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·*Original Article*

Establishment of a high-resolution 2-D reference map of human spermatozoal proteins from 12 fertile sperm-bank donors

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Abstract

Aim: To extend the analysis of the proteome of human spermatozoa and establish a 2-D gel electrophoresis (2-DE) reference map of human spermatozoal proteins in a pH range of 3.5–9.0. **Methods:** In order to reveal more protein spots, immobilized pH gradient strips (24 cm) of broad range of pH 3–10 and the narrower range of pH 6–9, as well as different overlapping narrow range pH immobilized pH gradient (IPG) strips, including 3.5–4.5, 4.0–5.0, 4.5–5.5, 5.0–6.0 and 5.5–6.7, were used. After 2-DE, several visually identical spots between the different pH range 2-D gel pairs were cut from the gels and confirmed by mass spectrometry and used as landmarks for computer analysis. **Results:** The 2-D reference map with pH value from 3.5 to 9.0 was synthesized by using the ImageMaster analysis software. The overlapping spots were excluded, so that every spot was counted only once. A total of 3 872 different protein spots were identified from the reference map, an approximately 3-fold increase compared to the broad range pH 3–10 IPG strip (1 306 spots). **Conclusion:** The present 2-D pattern is a high resolution 2-D reference map for human fertile spermatozoal protein spots. A comprehensive knowledge of the protein composition of human spermatozoa is very meaningful in studying dysregulation of male fertility. *(Asian J Androl 2007 May; 9: 321–329)*

Keywords: human spermatozoal proteins; ImageMaster; mass spectrometry; overlapping narrow range; 2-D gel electrophoresis

1 Introduction

The mammalian spermatozoa is a highly differentiated cell, produced through a complex series of morphogenetic events. In the later stages of spermatogenesis, unique cellular components, such as the acrosome, the outer dense fibers, and longitudinal columns and ribs of the fibrous sheath are formed. Also, the nuclear chromatin becomes highly condensed and transcriptionally inactive [1]. For this reason, research on spermatozoal mRNA has limits. However, with the recently developed proteomic technology, which takes 2-D gel electrophoresis (2-DE) and mass spectrometry as key techniques, we have the advantages of high through-put analysis and an integrated view of the studied target cell to study properties of spermatozoa at the molecular level. A compre-

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hensive knowledge of the protein composition of human spermatozoa is useful to study dysregulation of male fertility.

Some previous investigations have applied 2-DE and accessory techniques to study human spermatozoal proteins, but the number of protein spots distinguished in two-dimensional maps thus far is rather low. In 1990, Naaby-Hansen *et al*. [2] acquired an electrophoretic map of acidic and neutral human spermatozoal proteins and obtained over 260 protein spots. In 1992, Kritsas *et al*. [3] obtained over 500 spermatozoal protein spots with molecular mass ranging from 12 to 105 k and isoelectric points from 5.0 to 8.5. In 1994, Xu *et al*. [4] acquired more than 600 spermatozoal protein spots with molecular mass ranging from 7.9 to 93.5 k and isoelectric points between 4.0 and 7.0. In 1997, Naaby-Hansen *et al*. [5] used isoelectric focusing (IEF)/polyacrylamide gel electrophoresis (PAGE) and non-equilibrium pH gradient electrophoresis (NEPHGE)/PAGE in the research of vectorially labeled surface proteins of human spermatozoa, and acquired a composite 2-DE image showing 1397 human spermatozoal proteins that belong to the membrane protein fraction. In 2005, Daniel *et al*. [6] identified more than 1 760 human sperm proteins by liquid chromatography and tandem mass spectrometry. In the present study, to obtain a high resolution 2-DE map of human spermatozoal proteins, we used multiple overlapping narrow immobilized pH gradients (IPG) and computerized 2-D reference map synthesis.

2 Materials and methods

2.1 Preparation of spermatozoa

The semen samples were collected from twelve eligible sperm donors (aged 21.38 ± 2.39 years) from the Human Sperm Bank Department of Citic Xiangya Reproductive and Genetic Hospital (Hunan, China), during the period of March to June 2005. All donors underwent a standardized screening protocol, which included physical examination, review of medical and family history, and infectious disease screening according to the standards of the Ministry of Health of China. After a 3–5 days abstinence period, ejaculates were collected via masturbation, and only the ejaculates meeting the criteria of the World Health Organization (WHO) for normal semen parameters were included in this study [7]. All donors have proven fertility, and donated sperm five times at intervals of 4.11 ± 0.56 days. This was approved by the National Human Reproduction Ethic Investigation Committee, and all donors gave written consent.

