Comparative Proteome Analysis of Porcine *Longissimus dorsi* on the Basis of pH24 of Post-mortem Muscle

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Abstract

To identify proteins contributing to meat quality, a comparative shotgun proteomic profiling of Berkshire *longissimus dorsi* muscles was conducted in high pH group (HpHG) and low pH group (LpHG) based on 24 hour post-mortem pH. Triplicate liquid chromatography–tandem mass spectrometry analysis identified a total of 208 and 204 proteins in the HpHG and LpHG, respectively. A total of 128 proteins were classified on the basis of molecular function, cellular components, and biological process by gene ontology analysis, of which 13 and 21 proteins were exclusively found in the HpHG and LpHG, respectively. A total of 15 proteins, of which 6 proteins belonged to the LpHG and 2 to the HpHG, were assigned to the *Sus scrofa* genomic database. The dominant expressions of Igc, Prep, Ldhb, and Aco2 were identified in the LpHG by shotgun proteomic analysis, and confirmed by reverse transcriptase–mediated polymerase chain reaction analysis. These protein markers are suitable for determining meat quality.

Keywords: meat quality, *longissimus dorsi* muscle, protein biomarker, shotgun proteomics

1. Introduction

Porcine meat quality depends on meat color, hardness and the water and fat contents of muscle tissues (Hocquette et al., 2005). In general, the intramuscular fat content and tenderness of meat can affect its taste. Thus, the relation of these factors to the marbling score and Warner-Bratzler shear force is considered to be one of the most important factors in meat production and marketing (Kim et al., 2008). In addition to direct meat-quality factors, the water-holding capacity (WHC) of the muscle affects meat production, and the WHC is closely related to the post-mortem biochemical process (McIntosh, Berman, & Kench, 1977). Recently, traits correlated

with expression QTL (quantitative trait loci) analysis identified several biochemical processes involved in live skeletal porcine muscle and meat quality, including oxidative phosphorylation, mitochondrial pathways, and transporter activity (Ponsuksili et al., 2008). WHC correlates negatively with drip loss, which is primarily caused by the shrinkage of myofibrils due to a reduction in the intramuscular energy reservoir, pH, and temperature (Offer & Knight, 1988). The ultimate pH of post-mortem muscle, corresponding to the pH (pH_{24hr}) 24 hours post-slaughter, can be simply determined as a meat-quality trait that is closely related to WHC (Warner, Kauffman, & Greaser, 1997). Fast post-mortem degradation of glycogen leads to a rapid decline in muscular pH, resulting in poor meat quality (known as acid meat), whereas slow degradation of intramuscular glycogen results in a relatively slower decline in pH, suggesting good meat quality (Le Roy, Naveau, Elsen, & Sellier, 1990). Thus, high pH group (HpHG) and low pH group (LpHG) were classified on the basis of muscular pH_{24hr} after slaughter.

Proteomics allows the identification of potential protein markers involved in meat-quality traits. Recently, two-dimensional gel electrophoresis (2-DE) was used to identify differentially expressed proteins in Meishan and Large White pigs (Xu et al., 2009). The identification of proteins potentially related to a high level of marbling was attempted for the purpose of genetic design, using 2-DE–based proteomics (Liu et al., 2009) and the natural variations of synthetic lines from Duroc, Hampshire and Large White pigs. We performed large-scale proteomic profiling of *longissimus dorsi* muscle (LDM) from HpHG and LpHG of Berkshire pigs using a gel–based shotgun proteomic approach (Wang et al., 2004). In the present study, in order to identification of meat quality related proteins, high pH and low pH groups LDM of porcine 24 hours post-slaughter were analyzed by 1-DE/LC-MS/MS. The possible roles of the identified proteins as potential protein markers for meat quality investigation are discussed.

2. Method

2.1 Animals and Longissimus Dorsi Muscle Preparation

The pigs originated from a population of 102 male Berkshires (80~100 kg, 120 d old) provided by Da-San-Jong-Don Co. Ltd. (Namwon-city, Korea). Pigs were slaughtered following standard slaughtering procedures. A *longissimus dorsi* muscle (LDM) sample of approximately 10 gram was collected at the 5th thoracic vertebrate shortly after slaughter and immediately frozen in liquid nitrogen prior to shotgun proteomic analysis.

