



Proteomic Approach Analysis of Mammary Membrane Proteins Expression Profiles in Holstein Cows*

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ABSTRACT : To investigate host defense mechanisms for protecting the mammary gland from mastitis infection, the membrane fraction of mammary tissues from Holstein cows was purified by differential velocity centrifugation, and then the sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) separated proteins were identified by ion trap mass spectrometer equipped with a Surveyor high performance liquid chromatography (HPLC) system. A total of 183 proteins were identified. Bioinformatics software was applied to analyse physicochemical characteristics of the identified proteins and to predict biochemical function. These data may provide valuable information to investigate the mechanisms of mammary gland milk secretion and infectious disease, and enable a clear identification of proteins and potential protein targets for therapies. (**Key Words :** Holstein Cows, Mammary Gland, Proteome, Bioinformatics Software)

INTRODUCTION

Sequencing of several available genome information estimated that 15%-20% of ORFs coded for membrane proteins irrespective of the species and the genome size (Mitaku et al., 1999). Membrane proteins have close contact with membrane system that in the interface between the outer and inner worlds across the membrane. Such a membrane system contains many kinds of receptor proteins, transporter proteins and channel proteins which have critical roles in the interaction of the cell with its environment and in the function of subcellular organelles (Kashino, 2003). Membrane proteins are also important for pharmacological action, and many successful drugs act by modulating the activity of membrane proteins (Patton, 1999). Thus, the comprehensive and reliable identification and characterization of the expressed membrane proteins will reveal yet undiscovered, but highly specific features of these membranes that therefore used to elucidate and

understand cellular mechanisms, to account for substantially more cellular functions.

Generally, membrane proteins are very hydrophobic, and have an alkaline pH and have single or several transmembrane domains. This leads to membrane proteins less amenable to solubilization by conventional extraction buffers and also susceptible to precipitation during isoelectric focusing (IEF) separation. Proteomic analysis of membrane proteins has been considered to be difficult of using two-dimensional gel electrophoresis that offers the highest resolution available to date. Thus, many studies demonstrated that optimization of extraction conditions by using alteration of buffers, chaotropes, and detergents is sufficient to reliably achieve high-resolution maps of membrane proteins (Rabilloud et al., 1999; Luche et al., 2003; Churchward et al., 2005). While the restriction with regard to these limitations mentioned above is usually compensated for by particular biochemical techniques, mainly liquid chromatography tandem mass spectrometry (LC-MS/MS), exploiting LC-MS/MS for comparative investigations of integral membrane proteins are currently raised (Wu and Yates, 2003). LC-MS/MS takes advantage of MS-based procedures for automatic peptide fragmentation and the enormous amount of available genome data. Otherwise, highly efficient membrane protein extractions are carried out with a strong ionic detergent of sodium dodecyl sulfate (SDS) that solubilizes membrane

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proteins to protein-SDS complexes for one-dimensional PAGE. This is a robust method that resolves proteins of different molecular weights generally independent of their biochemical properties. Up to the present time, analysis of membrane proteome pattern based on the SDS-PAGE combination LC-MS/MS was widely used in mammalian tissue, isolated cell lines, yeast and bacteria (Rahbar and Fenselau, 2004; Wehmhoner et al., 2005; Zhang et al., 2005).

The mammary gland is a complex organ that provides neonatal offspring with milk for nourishment and disease resistance. It is protected by a variety of defense mechanisms, specific and innate immune factors associated with mammary gland tissues and secretion also play a vital role in protecting the gland from infectious disease (Sordillo et al., 1997). Previous the researches investigated the proteins in milk (skim milk, whey, and milk fat globule membrane) using proteomic approach, some proteins were identified that are involved in host defense to protect against infection and associated with disease (Baeker et al., 2002; Hogarth et al., 2004; Reinhardt and Lippolis, 2006; Smolenski et al., 2007). Besides, more recently proteomics approach has been widely used in characterized protein expression profiles in dairy cows for cellular and molecular mechanisms (Kim et al., 2006; Daniels et al., 2006; Ohsaki et al., 2006). However, little attention used proteomic analysis of membrane proteins of mammary tissues from Holstein cows. Here we systematically applied the SDS-PAGE separated proteins and followed by ion trap mass spectrometer equipped with a Surveyor HPLC system identified, in order to investigated the membrane protein composition. Further, to elucidate the mechanisms of mammary gland tissue infected disease and hope toward a clear identification of protein and potential protein targets for therapies and immune manipulations.

