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A Proteome Reference Map for Porcine Plasma Proteins

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ABSTRACT

To profile the proteome in porcine plasma, blood samples were collected from adult male barrows and those plasma were retrieved. For the depletion or pre-fractionation of high-abundance proteins, plasma samples were treated with commercial kits. Then, protein profiling was initiated using one and two-dimensional electrophoresis. Proteins were spotted and then identified by MALDI-TOF-TOF and LC-MS-MS. In the results, more than forty six proteins were identified and the reference map was constructed. The pre-treatment for the removal of high-abundance proteins caused the changes in 2-DE images and some of the proteins were newly uncovered after the most of high abundant proteins were removed. However, it is expected for further steps necessary to identify more low-abundance proteins that may contain potential bio-markers.

(Key words : 2-DE, Proteome, Porcine, MALDI-TOF-TOF, LC-MS-MS)

INTRODUCTION

Bio-markers allow early detection of disease, evaluation of health condition or animal selection with high production potentials. Although a wide variety of methods have been applied to find bio-markers in blood for human and live animals, the approaches considering proteins as potential bio-marker candidates have the advantage that proteins are more reflective of a biological system. It was demonstrated that some serum biochemical values including lactate dehydrogenase, total cholesterol, magnesium, and VA changed significantly with the fattening stage advanced and those were proposed as metabolic biomarkers (Adachi *et al*., 1997). Indeed, a proteomic approach has been continuously developed and improved technically to assess the medical, physiological and metabolic condition of human individual body and its progress has been slowly applied to other animals and livestock. There has been growing interest in pig proteome for the use of growth performance prediction and early disease diagnosis, as well as, of clinical applications for human transplantation. Some basic studies of different organs or cell types have been performed, including 2-DE maps of components of the photoreceptor matrix (Hauck *et al*., 2005), of testis (Huang *et al*., 2005) and prostate (Manaskova *et al*., 2002), of congestive organs of selected pig breeds (Park *et al*., 2005), and of alveolar macrophages (Rodriguez *et al*., 2001). In all cases, knowledge of protein dynamics in blood will improve our understanding of metabolic regulatory processes, growth and immune status of individual pig. Here, we present an approach of blood plasma proteome reference map as prerequisite for the identification of bio-markers related to the growth performance, meat quality and disease condition.

MATERIALS AND METHODS

Animals

Blood were sampled from five heads of crossbred pigs weighing over 30 kg up to 40 kg which were managed at feeding barn in the National Institute of Animal Science. All experimental procedures and the care of animals were conducted in accordance with the guidelines of the Animal Care and Use Committee (IACUC)

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of the National Institute of Animal Science in Korea.

Protein Preparation for 2-DE

Animal experimentation was approved by the Animal Care and Concern committee of the National Institute of Animal Science, in Suwon. To profile the proteome in porcine plasma, blood was collected from the assigned pigs above and the plasma was retrieved and kept in −80℃ until proteome analysis. Proteins were extracted from plasma in lysis buffer containing 8 M urea and 10 mM DTT as previously described (Wang *et al*., 2008). High-abundance proteins in the plasma samples were depleted by commercial kits (Bio-Rad, ProteoMiner Protein Enrichment Kit). The proteins collected by centrifugation were completely dried using speed-vac and re-dissolved in the 2-DE sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.5 % pH 3~10NL IPG buffer) for isoelectric focusing (IEF). The concentration of the total protein in the sample was determined by Bradford's protein assay method.