2.2 Physical Measurement of Meat Quality

After slaughtering, the pH values (pH_{45min} and pH_{24hr}) of the LDM samples at the 5th thoracic vertebrate were determined in triplicate, using a portable needle-tipped electrode (pH-K21; NWKbinarGmbH, Landsberg, Germany). The color (*L*, lightness; *a*, redness; *b*, yellowness) of each LDM surface was recorded after a 30-min blooming at 1°C using a Minolta Chromameter (CR400; Minolta, Japan). The WHC of the LDM samples was determined as described previously (Kristensen & Purslow, 2001). The fat and collagen contents (%) of the LDM samples were determined using a Foodscan (Food Scan[TM] Lab, type 78810; Foss Co., Denmark) (Anderson et al., 2007). Warner-Bratzler shear force, drip loss, and cooking loss were measured as previously described (Kauffman, Eikelenboom, Van der Wal, Engel, & Zaar, 1986; Wheeler, Shackelford, & Koohmaraie, 2000).

2.3 Extraction of Muscular Proteins

Protein samples were collected from three male Berkshire pigs randomly chosen from the upper 10% pH_{24hr} (pH 5.73~6.17, 10 pigs) and lower 10% pH_{24hr} (pH 5.37~5.47, 10 pigs) of the total group of 102 pigs. Porcine LDM proteins were prepared by using a previously described method (Lametsch & Bendixen, 2001). In brief, the 1.5-gram LDM tissue samples were ground with a mortar in liquid nitrogen, and the proteins were extracted with extraction buffer containing 0.3% (w/v) sodium dodecyl sulfate (SDS), 0.2 M dithiothreitol, and 1 M Tris-HCl (pH 8). The crude extracts of individual LDM samples were centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was treated with 20 kilounits of DNase and 7.5 kilounits of RNase and centrifuged at 14,000 rpm for 30 min to obtain the solubilized supernatant. The final supernatant was precipitated by incubation with 10% (w/v) trichloroacetic acid at room temperature for 1 hr. The mixture was centrifuged at 14,000 rpm for 30 min to obtain the precipitate and subsequently washed three times with 100% (v/v) acetone. The precipitate was freeze-dried by Speed Vacuum (SC110A; GMI Inc., MI) and dissolved with 20 mM Tris-HCl buffer (pH 8) and adjusted to a final concentration of 1 μg/μl, as measured by the Bradford protein assay.

2.4 One-dimensional Gel-based Proteomics Coupled with Liquid Chromatographytandem Mass Spectrometry

For the shotgun proteomic analysis based on SDS-polyacrylamide gel electrophoresis separation (SDS-PAGE) and liquid chromatography–tandem mass spectrometry (LC-MS/MS), the LDM proteins (15 μg/per lane) were loaded on 12% SDS-polyacrylamide gel (10 × 8 cm). After electrophoresis, the gels were stained with

Coomassie Brilliant Blue R-250 and then separately cut into 8 slices according to the stained gel band intensity, as shown in Figure 1. Each gel slice was transferred into a new Eppendorf tube. In-gel tryptic digestion was conducted as previously reported (Wang, et al., 2004). The tryptic digests were extracted with 0.02% (v/v) formic acid in 0.5% (v/v) acetic acid. The extracts were freeze-dried for later LC-MS analysis. The 10μl peptide samples were concentrated on an MGU30-C_{18} trapping column (LC Packings, Dionex, Sunnyvale, CA) and analyzed using the nano-column (10 cm × 75 mm internal diameter, C_{18} reversed-phase column; Proxeon, Odense, Denmark) at a flow rate of 120 nl/min. The peptides were eluted from the column by applying gradient (0-65%, v/v) acetonitrile for 80 min at a constant flow rate. All MS and MS/MS spectra were obtained in a data-dependent mode by the electrospray ionization ion trap MS (LCQ-Deca XP; Thermo, Waltham, MA). MS analysis was performed in triplicate using a different batch of samples.

