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Original article

Mapping of polar fox renal cortex proteins using two-dimensional gel electrophoresis and mass spectrometry – a preliminary study

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Abstract

The aim of the present study was to establish protein map of polar fox (*Alopex lagopus*) renal cortex. Kidney cortex proteins of isoelectric point ranging from 3 to 10 were analysed using two-dimensional electrophoresis and MALDI-TOF mass spectrometry. Sixteen protein spots corresponding to thirteen different gene products were identified. These proteins were divided into following groups: lipid and fatty acid metabolism, amino acid metabolism, energetic pathways, regulatory proteins, transport proteins and structural proteins. This is the first attempt to create reproducible 2-D map, of renal cortex proteins characteristic for polar foxes, used as animal model for carnivores. It is worth emphasizing that the results of this study may broaden currently available protein databases.

Key words: polar fox, kidney, cortex, proteome, two-dimensional electrophoresis, mass spectrometry

Introduction

The kidneys are paired organs that play a key role in the maintenance of a stable internal environment with regard to the system volume and composition of the body fluids. The complex renal functions are carried out by the basic morphological and functional unit – nephron, which consists of several specialized types of cells. Functional specialization along the nephron cells is reflected in specific gene and protein expression in different structural renal regions. Filtration takes place in the glomerulus within the renal cortex and is subsequently followed by intense absorption of ultrafiltrate components (glucose, amino acids, water and electrolytes) along the renal proximal tubule. Moreover, hormones responsible for modulating blood pressure, haemopoesis and calcium homeostasis are produced by the renal cortex. Renal medulla is responsible for urine concentration, mainly due to high osmolality of the surrounding environment. That environ-

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mental conditions have a great influence on the expression of highly specific proteins in the renal medullary cells. Differences between renal cortex and medulla functions are mirrored by a various protein expression pattern in both of the abovementioned regions (Arthur et al. 2002, Ożgo et al. 2007).

Proteomic analyses are focused on both qualitative and quantitative differences in protein expression. It is noteworthy that there is a lack of correlation between transcriptional profile and actual protein content in many various cells (Jaśkowski et al. 2010). Thus, complicated analysis of protein profile changes may lead to a better explanation of gene functions and mechanisms of posttranscriptional regulation (Kempisty et al. 2011). Proteomic analyses have been successfully applied in a large number of studies including biological and medical ones. Most of them have been focused on protein changes that occurred in tissue samples and/or in body fluids (e.g. serum, plasma, urine, saliva) in response to cancer or other diseases (Herosimczyk et al. 2006). Nevertheless, studies conducted on polar foxes (*Alopex lagopus*) are sparse. To survive in unfavorable conditions, the polar fox reveals numerous features including sophisticated water and electrolyte balance mechanisms described by Zhan et al. (1991). Moreover, only morphological features of polar fox kidneys in comparison to a human have been listed by Hadziselimovic and Cus (1975). Thus, proteomic studies may be useful in better understanding the kidney function in this suborder, as they allow for analysis, identification, and determination of function of a wide protein groups, which directly affect kidneys or are related to their activity. Moreover, protein databases such as SwissProt or NCBI are incomplete in case of protein sets of carnivorous animals. Proteomic techniques also enable to broaden the knowledge of renal pathogenesis. It should also be emphasized that renal cortex is a place for progressive development of many renal diseases (Ożgo et al. 2007). Thus, proteomic approaches may be an alternative for other, more invasive, methods like biopsy, which might be interrupted by incorrect renal position or too high adipose tissue content (Nowicki et al. 2005). Moreover, renal biopsy may lead to many complications like perirenal hematomas, lumbar area pain, urinary tract infection or numerous injuries of other organs (Jankowski 2003).

In the available literature there are not many informations concerning the application of proteomic tools for the assessment of renal functions in carnivores. Thus, creation of a reproducible 2-D protein map of polar fox renal cortex and in the future studies of renal medulla will enable to obtain a pattern of renal proteins characteristic of carnivorous animals and will also allow to compare the differences in protein expression patterns between these two regions. The aim of the present study was to establish 2-D protein map of renal cortex characteristic of healthy nine-month-old polar foxes used as a model for carnivorous animal's. This map might be useful for designing further proteomic studies aimed at elucidating the patterns of renal adjustments to various physiological and/or pathophysiological factors.