MATERIALS AND METHODS

Samples preparation

Samples of the mammary gland tissues were prepared from a dairy farm in the west of China. The current study comprised 8 healthy Holstein Cows uninfected with mastitis during the phases of late lactation that were determined according to LMT (Lanzhou Mastitis Test, Lanzhou, China) with similar to CMT method. The mammary gland was bisected in the midsagittal plane, mammary tissue samples about 9 cm³ (3 cm×3 cm×1 cm) were prepared from freshly obtained cistern tissues under 2 cm of the mammary epithelium that located in the central portion, to remove milk by washing with PBS, then tissue samples were frozen and stored in liquid nitrogen until needed. All animal care and handling procedures were approved by the state, local and experimental station guidelines and regulations.

Preparation of membrane protein

All the procedures were carried out for protein extraction at 4°C to minimize protease activity until otherwise stated. Membrane fractions were isolated as previously described by Butt and Coorsen (2005) with the modifications to separate the cellular membranes. Frozen mammary gland samples (about 1 g) were pooled and manually homogenized with a mortar and pestle in liquid nitrogen, the tissue powder was resuspended in a hypotonic lysis buffer consisting of 50 mM HEPES pH 7.4, 5 mM EDTA, and Protease Inhibitors (Cat. No. 80-6501-23, Amersham Biosciences, Sweden), then transfer into a glass homogenizer and homogenized on the ice. To ensure thorough homogenization, the homogenate was subjected to two round of freeze-thaw and centrifuged at 1,000 g for 10 min at 4°C, the suspension was collected and pellet was homogenized again, then supernatants from each centrifugation-step were pooled and equal volume of 2×PBS was added to restore isotonicity. Sequently, membrane fractions were collected by ultracentrifugation at 120,000 g for 2 h at 4°C using an Optima™ Max Ultracentrifuge with the MLA-80 rotor (Beckman-Coulter, USA). In order to removed soluble/cytosolic proteins from membrane/membrane associated proteins, the membrane pellet was gently resuspended in ice-cold 1×PBS with protease inhibitors, and collected as described above. That step was repeated twice for collected purified membrane pellets. Washed membranes were dissolved in an SDS-containing solution (63 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol) by pipetting and vortexing, and incubated for 1 h with periodic vortexing. Any small insoluble fraction by centrifugation at 10,000 g for 10 min, the suspension was collected and used as membrane protein samples were stored in aliquots at -75°C until use.

Separation of membrane proteins

The samples of membrane proteins were mixed with an equal volume SDS sample buffer and heated to 95°C for 5 min. Separation of membrane proteins was carried out in a Protean II xi (Bio-Rad, USA) electrophoresis system, using 4% stacking and 12% separating polyacrylamide gels and Tris-glycine-SDS pH 8.3 as the electrode buffer. The gels were started with 50 V and continued with 200 V until the bromophenol blue dye marker reached the bottom of the gel. The protein lanes were visualized using conventional Coomassie Brilliant Blue R-250 staining, then the gels were shaken in MilliQ water until the background staining was removed. The gel lane of each sample was sliced by following visualized positions, total of 19 gel sections were excised from the gel corresponding to their apparent mass regions. Then, the gel slices were placed into 1 mm³ cubes for further destaining and digestion.

Digestion and extraction peptide

The small cubes from Coomassie-stained gels were washed twice with 400 μ l MilliQ water for 30 min, and then washed twice with 200 μ l acetonitrile/25 mM ammonium bicarbonate (1:1 v/v) for 30 min at room temperature. Subsequently, the gel pieces were shrunk in acetonitrile and completely dried. Obtained particles were rehydrated with 10 mM dithiothreitol to reduce the proteins for 1 h at 56°C, and then washed twice with 100 μ l acetonitrile/25 mM ammonium bicarbonate (1:1 v/v). After the gel pieces were shrunk in acetonitrile, reduction proteins in gel were followed by alkylation using 55 mM iodoacetamide for 1 h in the dark, this procedure was repeated. Subsequently, the dried gel pieces were subjected to incubation in 25 mM ammonium bicarbonate containing 0.01% sequence-grade trypsin at 37°C overnight. Then, the peptides were extracted with 50% acetonitrile and 0.2% formic acid, and applied one round of vortexing and sonication (30 min), the supernatant was transferred to a clean Eppendorf, this step was repeated, the supernatant was pooled. For reproducibility, duplicate lanes were prepared for membrane fraction.

