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Comparison of Plasma Proteome Expression between the Young and Mature Adult Pigs

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ABSTRACT

Here, we present an approach of blood plasma proteome profiling and their comparisons between the young and the adult pigs as prerequisite for the identification of bio-markers related to the health conditions, growth performance and meat quality. To profile the proteome in porcine plasma, blood samples were collected from 19 young piglets and 20 adult male barrows and the plasma was retrieved. 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 thirty-six and twenty eight protein spots were selected in young piglets and adult pigs, respectively and twenty three proteins were identified. The proteome profile images were compared between those ones using Image Master Version 7.0. The image of expressed proteome showed that most of proteins from plasma of young piglet separated clearly and concentrated in 2DE display compared to ones from adult. Image analysis in detail was carried out to look for the specific proteins related to age progression. It demonstrated that the characteristics of proteome expression could be distinct to their age stages. Further investigations needed to proceed to understand the age dependent change of protein conformation and biological meaning of those differences in proteome expression between young and mature adult pigs.

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

INTRODUCTION

To maximize economic efficiency, supplying nutrients as required closely as possible, but not exceeding the requirements of the pig would be advantageous. Understanding the pigs with distinct physiological and metabolic condition is important in developing environmentally friendly, optimal feeding strategies for efficient and sustainable pig production (Chiba, 2000).

Bio-markers allow early detection of disease, evaluation of health condition or animal selection with high production potentials. It was demonstrated that some serum biochemical values, such as lactate dehydrogenase (LDH), total cholesterol (TC), magnesium (Mg), and VA changed significantly with the fattening stage advanced and those were proposed as metabolic biomarkers (Adachi *et al.*, 1997). Apart from the blood metabolites, the approaches considering blood proteins as potential biomarker candidates for the evaluation of individual health and growing conditions have the advantage that proteins are more reflective of a biological system. From the birth through the growing and fattening stages, dynamics of pig physiology change dramatically, supposed to be more than simple complexity, reflected in blood proteome profiles.

Literature dealing with proteomic investigations on pig proteins is sparse. Recently, 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). Porcine

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plasma has not yet been of major interest, except in studies on inflammation-induced changes of single proteins. Hence, in this study, we present an approach of blood plasma proteome profiling and their comparisons between young and adult pigs as prerequisite for the identification of bio-markers related to the health conditions, growth performance and meat quality.

MATERIALS AND METHODS

Animals

Blood samples were collected from 19 heads of crossbred 2 week old young male pigs weighing under 2 kg and 20 heads of 5 month old mature adult male barrow pigs weighing over 90 up to 120 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) 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) and the quality of 2-DE results were improved by 2-D Clean-Up kit (Amersham Biosciecne). 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 3~10 NL 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 drystrips (IPG) 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 Coomassie Brilliant Blue (CBB) G-250 staining method.

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 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 CBB-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 (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

The Young and Mature Adult Pig Plasma 2-DE Image

Young piglets were grouped as A (1.0~2.5, AVG 1.8 kg), B (3.2~4.0, AVG 3.6 kg), C (4.5~4.0, AVG 5.9 kg), D (17~35.0, AVG 26 kg) according to their weights. Blood plasma were collected and the proteins were extracted and separated by 2-DE as described in materials and methods. Expressed proteome spot images in 2-DE gel were shown to be greatly different between the young and the mature adult pig plasma (Fig. 1, 2). Image patterns of plasma proteome within young and mature groups seemed to be similar, however, the different expression pattern was shown to start from plasma of Group D, which consisted of a month old growing pigs, 35 kg indicating the changes of proteome profile implicating the metabolic turning point from the young to the mature.

Proteins Identification in Young and Mature Adults Pig Plasma

For the comparison of proteome profiles between the young and the mature, 2-DE images were compared among groups. There were not characteristic differences within A, B, C, D young pig groups, and E, F, G, H mature pig group. However, the patterns of expressed proteins in some regions in gel were quite different (Fig. 3). The prominent decreases in the intensity of the spots in low pH and high MW region and middle pH and low MW regions were shown in adult pig groups.