2.5 Bioinformatic Analysis

For protein identification, MS/MS spectra were searched using an internal Mascot server (version 2.2; Matrix Sciences Inc, London, UK) against a SwissProt 57.5 mammalian database. Search parameters allowed for methionine oxidation (+16 Da), cysteine carbamidomethylation (+57), and one missed trypsin cleavage within 1.5 Da for peptide tolerance and within 1.5 Da for fragmented peptide. The False discovery rate (FDR), up to 10%, was permitted to validate the final proteomic data. The exponentially modified protein abundance index (emPAI) values of identified proteins at the specific group were recorded by the triplicate LC-run, and their abundance was semi-quantified to present the average mol% using statistical analysis by Student's t-test, as described previously (Ishihama et al., 2005). The gene ontology of the identified proteins was searched using QuickGO (http://www.ebi.ac.uk). Each histogram and box plot was calculated using the statistical package R program.

2.6 Reverse Transcriptase–Mediated PCR Analysis

Total RNA in LDM was isolated with TriZol (Invitrogen, Seoul, Korea) and RNase-free DNase (Promega, Seoul, Korea) according to the manufacturer's protocol. The RNA was quantified with a Nanodrop.The complementary DNA (cDNA) was synthesized in a 20-μl volume, with 5 μg total RNA as a template, using oligo-dT primers and reverse transcriptase (Invitrogen, Seoul, Korea). The specific primers were for *igc* (sense: 5′-GCC ACC CTG GTG TGT CTA AT-3′, antisense: 5′-GCG TCA CTG TCT TCT CCA CA-3′), *prep* (sense: 5′-GAT GGC TCT CAT CCT GCC TT-3', antisense: 5′-ACC ACC TTT GTG CCA CGT CT-3'), *ldhb* (sense: 5′-CAC GGA AGC TTG TTC TTT CA-3′, antisense: 5′-CAT TGA CGT TCC TCT GCA CC-3′), and *aco2* (sense: 5′-GCT GGA CCT CAC CCA AAG AT-3′, antisense: 5′-ATG TTG CAG ATT GTC GCC AT-3′). The primers for β-actin (sense: 5′-AGG TCA TCA CTA TTG GCA AC-3′; antisense: 5′-ACT CAT CGT ACT CCT GCT TG-3′) were used as an internal control. PCR was performed using 1 μl of synthesized cDNA from four individual samples from the HPHG and LPHG and from 5 pmol of each primer set in a premixed PCR reaction kit (Bioneer, Daejeon, Korea) in a GeneAmp® PCR system 9700 (Applied Biosystems, CA). PCR amplification was carried out according to the following procedure: initial denaturation at 94°C for 5 min, followed by 30 cycles (28 cycles in case of *igc* and *ldhb*) at 94°C for 15 sec, 55°C for 15 sec (58°C in case of *prep*), and 72°C for 20 sec and the extension at 72°C for 5 min. The ethidium bromide–stained PCR products were electrophoresed in 2% agarose gel. The statistical significance of relative abundance was assessed using a paired Student's t-test at $P<0.001$.

3. Results

3.1 Comparison of Meat-quality Traits between the HpHG and LpHG

Post-mortem LDM pH_{24hr} values were measured from 102 male Berkshire pigs representing the highest 10% group (HpHG) and the lowest 10% group (LpHG). The overall distribution of post-mortem LDM pH_{24hr} values is shown in Supplementary Figure 1. The physical traits of meat quality were compared, as shown in Table 1. The initial difference in $pH_{45 min}$ after the onset of slaughter was not significant. However, the meat from the HpHG was slightly brighter, less reddish ($P<0.05$) and less yellowish on the surface than that of the LpHG. The WHC of the HpHG was higher than that of the LpHG, but the difference was not significant. Drip loss, however, was significantly lower for the HPHG than for the LpHG ($P<0.05$). This result coincides with the results of previous reports showing that similar to the drip loss factor, the extent of the pH decrease is an indicator of the meat quality (Fischer, 2007).

3.2 Differentially Expressed Proteins of LDM from the HpHG and LpHG

To identify the differentially expressed proteins from the HpHG and LpHG, the soluble proteins from the meat of both groups were extracted and separated by SDS-PAGE. On the basis of molecular weight and band intensity, the sliced gels were excised, digested with trypsin, followed by LC-MS/MS analysis. This shotgun proteomic

method is advantageous for identifying the proteome at a large scale - over 2-DE/MALDI-TOF MS (Wang, et al., 2004). Results obtained from triplicate analyses showed a total of 128 proteins both HpHG and LpHG. The identified proteins by at least two hits were analyzed for the specific proteins exclusively expressed in each group. As a result, 13 and 21 proteins were exclusively found in the HpHG and LpHG, respectively, whereas 94 proteins were commonly found in both groups (Figure 2). A total of 128 proteins were classified on the basis of molecular function, cellular components, and biological process by gene ontology analysis (Figure 3).