Protein identification and database search

Peptide mass was determined on an ion trap mass spectrometer (LCQ Deca XP, Thermo Finnigan) equipped with a Surveyor HPLC system (Thermo). Peptide mixtures were diluted to 0.05-0.1 mg/ml and 20 μ l was injected onto a 150 \times 0.18 mm BioBasic-18 column (Thermo) at a flow rate of 120 μ l/min. Mobile phase A consisted of water and 0.1% formic acid, and mobile phase B consisted of acetonitrile and 0.1% formic acid. Peptides were eluted with a 0.1% formic acid/acetonitrile linear gradient. MS spectra

for all samples were measured with an overall mass/charge (m/z) range of 400-2,000 with a maximum ion injection time of 200 ms followed by MS/MS scans of the three most abundant ions in each MS scan, MS/MS was performed in data-dependent mode. Peptides were identified using SEQUEST software (Bioworks 2.0, Thermo Finnigan), which used the tandem mass spectra of peptide ions to search against the publicly available NCBI nonredundant protein database (<http://www.ncbi.nlm.nih.gov>). The protein identification criteria that were based on delta correlation score Delta CN (≥ 0.1) and cross-correlation score Xcorr (one charge ≥ 1.9 , two charges ≥ 2.2 , three charges ≥ 3.75). Proteins were considered identified when at least two peptides met these requirements per protein, additional peptides were then included to maximize the peptide coverage of the identification. All protein identifications with an error rate of less than 10% were summarized and directly exported into Excel.

RESULTS

Membrane proteins were separated by SDS-PAGE. For subsequent analysis, the gels from three independent experiments were cut into total of 19 consecutive sections according to stained density. Reducing and alkylating the protein complexity in gels were performed and prepared for LC ion trap MS analyses as described in the methods. Tryptic peptides from the in-gel digested and extracted, and then subjected to ion trap mass spectrometry, resulted in the identification of 183 proteins (Supplementary Table I) based on the SEQUEST software (Bioworks 2.0, Thermo Finnigan). As seen in our dataset, many hypothetical/prediction proteins were found to be expressed in the

Table 1. i) Characterization of identified proteins with transmembrane domain

Protein name	Accession	Mw (Da)	pI	GRAVY	Transmembrane domain
2'-5' oligoadenylate synthetase 3	BAB18647	121,236.2	8.77	-0.251	1
Ab1-233	AAP92556	79,122.7	9.39	-0.424	1
ABCA3 variant protein	BAE06126	191,317.9	7.39	0.082	9
alpha 3 type VI collagen isoform 2 precursor	NP_476505	325,293	6.12	-0.236	1
At1g19700-like protein	AAZ73651	27,292.1	9.6	-0.776	1
Cytochrome c-type biogenesis protein comE	Q5NN30	15,922.3	9.52	-0.036	1
Chain B, Structure of Human Placental S-Adenosylhomocysteine Hydrolase	1A7AB	47,427.2	6.04	-0.172	1
Chain E, The Structure of Bovine F1-ATPase Covalently Inhibited with 4-Chloro-7-Nitro benzofurazan	1NBME	51,431.5	4.97	0.011	1
CYTOCHROME P450 71B5	CAB64231	56,373.7	6.52	-0.029	1
D-galactarate dehydratase	NP_533487	55,971.4	5.83	-0.18	1
DNA maturation protein	Np_042010	66,260.6	5.31	-0.296	2
envelope glycoprotein	ABE03113	95,988.1	8.48	-0.222	3
exported copper resistance protein	CAE51680	33,232.2	5.24	-0.373	1
FASN variant protein	BAE06070	277,370.1	6.2	-0.08	1
histone macroH2A1.2	AAC33433	39,601.7	9.8	-0.263	1