Materials and Methods

Animals and sample collection

A total of six, nine-month old, healthy males of polar fox (*Alopex lagopus*) were used. Animals were kept in the individual metabolic pens under unified controlled environmental conditions (temperature, humidity, air movement). During the experiment animals received a standard feed for polar foxes (90 kcal EM/kg of body weight, NRC 1982), once a day. The animals had free access to water. After animals were euthanized, the kidney samples were dissected in order to separate cortex and medulla. The use and handling of animals for this experiment was approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals (No. 2/2010 of 20.01.2010 and No. 26/2010 of 21.07.2010).

Dissected tissue fragments were washed twice with 0,9% NaCl (0°C) and thereafter twice with 20 mM Krebs-HEPES buffer (0°C, pH 7.4). Subsequently, tissue fragments were placed in liquid nitrogen, homogenized and dissolved in lysis buffer containing 5M urea, 2M thiourea, 4% CHAPS, 40mM Tris, 0.2% carrier ampholytes. The samples, prepared in that manner, were then stored (-80°C) until further analysis.

Two-dimensional electrophoresis (2-DE)

Prior to analysis the samples were thawed at 0°C, precipitated with four volumes of cold acetone (-20°C) and centrifuged (15000 rpm/30 min). The protein pellets obtained in this way were later dissolved in the lysis buffer (5 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris, 0.2% w/v 3-10 ampholytes and 2 mM TBP). Isoelectric focusing was run (Protean® IEF Cell, Bio-Rad) using 3-10, 17 cm NL ReadyStrip™ IPG Strips (Bio-Rad) (400 µg protein/400 µl rehydratation buffer) in total 92400 Vh. The focused IPG strips were reduced with DTT in equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% w/v glycerol and 1% w/v DTT) for 15 minutes and then alkylated with iodoacetamide (2.5% w/v) for 20 minutes at ambient temperature. After equilibration process SDS-PAGE was performed on large format 12% SDS