After liquefaction, the semen were separated by Percoll (Sigma, Missouri, USA) density centrifugation as described by Naaby-Hansen *et al*. [5] with some modifications. In brief, the sample was overlaid on a two-layer Percoll density gradient consisting of 90% and 45% isotonic Percoll solutions prepared in Ham's F-10 medium (HyClone, Utah, USA), the latter forming the upper layer. After centrifugation at 300 × *g* for 30 min at room temperature, the sperm pellet was collected at the bottom of the 90% layer, then washed with PBS three times by centrifugation at 450 × *g* for 10 min at room temperature. All samples showed > 90% spermatozoa with good motility. The spermatozoa were pooled and frozen immediately in the liquid nitrogen until use.

2.2 Solubilization of spermatozoa

All samples were prepared at the same time. Spermatozoa were routinely solubilized in lysis buffer consisting of 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L Tris, 75 mmol/L DTT, and 2 mmol/L PMSF, as previously described but with some modifications (75 mmol/L DTT instead of 65 mmol/L DTT, additional 40 mmol/L Tris) [8, 9]. This was followed by centrifugation at 10 000 × *g* for 30 min at 4ºC. After protein concentration determination by the 2-D Quant Kit (Amersham Bioscience, Uppsala, Sweden), the supernatant was applied to the first dimensional electrophoresis or stored in –80ºC freezer for further use. In addition, parts of the supernatant were processed by the 2-D Clean Up Kit (Amersham Bioscience, Uppsala, Sweden) for protein precipitation, or by trichloroacetic acid (TCA) in acetone solution for use in pH 6–9 IPG strips [9].

2.3 2-D gel electrophoresis

The first dimensional electrophoresis was performed on an Ettan IPGphor II isoelectric focusing apparatus (Amersham Bioscience, Uppsala, Sweden). The broad range pH 3–10 IPG strip (24 cm long) and the narrower range (pH 6–9) strip and acidic narrow pH range strips cover only one pH unit, 3.5–4.5, 4.0–5.0, 4.5–5.5, 5.0–6.0 and 5.5–6.7, respectively. The strips are commercially available from Amersham Bioscience. The rehydration buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L DTT, 0.5% IPG buffer and a few grains of bromophenol blue was used. For the pH 6–9 IPG strip, rehydration buffer was modified with ad-

ditional 10% isopropanol and 5% glycerol, and with replacement of DTT by Destreak (Amersham Bioscience, Uppsala, Sweden) [9–11]. The sample-loading amount and electrophoresis-running parameters are given in detail in Table 1.

After the isoelectric focusing electrophoresis, the IPG strips were equilibrated in the equilibration solution (6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS] and 50 mmol/L Tris-HCl, pH8.8) containing 1% DTT for 15 min and then in the same solution without DTT but with 2.5% iodoacetamide (IAA) for 15 min [9]. The second dimensional electrophoresis was carried out in a homogeneous SDS-PAGE (12.5%) on Ettan DALT*six* electrophoresis unit (Amersham Bioscience, Uppsala, Sweden). The electrophoresis conditions were modified to 15ºC, 2.5 watt (W)/gel for 30 min and then 20 W/gel for 4 h.

The protein pattern was visualized by mass spectrometry-compatible silver staining, or Coomassie Brilliant Blue (CCB) staining, which is better than the former for the MS analysis [12]. All the stained gels were scanned at 300 dpi resolution using ImageScanner (Amersham Bioscience) and digitized and analyzed using 2-D ImageMaster software (Amersham Bioscience). Parameters of detection of spots by the software are as follows: Smooth 4, MinArea 6 and Saliency 2.00000.

2.4 In-gel digestion

Some mass spectrometry-compatible silver and CCB-stained spots correspondent among different strips were randomly selected, including the spots acting as the landmarks and cut from the gels for data analysis. The silver-stained spots were destained by 30 mmol/L potassium ferricyanide and 100 mmol/L sodium thiosulfate for 2 min and the CCB stained spot samples by 50% acetonitrile (made up with 25 mmol/L NH_4HCO_3), respectively. All destained spots were dried in a vacuum pump, reduced by 10 mmol/L DTT (made up with 50 mmol/L NH_4HCO_3) for 30 min at 56ºC, followed by the replacement of DTT and alkylation for 30 min in dark with 55 mmol/L IAA solution (made up with 50 mmol/L NH_4HCO_3), then dehydrated in 100 mmol/L NH_4HCO_3 and digested by adding the trypsin digestion work solution (T 6567; Sigma, Missouri, USA)) and incubation for 15–18 h at 37ºC.