Table 1. ii) Characterization of identified proteins with transmembrane domain

Protein name	Accession	Mw (Da)	pI	GRAVY	Transmembrane domain
Homo sapiens eukaryotic translation initiation factor 3, subunit 5 epsilon, 47kDa	AAP36731	37,667	5.24	0.054	1
Homo sapiens tubulin, beta, 4	AAP36356	88,494.9	5.55	0.129	9
isocitrate dehydrogenase 1 (NADP+), soluble	NP_005887	46,660	6.53	-0.392	1
KIAA0866 protein	BAA74889	228,722.2	5.4	-0.856	1
methionyl-tRNA synthetase-like protein	CAB78420	89,853.6	6.65	-0.431	2
mevalonate kinase	CAA54820	38,798.6	6.35	0.048	2
mitochondrial ATP synthase, H ⁺ transporting F1 complex beta subunit	ABD77240	48,113.8	4.95	0.003	1
Myb-binding protein 1A	O35281	152,286.1	9.07	-0.458	2
neoplasm-related C140 product	AAB30819	24,044.3	10.41	-0.7	1
nitrate transporter	YP_544433	42,508.1	9.8	0.808	12
NudC domain-containing protein 3	Q81VD9	40,769	5.1	-0.591	1
otopettrin 3	NP_839947	66,295.9	8.96	0.376	12
myosin, heavy chain 9, non-muscle	CAB05105	226,531.7	5.5	-0.854	1
PREDICTED: hypothetical protein	XP_001069125	12,453.3	4.63	0.424	2
PREDICTED: hypothetical protein	XP_001078693	183,437.5	8.63	-0.287	3
PREDICTED: prohibitin isoform 7	XP_511949	29,804.1	5.57	0.024	1
PREDICTED: similar to Calgranulin B	XP_547585	38,628.1	5.59	-0.619	1
PREDICTED: similar to	XP_001058641	210,918.9	8.15	-1.121	2
Chromodomain-helicase-DNA-binding protein 2					
PREDICTED: similar to DNA-binding protein TAXREB107 isoform 12	XP_509392	32,728.7	10.59	-0.732	1
PREDICTED: similar to keratin 6 irs3	XP_610486	58,835.6	6.96	-0.384	2
PREDICTED: similar to leucine rich repeat and sterile alpha motif containing 1	XP_231157	83,931.6	5.71	-0.555	1
PREDICTED: similar to predicted CDS, polyprotein family member	XP_786079	118,114.9	9.64	-0.487	2
PREDICTED: similar to signal-induced proliferation-associated 1 like 2	XP_392312	174,222.3	8.13	-0.767	1
PREDICTED: similar to tetratricopeptide repeat domain 21B, partial	XP_650803	165,164.9	7.79	-0.329	1
PREDICTED: similar to toll-like receptor 3	XP_782579	101,682.7	6.42	-0.027	2
ribosomal protein L3 isoform b	NP_001029025	40,152.5	10.23	-0.599	1
ribosomal protein L4	AAH66925	47,698	11.07	-0.611	2
ribosomal protein L8	BAA25829	14,254.5	10.22	-0.356	1
unknown (protein for IMAGE:3138929)	AAH07267	101,465.2	6.46	-0.049	1
unnamed protein product	BAC05073	56,309.4	5.17	-0.719	1
unnamed protein product	CAA27246	57,955.6	5.8	-0.03	1
vesicle amine transport protein 1 homolog	AAI01883	43,119.4	6.17	-0.048	1
voltage-dependent N-type calcium channel subunit alpha-1B	Q02294	262,256.1	8.82	-0.229	18

membrane fraction. However, there were some MS/MS spectra that did not result in any identifications of proteins or gave out ambiguous database search results and therefore did not pass the threshold for positive identification.

Physicochemical characteristics of the identified proteins

The 183 identified proteins were categorized according to their different physicochemical properties by using bioinformatic software tools ProtParam (<http://us.expasy.org/tools/protparam.html>) analysis, such as molecular weight (Mw), isoelectric point (pI), and hydrophobicity (Grand

average of hydrophobicity, GRAVY). The GRAVY values determined according to Kyte and Doolittle provide an image of the hydrophobicity of the whole protein. Generally proteins exhibiting positive values are considered hydrophobic and negative scores are deemed hydrophilic. The membrane protein distribution patterns were compared resulted from the above characteristics listed Figure 1.

