CHAPTER III
RESEARCH METHODOLOGY

3.1 Chemicals and reagents

The chemicals and reagents used in this study were analytical grade and commercially available as shown in Table 4.

Table 4 List of the chemicals and reagents used in this study

Chemicals	Catalog Number	Company
Sample preparation		
2-D clean-Up Kit	80-64851	Amersham Bioscience ,USA
Determination of protein		
Protein standard (Bovine serum albumin)	P0914	Sigma, USA
Bradford reagent	B6916	Sigma, USA
Isoelectric focusing		
CHAPS	13361	usb, USA
Dithiothreitol (DTT)	15397	usb, USA
Iodoacetamide	RPN6302	GE Healthcare, UK
Protease inhibitor mix	80-6501-23	Amersham Biosciences, USA
Immobiline DryStrip Cover Fluid	17-1335-01	Amersham Biosciences, USA
Immobiline DryStrip, pH3-10L, 7 cm	17-6001-11	GE Healthcare, Sweden
IPG Buffer, pH 3–10	17-6000-87	Amersham Biosciences, Sweden

Table 4 List of the chemicals and reagents used in this study (Cont.)

Chemicals	Catalog Number	Company
Bromophenol blue	B8026	Sigma, USA
Glycerol	17-1325-01	Amersham Biosciense, Sweden
Tris (Trizma base)	T1503	SAFC, USA
Thiourea	628K2499179	SAFC, USA
Urea	ITEM75826	usb, USA
Acrylamide PAGE	17-1302-02	Amersham Biosciences, Sweden
Ammonium persulfate	A-3678	Sigma, USA
N,N'-methylene bis acrylamide (Bis)	17-1304-02	Pharmacia Biotech, Sweden
Agarose	A-3768	Sigma, USA
TEMED	17-1312-01	Amersham Biosciense, Sweden
Glycine	16407	usb, USA
n-butanol	K295790	Merck, Germany
Low molecular weight markers	17-0446-01	Amersham Biosciense, Sweden
Coomassie Blue staining solution		
Trichloroacetic acid	91230	Fluka, Germany
Coomassie Brilliant Blue R-250	32826	usb, USA
Glacial acetic acid	10015N	BDH, UK

Table 4 List of the chemicals and reagents used in this study (Cont.)

Chemicals	Catalog Number	Company
Methanol	K35091309	Merck, Germany
In-gel Tryptic protein digestion		
Trypsin	-	
Zip TipC18	-	

3.2 Instruments

The instruments used in this study are listed in Table 5.

Table 5 List of the instruments used in this study

Instrumentation	Model	Company
Automatic Pipettes	-	Finpipettes, Finland
Centrifuge	H-103N series	Kokusan, Japan
Centrifuge	3K 30	Sigma, USA
Microcentrifuge (1.5 ml)	Microfuge E ™	Beckman, UK
Deepfreezer	Ultra Low	Sanyo, Japan
Freezer	Fresh cool	Sanyo, Japan
Weighting machine(d=0.0001g)	XT120 A	Precisa, Switzerland
Visible spectrophotometer	Spectronic 20® Genesys ™	Spectronic Unicam, USA
Isoelectric focusing and SDS-PAGE		
Isoelectric Focusing System	Ettan IPGphor II	Amersham Biosciense, Sweden
Vertical electrophoresis system	Hoefer miniVE	Amersham Biosciense, Sweden
Electrophoresis power supply	EPS 301	Amersham Biosciense, Sweden
Image analysis system and software		
ImageScanner	ImageScanner II	Amersham Biosciense, Sweden
ImageMaster 2D platinum	ImageMaster 2D Platinum 6.0	Amersham Biosciense, Sweden
Protein identification		
Speed Vac concentrator	Savant	Holbrook, NY
Q-TOF Ultima MALDI instrument	Micromass	Manchester, UK

3.3 Subjects and sample collection

The kidney tissues from the left upper quadrant were obtained the twelve vehicle accident males. The cadaveric donors who had one or more of the following conditions were excluded from the study, including (1) underlying medical illness prior to the death (2) evidence of kidney injury (3) no health information in the past (4) no permission from the cadaveric relatives. All autopsies were collected within 6 hours of the death to obtain the most reliable data (Tavichakorntrakool et al., 2007). This human study were reviewed and approved by the Khon Kaen University Ethics Committee for human Research and the consent was obtained from the cadaveric's family (HE481140). The cadaveric subjects were divided into two groups, based on their muscle K content, NKD (K= 90.03 ± 1.42 μmol/g wet weight; n=6) and KD (67.88 ± 0.79μmol/ g wet weight ;n= 6) groups (Tavichakorntrakool et al., 2007).

