Differential expression of liver proteins in Chianina and Holstein young bulls

M. Miarelli and F. Signorelli
Agricultural Research Council, Research Centre for Meat Production and Genetic Improvement, Via Salaria, 31 00016 Monterotondo (Rome), Italy

ABSTRACT: The purpose of this work was to produce and compare liver proteomic maps of cattle breeds that have been selected for different purposes: dairy (Holstein) and beef (Chianina). Liver was chosen because it displays functions of synthesis, homeostasis, excretion, and defense. Two dimensional electrophoresis analyses were performed on bovine livers collected from 4 individuals of each breed to evaluate differential protein expression. We found 9 spots differing in photodensity by ≥2-fold between the 2 breeds. Spots were analyzed through matrix-assisted laser desorption ionization mass spectrometry-time of flight (MALDI-TOF MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS). A search against protein sequences in the National Center for Biotechnology Information databases indicated that the differentially expressed proteins correspond to liver-specific enzymes, with a major role in the metabolism of fatty acids, carbohydrates, AA, and the urea cycle.

Key words: cattle breed, liver, proteome, two-dimensional electrophoresis

INTRODUCTION

Proteomics is a powerful tool for generating analytical data to discover differentially expressed proteins and to elucidate the biochemical and physiological mechanisms that control animal production and quality. Currently, 2-dimensional electrophoresis (2-DE) is the only technique that can be routinely applied for parallel quantitative expression of complex protein mixtures (Gorg et al., 2004). Several high-resolution reference electrophoretic maps of liver, kidney, muscle, plasma, and red blood cell samples from Holstein individuals were produced by Talamo et al. (2003), who also established a 2-DE bovine database. These analytical data may contribute to the understanding of relevant biological processes and serve as a tool in future studies aimed at the evaluation of changes in response to various internal or external factors (Fountoulakis and Suter, 2002), as well as assist in the characterization of protein modifications due to technological processes used in the food industry.

The present work represents a preliminary approach to a comparative study of bovine proteome in 2 breeds that have been selected for different purposes: dairy (Holstein) and beef (Chianina). The study focuses on characterizing liver protein diversity between breeds.

MATERIALS AND METHODS

All animals used in this study were treated according to the guidelines stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1988).

The trial was carried out with 4 Chianina and 4 Holstein young bulls reared in the same farm. Animals were offered feed [alfalfa hay 14%, maize silage 60%, barley and maize grains 16%, and protein supplement 0.7% (DM basis)] ad libitum and were slaughtered at commercial maturity: age 570 d ± 15; BW 670 kg ± 40 for the Chianina; age 480 d ± 30; BW 530 kg ± 45 for the Holstein.

Liver tissue samples were collected, immediately frozen in liquid nitrogen, and stored at –80°C. After homogenization, 50 mg of each single sample was lysed in 5-mL lysis buffer {7 M urea, 2 M thiourea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 1% 1,4-dithio-DL-threitol, 0.8% Pharmalite, 40 mM Tris, and the protease inhibitors cocktail, 50 µL/g of tissue (cod P8340, Sigma, St. Louis, MO)}, sonicated for 10 cycles for 1 s, shaken for 1 h at 15°C,
and then centrifuged for 5 min at 20,000 × g at 15°C. In this work, we ensured that each tissue sample was submitted to 2-DE analysis individually, rather than as a pool of samples of the individuals of the same breed, as is commonly performed in this type of study (Guelfi et al., 2006). Although both approaches should result in comparable average spot volumes, only the former enables the precise identification of significant differences among experimental factors.

Total protein concentration was quantified by the 2-D Quant Kit (GE Healthcare, Niskayuna, NY) and 750 µg were loaded on 17-cm strips (pH 3 to 10, nonlinear) for isoelectric focusing using a Protean IEF cell (Bio-Rad Laboratories Inc., Hercules, CA). Each sample was analyzed in triplicate. Strips were applied onto 12% SDS-PAGE in a Protean II XL multicell (Bio-Rad Laboratories). The 2-D gels were stained with colloidal Coomassie blue (Neuhoff et al., 1988). The proteins were visualized by gel scanning with the Molecular Image Phoros FX (Bio-Rad Laboratories) scanner, then detected and quantified using the PDQuest Advanced software package (Bio-Rad Laboratories). To identify valid spots, PDQuest spot detection software was used with appropriate selection of the faintest and the smallest spots and a large representative section of the image containing spots, streaks, and background gradation to make corrections for noise.