36 spots in young and 28 spots in mature adult pig plasma were selected and analyzed (Fig. 2). Among them, twenty three proteins were identified by mass spectrometry, MALDI-ToF-ToF (Table 1, 2). Considered the overlapped proteins in spite of pretreatment of abundant protein depletion, it has shown that the depletion method used was not enough to be satisfactory, staying limited use, and more technologies and methods for major protein separation is required. Even the fact

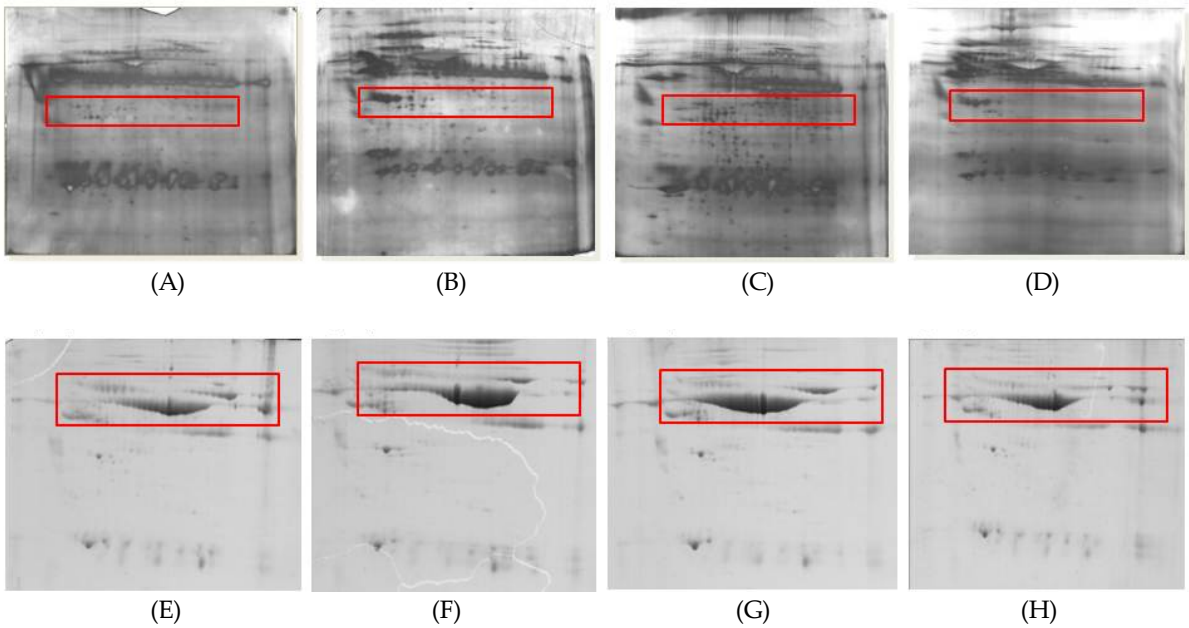

Fig. 1. 2-DE images of expressed proteome of the young and the mature adult pig plasma. (A) 1 to 2 kg piglets, (B) 2 to 3 kg, (C) 4 to 5 kg, (D) 35 kg, (E) 90 kg mature pig, (F) 100 kg pig, (G) 110 kg pig, and (H) 120 kg pig.