2-DE and Image Analysis

500 μg of protein was loaded onto the immobiline dry strips pH 3-10 NL (GE Health care). The rehydrated strips were focused on IEF system (AP Biotech, Sweden) for ~80 kVh at a maximum of 8,000 V in a rapid ramping mode with maximum current per strip of 50 μA. Equilibration of the immobilized pH gradient strips was performed in two steps: reduction followed by alkylation (Ahmed and Bergsten, 2005). The second dimension was run on 12.5% polyacrylamide sodium dodecyl sulphate gels (26×20 cm) with a constant voltage of 100 V for 30 min, 250 V for 6 hr using the EttanDALT II system (Amersham Bioscience, Piscataway, USA). The proteins were visualized using silver or Coomassie Brilliant Blue (CBB) G-250 staining method. The silver-stained gels were scanned using GS-800 scanner (Bio-Rad) at an optical resolution of 300 dpi. Spot detection, quantification and matching were performed using Image Master Version 7.0 (GE healthcare). A match set consisting of three images, each from one depot was created. To correct the variability due to silver staining and to reflect the quantitative variations of protein spot, the individual spot volumes were normalized by dividing their optical density (OD) values by the total OD values of all the spots present in the gel. The significance of the expression difference of proteins between samples was estimated by student's *t*-test, $p<0.05$ using Image Master (ver 7.0) software.

Protein Identification

The stained protein spots were excised from gels using a punch and placed in 500 μl Eppendorf tubes. The proteins were digested in-gel with trypsin as described by (Hellmann *et al*., 1995). Briefly, each spot was destained with 50 μl 50% acetonitrile (ACN) in 50 mM NH_4HCO_3, incubated at 37℃ for 30 min and repeated once. Then the gels were reduced and alkylated. The gel pieces were digested overnight with trypsin (20 μg/μl) in 50 mM NH_4HCO_3 containing 10% ACN. The digest was then vortexed for 30 min and dried using speed vac. The dried extracted peptides were resuspended in 1 μl solution containing pure water:ACN:trifluoracetic acid (TFA) (93:5:2). Solution-phase nitrocellulose target preparation was used according to the method reported by Landry *et al.* (2000). α-cyano-4-hydroxycinnanic acid (CHCA) (40 mg/ml) and nitrocellulose (20 mg/ml) were prepared separately in acetone and mixed with 2-propanol at a ratio of 2:1:1. The matrix solution mixed with the sample at a ratio 1:1, 0.5-0.3 μl was spotted onto the target and dried. The immobilized samples were washed with 1% formic acid twice and dried prior to the MALDI-TOF-MS/MS analysis. Sample peptide masses were obtained using Applied Biosystems 4700 Proteomics analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) in the positive ion reflector mode. MS/MS analysis was performed on the 5 most abundant ions and the proteins were identified by searching the SWISS-PROT and National Center for Biotechnology Information databases using the Mascot programs (Matrix Science, London, UK). Mass accuracy was considered to be within 50 ppm for peptide mass analysis and within 100 ppm for MS/MS analysis. For protein identification, known contamination peaks such as those of keratin and autoproteolytic were removed, and molecular weight, pI and protein scores were considered. For LC-MS-MS, 1-DE protein bands were incised and injected onto an MDLC system (GE healthcare) with a C18 capillary column. Proteins were eluted with a linear gradient from 5 to 45% acetonitrile developed over 120 min at a flow rate of 500 nl/min, and effluent was electrosprayed into the LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Data were collected in the "Triple Play" (MS scan, Zoom scan, and MS/MS scan) mode and filtered and analyzed by a proprietary algorithm. Database searches against a porcine database (derived from sequence data available at Pubmed Protein) were carried out using both the X! Tandem and SEQUEST algorithms.

RESULTS AND DISCUSSION

Proteomic Profiling: Forty Six Proteins Identified (MALDI-ToF-ToF, 19 Proteins; LC-MS-MS, 37 Proteins)