In the present work, for the total 183 proteins, 134 proteins distribute among 10 kDa-60 kDa Mw intervals, which are compatible with general SDS-PAGE, the smallest and the largest molecular mass obtained are 4.87 kDa and

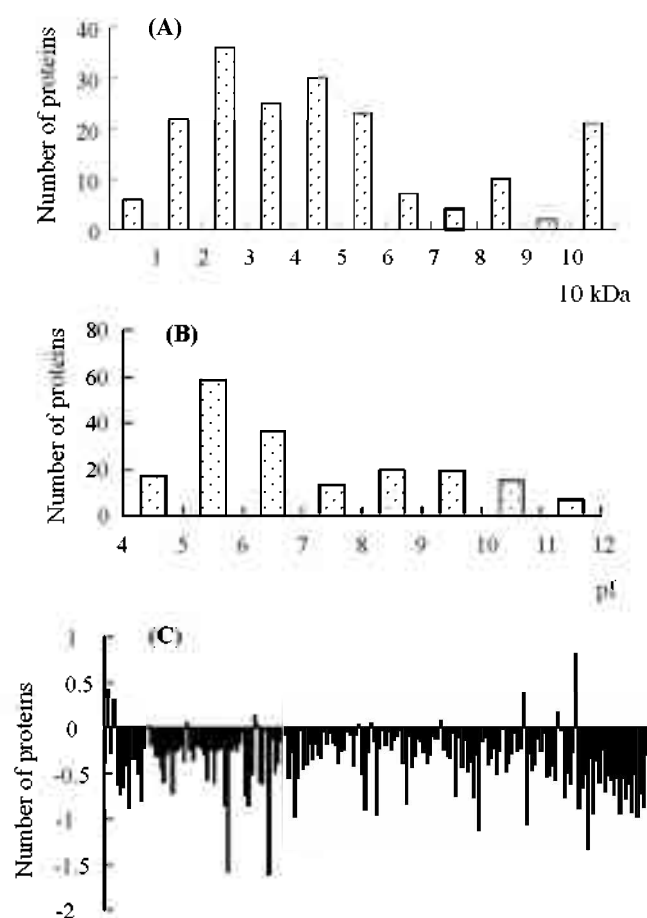


Figure 1. Physicochemical characteristics of the identified proteins, distribution of the proteins in relation to their theoretical molecular mass (A), pI (B), GRAVY values (C).

325.29 kDa, respectively. The protein with the minimum Mw was the casocidin-I (AAB35385), and the maximum Mw was the alpha 3 type VI collagen isoform 2 precursor (NP_476505). Regarding the pI distribution, the 183 proteins distributed cross a wide pI range from 4.27 to 11.75. 162 proteins distribute among pI 4-10 intervals but 21 proteins with pI >10 were identified that beyond the 2D-PAGE separation capability. The protein with the maximum pI at 11.75 was the ribosomal protein L13 (NP_990330).

For the 183 identified proteins, their GRAVY values vary in the range of -1.61 - +0.808. 13 proteins have positive values. In addition, 65 proteins had GRAVY values >-0.25 that have been identified. These proteins hardly detected in 2D-PAGE that are generally separation hydrophilic proteins, also with negative GRAVY values.

Transmembrane proteins prediction

Transmembrane domain of identified proteins was predicted by TMHTOP (<http://www.enzim.hu/hmmtop/>). There are 48 possible transmembrane proteins with one to eighteen trans-membrane regions. Among them, 31 proteins

with one trans-membrane region, 10 with two transmembrane regions, and 7 proteins with three or more transmembrane regions. Those proteins listed in Table 1.

Functional annotation

As an approach to understanding mammary gland membrane protein biological process and molecular functions, we searched for known Gene Ontology categories within identified proteins. 152 of proteins were detected and reflected a broad range of molecular functions, and can be categorized in nine major functional groups. The category having the greatest number of proteins was binding activity, others proteins involved in structural molecule activity, catalytic activity, and transporter activity (Figure 2A). According to their biological process, associated with physiological process, cellular process, and developmental process are main biological function (Figure 2B). Therefore, categories may appear to be overrepresented just because they belong to more abundant protein classes. Such as sulfonate/nitrate transport system ATP-binding protein (YP_172698), its molecule function was categorized binding and catalytic activity. 14-3-3 protein zeta chain (AAB22943) involved in biological process contained cellular process, developmental process and biological regulation.