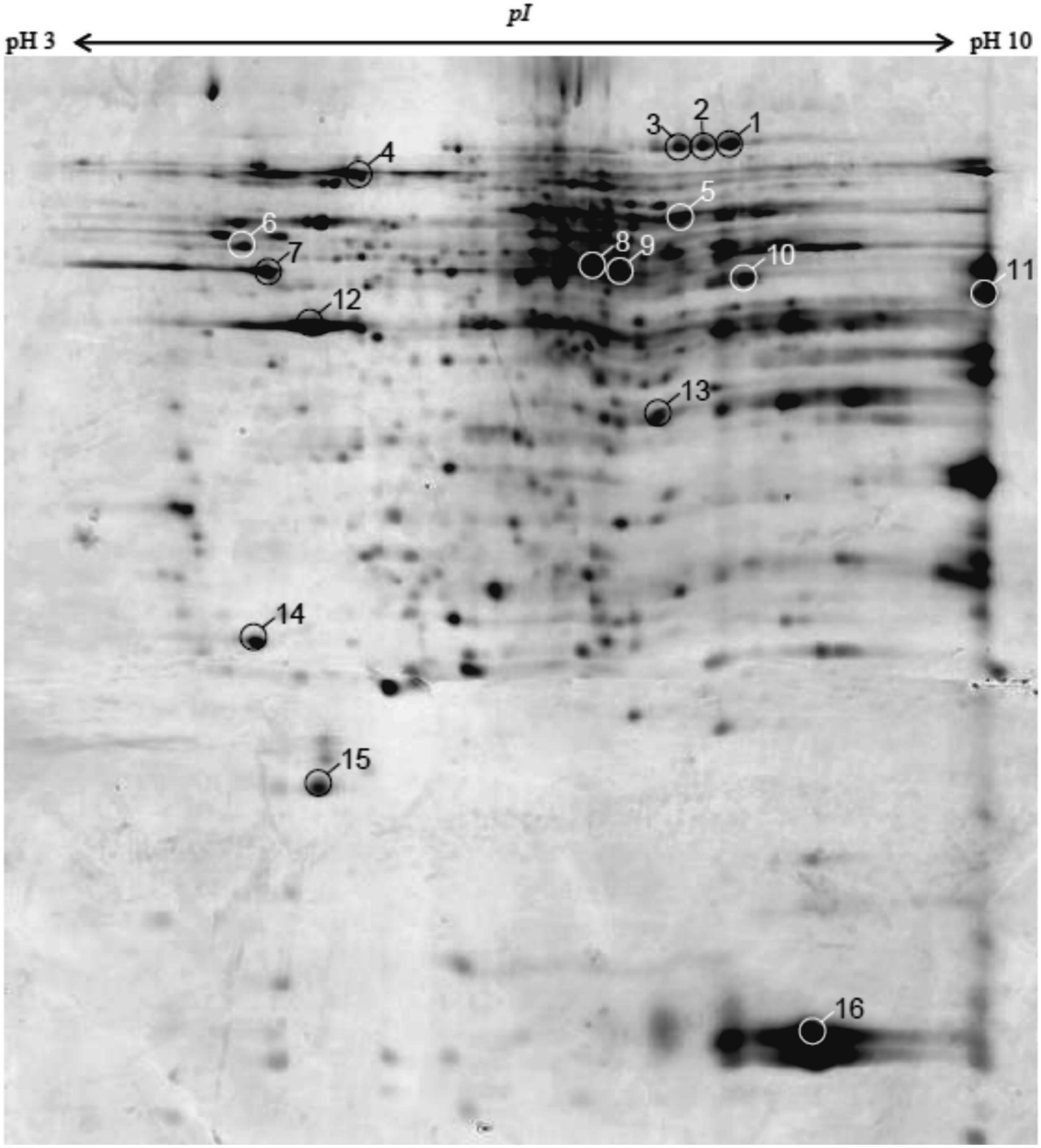

Fig. 1. Two-dimensional map of polar fox (*Alopex lagopus*) renal cortex proteome. Proteins (400 µg) were separated in the first dimension by IEF using 3-10 non linear IPG strips and followed by the second dimension on a 12% SDS-PAGE gels. The proteins were visualized by Coomassie colloidal blue G-250. The numbered spots were identified by MALDI-TOF MS.

polyacrylamide gels at 40V for 1h and subsequently at 120V for 18h at 10°C. After 2-DE separation, the gels were stained for 72 h with colloidal Coomassie Brilliant Blue G-250. The gel images were acquired (GS-800™ Calibrated Densitometer, Bio-Rad) and analysed using PDQuest 8.0. software (Bio-Rad).

Mass spectrometry (MS)

Protein spots, with altered expression, were manually excised from coomassie stained gels and decolorized (25 mM NH_4HCO_3 in 5% v/v ACN) then dehydrated with 100% ACN and vacuum dried (Con-

Table 1. The summary of the identified renal cortex proteins from polar fox (*Alopex lagopus*) with the aid of MALDI-TOF MS.

Spot	Protein information	Accession No.	Theoretical Mr [kDa]	Theoretical p*I*	Score	Sequence coverage [%]
1.	Aconitate hydratase, mitochondrial precursor	P16276	86.449	8.24	98	30
2.					125	26
3.	Putative potassium channel regulatory protein	Q2TUM3	29.569	7.10	61	27
4.	Serum albumin precursor	P49822	70.566	5.52	63	17
6.		PO2769	71.244	5.82	80	23
9.		PO2769	71.244	5.82	105	19
5.	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial precursor	P0C2X9	62.286	7.14	79	19
7.	ATP synthase subunit beta	P56480	56.265	5.19	173	62
8.	Glutamate dehydrogenase 1, mitochondrial precursor	P26443	61.640	8.05	106	26
10.	4-aminobutyrate aminotransferase, mitochondrial precursor	P80404	57.087	8.17	107	32
11.	Trifunctional enzyme subunit beta, mitochondrial precursor	O46629	51.598	9.37	65	18
12.	Actin, cytoplasmic 1	O18840	41.923	6.30	131	53
13.	Alcohol dehydrogenase [$NADP^+$]	Q9JII6	36.792	6.90	65	23
14.	Apolipoprotein A – I precursor	PO2648	30.178	5.20	156	54
15.	Plasma retinol-binding protein precursor	P27485	23.394	5.41	64	47
16.	Hemoglobin subunit beta	P21201	16.084	7.96	173	86