Ethic permission (HE 481140)

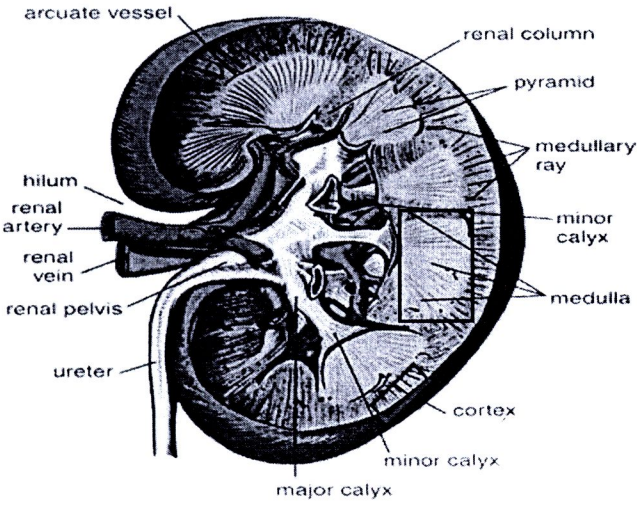

Figure 7 Sites of kidney tissues used in this study

3.4 Experimental protocol for proteomic analysis

3.4.1 Sample preparation

Kidney tissues were pulverized into powder under liquid nitrogen. The ground samples were solubilized in a lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethyl-ammoniol]-propanesulonate (CHAPS), 2% (v/v) ampholyte (pH 3-10) and 40 mM dithiothreitol (DTT), and mixtures were incubated at 4 °C for 30 min. After centrifugation at 12000*g* for 10 min, the supernatants then were processed by the 2-D cleanup kit according to manufacturer's protocol and the protein concentration were determined by spectrometry based on Bradford's method (Bradford, 1976).

3.4.2 Two-Dimensional Gel Electrophoresis (2-DE)

(1) First dimension: isoelectric focusing (IEF)

Immobilized pH gradient (IPG) strips (linear pH3-10,7cm long) (GE Healthcare;Uppsala, Sweden) were rehydrated overnight with equal amount protein (200 µg) extracted from renal cortex and medulla derived from individual subjects and premixed with a rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) ampholytes (pH3-10), 18 mM DTT, and 0.002% bromophenol blue (to make the final volume of 150 µL per strip). The first dimensional separation was performed in Ettan IPGphor II Isoelectric Focusing Unit (GE Healthcare) at 20 °C, using stepwise mode to reach 9000 Vh as shown in table below.

pH interval	Voltage mode	Voltage (V)	Time(h:min)	kVh
3-10	1 Step and Hold	500	1: 00	0.5
	2 Gradient	1000	1: 00	0.8
	3 Gradient	6000	2:00	0.7
	4 Step and Hold	6000	0:10-0:40	0.7-3.7
	Total		4:05-4:40	9.0-12.0

(2) Equilibration of IPG strip for SDS-PAGE

After completion of the isoelectric focusing, the separated proteins were equilibrated with a buffer containing 6 M urea, 65 mM DTT, 29.3% glycerol, 75 mM Tris-HCl ; pH8.8, 2% SDS, 0.002% bromophenol blue for 20 minutes, and then with another buffer containing 6 M urea, 135 mM iodoacetamide, 29.3% glycerol, 75 mM Tris-HCl, 2% SDS, 0.002% bromophenol blue for further 20 minutes.

(3) Second dimension: sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The equilibrated IPG strips were then transferred onto 12% acrylamide slab gels (8×9.5 cm) and the second dimensional separation was performed in Hoefer miniVE system (GE Healthcare) with the current of 20 mA/gel for approximately 1.5 hour. The resolved protein spots were then visualized using Coomassie Brilliant Blue R-250 stain.

3.4.3 Coomassie Brilliant Blue Staining

The gels were fixed in 20% TCA for 40 minutes. The gels were submerged in Coomassie Brilliant Blue staining solution and shaked slowly for 6 hours. After staining, they were washed with destain solution I and shaked slowly for 30 minutes,then they were washed again with destain solution II and shaked slowly for 30 minutes. They were stored in destain solution II containing 1% glycerol.

3.4 4 Image record and data processing

The images of protein pattern in each gel were scanned with ImageScanner (Amersham Biosciences, Taiwan). Each gel was scanned with transparent mode 600 dpi. red filter and saved the image file format as.*mel and *tif.

3.4.7 Protein identification by Q-TOF MS and MS/MS

The proteolytic samples were premixed 1:1 with the matrix solution (5 mg/ml R-cyano-4-hydroxycinnamic acid in 50% AACN,0.1% v/v TEF and 2% w/v ammonium citrate) and spotted onto the 96-well-MALDI sample stage.

The samples were analyzed by the Q-TOF Ultima MALDI instrument (Micromass, Manchester,UK), which was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another. Within each sample well, parent ions that met the predefined criteria (any peak within *m/z* 800-3000 range with intensity above ten count ± include/exclude list) were selected for CID MS/MS using argon as the collision gas and mass-dependent ± 5V rolling collision energy until the end of the probe pattern was reached (http://proteome.sinica.edu.tw). For PMF, both ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) and MASCOT (http://www.matrixscience.com) search engines were used. Proteins were identified based on the assumptions that peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Both NCBI and Swiss-Prot databases were used and the searches were restricted to mammalians. A mass tolerance of 25 ppm was used and up to one missed trypsin cleavage was allowed. Identities with probability-based MOWSE scores> 68 (for MASCOT) and/or Z scores > 1.65 (for Profound) were considered as "significant hits." In the case of nonsignificant hits with the mass tolerance of 25 ppm, the mass tolerance was changed to 50, 100, and 150, and the searches were again performed. For MS/MS peptide sequence identification, the MASCOT search engine was employed. Search parameter allowed were similar to those for PMF. Peptides with ions scores > 44 were considered as "significant hits." Only significant hits from peptide mass fingerprinting and/or MS/MS peptide ion search were reported in the results (Thongboonkerd et al., 2006).

3.5 Statistical analysis

All data are reported as mean ± SD. Statistical analysis was performed with SPSS software (version 16.0). To test for the differences between two groups, an unpaired *t*-test was used. *P* value < 0.05 were considered statically significant.