3.2.1 Validation of Protein Biomarkers Responsible for Meat Quality

LpHG-specific proteins (Ldhb and Prep) and LpHG-abundant proteins (Igc and Aco2), identified by LC-MS/MS based shotgun proteomics, were chosen for the biochemical validation to determine whether these proteins are indicators of meat quality, as based on post-slaughter pH_{24hr}. The relative expressions of the four targeted genes were determined by using reverse transcriptase–mediated PCR analysis. As shown in Figure 4, randomly selected muscle samples from the HpHG or LpHG were used for a comparative analysis of mRNA abundance. The expression levels of *igc, prep, ldhb*, and *aco2* were dramatically higher in the LpHG than in the HpHG, thus showing good agreement with mRNA and protein levels.

Table 1. Characteristics of physical meat traits from two Berkshire male meat quality groups

Component		Low pH Group	High pH Group
Sample number		10	10
pH45min		6.05±0.23	6.12±0.26
pH24hrs		5.43±0.04	5.86±0.14[b]
	L	50.36±3.13	47.00±4.31
CIE	a	6.29±0.87	5.42±0.83[a]
	b	2.58±0.95	1.78±0.99
Water Holding Capacity (%)		55.62±1.80	56.21±1.33
Intramuscular fat contents (%)		2.29±1.23	1.70±0.74
Collagen contents (%)		0.89±0.10	0.86±0.09
Warner-Bratzler shear force (kg/0.5 in^2)		3.26±0.53	3.35±0.74
Drip loss (%)		6.81±1.39	4.57±2.48[a]
Cooking loss (%)		28.07±3.25	24.62±5.02

[a]$P<0.05;$[b]$P<0.01$

Table 2. Identified proteins from high and low meat quality groups

Acc No.	Protein name	Gene Name	Organism	Average Mol%[a]		P value[b]
				HpHG	LpHG	
Q00004	Signal recognition particle 68 kDa protein	SRP68	*Canis familiaris*	0.07	N.D.[c]	0.000
Q8K2B3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Sdha	*Mus musculus*	0.07	N.D.	0.000
P50828	Hemopexin	HPX	*Sus scrofa*	0.09	N.D.	0.000
Q8BZF8	Phosphoglucomutase-like protein 5	Pgm5	*Mus musculus*	0.08	N.D.	0.000
Q2KPA3	Hemoglobin subunit beta	HBB	*Scapanus orarius*	0.44	N.D.	0.026
A2ASS6	Titin	Ttn	*Mus musculus*	0.00	N.D.	0.032-
Q7TQ48	Sarcalumenin	Srl	*Mus musculus*	0.09	0.03	0.046
Q2KJJ9	Fructose-1,6-bisphosphatase isozyme 2	FBP2	*Bos taurus*	0.58	0.29	0.024
O18751	Glycogen phosphorylase, muscle form	PYGM	*Ovis aries*	5.37	3.06	0.006
Q3ZC07	Actin, alpha cardiac muscle 1	ACTC1	*Bos taurus*	0.04	0.24	0.017
Q2HYU2	6-phosphofructokinase, muscle type	PFKM	*Sus scrofa*	0.02	0.49	0.000
P23109	AMP deaminase 1	AMPD1	*Homo sapiens*	N.D.	0.11	0.000
Q75NG9	Troponin T, fast skeletal muscle	TNNT3	*Sus scrofa*	N.D.	0.15	0.000
P00336	L-lactate dehydrogenase B chain	LDHB	*Sus scrofa*	N.D.	0.79	0.002
Q5S1U1	Heat shock protein beta-1	HSPB1	*Sus scrofa*	N.D.	1.03	0.031
Q8VHX6	Filamin-C	Flnc	*Mus musculus*	N.D.	0.03	0.046

HpHG, high pH24 group; LpHG, low pH24 group

[a] Average Mol% is based on emPAI that is calculated by MASCOT v. 2.2.