Mean values and CV of the triplicates for each protein spot of each individual were calculated. Only the spots with an intrariplicate CV ≤10% were further taken into account. Differences in protein expression were defined as ≥2-fold change of photodensity between each of the 4 Chianina and each of the 4 Holstein averaged spots. If any 1 of the 4 individuals of a single breed did not fulfill this requirement, then the spot was not considered further. An exception was made for 1 protein spot that was absent in all the 4 Chianina individuals, although present in 3 of the 4 Holsteins, because it was the only detected case of absence of a spot in all individuals of the same breed.

In this work we arbitrarily decided to establish a significance level at ≥2-fold change in photodensity of the spots of each individual of 1 breed vs. every individual of the second breed. This significance level is greater than the one used in Xu and Wang (2008), but we preferred to avoid the evaluation of results that might be misleading or not representative of the differences between the considered breeds. By establishing a significance level at ≥2-fold change in photodensity, and by comparing each individual of 1 breed vs. every individual of the second breed, we coped with the occurring artifacts of the 2-DE that might give multiple spots for the same protein (Fountoulakis and Suter, 2002). It is unlikely that this occurred exactly in the same way in all individuals of 1 breed and in none of the individuals of the second breed.

The selected protein spots were picked and submitted to trypsin digestion. Briefly, gel pieces were washed in 50 mM ammonium bicarbonate pH 8.0 in 50% acetonitrile to achieve a complete destaining, then resuspended in 50 mM ammonium bicarbonate pH 8.0 containing 100 ng of trypsin and incubated for 2 h at 4°C, then overnight at 37°C. The supernatant containing the resulting peptide mixtures was removed, and the gel pieces were reextracted with acetonitrile. The 2 fractions were then collected, freeze-dried, and analyzed by matrix-assisted laser desorption ionization mass spectrometry-time of flight (MALDI-TOF MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS); MALDI mass spectra were recorded on an Applied Biosystem Voyager DE-PRO mass spectrometer equipped with a reflectron analyzer and used in delayed extraction mode. One microliter of peptide sample was mixed with an equal volume of α-cyano-4-hydroxy-cinnamic acid as matrix (10 mg/mL in 0.2% trifluoroacetic acid in 70% acetonitrile), applied to the metallic sample plate, and air-dried. The acceleration voltage was 20 kV, delay time was 100 ns, and the grid voltage was set to 75%. Spectra were acquired from 600 to 5,000 Da using 1,000 laser shots/spectrum. Mass calibration was performed by using the standard mixture provided by the manufacturer. Typical error was in the 50 to 100 ppm range.

When the identity of the proteins could not be established by peptide mass fingerprinting, the peptide mixtures were further analyzed by LC-MS/MS using the LC/MSD Trap XCT Ultra (Agilent Technologies, Palo Alto, CA) equipped with a 1100 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture (7 µL in 0.5% trifluoroacetic acid) was first concentrated at 4 µL/min in 40-nL enrichment column (Agilent Technologies chip), with 0.1% formic acid as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (75 × 43 mm in the Agilent Technologies chip) at a flow rate of 300 nL/min, with a linear gradient of eluent B (0.1% formic acid in acetonitrile) in A (0.1% formic acid) from 7 to 50% in 35 min. Elution was monitored on the mass spectrometers without any splitting device. Peptide analysis was performed using data-dependent acquisition of 1 MS scan (m/z range from 400 to 2,000 Da/e) followed by MS/MS scans of the 3 most abundant ions in each MS scan. Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (2 min) of ions from which definitive mass spectral data had previously been acquired. Moreover, a permanent exclusion list of the most frequent peptide contaminants (keratins and trypsin peptides) was included in the acquisition method to focus the analyses of significant data. Mass spectral data obtained from the MALDI-MS and the LC-MS/MS analyses were used to search a nonredundant protein database (National Center for Biotechnology Information mammals database) using an in-house version of the Mascot (Matrix Science, Boston, MA) software.