DISCUSSION

Membrane proteins present in the interface between the outer and inner worlds across the membrane that have critical roles in the cell important processes and events to fulfill key functions, as signal transduction, energy conversion, transport and recognition. They are also involved in a wide range of diseases, and become large majority targets for the diagnostic and therapeutics. Thus, knowledge of membrane proteome is urgently required. The objective of this study was to identify the membrane proteins of mammary gland from Holstein cows through SDS-PAGE combination ion trap mass spectrometry. SDS is a strong anionic detergent which denatures proteins and especially solubilizes hydrophobic membrane proteins, and helps to keep them moving in the gel with equal charge or charge densities that separates proteins without any pI discrimination. Inevitably, per gel band may contain several denatured proteins or polypeptides that lead to a mixture of peptides in in-gel digestion. However, ion trap mass spectrometer equipped with a Surveyor HPLC system provides the high resolution peptide separation and detection sensitivity required for the analysis of complex peptide mixtures. As a result, only 183 proteins were identified. This phenomenon was probably a consequence of the limited number of bovine sequences present in

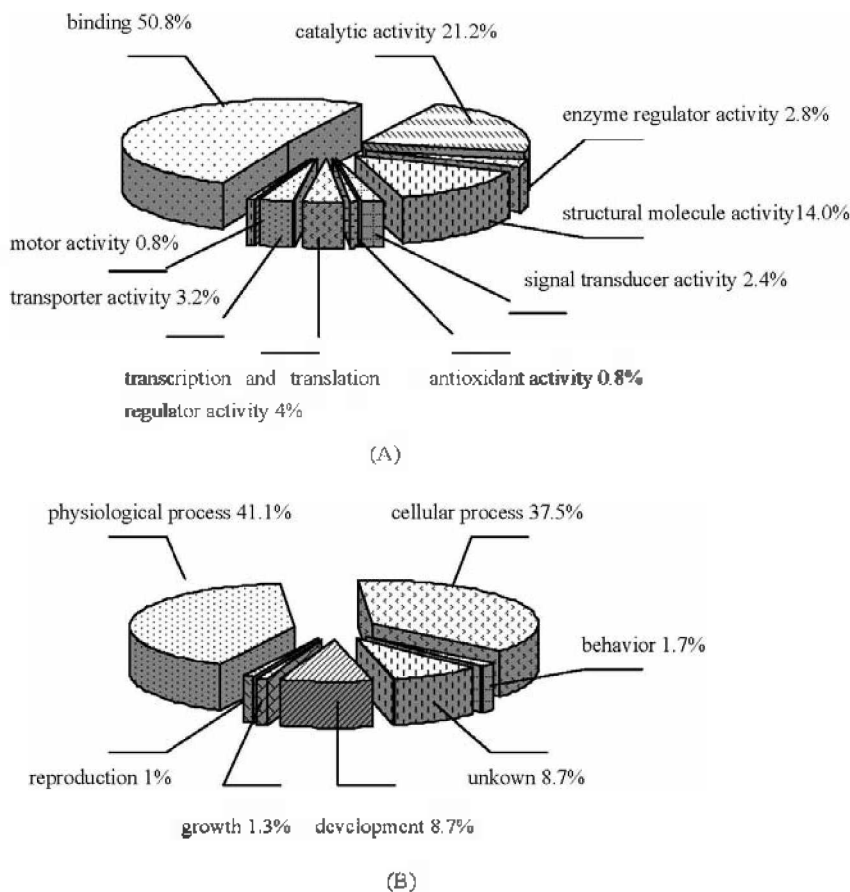


Figure 2. Biological function classification of the identified proteins based on the Gene Ontology annotation, (A) shows molecule function category, (B) shows biological process category.

databases.