Table 1. The porcine plasma protein identified by MALDI-ToF/ToF

No.	Protein names and accession numbers[1)]	Species	M.W[2)]	PI[2)]	Count	Score
6	(P06867) Plasminogen precursor (EC 3.4.21.7) [Contains: Plasmin heavy chain A; Activation peptide	PLMN_PIG	91272	7.2	25	328
7	(P06867) Plasminogen precursor (EC 3.4.21.7) [Contains: Plasmin heavy chain A; Activation peptide	PLMN_PIG	91272	7.2	20	221
10	(P00734) Prothrombin precursor (EC 3.4.21.5) (Coagulation factor II) [Contains: Activation peptide	THRB_HUMAN	71474.7	5.6	4	62.9
12	(P48819) Vitronectin precursor (Serum-spreading factor) (S-protein)	VTNC_PIG	53336.9	5.6	10	196
19	(P01790) Ig heavy chain V region M511	HV21_MOUSE	31.7	85.4	13757.7	8.01
22	(P20305) Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin) (Fragment)	GELS_PIG	85064.9	5.9	17	128
24	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	22	185
25	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	29	333
25	(P49064) Serum albumin precursor (Allergen Fel d 2)	ALBU_FELCA	70610.7	5.5	9	109
25	(P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	ALBU_BOVIN	71244.2	5.8	10	78.1
25	(P35747) Serum albumin precursor (Allergen Equ c 3)	ALBU_HORSE	70550.1	6	6	60.8
25	(P49822) Serum albumin precursor (Allergen Can f 3)	ALBU_CANFA	70555.7	5.5	6	58.5
26	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	28	329
26	(P49064) Serum albumin precursor (Allergen Fel d 2)	ALBU_FELCA	70610.7	5.5	8	102
26	(P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	ALBU_BOVIN	71244.2	5.8	10	75.8
26	(P49822) Serum albumin precursor (Allergen Can f 3)	ALBU_CANFA	70555.7	5.5	6	65.8
27	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	28	352
27	(P49064) Serum albumin precursor (Allergen Fel d 2)	ALBU_FELCA	70610.7	5.5	9	104
27	(P35747) Serum albumin precursor (Allergen Equ c 3)	ALBU_HORSE	70550.1	6	7	73
27	(P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	ALBU_BOVIN	71244.2	5.8	10	72
27	(P49822) Serum albumin precursor (Allergen Can f 3)	ALBU_CANFA	70555.7	5.5	7	59.8
28	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	28	391
28	(P49064) Serum albumin precursor (Allergen Fel d 2)	ALBU_FELCA	70610.7	5.5	8	119
28	(P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	ALBU_BOVIN	71244.2	5.8	9	89.8
28	(P49822) Serum albumin precursor (Allergen Can f 3)	ALBU_CANFA	70555.7	5.5	7	86.5
28	(P35747) Serum albumin precursor (Allergen Equ c 3)	ALBU_HORSE	70550.1	6	6	63.4
28	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	26	281