[b]P value was obtained by Student *t*-test with Mol% of each LC-MS/MS run.

[c] Not detectable

Table 3. Meat quality-determining protein biomarker candidates assigned to *Sus scrofa*

Acc No.	Protein name	Gene Name	Average Mol%[a]		P value	Ratio[c]
			HpHG[b]	LpHG[b]		
Q75NG9	Troponin T, fast skeletal muscle	TNNT3	N.D.[d]	0.15	0.000	LpHG
P00336	L-lactate dehydrogenase B chain	LDHB	N.D.	0.79	0.002	LpHG
Q5S1U1	Heat shock protein beta-1	HSPB1	N.D.	1.03	0.031	LpHG
P23687	Prolyl endopeptidase	PREP	N.D.	0.04	0.092	LpHG
Q9TSX9	Peroxiredoxin-6	PRDX6	N.D.	0.13	0.092	LpHG
Q7M2W6	Alpha-crystallin B chain	CRYAB	N.D.	0.54	0.163	LpHG
Q2HYU2	6-phosphofructokinase, muscle type	PFKM	0.02	0.49	0.000	0.04
P01846	Ig lambda chain C region	IGC	0.17	0.48	0.098	0.34
Q2XQV4	Aldehyde dehydrogenase, mitochondrial	ALDH2	0.06	0.11	0.221	0.50
Q9GJT2	S-formylglutathione hydrolase	ESD	0.06	0.11	0.268	0.51
P16276	Aconitate hydratase, mitochondrial	ACO2	0.12	0.17	0.130	0.69
P00339	L-lactate dehydrogenase A chain	LDHA	3.48	2.49	0.108	1.40
P52552	Peroxiredoxin-2 (Fragment)	PRDX2	0.58	0.40	0.334	1.44
P50828	Hemopexin	HPX	0.09	N.D.	0.000	HpHG
P00346	Malate dehydrogenase, mitochondrial	MDH2	0.09	N.D.	0.092	HpHG

[a] Average Mol% is based on emPAI that is calculated by MASCOT v 2.2.

[b]HpHG, high pH group; LpHG, low pH group.

[c]Ratio is calculated by the average Mol% of HpHG divided by that of LpHG.

[d] Not detectable

4. Discussion

The decreased rate of pH decline after slaughter reflects a slightly high WHC and a low drip loss, which are influenced by biochemical processes such as ultimate muscle pH, protein denaturation, and intramuscular sarcomere length (Offer & Knight, 1988). The Rendement Napole (*RN*) gene affects the glycogen content of muscle and a mutation at the *RN* gene results in poor meat quality due to post-mortem degradation of glycogen, which leads to a lowering of muscle pH, associated with a sub-optimal WHC (Le Roy, et al., 1990; Milan et al., 2000).

When categorized by molecular function, the proteins identified by LC-MS/MS analysis functioned in enzyme regulation (49%), extracellular space (33%), macromolecular complex (10%), membrane-bound organelles (3%).When categorized by cellular process, the high-ranking groups were protein complex (28%), rhythmic process (17%), structural molecular activity (12%), response to stimulus (11%), metabolic process (10%). When categorized by biological process, the proteins were identified as belonging to the following classes: metabolic process (46%), multi-cellular organism process (15%), response to stimuli (14%), and others (25%) (Figure 3). The differentially expressed proteins identified from either the HpHG or LpHG are shown in Table 2; the proteome data from triplicate LC-runs were considered significant if the *P* value is less than 0.05. Due to the incomplete genomic database for pigs, all of the proteomic data were not assigned to *Sus scrofa*. Sixteen proteins listed in Table 3 belonged to *S. scrofa* exactly, for which the semi-quantitative abundance is presented as the average mol% based on the exponentially modified protein abundance index (PAI), as described previously (Ishihama, et al., 2005). In particular, 12 proteins were increased in LpHG, whereas 4 proteins increased in HpHG.

Theporcine immunoglobulin lambda gene (*igl*) was mapped on chromosome 14q17-q21 by fluorescence *in situ* hybridization (Slingsby, Dyson, Morley, Walport, & Simpson, 1996). The expression of *Igl*was more highly up-regulated in the skeletal proteome of male pigs than that of female pigs (Hakimov et al., 2009). Our finding was similar to that reported by Medina et al. (2000)(Medina, Strasser, & Kincade, 2000), who showed that the expression of *igc* was up-regulated in murine males, resulting in the suppressed production of new B-lineage precursor lymphocytes, caused by different exposures to estrogen.