centrator 5301, Eppendorf). Next, samples were incubated with trypsin (20 µl/spot of 12.5 µg/ml in 25 mM NH_4HCO_3; Sigma-Aldrich, St. Louis, MO) for 16 h at 37°C. Resulted peptides were extracted with 100% ACN, combined with an equal volume of matrix solution (5 mg/ml CHCA, 0.1% v/v TFA, 50% v/v ACN) and loaded onto a MALDI-MSP AnchorChip™ 600/96 plate (Bruker Daltonics, Germany). For calibrating mass scale Peptide mass standard II (Bruker Daltonics, Germany within mass range 700-4000 Da) was used. Mass spectra were acquired in the positive-ion reflector mode using Microflex™ MALDI TOF mass spectrometer (Bruker Daltonics, Germany). The PMF (peptide mass fingerprinting) data were compared to mammalian databases (SWISS-PROT; http://us.expasy.org/uniprot/) by the aid of MASCOT search engine (http://www.matrixscience.com/). The database search in MASCOT was conducted using a mass accuracy of 150 ppm, one missing peptide cleavage site, partial carbamidomethylation of cysteine and partial methionine oxidation. A protein was successfully identified when a significant MASCOT score was achieved ($p<0.05$).

Results

The aim of the present study was to obtain protein profile of renal cortex characteristic for healthy polar foxes. Protein samples (in total six) were separated using high resolution two-dimensional electrophoresis. All gels were scanned with the aid of GS-800™ calibrated densitometer (Bio-Rad). The 2-D image computer analysis was performed using PDQuest Analysis software version 8.0. Advanced (Bio-Rad). Gel images were cropped prior to analysis according to the same pattern. The following analysis were performed: spot background substraction, spot detection and matching. The parameters used for between-gel comparison were: the size of the most faint spot, the smallest spot, the size of the largest spot. Normalization of each individual spot was performed using local regression model (LOESS). These analyses allowed us to detect 392 protein spots which were reproducible on each 2-D gel, with regard to its location and stain intensity. On that basis we selected one, representative 2D image of resolved proteins (400 µg) in the pH range of 3-10 (Fig. 1). All reproducible protein spots (392) were

excised from the gels and subjected to mass spectrometric analysis. Only those proteins whose isoelectric points and molecular masses correspond to its actual location on the 2-D gels were finally approved. As a result only sixteen proteins were successfully identified with the aid of MALDI-TOF MS and they are listed in Table 1. The following criteria for protein identification were taken into consideration: level of amino acid sequence coverage, number of matched peptides and the highest score from at least two databases: Swiss-Prot, NCBI.

Unfortunately, the majority of excised spots remained unidentified despite repeated analysis. The main reasons of failed identification could be attributed to low protein content and to intrinsic limits of PMF e.g. inability to detect low molecular weight proteins, and most importantly, to limited number of entries present in the available protein databases for carnivore species.

Discussion

The proteins identified in the current study were divided into six groups based on their conjoined role in the biological functions. These groups include: lipid and fatty acid metabolism (apolipoprotein A-I precursor, 4-aminobutyrate aminotransferase, trifunctional enzyme subunit beta), amino acid metabolism (glutamate dehydrogenase 1, delta-1-pyrroline-5-carboxylate dehydrogenase), energetic pathways (ATP synthase subunit beta, alcohol dehydrogenase [$NADP^+$], aconitate hydratase), regulatory proteins (putative potassium channel regulatory protein, serum albumin precursor, glutamate dehydrogenase 1), transport proteins (serum albumin precursor, hemoglobin subunit beta, plasma retinol-binding protein precursor) and structural proteins (actin, cytoplasmic 1).