2.5 TOF/TOF mass spectrometry (MS) analysis

MS analysis was performed according to Myung *et al*. [13] after in-gel digestion. The extracted peptides were directly applied onto a target (AnchorChip, Bruker Daltonics, Germany) that was loaded with α-cyano-4-hydroxy-cinnamic acid (CHCA) (Bruker Daltonics, Germany) matrix thin layer. The mass spectrometer used in this work was an Ultraflex TOF/TOF (Bruker Daltonics, Germany) operated in the reflector for matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) peptide mass fingerprint (PMF) or in LIFT mode for MALDI-TOF/TOF with a fully automatic mode using the FlexControl software. Database searches were performed through Mascot using combined PMF and tandem mass spectrometry (MS/MS) datasets via BioTools 2.2 software (Bruker Daltonics, Germany). Two parameters were modified according to Myung *et al*. [13], a maximum of four instead of three precursor ions per sample were chosen for MS/MS analysis, and MS/MS tolerance of 1.0 Da instead of 0.5 Da for MS/MS search.

3 Results

Table 1. Sample-loading amount and electrophoresis-running conditions used for the isoelectric focusing electrophoresis with broad and narrow range immobilized pH gradient gels. IPG, immobilized pH gradients. Vhr., volt · hour.

IPG dry strips	Rehydration	Sample loading	Total Vhr.
pH 3–10	12 h, 30 V, with sample	0.5 mg in-gel rehydration loading	50 000
pH 3.5–4.5	12 h, 30 V, without sample	1.5 mg anodic cup loading	100 000
pH 4.0–5.0	12 h, 30 V, without sample	1.5 mg anodic cup loading	100 000
pH 4.5–5.5	12 h, 30 V, without sample	1.5 mg anodic cup loading	100 000
pH 5.0–6.0	12 h, 30 V, without sample	1.5 mg anodic cup loading	100 000
pH 5.5–6.7	12 h, 30 V, without sample	1.5 mg anodic cup loading	100 000
pH 6–9	12 h, 30 V, without sample	0.8 mg anodic cup loading	80 000

3.1 Analysis of 2-D gels

The samples loaded for electrophoresis in pH 3–10, 6–9, and acidic narrow pH gels, contained 0.5 mg, 0.8 mg, and 1.5 mg of protein, respectively. Analysis of spots with mass spectrometry was performed after silver-staining. After establishment of a reliable protocol, the analysis of all 2-D maps revealed excellent reproducibility. All the kinds of pH 2-DE were repeated at least five times, and the best 2-D patterns were used for analysis. 2-D maps of different pH gradient ranges of human spermatozoal proteins are shown in Figures 1 and 2. The number of spots detected by software analysis in pH 6–9 gel, each narrow pH range gel, and the corresponding zones of the pH 3–10 gel, are presented in the histogram of Figure 3.

Total of 1 306 distinct protein spots were detected in the broad pH range 2-DE map. Compared to the map, the sensitivity was enhanced by using narrower and narrower pH IPG strips. About 168 distinct spots were