For the 183 identified proteins in this research, that contained 21 proteins with basic pIs higher than 10, and 13 proteins with GRAVY positive values, and 48 transmembrane proteins with one or more trans-membrane region, which usually are undetectable by 2D-PAGE approach. As previous observations, the proteins detected in 2D-PAGE gels are in general hydrophilic with negative GRAVY values and few transmembrane regions in membrane proteins (Lehner et al., 2003; Zhang et al., 2005). Fountoulakis and Suter (2002) identified 170 proteins from 2D-PAGE gels of rat liver, only 14 proteins had low positive GRAVY values with maximum 0.21, and only one protein with three transmembrane domains. Later, Zhang et al. (2005) used different lysis buffers and different procedures for PM protein treatments of mouse liver to extend the solubilization of membrane proteins, thus, they found approximately 28% of the 175 proteins detected by 2D-PAGE-MS in the membrane fraction carried at least one transmembrane domain. 8% of the proteins have positive GRAVY values. Considering the 1D-PAGE separation, this method increased the number of membrane proteins with

two or more transmembrane domains (up to 88). So, compared with 2D-PAGE, 1D-PAGE separation combination LC-MS/MS could identify more integral membrane proteins. In our study, without specific methods for enrichment or treatment of membrane proteins, 48 of the 183 proteins with one or more transmembrane proteins were identified, in which 7 proteins have three or more transmembrane domains.

Furthermore, identified proteins were compared against a reference set of complete Gene Ontology annotations. These proteins were associated with physiological process, cellular and developmental process in biological process, and involved in binding, catalytic activity, and structural molecule activity according to molecule function. From their functional categories, it can be postulated that these proteins are intensively associated with membrane and membrane-associated proteins and participated in membrane biochemical processes. For example, 127 proteins were identified as being involved in binding activity, participating in protein, ion, lipid, and DNA binding activity that associated with cell adhesion, cell-cell signaling, signal transduction. 53 proteins could be

functional in structural molecule activity. In particular, using the Gene Ontology criteria, some proteins were detected that classified as host defense function, such as Crystal Structure Of Bovine Cu Zn Sod (IE9QA), microsomal epoxide hydrolase (AAF87734), and Ig gamma-2 chain C region (S06611), etc. They were involved in antigen recognition, inflammatory response, and regulating of binding that associated with protecting the mammary gland from infection. However, the Gene Ontology annotations do not fully account for the multiple functions of proteins in reported articles. For example, casocidin-I is casein-alpha s2 derived antibacterial peptide that is not in annotations and classed as host defense-related, despite clearly having such a role (Zucht et al., 1995). The S100 calcium binding protein family, classed by Gene Ontology as binding proteins, that may also have a role in host defense (Cole et al., 2001). In our study, proteins similar to S100 protein family were also identified in mammary gland of dairy cows contained hypothetical protein LOC532569 (NP_001039793), PREDICTED: similar to Calgranulin A (XP_547583), PREDICTED: similar to Calgranulin B (XP_547585), PREDICTED: similar to S-100 calcium-binding protein A2 - human isoform 5 (XP_513816). Interestingly, these proteins were only predicted by MS/MS analysis and research, this result may be those proteins contained other function domains except for S-100/ICaBP-like conserved domain in amino acid sequence of proteins. Moreover, some proteins participated in multiple key functions in cell process. Annexin V belongs to annexin family of protein associated with membrane-binding involved in a variety of physiological and pathological processes, as structural organization of the cell, intracellular signaling by enzyme modulation and ion fluxes, and growth control (Benz and Hofmann, 1997; Moss and Morgan, 2004). 14-3-3 protein present in multiple isoforms form a family of highly conserved cellular proteins that play key roles in the regulation of central physiological pathways, including proteins involved in signal transduction, cell cycle control, apoptotic cell death and malignant transformation (Darling et al., 2005; van Heusden, 2005; Tzivion et al., 2006).

In summary, this study first used SDS-PAGE combination ion trap mass spectrometry identified membrane proteins that can help us to understand the proteomic profile for the membrane composition of the mammary gland tissue from Holstein Cows. The results may provide valuable information for the investigating of the host mammary immune system molecular mechanism and response to defense against pathogens at the protein level, and certain membrane proteins may become important targets for defense and therapeutics through protein interaction analysis.

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