29	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	27	344
29	(P49064) Serum albumin precursor (Allergen Fel d 2)	ALBU_FELCA	70610.7	5.5	10	106
29	(P02769) Serum albumin precursor (Allergen Bos d 6) (BSA)	ALBU_BOVIN	71244.2	5.8	11	71.7
29	(P35747) Serum albumin precursor (Allergen Equ c 3)	ALBU_HORSE	70550.1	6	6	63.9
37	(P29700) Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Fragment)	FETUA_PIG	39198.7	5.5	10	72.8
39	(P29700) Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Fragment)	FETUA_PIG	39198.7	5.5	8	56.4
40	(Q3MHN5) Vitamin D-binding protein precursor (DBP) (Group-specific component) (Gc-globulin) (VDB)	VTDB_BOVIN	54903.7	5.4	6	97.7
40	(P48819) Vitronectin precursor (Serum-spreading factor) (S-protein)	VTNC_PIG	53336.9	5.6	6	63.3
41	(P48819) Vitronectin precursor (Serum-spreading factor) (S-protein)	VTNC_PIG	53336.9	5.6	13	278
42	(P48819) Vitronectin precursor (Serum-spreading factor) (S-protein)	VTNC_PIG	53336.9	5.6	9	197
44	(P48819) Vitronectin precursor (Serum-spreading factor) (S-protein)	VTNC_PIG	53336.9	5.6	8	101
45	(P48819) Vitronectin precursor (Serum-spreading factor) (S-protein)	VTNC_PIG	135.4	100	53336.9	5.6
45	(P00761) Trypsin precursor (EC 3.4.21.4)	TRYP_PIG	41.7	98.3	25078.1	7
48	(P01027) Complement C3 precursor (HSE-MSF) [Contains: Complement C3 beta chain; Complement C3 alpha	CO3_MOUSE	35.5	93.5	187904	6.39
51	(P01027) Complement C3 precursor (HSE-MSF) [Contains: Complement C3 beta chain; Complement C3 alpha	CO3_MOUSE	47.4	99.72	187904	6.39
52	(O46409) Apolipoprotein A-IV precursor (Apo-AIV) (ApoA-IV)	APOA4_PIG	43267.7	5.7	18	145
53	(O46409) Apolipoprotein A-IV precursor (Apo-AIV) (ApoA-IV)	APOA4_PIG	43267.7	5.7	17	128
54	(O46409) Apolipoprotein A-IV precursor (Apo-AIV) (ApoA-IV)	APOA4_PIG	43267.7	5.7	19	165
64	(P18650) Apolipoprotein E precursor (Apo-E)	APOE_PIG	36633.8	5.6	12	68
65	(P18650) Apolipoprotein E precursor (Apo-E)	APOE_PIG	36633.8	5.6	14	107
70	(P37109) Serine protease inhibitor Kazal-type 4 precursor (Peptide PEC-60)	ISK4_PIG	9970.9	4.7	5	55
73	(P18648) Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)	APOA1_PIG	30306.7	5.5	13	100
74	(P18648) Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)	APOA1_PIG	30306.7	5.5	11	85
77	(Q03472) Apolipoprotein R precursor (Apo-R)	APOR_PIG	23450.8	5.9	8	81
79	(P08835) Serum albumin precursor	ALBU_PIG	71642.8	6.1	12	83
97	(Q29549) Clusterin precursor (Complement cytolysis inhibitor) (CLI) (CP40) [Contains: Clusterin bet	CLUS_PIG	52311.6	5.6	8	65

