse data based search of GeneBank and probability matches searched by a high protein score (>64; <http://www.matrixscience.com>) and a low expectation (E) calculated.²⁰ The lower the E-value, the higher is the probability of identification (E 10⁻³ as a threshold value).

Western Blot Analysis

Expression of selected proteins identified by 2-DE and MS was confirmed by Western blot analysis using respective antibodies and standard immunoblotting protocols.^{4,21} For Western blot analysis, three animals per group were used. Pancreatic homogenate was prepared, and 45 µg of pancreatic protein was subjected to SDS PAGE (10 Well NuPAGE™ Novex™ 4–12% Bis-Tris Protein Gels, 1.5 mm thick, 10 well). The gels were washed with washing buffer and the proteins were transferred onto nitrocellulose membrane. Membrane was

incubated either with rabbit polyclonal antibodies against lipoamide acyltransferase component of branched-chain α -keto acid dehydrogenase complex, mitochondrial (dihydrolipoamide branched chain transacylase E2, DBT; 1:500 dilution, MW: 55kDa) or mouse monoclonal antibodies against amylase (1:1000 dilution, 53 kDa) were procured from ThermoFisher Scientific (Houston, Tex; Cat # PA-529727) or Santa Cruz BioTechnology Inc. (Dallas, Tex; Cat #Sc-46657), respectively. β -Actin (rabbit mAb Cat #8457; MW: 45 kDa) from cell signaling with 1:1000 dilution was used as individual loading control to normalize the band intensities.

After washing, the blots were again incubated with horseradish peroxidase conjugated secondary antibodies for 1 hour, and the target proteins were visualized using a commercially available ECL detection system. The protein band intensity was assessed by ImageJ software v1.50i (NIH, Bethesda, Md) and proteins normalized by individual loading control (β -actin) values.

Statistical Analysis

The data sets were analyzed for statistical significance using Student's *t*-test and the results are expressed as mean $\pm$ SEM (standard error of mean) using five animals/group for BAC and four animals/group for proteomics, and three animals/ group for Western blot analysis. P Value <0.05 was considered statistically significant.

RESULTS

Blood alcohol concentration averaged 180 mg% in ethanol fed mice as compared to 13 mg% in peer-fed controls (Fig. 1). The pancreas of ethanol fed mice showed histological changes including cellular disarray along with necrosis and atrophy of acinar cells, and disorganization of acinar structure and interstitial edema (Fig. 2). Analysis of pancreatic proteome revealed several differentially altered proteins as shown by representative 2D electrophoretic (2-DE) patterns at various pH ranges (3 – 5.4, 5.4 – 7.0 and 7–10) (Fig. 3). SameSpots image analysis of 2-DE gels showed 94 differentially expressed proteins in the pancreatic tissues of ethanol-fed group. A large number of these proteins were either unknown, not mapped in UniProt database or post-translationally modified (PTMs). However, only 19 proteins differentially expressed proteins out of the 94 proteins identified are summarized in Table 1 based upon well-established proteomic criteria (>1.5 fold change along with p value <0.05; PI, MW, best protein score of >64, and a good protein match having MS expectation value of 10exp^{-3} or less). Out of nineteen differentially expressed proteins identified in the pancreas, ten proteins were from fractions 1 (pH 3.0 – 5.4), eight from fraction 2 (pH 5.4 – 7.0) and one from fraction 3 (pH 7 – 10).

Out of 19 identified proteins, ten proteins were upregulated and remaining nine down-regulated in the pancreas of ethanol-fed group (Fig. 3; Table 1). Upregulated proteins identified in this study were either Ca binding proteins, structural proteins in nucleosome, chromosomal fiber and ribosome; heat shock protein, branched-chain α -keto dehydrogenase (key enzyme in Krebs cycle) and pre-mRNA processing nucleotide binding proteins or those involved in ER homeostasis and anti-apoptotic process. The down regulated proteins were identified to be those involved in apoptosis, ATP synthesis, cell regulation and metabolism,

regulation of colloidal osmotic pressure, carbohydrate metabolism and cell motility, as well as structural and conformational proteins. Overall, ADH⁻ deer mice fed ethanol up-regulates pancreatic proteins related to ER membrane and its homeostasis; nucleosomal, chromosomal and ribosomal proteins, and those associated with membrane structure and cellular homeostasis and conversion of α -keto acids to acyl CoA. The proteins involved in apoptosis, carbohydrate metabolism, ATP synthesis were down regulated. Two of the differentially expressed proteins, amylase 2b isoform 1 precursor and lipoamide acyltransferase component of branched-chain α -keto acid dehydrogenase complex, mitochondrial, DBT, identified by proteomic studies were also confirmed by Western blot analysis (Fig. 4).