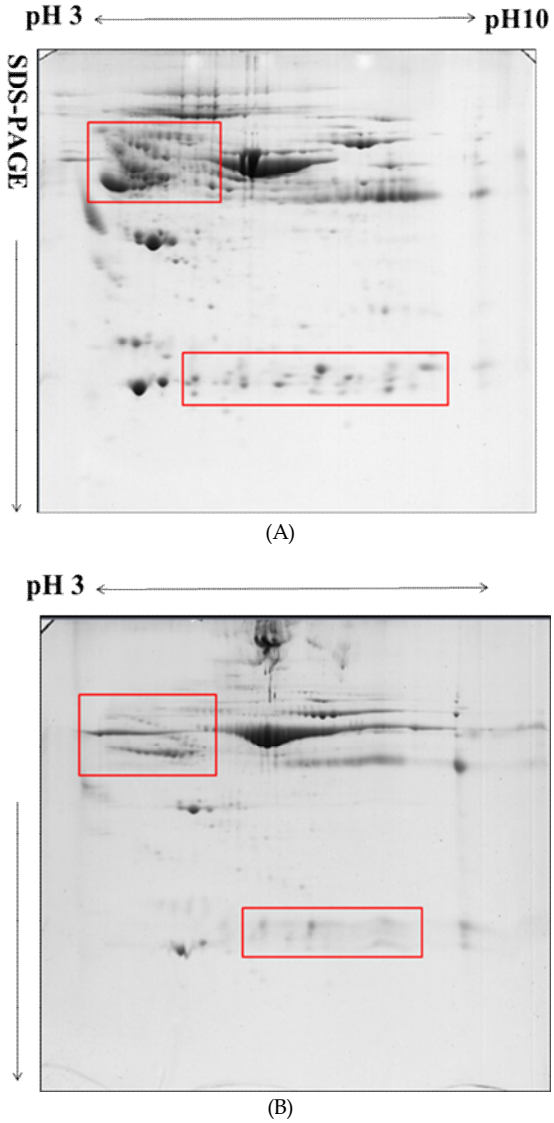

Fig. 2. Expression pattern of porcine plasma proteome by two-dimensional electrophoresis analysis. Protein (500 μg) was loaded and separated in the pH 3~10, 18 cm IPG strip and 12.5% SDS-PAGE gels. The gels were stained the with coomassie G-250. The young (A) and the mature adult (B) pig plasma 2-DE image.

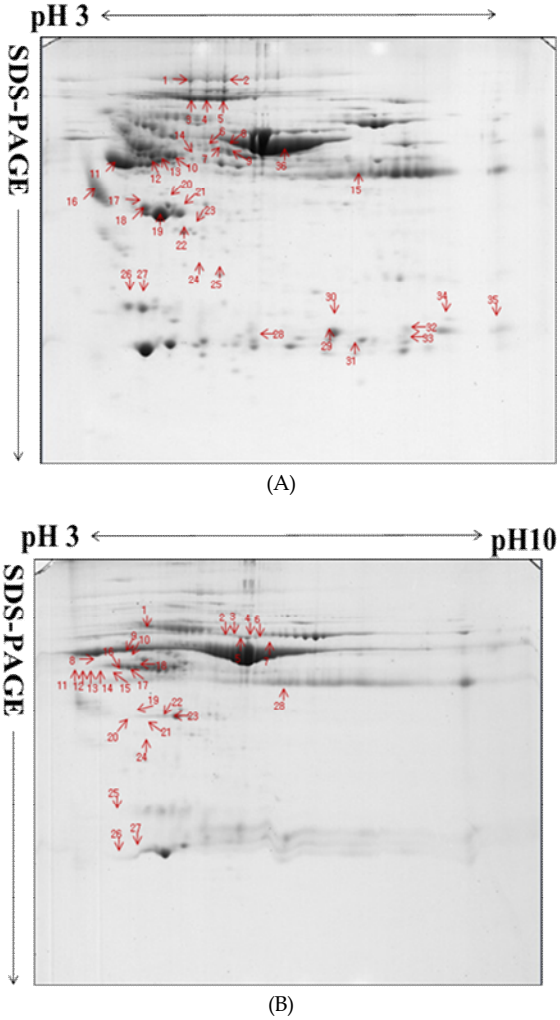

Fig. 3. Arrows indicate the identified proteins in young (A) and adult (B) pig plasma.

that clear separate independent spots in 2-DE gel were frequently found to be the same proteins indicated that one of the characteristics of plasma proteins is existence of multiple isoforms playing various but similar roles in blood. The same time some of protein couldn't be identified due to insufficient pig protein DB.