[1)] Protein names and accession numbers were derived from the SWISS-Prot database.
[2)] Mw and pI theoretical (recorded in SWISS-Prot database).

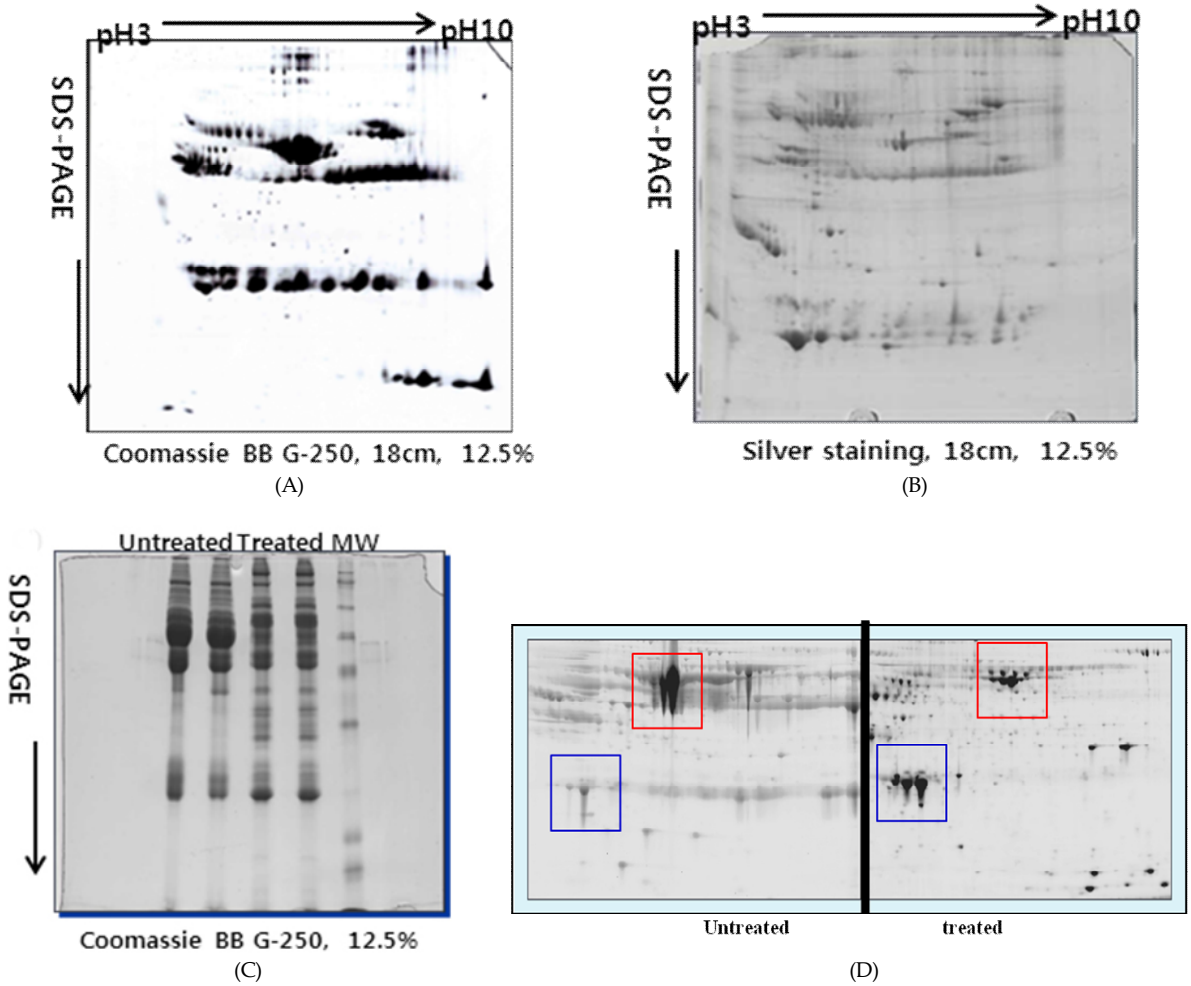

Fig. 1. 1- and 2-DE analysis of ProteoMiner Enrichment Kit-treated porcine plasma. A plasma sample was treated with the ProteoMiner Enrichment Kit (Bio-Rad), and protein from the original sample (untreated) and eluted sample (treated) were analyzed by SDS-PAGE. (A) 1-DE Coomassie BB stained, (B) 2-DE Coomassie BB stained, (C) and 2-DE and silver stained, (D) protein spots magnifified of (B).

Here, we removed major abundant protein for optimization of 2DE analysis to uncover minor proteins of porcine plasma. 1-DE and 2-DE separation of porcine plasma are shown in Fig. 1. Abundant protein depletion treatment has shown to improve 2-DE image and subsequent protein identification with mass spectrometer. This image is representative for the plasma pattern of the group of healthy pigs we have analysed. Fig. 1 is a composite image of 5 gels of different individuals. Nineteen proteins were identified by mass spectrometry (MS), MALDI-TOF/TOF mass spectrometer (Applied Biosystems 4700 Proteomics analyzer). Names and functions are given in Table 1 according to SWISS-PROT (http://us.expasy.org/uniprot/). Classical 2-DE is performed under denaturing and reducing conditions (urea, DTT) which break protein complexes and split multi-chain proteins into their subunits. Therefore, proteins may appear in the 2-DE pattern as single spots (with spots of different pI but almost same molar mass) or several spot chains (subunits) (Fig. 2). Blood plasma whose abundant proteins were depleted was loaded to 1-DE system and distinct band was incised and applied to LC- MS-MS (MDLC system and LTQ, GE healthcare and Thermo Fisher Scientific). Forty two proteins were identified, thereof 14 from abundant protein undepleted samples and 28 from depleted ones by mass spectrometry, LC-MS-MS (Table 2, 3). Considered the overlapped proteins in between treated and untreated sample (Fig. 3) total thirty seven proteins were identified by LC-MS-MS (MDLC system and LTQ, GE healthcare and Thermo Fisher Scientific). It has shown that pretreatment with abundant protein depletion kit improve protein identification as well as protein separation (Fig. 3). Porcine plasma protein map was compared to the human serum/plasma protein map