Propyl endopeptidase (Prep) is a key player in the degradation of peptides and neuropeptides, although its biological function is not known. Prep, however, is widely distributed across mammalian tissues, plants and bacteria (Cunningham & O'Connor, 1997). Because the protein level of Prep, as a proline-specific peptidase, is more highly expressed in LpHG than in HpHG, Prep is presumably involved in the retarded degradation of

muscle proteins in HpHG. Up-regulation of lactate dehydrogenase in LpHG suggests higher oxidative muscle metabolism, which in turn is related to a lower pH_{24hr}. This phenomenon was likely observed in previous reports (Bee, Guex, & Herzog, 2004; Gondret & Lebret, 2002). Aconitate hydratase functions to catalyze the isomerization of the mutual conversion of citrate to *cis*-aconitate in the tricarboxylic acid cycle. In rats, under conditions of food-deprivation, the activity of aconitate dehydratase in rat hepatocytes is known to increase (Eprintsev, Semenova, & Popov, 2002). This suggests that the mobilization of the reserved nutrients to supply energy-consuming organs, such as skeletal muscle, results in the activation of citric acid–catalyzing enzymes. Likewise, the increased expression level of aconitate dehydratase in LpHG suggests that it is related to the higher energy conversion that occurs to ensure gluconeogenesis, due to the low capacity of direct glucose utilization.

In conclusion, SDS-PAGE was used to extract and separate LDM proteins from the two meat-quality groups (HpHG and LpHG), based on muscular pH_{24hr} after slaughter. This extraction and separation was followed by protein analysis using LC-MS/MS. Shotgun proteomic analysis by SDS-PAGE/LC-MS/MS identified a total of 128 proteins. Most of the proteins were grouped in the categories "metabolic process" and "structural" proteins. Of the 16 proteins assigned to *S. scrofa*, those expressed either abundantly or exclusively in the LpHG were chosen for analysis of mRNA levels using RT-PCR. Taken together with the proteomic analysis, the expression levels of *igc*, *prep*, *ldhb*, and *aco2* were higher in the LpHG. The result of the present investigation is supported by the previously established concept that mRNA expression patterns are able to represent with protein expression levels (Ahsan, et al., 2008). These results provided additional evidence of the identified proteins, as well as indicating that these proteins are highly related meat quality among high and low pH groups. Thus, the meat quality–determining proteins in Berkshire pigs can be easily used in the agricultural industry as a marker for meat quality.
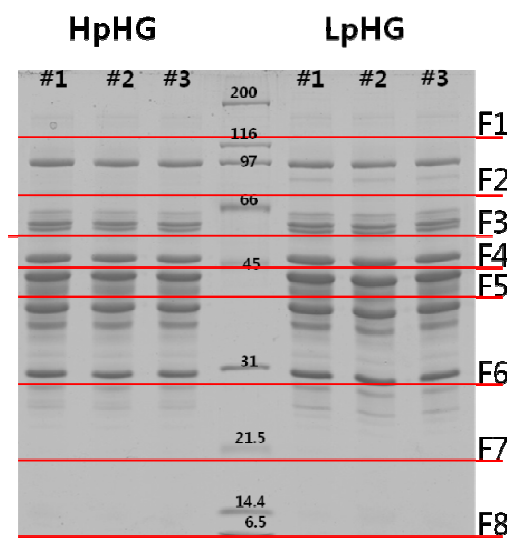

Figure 1. Coomassie-stained SDS-polyacrylamide gel of porcine LDM proteins from HpHG and LpHG. Central lane, molecular weight markers
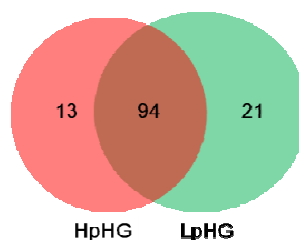