The proteins which showed differential expression between the young and adult were identified as inter- alpha-trypsin inhibitors, Apolipoprotein, C-reactive proteins and etc. Those proteins were also found to be expressed differently according to ages implicating that expressions of some proteins are regulated by age dependent body metabolism (Villanueva *et al.*, 2006; Lopez *et al.*, 2007; Nomura *et al.*, 2004). Recently, 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). Porcine plasma has not yet been of major interest, except in studies on inflammation-induced changes of single proteins. In conclusion, in this study, we present an approach of blood plasma proteome profiling and their comparisons between young and adult pigs as prerequisite for the identification of bio-markers related to the health conditions, growth performance and meat quality.

Table 1. Proteins in the plasma of young pigs aged two weeks

Spot No.	Protein name[a]	Entry name[b]	P(pro)	Score	MW	Peptide (Hits)
			P (pep)	XC	Sp	Ions
1	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_PIG	1.47E-05	10.14	102083	1 (10000)
2	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_PIG	2.89E-06	30.19	102083	4 (40000)
3	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_PIG	7.83E-11	170.27	102083	110 (1100000)
4	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_PIG	6.29E-09	120.21	102083	204(2040000)
5	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_PIG	1.88E-13	270.3	102083	279(2790000)
6	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG	5.92E-05	10.1	40591	3(30000)
7	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG	3.63E-05	10.1	40591	2(20000)
8	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG	6.27E-05	20.3	40591	5(50000)
9	Serum albumin	ALBU_PIG	9.11E-07	20.17	69647	3(30000)
10	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG	7.28E-04	10.1	40591	2(20000)
11	Alpha-2-HS-glycoprotein (Fragment)	FETUA_PIG	1.00E-30	50.34	38400	435(4350000)
12	Alpha-2-HS-glycoprotein (Fragment)	FETUA_PIG	4.82E-11	20.25	38400	6(60000)
13	Alpha-2-HS-glycoprotein (Fragment)	FETUA_PIG	2.31E-06	10.28	38400	1(10000)
14	Thyroxine-binding globulin	THBG_PIG	5.08E-04	10.11	46185	1(10000)
15	Serum albumin	ALBU_PIG	2.20E-06	10.16	69647	2(20000)
16	Alpha-2-HS-glycoprotein (Fragment)	FETUA_PIG	3.28E-04	10.12	38400	1(10000)
17	Haptoglobin	HPT_PIG	2.18E-10	50.17	38457	47(470000)
18	Haptoglobin	HPT_PIG	7.39E-06	20.16	38457	75(750000)
19	Haptoglobin	HPT_PIG	2.02E-13	60.25	38457	82(820000)
20	Haptoglobin	HPT_PIG	3.15E-11	40.26	38457	53(530000)
21	Haptoglobin	HPT_PIG	1.48E-10	50.21	38457	18(180000)
22	Apolipoprotein A-IV	APOA4_PIG	1.68E-12	150.27	43268	129(1290000)
23	Apolipoprotein A-IV	APOA4_PIG	1.11E-15	120.24	43268	74(740000)
24	Apolipoprotein E	APOE_PIG	3.48E-06	30.15	36577	17(170000)
25	Apolipoprotein E	APOE_PIG	1.76E-07	20.21	36577	62(620000)
26	Ig lambda chain C region	LAC_PIG	7.50E-08	20.17	10996	13(130000)
27	Ig lambda chain C region	LAC_PIG	3.08E-13	30.24	10996	71(710000)
28	C-reactive protein	CRP_PIG	3.55E-07	10.13	24909	2(20000)
29	Ig lambda chain C region	LAC_PIG	2.64E-10	10.18	10996	16(160000)
30	Secretogranin-1	SCG1_PIG	3.44E-04	10.12	74348	2(20000)
31	Hemoglobin subunit beta	HBB_PIG	3.39E-04	10.16	16156	2(20000)
32	Serum albumin	ALBU_PIG	4.25E-10	40.24	69647	144(1440000)
33	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG	7.45E-04	10.09	40591	1(10000)
34	Ig lambda chain C region	LAC_PIG	6.68E-07	20.17	10996	11(110000)
35	Ig lambda chain C region	LAC_PIG	6.14E-08	20.16	10996	14(140000)
36	Serum albumin	ALBU_PIG	1.47E-10	70.27	69647	561(5610000)