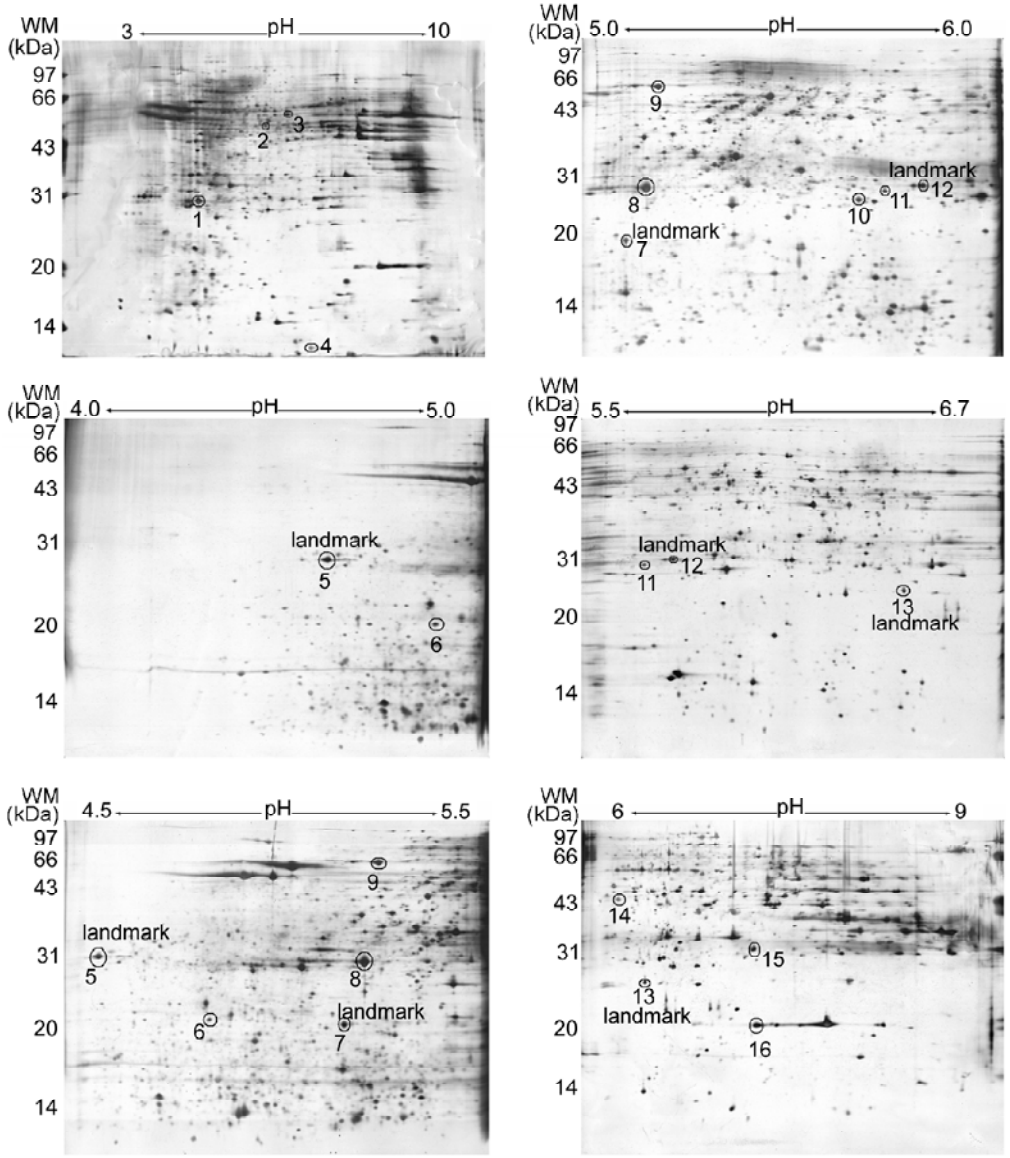

Figure 1. 2-D pattern of the proteins of human fertile spermatozoa achieved using different pH gradients, the outlined spots were identified by mass spectrometry (MS) (shown in Table 2), including the 4 landmarks which were used in the process of composing reference map (Figure 5). No. 5, No. 7, No.12 and No. 13 are the landmarks between pH 4.0–5.0 and pH 4.5–5.5, pH 4.0–5.5 and pH 5.0–6.0, pH 4.0–6.0 and pH 5.5–6.7, and pH 4.0–6.7 and pH 6–9, respectively. WM, weight mass.

resolved in the pH 3.5–4.5 2-D gel compared to only 75 spots in the corresponding zones of the pH 3–10 2-D gel; 572 in the pH 4.0–5.0 2-D gel compared to only 191 spots; 1 463 in the pH 4.5–5.5 2-D gel compared to 313; 1 370 in the pH 5.0–6.0 2-D gel compared to 344; 1 121 spots in the pH 5.5–6.7 2-D gel compared to only 347; and 1 061 in the pH 6–9 2-D gel compared to only 631.

Our results also show that, in comparison with broad pH range IPG strips, in the narrow pH range gels not only the spots became further apart, but several visually single spots were divided into two or more protein spots [14]. These apparent improvements are shown in Figure 4. There are a total of 5 755 spots detected from the different pH gradient 2-D maps, excluding the broad pH range map. Of these 5 755 spots, approximately 3 872 independent spots could be detected on the composite narrower and narrow pH range gels after excluding the overlapping spots (1 883) by data analysis.