Table 2. Protein identification by LC-MS-MS

Band No.	Protein name	Entry name
47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 65, 66, 67, 68,69, 71, 72, 73, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 91, 92, 93, 95	Serum albumin	ALBU_PIG
47, 48, 49, 51, 52, 56, 57, 58, 59, 67, 68, 69, 70, 71, 73, 74, 75, 76, 77, 78, 80,81, 85, 91, 93, 95	Trypsin	TRYP_PIG
48, 56, 75	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1_PIG
49, 50, 51, 53, 54, 55, 56,57, 59, 60, 61, 62, 63, 64,66, 68, 75, 76, 77, 79, 80, 83, 85, 88, 91, 93	Alpha-fetoprotein	FETA_PIG
49, 50, 51, 52, 53, 54, 55, 57, 58, 59, 60, 64, 67, 68, 69, 70, 71, 73, 74, 75, 76, 77, 80, 81, 83, 84, 85, 86, 95	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG
57	PIG plasminogen	PLMN_PIG
62	Inhibitor of carbonic anhydrase	ICA_PIG
64	Alpha-2-HS-glycoprotein (Fragment)	FETUA_PIG
64	Alpha-1-antitrypsin	A1AT_PIG
66	Haptoglobin	HPT_PIG
67	Tctex1 domain-containing protein 4	TC1D4_PIG
70, 71, 72, 95	Ig lambda chain C region	LAC_PIG
72, 96	Apolipoprotein A-I	APOA1_PIG
87	Serotransferrin	TRFE_PIG

Table 3. Protein identification by LC-MS-MS after abundant protein treatment

Band No.	Protein name	Entry name
1, 3, 9, 21, 25, 27, 34, 41, 43, 44, 45, 46	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG
1, 10, 11, 12, 13, 14, 24, 25, 29, 33, 34, 35, 36, 37	Vitronectin	VTNC_PIG
3, 8, 10, 13, 14, 25, 28, 31, 31, 33, 34, 36, 38, 40	Serum albumin	ALBU_PIG
6, 28	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_PIG
7, 9, 29, 30, 31	Plasminogen	PLMN_PIG
7	Coagulation factor V	FA5_PIG
8, 13, 15, 16, 18, 19, 20, 22, 36, 38, 40, 41, 42, 43,44, 45, 46	Trypsin	TRYP_PIG
10, 33	Alpha-fetoprotein	THRB_PIG
13, 14, 15, 16, 17, 18, 19, 36, 37, 38, 39, 41, 42	Prothrombin	
15	Vitamin K-dependent protein C	PROC_PIG
15, 17, 18, 22, 39, 46	Apolipoprotein	APOA4_PIG
16, 38	Apolipoprotein A-IV	APOE_PIG
21, 23, 24, 26, 45	Apolipoprotein A-I	APOA1_PIG
41, 42	Apolipoprotein E	APOE_PIG
20, 44	Complement C1q subcomponent subunit A	C1QA_PIG
20, 21, 45	Ig lambda chain C region	LAC_PIG
21	Serum amyloid P-component	SAMP_PIG
16, 39, 41	Clusterin	CLUS_PIG
22	C-reactive protein	CRP_PIG
22	Insulin-like growth factor-binding protein 3	IBP3_PIG
27	Myoglobin	MYG_PIG
27	Myosin-2	MYH2_PIG
27	Actin, alpha skeletal muscle	ACTS_PIG
27	Tropomyosin alpha-1 chain	TPM1_PIG
28	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_PIG
37	Actin, cytoplasmic	ACTB_PIG
37	Haptoglobin	HPT_PIG