DISCUSSION

Both, pancreas and liver are the target organs of alcohol abuse and share striking functional similarities including embryological development^{22–26}. Clinically, significant alcoholic pancreatitis occurs quite frequently in association with alcoholic liver disease^{27,28}. Therefore, identification of proteins differentially expressed in the pancreatic tissue of chronic ethanol feeding model of hepatic ADH⁻ deer mice could lead to a better understanding of metabolic basis and mechanism(s) of alcoholic pancreatitis. Such efforts can also identify early biomarker(s) and therapeutic targets of chronic alcohol abuse^{29,30}. Our findings in ADH⁻ deer mice suggest that chronic ethanol feeding alters pancreatic lipid regulation, increases ER stress and tissue injury, decreases ATP synthesis, and affects cell growth, cellular architecture and adhesion properties.

Previous proteomic studies in pancreatic tissue were mainly focused on finding biomarkers of pancreatic cancer^{31,32}. At least 40% of the differentially expressed proteins were common in chronic pancreatitis and pancreatic ductal adenocarcinoma (PDAC), a common pancreatic cancer^{33,34}. MicroRNA signatures identified in patients diagnosed with PDAC or chronic pancreatitis varied from one study to other^{35–37}. Some common target proteins including those related to stress and cell death have been identified in ethanol-induced acute and chronic pancreatitis^{4,38–40}. Up regulation of calreticulin “chain A, crystal structure of the calreticulin lectin domain” (calcium binding chaperone), translocon-associated protein subunit α (regulates retention of ER resident proteins), calmodulin (calcium binding protein) and “ribosome binding protein 1 isoform” (a receptor protein that mediate interaction between microsomal and ER membrane) suggest that ER membrane proteins are targeted in our chronic ethanol feeding mouse model. Such changes can alter structural and functional integrity of ER membrane and its homeostasis as evident by the induction of glucose-regulated protein (GRP) 78 and edema as characterized by electron microscopy in chronic ethanol feeding model of ADH⁻ deer mouse^{4,15}.

Calmodulin mediates the control of a large number of enzymes, ion channels, aquaporins and other proteins by Ca^{2+} . Calreticulin is a cellular component of ER lumen, calcium binding chaperone, which interacts with glycoproteins synthesized in the ER membrane. Increased expression of calreticulin as found in this study could be related to ethanol-induced ER stress⁴. Up regulation of translocon-associated protein subunit α , which regulates the retention of ER resident proteins by binding to calcium in the ER membrane could be involved in accumulation of proteins including misfolded proteins in the ER

membrane resulting in ER stress. This is parallel to our earlier findings on ER stress and increased expression of GRP 78, an ER stress marker in chronic ethanol feeding model of ADH⁻ deer mouse⁴. Similarly, increased expression of 60kDa heat shock protein in ethanol-fed mice could be a natural biological response against ER stress generated in the pancreas⁴. Early stages of acute pancreatitis involve differential expression of multiple rough ER proteins that effect synthesis and processing of digestive enzymes⁴¹. Although acute pancreatic injury can increase the output of digestive enzymes, but a decreased expression of amylase 2b isoform 1 precursor as observed in the present study indicates a slow destruction of the gland by chronic ethanol feeding. Up-regulation of structural constituents such as “60S ribosomal protein L31 like isoform 1” and histone H4 or Hist1h4f (nucleosome structure of the chromosomal fiber) as found in this study could reflect an ongoing tissue remodeling and reorganization in the pancreas during chronic ethanol injury.

60 kDa heatshock protein is highly expressed in inflammatory disease like insulinitis related to pancreas, rheumatoid arthritis and atherosclerosis^{42–44}, and plays a key role in T cell activation, positive regulation of interferon- α and γ production and ATP binding and also involved in the transportation and degradation of proteins^{45–47}. An increased expression of 60 kDa heatshock protein has been shown during folding and unfolding and aggregation of newly synthesized proteins⁴⁸. Therefore, an up-regulation of HSP60 appears to be protective response against ethanol-induced ER stress.

Over expression of “lipoamide acyltransferase (component of mitochondrial branched-chain α -keto acid dehydrogenase complex)” which converts α -keto acids to acyl-CoA, and an anti-apoptotic translationally controlled tumor protein have not been reported previously in ethanol-induced pancreatitis. Over expression of lipoamide acyltransferase could be a result of impaired β -oxidation of fatty acids by chronic ethanol feeding. This needs to be supported by assessing the lipid phenotype of the pancreas, although we have previously reported an increased lipid phenotype in the pancreas of ADH⁻ deer mice fed ethanol for two months⁴. Up-regulation of anti-apoptotic protein can also promote necrotic cell death and inflammation leading to chronic pancreatitis, and/or may negatively regulate intrinsic apoptotic signaling pathway involved in microtubule stabilization and calcium binding.