3.2 Protein identification from 2-D gel spots by MS

To confirm the visually identified landmarks for different pH range gels, we selected several correspondent spots from the silver-stained gels and CCB-stained gels that were used in the data analysis. Both PMF and MS/MS were performed and all the outlined spots (selected as shown in Figure 1, the CCB-stained gel is not shown) have been identified and listed in Table 2.

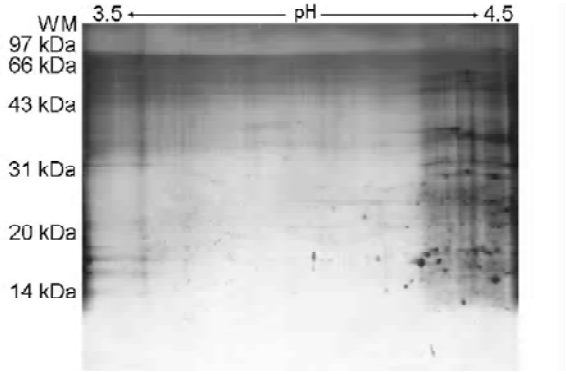

Figure 2. 2-D pattern of the proteins of human fertile spermatozoa (pH 3.5–4.5). WM, weight mass.

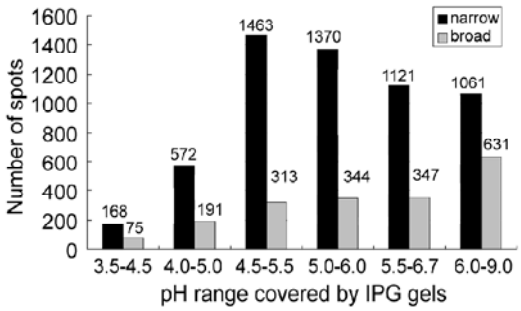

Figure 3. Comparison of the number of individual spots detected in the narrow pH range 2-DE map and in the corresponding regions of the broad rang (pH 3–10) 2-DE map. 2-DE, 2-D gel electrophoresis. IPG, immobilized pH gradients.

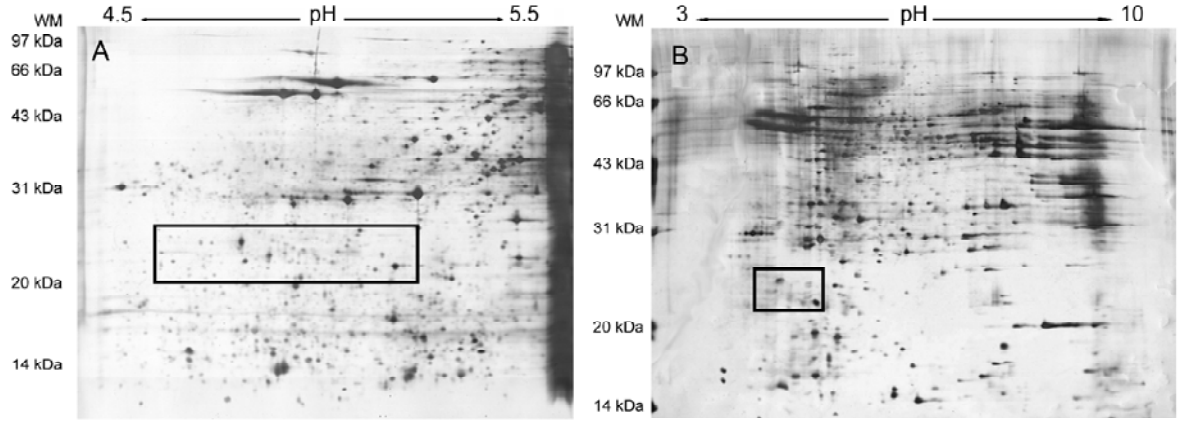

Figure 4. Comparison of the resolution of the narrow pH range (pH 4.5–5.5) 2-DE map (A) with the broad pH range (pH 3–10) 2-DE map (B), the corresponding area encircled by rectangle for comparison. (A): 2-D pattern of increased separation of human fertile spermatozoal protein achieved using progressively narrower pH 4.5–5.5 gradients, the encircled region was represented and compared to the corresponding area by rectangle in (B). (B): 2-D pattern of human fertile spermatozoal protein by the broad pH range (pH 3–10). 2-DE, 2-D gel electrophoresis.