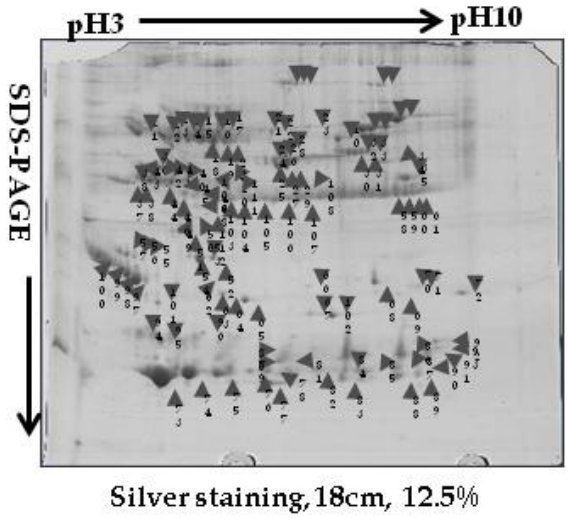

Fig. 2. Expression pattern of porcine plasma proteome by two-dimensional electrophoresis analysis. Protein (500 μg) was loaded and separated in the pH 3~10, 18 cm IPG strip and 12.5% SDS-PAGE gels. The gels were stained with the silver staining. Arrows indicate the identified that were expressed abundant depleted of the abundant proteins.

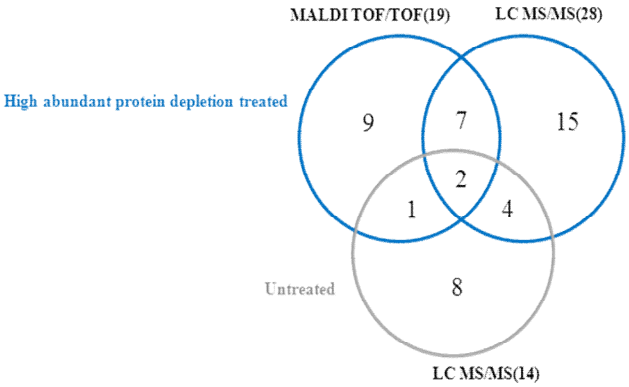

Fig. 3. Porcine plasma reference proteome identified by MALDI-ToF/ToF and LC-MS-MS. 1-DE and 2-DE are a high enough resolution method that allows detailed insight into the properties of single proteins in a complex mixture. It can resolve proteins into its isoforms reflecting post-translation modification such as altered glycosylation and phosphorylation patterns (Parekh *et al.*, 1985). The 1-DE, 2-DE pattern of pretreated plasma gives an overview on all proteins of medium to major concentration at one glance and allows qualitative evaluation of single proteins. It may give a hint which proteins are involved in the pathology; this is especially useful in multifactorial diseases. With 2-DE well established, this approach is more straight-forward than are single protein determinations which need specific assays for each of them.

in which there are a larger number of database entries for single human proteins. As expected, a closer comparison of our pig map (Fig. 2) with the existing human map (SWISS-2DPAGE database http://us.expasy.org/ch2d/publi/inside1995. html) shows differences in homologous proteins, isoforms in respect to MW and pI reflecting their different physicochemical properties.

Abundant Protein Removals 2-DE Proteome Image of Plasma Partially Depleted with Abundant Proteins

Major challenges in proteomics are the large number of proteins and their broad dynamic range in the tissue and cell. These high abundant proteins, and in particular albumin, produce large signals in most proteomics approaches and they mask or interfere with the detection of the other low amount protein components. In a typical plasma sample, the 10 most abundant proteins including albumin will compose ~95% of total protein content, but they represent less than 0.1% of the total number of proteins (Miller *et al.*, 2009). Different organs or cell types have been performed, including 2-DE maps of components of the photoreceptor matrix (Hauck *et al.*, 2005) and prostate (Manaskova *et al.*, 2002), of congestive organs of selected pig breeds (Park *et al.*, 2005). This situation makes it difficult to discover new proteins or peptides biomarkers in blood. To minimize these problems, techniques are constantly developed to provide a wider range and an optimized detection of low concentration. In conclusion, our results demonstrated that proteomics were profiled in plasma protein of postnatal pig; nineteen proteins identified using MALDI-ToF-ToF and thirty seven proteins identified using LC-MS-MS. Further closer investigations for those homologus isoform proteins are required.

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