The accurate peptide mass values from MALDI-MS analyses were used in the peptide mass fingerprint type
of search taking into account the carbamidomethyl-Cys as fixed modification, a peptide mass tolerance of ±100 ppm, and several missed cleavages of 2. Peptide mass values and sequence information from LC-MS/MS experiments were used in the MS/MS ion search, taking into account the carbamidomethyl-Cys as fixed modification, a precursor ion and a fragment ion mass tolerance of ±600 ppm and 0.6 Da, respectively.

RESULTS AND DISCUSSION

In this study, we wanted to elucidate the differential expression of liver proteins in young Holstein and Chianina bulls. Liver was chosen because it is the central tissue for metabolism, displaying functions of synthesis, homeostasis, excretion, and defense. A total of 649 individual protein spots were detected and compared. Nine protein spots fulfilled the established requisites for differentiating the 2 breeds, as indicated above. The combined MALDI-MS and LC-MS/MS system allowed identification of all 9 spots. The search against the National Center for Biotechnology Information mammalian database matched all of the proteins to the *Bos taurus* species.

Figure 1 shows the expansion of the map area of the 2-DE Coomassie-stained gels of Chianina (A) and Holstein (B) liver, where differentially expressed proteins were detected. Spot numbers correspond to the first columns of Tables 1 and 2 that show the identified bovine liver proteins. The 2 tables report the list of the upregulated proteins in each breed with the average fold change compared with the other breed. One protein (sulfotransferase family, cytosolic, 1C, member 2) was identified in 2 different individual spots (5 and 9). Spot 5 was present in all the individuals of the 2 breeds and upregulated in the Chianina. Spot 9 was missing in all Chianina individuals, and in 1 of the 4 Holsteins. It is therefore unlikely that the separation of this protein was due to an artifact occurring during the analysis, but we can infer that this protein is present in different isoforms (i.e., variable spliced forms of the same gene product), or posttranslational modifications, as reported by Harry et al. (2000) and Fountoulakis and Suter (2002).

**Proteins Upregulated in the Chianina Livers**

**Spot 1—Chain C, the Crystal Structure of Modified Bovine Fibrinogen.** This key component of hemostasis is a plasma glycoprotein that participates in blood coagulation processes of all vertebrates, producing the fibrin clot upon activation by thrombin. Fibrinogen is also a positive acute-phase protein. In an inflammatory state, fibrinogen synthesis is under the control of glucocorticoids that act as mediators in the liver (Nicodeme et al., 1995).

**Spot 2—Galactose Mutarotase.** Mutarotase is a key enzyme in carbohydrate metabolism, catalyzing the interconversion of the α- and β-anomers of hexose sugars as galactose. In most organisms, the conversion of β-d-galactose to the more metabolically useful glucose 1-phosphate is carried out by the action of 4 enzymes that constitute the Leloir pathway. In the first step of this pathway, β-d-galactose is epimerized to α-d-galactose by galactose mutarotase (Holden et al., 2003).

**Spot 3—Fumarylacetoacetate Hydrolase Domain Containing 2A.** Fumarylacetoacetate hydratase catalyzes the final step of tyrosine and phenylalanine catabolism, the hydrolytic cleavage of a carbon-carbon bond in fumarylacetoacetate, to yield fumarate and acetoacetate (Timm et al., 1999). This enzyme is then involved in tyrosine catabolism, which
is a well-characterized pathway in mammals and is essential for the breakdown of aromatic AA derived from the diet and from protein turnover (Dreumont et al., 2001). Schaefer and Krishnamurti (1984) associated the efficiency of protein synthesis in ovine fetuses to tyrosine concentration in different tissues including liver and concluded that efficiency was greater in the liver with the greatest tyrosine concentrations.

**Spot 4—Fructose-1,6-Bisphosphatase.** This is a key enzyme in gluconeogenesis. Ruminant glucose production from propionate, valerate, AA, lactate, and glycerol is relevant at all physiological stages, and gluconeogenesis rate increases after feeding, particularly when ruminants are in a positive energy balance. Furthermore, glucose availability or gluconeogenic capacity of liver represents a limitation to milk production in dairy cattle (Naifkov and Beitz, 2007).