3.3 Composing a 2-D reference map of human fertile spermatozoal proteins

All spermatozoal proteins were extracted at the same time, to try to exclude experimental variation. First, the identical landmarks between two consecutive pH range gels (for example, pH 4.0–5.0 and pH 4.5–5.5) were matched, and the other overlapping identical spots were deleted. Then, the composite 2-D reference map that has a new large pH range (for example, pH 4.0–5.5) was composed. Other consecutive pH range gels can be composed in the same way. Finally, a 2-D reference map of human fertile spermatozoal proteins in the pH range 3.5–9.0 was composed through image analysis by using 2-D ImageMaster software (Figure 5). In the composite narrower and narrower pH ranges map 3 872 independent spots could be detected, excluding the overlapping spots (1 883). The results of 2-D gel were reproductive in several times, so we think this 2-D reference map could be used for comparison with 2-D analysis of other sperm samples by different researchers.

4 Discussion

The 2-DE is a useful technique in proteomics, giving reproducible and high-resolution data of complex protein mixtures [14, 15]. In the present study, we have attempted to improve resolution, to obtain more protein spots in a 2-D pattern of human spermatozoal proteins, by employing currently available overlapping acidic narrow pH range IPG gels strips. Unfortunately, the narrow-pH range gels strips in the basic range are still unavailable from Amersham Bioscience. We achieved a high resolution 2-DE map of human spermatozoal proteins, identifying a total of 3 872 distinct protein spots by using multiple overlapping narrow IPG. To our knowledge, the present high resolution 2-D reference map contains the highest number of human spermatozoal protein spots reported so far.

For the pH 6–9 IPG strip, the replacement of the charged reductant DTT in the rehydration solution by DeStreak or by uncharged reductant tributyl phosphine (TBP) can significantly reduce horizontal streaking, resulting in 2-D maps with spot patterns more simplified and reproducible [15, 16]. Our experiments also showed significant improvement by using TBP, especially by using DeStreak (data not shown). There was still much horizontal streaking in pH 6–9 2-DE map, and we suppose that this streaking may come from insolubility, migration or precipitation of some proteins [11, 17], and high sample-loading may also contribute to the horizontal streaking.

It is very difficult to acquire a perfect 2-D pattern for the pH 3.5–4.5 IPG strip in our experiments (shown in Figure 2). According to previous studies [18], μsol-IEF (a sample-prefractionation method) may result in a 2-DE pattern rather better, with the major interfering proteins removed by the prefractionation procedure. This method can separate proteins using much higher protein-loading, such as on the extreme acid narrow pH range IPG gels, and obtain a good 2-D pattern. Furthermore, the detection of low abundance proteins was greatly enhanced by using the prefractionation method. The method can also improve the result for the 2-DE pattern of other pH gradient gels. We are now applying this to improve the 2-D map for pH 3.5–4.5 IPG strip.

Enrichment of spots can be obtained by loading different amounts of protein. High-sample loading can enrich more proteins that have a low abundance, while low

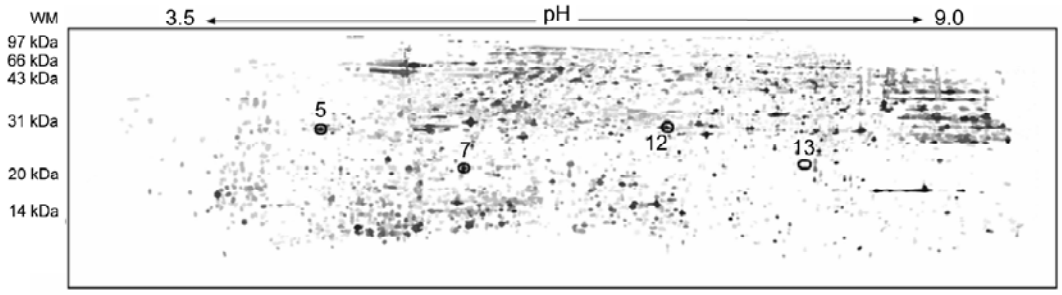

Figure 5. Composite 2-D referenced map of human fertile spermatozoal proteins (pH 3.5–9.0). All the different pH range gels were visualized by using mass spectrometry-compatible silver staining. The spots circled are the landmarks between different pH range gels. WM, weight mass.