Figure 2. Venn diagrams of identified proteins from high pH group (HpHG) and low pH group (LpHG) samples by shotgun proteomic analysis. Proteins separated on the SDS-polyacrylamide gel, in-gel digested, following LC-MS/MS analysis in which proteins were assigned to SwissProt 57.5 mammalian database. Exclusively found protein number with at least two or more hits in HpHG or LpHG samples were shown
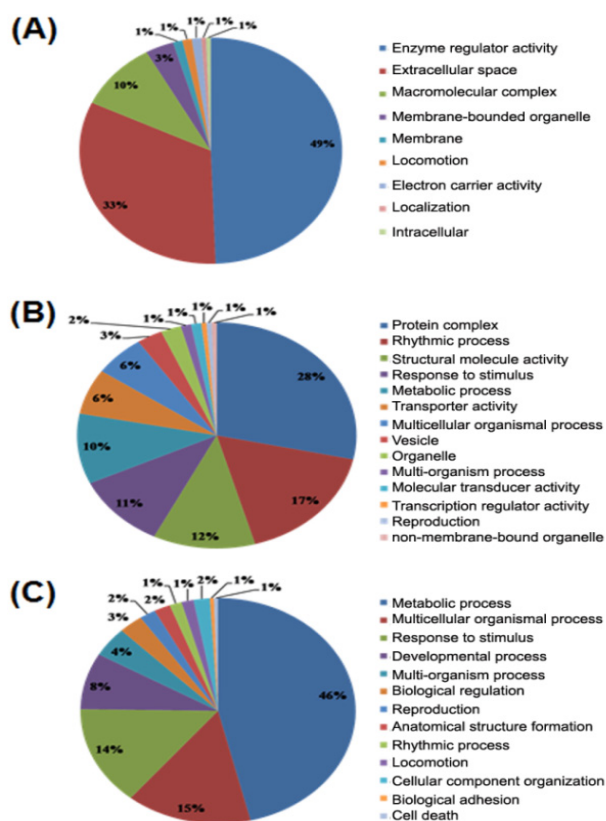
Figure 3. Gene ontology of a total of 128 identified proteins classified according to molecular function (A), cellular components (B), and biological process (C)
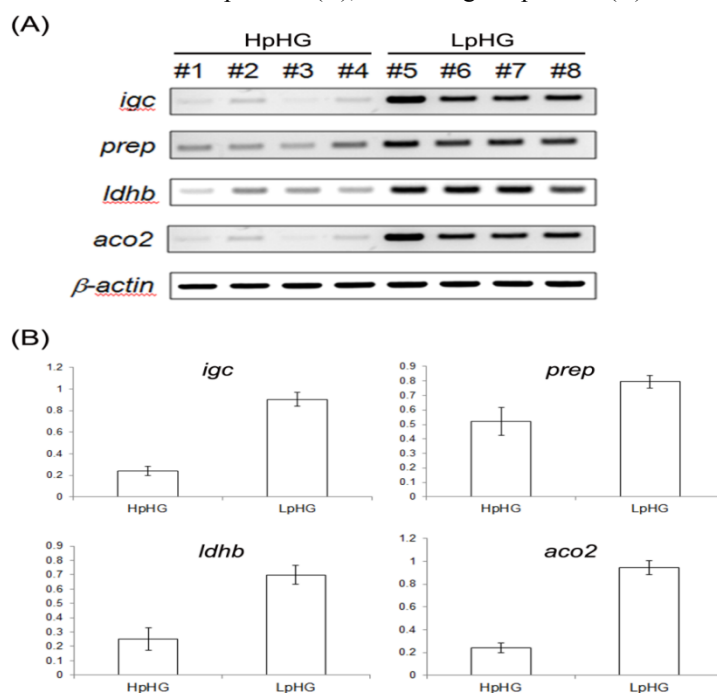

Figure 4. Relative expression levels quantified by reverse-transcriptase-mediated PCR analysis. (A) Individual RT-PCR of gene such as *igc* (Ig lambda chain C region), *prep* (propyl endopeptidase), *ldhb* (lactate dehydrogenase-B) and *aco2* (aconitate hydratase, mitochondrial) was presented as lane number 1-4 (HpHG); lane number 5-8 (LpHG). Beta-actin was used as internal standard expression marker. (B) The histograms representthe average of gene/*β-actin*ratios, measured with the ImageJ, densitometry software (http://rsbweb.nih.gov/ij/index.html)

Acknowledgements

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