Hist1h4f (histone H4) protein plays a central role in transcriptional regulation and could also be responsible for maintenance of nucleosome structure of the chromosomal fiber. Up regulation of histone H4 by ethanol exposure could be a response towards induction of DNA repair and DNA replication required for the chromosomal stability. Down regulation of keratine type II cytoskeleton 8 or EndoA' cytokeratin, related to structural and functional entities involved in apoptosis and carbohydrate metabolism in the pancreas, as found in the present study, has also been reported in ab/db mouse⁴⁹. Down-regulation of transitional ER ATPase or valosin-containing protein (VCP) as found in this study could be associated with ethanol-induced ER stress as well as apoptosis⁵⁰. ER membrane also acts as an internal calcium storage organelle and functionally synthesizes secretory membrane proteins and lipids. However, perturbation in ER homeostasis due to chronic ethanol insult might impair its calcium retention capacity and increase the load of unfolded proteins in the ER membrane⁵¹. VCP can also be related to ATPase activity and lipid binding properties, and may activate cysteine-type endopeptidase activity involved in apoptotic process. Similarly,

increased expression of ribosome-binding protein 1 or ribosome receptor protein (widely expressed in the pancreas, and interacts between the ribosome and endoplasmic reticulum membrane) may be a protective mechanism against ethanol toxicity.

Down regulation of “mitochondrial ATP synthase subunit β ” in ethanol fed mice perhaps indicates impaired ATP synthesis and possible shut down of a number of energy requiring cellular processes including execution of apoptotic cell death, lipid metabolic processes and regulation of intracellular pH. Decreased expression of 40S ribosomal protein SA or laminin receptor might indicate detachment of pancreatic acinar cells from the basement membrane as observed in histological examination of pancreatic tissues in ethanol fed deer mice. Similarly, decreased expression of serum albumin precursor in the pancreas of ethanol-fed mice suggests effect of ethanol and/or its metabolites on regulation of colloidal osmotic pressure, and binding and transport of endogenous substrates to and from target organs.

Actin, also known as β -actin, is localized in cytoplasmic ribonucleoprotein granule and known to be involved in various types of cell motility. Therefore, decreased expression of actin may reduce cellular motility and ATP binding activity. Increased expression of “heterogeneous nuclear ribonucleoprotein protein A”, a cytoplasmic protein, could enhance pre-mRNA processing and nucleotide binding activity. Although “predicted-60S ribosomal protein L31-like isoform 1”, is up regulated, some identified proteins such as mCG13192, isoform CRA b (hypothetical protein), amylase 2b isoform1 precursor, mCG17894, isoform CRA b are down regulated. However, a precise role of these proteins in alcoholic pancreatic disease is not clearly defined and also not mapped in UniprotKb.

Overall, chronic ethanol feeding in ADH⁻ deer mice targets proteins related to ER membrane and its homeostasis, and calcium binding and ribosomal proteins, and down-regulates the proteins involved in ATP synthesis, carbohydrate metabolism, cellular architecture and colloidal osmotic pressure. However, the differentially expressed proteins as found in this study need to be validated in the biopsy samples of pancreatic tissue and plasma of patients diagnosed with alcoholic pancreatitis to guide the biomarker discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Blood alcohol concentration (BAC) in ADH⁻ deer mice fed ethanol for three months. Values are mean $\pm$ SD of five mice in each group. * P $\leq$ 0.05

FIGURE 2.

Histological changes as shown in a representative H & E stained section of pancreas of ethanol-fed mice by arrows: cellular disarray of acinar cells along with necrosis and atrophy of cells (D), disorganization of acinar structure(DO), interstitial edema(E) and necrosis (N) of acinar cells (magnification $\times 200$).

FIGURE 3.

Representative 2D gels showing differentially altered proteins (>1.5 fold, $p < 0.05$) identified by MALDI-TOF/TOF at three different pH range in the pancreatic tissues of ADH⁻ deer mice fed ethanol as compared to pair-fed controls. The numbers in the figures are the spot identifiers given by the software. (n = four animals/group).

FIGURE 4.