Table 2. Identification of some human spermatozoal protein spots by mass spectrometry (MS). *The result was obtained by LCQ-DECA-XP plus (Finnigan) from Research Centre for Proteome Analysis, Key Lab of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Science, Chinese Academy of Science, Shanghai, China. # These spots were selected as landmarks in data analysis. PMF, peptide mass fingerprint. Mr., relative molecular mass. obs., observe. theor., theory.

Spot No.	Protein	Accession Code	Identification method	Sequence coverage(%)	m/z	Sequence	pI/Mr. (obs.)	pI/Mr. (theor.)
1	chain D, ligand-fFree heterodimerichuman glutathione S-transferase	gi|5822513	PMF	73			5.20/28.0	5.37/27.0
2	tektin 1	gi|16753231	PMF	–			6.20/50.0	5.98/49.0
3	cytosol aminopeptidase, (leucine aminopeptidase) (LAP)	gi|12643394	MS/MS	–	2096.00	AAGIDEQEN WHEGKENIR	6.60/55.0	6.30/53.0
4*	calgranulin A	SWISS-PROT: P05109		27	1273.46	KALNSIID VYHKY	6.80/12.0	6.51/11.0
5#	proteasome (prosome, macropain) subunit, alpha type 5	gi|7106387	MS/MS	51	1423.78	LFQVEYA IEAIK	4.60/30.0	4.74/26.5
6	splice isoform 2 of diablo homolog, mitochondrial precursor	IPI00219865	PMF	37			4.84/22.0	4.81/21.2
7#	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d isoform b	gi|51479152	MS/MS	70	1873.02 1932.05	TIDWVAFA EIIPQNQK LAALPENPP AIDWAYYK	5.20/20.0	6.60/15.8
8	glutathione S-transferase Mu 3	IPI00246975	PMF	64			5.18/29.5	5.37/26.8
9	60 kDa heat shock protein, mitochondrial precursor	IPI00472102	PMF	43			5.21/63.5	5.70/61.3
10	proteasome subunit beta type 7 precursor	IPI00003217	PMF	20			5.64/28.5	5.75/30.2
11	metaxin 2, isoform b	IPI00477773	PMF	16			5.75/31.0	6.09/29.1
12#	enoyl-CoA hydratase, mitochondrial precursor (short chain enoyl-CoA hydratase) (SCEH)	gi|2851395	MS/MS	60	1322.67 2125.09	SLAMEMVL TGDRAQFA QPEILIGTIP GAGGTQR	5.80/32.0	8.34/31.8
13#	proteasome beta 2 subunit	gi|4506195	MS/MS	31	1306.73 2020.00	FILNLPTFSVR APFAAHGYG AFLTLSILDR	6.37/26.5	6.51/22.9
14	glutamine synthetase	IPI00010130	PMF	49			6.21/45.0	6.43/42.6
15	proteasome subunit alpha type 4	IPI00299155	PMF	35			6.98/31.0	7.57/29.7
16	glutathione peroxidase 4	gi|4504107	MS/MS	51	1294.73 1575.80 1658.99	ILAFPCNQFGK DIDGHMVNLDKYR TEVNYTQLVDLHAR	7.05/21.0	8.69/22.6

sample-loading can reduce spot trains and hence acquire better separation of the proteins that have a high abundance. We obtained more than 1 350 spots of human spermatozoal proteins from broader pH range IPG strip (18 cm in length) of pH 3–10 by using different amounts of protein loading in our study (data not shown).

It is to be noted that the present computerized 2-D reference map cannot be regarded as perfect. First, there are still some flaws in the 2-D technique, such as the inherent complexity of the procedure, and the precision of analysis is dependent on the operator to some degree. Second, the 2-D analysis software still needs further improvement. In addition, the identification by MS of the landmark picked out from the pH 3.5–4.5 2-D gel is not convincing; the spot could not be identified by MS analysis and hence is not listed in Table 2, and cannot be used in data analysis. To avoid the repeated spots between pH 3.5–4.5 gel and pH 4.0–5.0 gel to be added to the composite gel, some independent spots were left out of the analysis subjectively. On all accounts, the computerized 2-D map of human spermatozoal proteins needs improvement.