**Spot 5 and Spot 9—Sulfotransferase Family, Cytosolic, 1C, Member 2.** The cytosolic sulfotransferase super family has a wide tissue distribution in plants and animals. In mammals these enzymes catalyze the sulfate conjugation to a multitude of substrates and act as a major detoxification enzyme system (Gamage et al., 2006).

### Proteins Upregulated in Holstein Livers

**Spot 6—Argininosuccinate Lyase.** Argininosuccinate lyase is an enzyme of the urea cycle. Excess ammonia is converted to urea in the liver and is excreted into urine. In ruminants, however, urea is partially transferred back into the digestive tract by secretion into saliva or transport across the rumen wall and is recycled for the synthesis of bacterial proteins. Thus, urea is an important reusable nitrogen source for ruminants. Takagi et al. (2008) demonstrated that the activity of urea cycle enzymes is regulated by several nutritional and hormonal factors in rats.

**Spot 7—Acetyl CoA Acyl Transferase-1.** Peroxisomes contain a battery of fatty acid oxidizing enzymes, one of which is acetyl-CoA acyltransferase-1, which catalyzes the cleavage of 3-ketoacyl CoA by the thiol group of another molecule of CoA in the final step of β-oxidation. For its involvement in fatty acid degradation metabolism, this enzyme has been investigated in cattle in veterinary studies and specifically associated with physiological and pathological conditions (Marton et al., 1998; Lockhart and Winzeler, 2000). To our knowledge, the only proteomics study on cattle liver where differential expression of this enzyme was made evident was performed by Xu and Wang (2008), who found that the expression of acetyl CoA acyl transferase was decreased in the liver tissues of ketotic cows, compared with normal cows, indicating that the ability to utilize fatty acids is decreased in ketotic cows.

**Spot 8—Annexin IV.** Annexin is a protein family characterized by a highly conserved fold. The physiological function is not completely clear; annexin IV, a Ca²⁺-dependent membrane-binding protein, modifies

<table>
<thead>
<tr>
<th>Spot</th>
<th>Fold change</th>
<th>Identification method</th>
<th>Accession number</th>
<th>Theor. Mr</th>
<th>Theor. pI</th>
<th>Mascot score</th>
<th>Sequence coverage, %</th>
<th>Protein name</th>
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<td>gi/6980816</td>
<td>47,116</td>
<td>5.59</td>
<td>21</td>
<td>103</td>
<td>Chain C, the crystal structure of modified bovine fibrinogen</td>
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<td>2.3</td>
<td>MF</td>
<td>gi/77735568</td>
<td>47,116</td>
<td>5.59</td>
<td>21</td>
<td>103</td>
<td>Galactose mutarotase</td>
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<td>3</td>
<td>5.4</td>
<td>TMS</td>
<td>gi/45430009</td>
<td>37,819</td>
<td>6.54</td>
<td>11</td>
<td>54</td>
<td>Fumarylacetoacetate hydrolase domain containing 2A</td>
</tr>
<tr>
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<td>TMS</td>
<td>gi/77735849</td>
<td>37,819</td>
<td>6.54</td>
<td>54</td>
<td>678</td>
<td>Fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>5</td>
<td>6.1</td>
<td>TMS</td>
<td>gi/124249242</td>
<td>35,062</td>
<td>6.00</td>
<td>54</td>
<td>678</td>
<td>Sulfotransferase family, cytosolic, 1C</td>
</tr>
</tbody>
</table>

1Theor. Mr = theoretical molecular weight values.
2Theor. pI = theoretical isoelectric point.
3Method of identification: MF = mass fingerprint; TMS = tandem mass spectrometry.
membrane bilayers by increasing rigidity, reducing wa-
ter and H+ permeability, promoting vesicle aggregation,
and regulating ion conductances (Li et al., 2003). An-
nexin IV also was identified by Talamo et al. (2003)
and included in the Holstein protein database.