Western blot analyses of differentially expressed proteins in the pancreas of pair-fed and ethanol fed ADH⁻ deer mice. Relative intensities of the protein bands were determined by ImageJ. * $P < 0.05$ as compared to the control (three animals/group).

Differentially Expressed Proteins in the Pancreas of Ethanol-fed ADH⁻ Deer Mice vs Pair-fed Controls

Table 1

UniProt KB Recommended Protein Name (alternate name)	Accession Number	Gel Spot No	PI	MW (kD)	MS ID Expectation value	Protein Abundance	Protein Function (cellular localization)
Fraction 1 (pH 3.0–5.4)							
Calreticulin (chain A, crystal structure of the calreticulin lectin domain)	gi 307776557	6	3.36	147	1.58E-04	1.66	Calcium-binding chaperone that promotes folding, oligomeric assembly and quality control in the endoplasmic reticulum (ER)
Transitional ER ATPase (valosin- containing protein)	gi 55217	90	4.25	89	6.30E-14	–2.11	ER function & homeostasis, endocytosis (cytoplasm, ER)
ATP synthase subunit beta, mitochondrial	gi 31980648	226	4.05	46	3.16E-64	–1.54	Synthesis of ATP from ADP to provide energy to the cell, and involved in lipid metabolic process
mCG13192, isoform CRA_b	gi 148673964	255	4.05	43	1.99E-25	–2.38	Hypothetical protein
40S ribosomal protein SA (laminin receptor)	gi 293694	272	3.6	41	3.97E-35	–1.63	Cell adhesion to the basement membrane (cell membrane, cytoplasm & nucleus)
Translocon-associated protein subunit α	gi 165377206	330	3.25	34	3.16E-04	1.51	Binds calcium and regulates retention of ER resident proteins
Translationally- controlled tumor protein	gi 6678437	435	3.48	23	6.30E-09	1.92	Anti-apoptosis, binds to calcium and stabilizes microtubules (extracellular)
Calmodulin (Chain A, structure of Apo-Calmodulin Bound to Unconventional)	gi 122920763	741	3.22	17	6.30E-15	1.51	Calmodulin mediates the control of a large number of enzymes, ion channels, aquaporins and other proteins by Ca ²⁺ (all components of the cell)
Histone H4 (hist1h4f protein)	gi 141794860	594	3.73	16	6.30E-07	2.02	Responsible for the stability of nucleosome structure of the chromosomal fiber (nucleus)
PREDICTED- 60S ribosomal protein L31-like isoform 1	gi 82898755	751	3.25	16	7.92E-12	1.6	Structural constituent of ribosome (ER)
Fraction2 (pH 5.4–7.0)							
Ribosome-binding protein 1	gi 124486712	149	5.75	91	3.97E-16	1.66	Acts as receptor and mediates in interaction between the microsome and ER membrane
Serum albumin	gi 163310765	902	6.05	78	9.98E-06	–2.17	Regulates colloidal osmotic pressure of blood and a carrier protein
60 KDa heat shock protein, mitochondrial (hspl protein)	gi 76779273	252	5.87	60	5.00E-14	1.79	T cell activation, positive regulation of interferon- α and γ production, ATP binding
Amylase 2b isoform 1 precursor	gi 298676467	263	5.68	57	1.99E-05	–2.05	Glycosidase, carbohydrate metabolic process in digestion (extracellular)

UniProt KB Recommended Protein Name (alternate name)	Accession Number	Gel Spot No	PI	MW (kD)	MS ID Expectation value	Protein Abundance	Protein Function (cellular localization)
Keratine Type II, cytoskeleton 8 (Endo' A cytokeratin)	gi 309215	334	5.82	50	1.58E-48	-1.65	Structural and conformational protein and involved in apoptosis (cytoplasmic),
Lipoamide acyltransferase component of branched-chain α -keto acid dehydrogenase complex, mitochondrial (DHT)	gi 170172520	306	6.13	50	3.16E-05	1.67	The branched-chain α -keto dehydrogenase complex catalyzes the overall conversion of α - keto acids to acyl-CoA and CO ₂ .
Actin cytoplasmic 1 (β -actin)	gi 49868	277	5.79	54	1.99E-04	-1.83	Cytoplasmic β -action for many aspects of cell motility
mCG17894, isoform CRA_b	gi 148700409	392	6.42	50	2.51E-07	-1.53	Hypothetical protein
Fraction3 (pH 7-10)							
Heterogeneous nuclear ribonucleoproteins A2/B1	gi 124028629	357	8.67	30	1.26E-16	1.85	Pre-mRNA processing and nucleotide binding (cytoplasmic)

Values are mean of 4 animals/group, p value <0.05.