We obtained 16 identified spots by MS, including four proteins of proteasome subunits (spot No. s 5, 10, 13 and 15). The human sperm proteasome plays an important role in fertilization [19]. A comprehensive knowledge of the protein composition of human fertile spermatozoa is useful in elucidating cellular processes at the level of the proteomics and studying dysregulation of male fertility. Differential extraction of proteins from spermatozoal cytoplasm and nucleus is difficult because of the complex cyto-architecture of the spermatozoa. In fact, the protein spots from the present 2-D pattern represent a very small part of the total proteins of the whole cell. In addition, because of differential mRNA splicing and extensive co-translational and post-translational modifications of proteins, more proteins and variant forms are expressed than the number of expressed genes [20]. So further work needed to study the proteome of human spermatozoa.

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References

1 Shetty J, Diekman AB, Jayes FC, Sherman, NE, Naaby-Hansen S, Flickinger CJ, *et al*. Differential extraction and enrichment of human sperm surface proteins in a proteome: Identification of immunocontraceptive candidates. Electrophoresis 2001; 22: 3053–66.
2 Nabby-Hansen S. Electrophoretic map of acidic and neutral human spermatozoal proteins. J Reprod Immunol 1990; 17: 167–85.
3 Kritsas JJ, Schopperle WM, Dewolf WC, Morgentaler A. Rapid high resolution two-dimensional electrophoresis of human sperm proteins. Electrophoresis 1992; 13: 445–9.
4 Xu C, Rigney DR, Anderson DJ. Two-dimensional electrophoretic profile of human sperm membrane proteins. J Androl 1994; 15: 595–602.
5 Naaby-Hansen S, Flickinger CJ, Herr JC. Two-dimensional electrophoretic analysis of vectorially labeled surface proteins of human spermatozoa. Biol Reprod 1997; 56: 771–87.
6 Johnston DS, Wooters J. Kopf GS, Qiu Y, Roberts KP. Analysis of the human sperm proteome. Ann NY Acad Sci 2005; 1061: 190–202.
7 World Health Organization. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. UK: Cambridge University Press; 1999. p19, 71.
8 Starita-Geribaldi M, Roux F, Garin J, Chevallier D, Fenichel P, Pointis G. Development of narrow immobilized pH gradients covering one pH unit for human seminal plasma proteomic analysis. Proteomics 2003; 3: 1611–9.
9 Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, *et al*. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 2000; 21: 1037–53.
10 Com E, Evrard B, Roepstorff P, Aubry F, Pineau C. New insights into the rat spermatogonial proteome: Identification of 156 additional proteins. Mol Cell Proteomics 2003; 2: 248–61.
11 Hoving S, Gerrits B, Voshol H, Mller D, Roberts RC, Ootrum J. Preparative two-dimensional gel electrophoresis at alkaline pH using narrow range immobilized pH gradients. Proteomics 2002; 2: 127–34.
12 Simpson RJ. Proteins and Proteomics: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press; 2003. p190–5.
13 Myung JK, Afjehi-Sadat L, Felizardo-Cabatic M, Slavc I, Lubec G. Expressional patterns of chaperones in ten human tumor cell lines. Proteome Sci 2004; 2: 8.
14 Wildgruber R, Harder A, Obermaier C, Boguth G, Weiss W, Fey SJ. Towards higher resolution two-dimensional electrophoresis of *Saccharomyces cerevisiae* protein using overlapping narrow immobilized pH gradients. Electrophoresis 2000; 21: 2610–6.
15 Görg A, Weiss W, Dunn MJ. Current two-dimensional elec-

trophoresis technology for proteomics. Proteomics 2004; 4: 3665–85.
16 Zuo X, Speicher DW. Comprehensive analysis of complex proteomes using microscale solution isoelectrofocusing prior to narrow pH range two-dimensional electrophoresis. Proteomics 2002; 2: 58–68.
17 Poland J, Cahill MA, Sinha P. Isoelectric focusing in long immobilized pH gradient gels to improve protein separation in proteomic analysis. Electrophoresis 2003; 24: 1271–5.
18 Olsson I, Larsson K, Palmgren R, Bjellqvist B. Organic disulfides as a means to generate streak-free two-dimensional maps with narrow range basic immobilized pH gradient strips as first dimension. Proteomics 2002; 2: 1630–2.
19 Morales P, Kong M, Pizarro E, Pasten C. Participation of the sperm proteasome in human fertilization. Hum Reprod 2003; 5: 1010–7.
20 Westbrook JA, Yan JX, Wait R, Welson SY, Dunn MJ. Zooming-in on the proteome: very narrow-range immobilised pH gradients reveal more protein species and isoforms. Electrophoresis 2001; 22: 2865–71.

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