To our knowledge, this is the first work aiming to
investigate liver proteomics in Holstein and Chianina
animals, 2 widely used cattle breeds representing an
important source of animal products, but selected for
extremely different purposes. Several proteomics studies
in livestock aimed to define optimal management sys-
tems for achieving better quality of production in the
same breed. For example, Mullen et al. (2006) and Hol-
lung et al. (2007) applied proteomic techniques to beef
muscle to understand the molecular mechanism behind
meat quality. In particular, Shibata et al. (2009) com-
pared skeletal muscle proteome of grass-fed and grain-
fed cattle and made evident that muscle fiber type con-
version to slow-twitch tissue occurred with change in
the energy metabolic enzymes when cattle were grazed
in the late fattening period. In addition to meat qual-
ity, other traits were studied through a proteomic ap-
proach. Kuhla et al. (2007) investigated the jejunal and
hepatic proteomes in young goats to determine whether
a milk diet partly containing soy protein could change
provided a detailed insight into protein expression pat-
terns in the liver of ketotic cows, to understand the
pathogenesis of bovine ketosis. An interesting example
of proteomic comparison between different breeds with
the aim to identify differentially expressed proteins was
given by Ohsaki et al. (2007). These authors identified
the proteins with different expression levels for the LM
and the subcutaneous adipose tissue between Japanese
Black and Holstein cattle.

Within the upregulated proteins in the Chianina,
we found fibrinogen and 2 key enzymes (galactose mu-
tarotase and fructose-1,6-bisphosphatase) of the path-
way glycolysis/gluconeogenesis (http://www.genome.
jp/kegg/pathway.html). Because animals of this trial
were in good health and maintained for production pur-
poses, we do not suspect that the upregulated fibrino-
gen found in the Chianina breed was due to an inflam-
matory state. It is more likely that this is a feature of
this breed.

Although differential gluconeogenesis between dairy
and beef cattle has not specifically been investigated,
gluconeogenesis does increase when ruminants are in
a positive energy balance (Murondoti et al., 2004).
Therefore, we can infer that Chianina in this study had
a more positive energy balance due to decreased energy
expenditure or because this breed has a different gluco-
neogenic capacity.

Within the upregulated proteins detected in the Hol-
steins relative to Chianina, we identified an enzyme
of the urea cycle (argininosuccinate lyase) and acetyl-
CoA acyltransferase-1. To our knowledge, no studies
have been performed to investigate differences in urea
cycle in dairy vs. beef cattle. However, evidence exists

<table>
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<td>3.6</td>
<td>Annexin IV</td>
<td>gi/1063258</td>
<td>TMS</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>Sulfotransferase family, cytosolic 1C</td>
<td>gi/124249242</td>
<td>TMS</td>
</tr>
</tbody>
</table>

2. Theor. pI = theoretical isoelectric point.
that urea metabolism is more pronounced in the Holstein cows with high vs. low milk yield (Gustafsson and Palmquist, 1993; Huntington and Archibeque, 2000), which could explain the greater expression of argininosuccinate lyase in Holstein vs. Chianina livers.

Xu and Wang (2008) suggested that the increased expression of acetyl CoA acyl transferase indicates a greater ability to utilize fatty acids and found a decreased expression in the liver of cows affected by ketosis, a disease directly associated with the low milk fat syndrome. Therefore, this ability to utilize fatty acids is likely to be more pronounced in dairy breeds, particularly those selected for greatest milk yield, corroborating our finding that this enzyme is upregulated in the liver of the Holstein breed.

In conclusion, in this study on differential expression of liver proteins in young beef and dairy bulls, we identified 5 differentially expressed proteins in the liver of bulls including fibrinogen, galactose mutarotase, fructose 1,6-bisphosphatase, argininosuccinate lyase, and acetyl CoA acyl transferase-1, which may represent the result of alternative selection goals to which the 2 breeds have been subjected. Proteomics can be used as a tool for understanding metabolic and biochemical mechanisms and consequently for defining parallel breed genetic fingerprints. Moreover, protein identification may provide the basis for discovering potential biomarkers of breed-specific quantitative phenotypes of breeding relevance (Talamo et al., 2003).

**LITERATURE CITED**