[a] Protein names and entry numbers were derived from the SWISS-Prot database.
[b] Mw theoretical (recorded in SWISS-Prot database).

Table 2. Proteins in the plasma of mature adult pigs aged five months

Spot No.	Protein name[a]	Entry name[b]	P (pro)	Score	MW	Peptide (Hits)
			P (pep)	XC	Sp	Ions
1	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG	4.64E-04	10.08	40591	1(10000)
2	cAMP-dependent protein kinase catalytic subunit alpha	KAPCA_PIG	1.33E-04	10.1	40591	1(10000)
3	Serotransferrin	TRFE_PIG	6.76E-10	20.18	76918.5	4(40000)
4	Serotransferrin	TRFE_PIG	1.04E-10	30.27	76918.5	6(60000)
5	Serotransferrin	TRFE_PIG	3.44E-10	10.19	76918.5	7(70000)
6	Serotransferri	TRFE_PIG	5.61E-11	88.22	76918.5	23(194000)
7	Serotransferrin	TRFE_PIG	4.36E-12	70.25	76918.5	34(340000)
8	Serotransferrin	TRFE_PIG	7.27E-13	120.28	76918.5	47(461000)
9	Serotransferrin	TRFE_PIG	1.11E-06	10.22	76918.5	3(30000)
10	Serotransferrin	TRFE_PIG	2.63E-08	20.2	76918.5	3(30000)
11	Serum albumin	ALBU_PIG	8.66E-14	230.27	69647.2	656(6560000)
12	Serum albumin	ALBU_PIG	2.89E-11	130.24	69647.2	115(1150000)
13	Serum albumin	ALBU_PIG	1.58E-09	150.23	69647.2	69(690000)
14	Serum albumin	ALBU_PIG	6.61E-13	70.23	69647.2	30(300000)
15	Serum albumin	ALBU_PIG	1.18E-08	50.22	69647.2	12(120000)
16	Serum albumin	ALBU_PIG	2.59E-10	30.21	69647.2	8(80000)
17	Serum albumin	ALBU_PIG	1.74E-10	20.18	69647.2	4(40000)
18	Serum albumin	ALBU_PIG	4.24E-08	20.19	69647.2	3(30000)
19	Alpha-1-antitrypsin	A1AT_PIG	6.66E-15	90.28	47164.3	50(500000)
20	Alpha-1-antitrypsin	A1AT_PIG	6.47E-05	20.16	47164.3	6(60000)
21	Haptoglobin	HPT_PIG	1.57E-10	30.16	38457.3	14(140000)
22	Alpha-1-antitrypsin	A1AT_PIG	2.41E-06	10.12	47164.3	1(10000)
23	Serum albumin	ALBU_PIG	1.38E-06	30.15	69647.2	7(70000)
24	Haptoglobin	HPT_PIG	5.89E-05	20.13	38457	4(40000)
25	Ig lambda chain C region	LAC_PIG	3.60E-06	10.21	10996.4	2(20000)
26	Ig lambda chain C region	LAC_PIG	1.64E-10	30.24	10996.4	17(170000)
27	Apolipoprotein A-I	APOA1_PIG	5.51E-13	180.27	30306.7	125(1250000)
28	Apolipoprotein A-I	APOA1_PIG	1.55E-13	150.26	30306.7	72(720000)

[a] Protein names and entry numbers were derived from the SWISS-Prot database.
[b] Mw theoretical (recorded in SWISS-Prot database).

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