Proteomic studies of rat renal cortex were also performed by Xing et al. (2006). The authors using western blot technique analysed changes in the expression of four renal cortex proteins, mainly nephrin, podocin, CD2AP and α-actinin-4 in Adriamycin-induced nephritic (ADR) rats. These proteins are responsible for maintaining structural and functional integrity of podocyte thus preventing the proteinuria. Velic et al. (2010) suggest that mass spectrometry-based proteomic analysis of kidney should rather focus on analysis of protein profiles in functional and morphological subunits – nephrons, rather than the whole tissue. This is related to the fact that many protein are localised to specific nephron segments. This may allow for the evolution and better understanding of its morphology and function.

Identified in the present study proteins from the lipid and fatty acid metabolism group are involved in various metabolic processes, associated with transport, conversion and/or degradation of lipids and fatty acids. The most abundant protein in this group is apolipoprotein A-I precursor (pro-ApoA-I), which participates in the reverse transport of cholesterol from peripheral tissues e.g. from kidneys to the liver. Thus, apoA-I promotes cholesterol efflux from tissues and acts as a cofactor for the lecithin cholesterol acyltransferase (LCAT). Moreover, it plays a role as a part of the sperm activating protein (SPAP) complex, and also activates spermatozoa motility (Akerlöf et al. 1991). ApoA-I is the major apoprotein constituent of high-density lipoprotein and it may also have anti-inflammatory properties (Yoo et al. 2009). The authors claim that decreased expression of apoA-I is connected with the appearance of cysts in the kidneys. The lack of pro-ApoA-I leads to deficiency of its mature form – apolipoprotein A-I (ApoA-I). Defects in ApoA-I or its lack, in turn, result in low HDL levels, which is a characteristic symptom observed in high density lipoprotein deficiency type 1 (HDLD1), also known as analphalipoproteinemia or Tangier disease (TGD). In HDLD1 patients, ApoA-I fails to associate with HDL, probably because of the faulty conversion of pro-ApoA-I molecules into mature chains (by the cleavage of 6 amino acids), either due to a defect in the converting enzyme (specific metalloproteinase) activity or a specific structural defect in Tangier ApoA-I. HDLD1 is characterized by the absence of plasma HDL, accumulation of cholesteryl esters, premature coronary artery disease, hepatosplenomegaly, recurrent peripheral neuropathy and progressive muscle wasting and weakness (Nakata et al. 1993, Ng et al. 1994). Moreover, defects in ApoA-I are the cause of amyloid polyneuropathy-nephropathy Iowa type (AMYLIOWA). AMYLIOWA is a hereditary generalized amyloidosis due to deposition of amyloid mainly constituted by apolipoprotein A-I. The neuropathy and nephropathy, which occur in the early or late stage of the disease, dominate the clinical picture of this disorder. In most cases death is due to renal amyloidosis (Nichols et al. 1988, Nichols et al. 1990, Nakata et al. 1993, Ng et al. 1994). Other disorders caused by a defect or lack of pro-ApoA-I include: nephrotic syndrome, arterial hypertension, hepatosplenomegaly, cholestasis, petechial skin rash (Soutar et al. 1992).

Proteomic tools have also been successfully employed to analyse proteins derived from intracellular structures within the glomerulus and tubules. It should be emphasized that glomerulus plays crucial role in the process of filtration and it may be a place for progressive development of many renal diseases. For example, Yoshida et al. (2005) created a profile of

the normal human glomerulus proteome using 2-DE, MALDI-TOF MS and LC-MS/MS (liquid chromatography-tandem mass spectrometry). All proteins identified by the authors were deposited in the database (http://www.sw.nec.co.jp/bio/rd/hgldb/index.html) and divided into the following categories: cell-cell signalling proteins, proteins involved in immune response and in developmental process, proteins engaged in DNA, RNA and protein metabolism, signal transduction, stress response, cell adhesion proteins, cell cycle and proliferation, cell death and cell organisation, motility and transport proteins.

Another group identified in the present study includes proteins which are involved in amino acid metabolism. Renal ammoniagenesis is a process whereby the kidney produces ammonia primarily via glutamine metabolism (Treberg et al. 2010). Deferarri et al. (1994) reported that over 50% of the total renal ammonia production was released into the renal vein in normal acid-base status and the remaining amount was excreted in the urine. Nevertheless, the authors observed increased ammonia production in chronic acidosis and that approximately 75% of this ammonia was excreted in the urine (Deferarri et al. 1994). The increased capacity for renal ammoniagenesis that occurs particularly during acidosis involves increased catabolism of glutamine (Treberg et al. 2010). As previously shown by Treberg et al. (2010), there exists an evidence that activation of renal glutamate dehydrogenase is an important element in the increased capacity to excrete ammonia during acidosis. Moreover, glutamate dehydrogenase 1 protein, which has homohexamer subunit structure and is localized in the mitochondrion matrix, may be involved in the learning processes and memory reactions by enhancing the turnover of the excitatory neurotransmitter – glutamate.

Proteins which are vital in energetic pathways are involved in the citric acid cycle, which plays a pivotal role in mitochondrial bioenergetics by providing the reducing equivalents, NADH and $FADH_2$ for the ATP synthesis, and NADPH for the reduction of H_2O_2 (Yarian et al. 2006). Yarian et al. (2006) claim that aconitate hydratase may undergo oxidative modification *in vitro* with a subsequent decrease in enzymatic activity, after the exposure of mammalian mitochondria to hydrogen peroxide. Aconitate hydratase has also been shown to be carbonylated *in vivo* in insects and mammals, resulting in a reduction of activity (Yan et al. 1997, Das et al. 2001, Yarian et al. 2005). The results presented by Yarian et al. (2006) demonstrate that the activities of specific citric acid cycle enzymes, such as aconitate hydratase, are lowered in the kidneys during the ageing process. The above may define the nature of the bioenergetic impairments observed during the ageing process. According to Yarian et al. (2006) a decrease in renal aconitate hydratase activity may be responsible for affecting the overall turnover efficiency of the citric acid cycle, and thus leads to citric acid accumulation in the cytosol. This would quite likely activate fatty acid synthesis. In addition, a decrease in aconitate hydratase activity would ultimately affect the products of the downstream reactions in the cycle.

Some of the identified proteins were included in the regulatory protein group: putative potassium channel regulatory protein, serum albumin precursor, glutamate dehydrogenase 1. The precise function of putative potassium channel regulatory protein is still not fully understood. It is only known that this protein inhibits potassium fluxes in the cells. The mature form of serum albumin precursor plays a role in the regulatory processes. The main function of the serum albumin is the regulation of the blood colloidal osmotic pressure. The transport proteins identified in this experiment are carriers and play a key role in maintaining proper function of the organism. Serum albumin precursor is considered to be the precursor form of the main plasma protein. It shows good binding capacity for water, Ca^{2+}, Na^+, K^+, bilirubin, hormones, fatty acids and drugs. This protein is a major zinc transporter in plasma, typically binds approximately 80% of all plasma zinc (Lu et al. 2008). Moreover, subunits of serum albumin, which are present in the urine (in pathological condition), might serve as biomarkers of renal diseases, such as IgA nephropathy, steroid-resistant and steroid-sensitive nephritic syndrome or diabetic nephropathy (Ciechanowicz et al. 2011). Plasma retinol-binding protein plays a role mainly in the liver, nevertheless it is catabolized in the kidney. Actin, cytoplasmic 1 (ACTB) is a member of actin family, involved in various types of cell motility and ubiquitously expressed in all eukaryotic cells. ACTB is mainly localized in the cytoplasm as a cytoskeleton structure.

Conclusion

This is the first study attempting to create 2-D map of renal cortex proteins characteristic for healthy polar foxes, serving as animal model for carnivores. Since there is very limited information on the expression of proteins in the kidneys of carnivorous animals, presented in the current paper 2-D profile may broaden the knowledge in the area of renal physiology in this group of animals. This protein map may broaden currently available protein databases and it may also be useful for designing further proteomic studies aimed at elucidating the patterns of

renal adjustments to various physiological and/or pathophysiological factors.

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