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ANALYSIS OF NICKEL-BINDING PROTEINS FROM VARIOUS ANIMAL SERA

Šimková, J.[1], Milkovičová, M.[1], Valko-Rokytovská, M.[1], Kostecká, Z.[1] Bencúrová, E.[2, 3], Pulzová, L.[2], Čomor, Ľ.[2], Bhide, M. R.[2, 4]

[1]Department of Chemistry, Biochemistry and Biophysics
University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
[2]Laboratory of Biomedical Microbiology and Immunology
University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
Slovakia
[3]Department of Bioinformatics, Julius-Maximilian University of Wuerzburg, Am Hubland, 970 74 Wuerzburg
Germany
[4]Instituteof Neuroimmunology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 10 Bratislava 45,
Slovakia

jana.simkova@uvlf.sk

ABSTRACT

Nickel-binding proteins play an important role in the biological processes and can also be utilized in several fields of biotechnology. This study was focused on analysing the nickel-binding proteins from the blood sera of humans (*Homo sapiens*), cattle (*Bos taurus*), sheep (*Ovis aries*), red deer (*Cervus elaphus*), mouflon (*Ovis orientalis*), fallow deer (*Dama dama*), horses (*Equus ferus caballus*), pigs (*Sus scrofa domesticus*), wildboars (*Sus scrofa*), brown bears (*Ursus arctos*) and pheasants (*Phasianus colchicus*). The presence of higher abundance proteins in the blood serum, such as albumins, may mask the detection of lower abundance proteins. The samples were depleted from these higher abundance proteins to facilitate the detection of those with lower abundance. For the characterization of these proteins, nickel cations bound to tetradentate ligand nitrilotriacetic acid(Ni-NTA)immobilized on agarose beads were incubated with animal sera to capture nickel-binding proteins and subsequently the proteins were eluted and fractionated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed a set of nickel-binding proteins with various molecular weights within different animal species. A unique ~42 kDa nickel-binding protein in the brown bear serum, which was not present in any of the other species, was further characterized and identified by matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS). This protein was identified as ahaptoglobin-like protein. This result may provide some valuable clue for the physiological difference in the metal binding proteins in the serum of Ursus arctos and other animals.

Key words: animal sera; mass spectrometry; nickel-binding proteins; proteomics

INTRODUCTION

Metalloproteomics is a rapidly developing field of science that involves the comprehensive analysis of all metal-containing or metal-binding proteins in a biological sample [13].

Metals play pivotal roles in a broad range of biological processes in all living organisms. Most of these metal ions are bound to specific proteins or enzymes, and exert their effects as active or structural centers of proteins [12]. It is estimated that approximately one-third of all proteins and enzymes require a metal cofactor for functionality; thus named "metalloproteins" [2, 37]. Among the metal-containing proteins, Mg and Zn belong to the most abundant elements; however, Ca, Mn, Fe, and Ni are also frequently observed [35].

Metal-binding proteins and metalloproteins are responsible for many metabolic processes, such as biological energy conversion in photosynthesis and respiration; they are also involved in signalling processes, gene expression and regulation. Metal sites in protein structures also control other processes such as catalysis, substrate binding and enzyme activation. Moreover, they can serve as the reservoir of metal ions and their storage for the cell maintenance [3]. The ability of proteins to bind essential and toxic metals plays an important physiological role in both normal and diseased states [4–7]. Therefore, the characterization of metalloproteins is important for understanding the structure and biological functions of such proteins, thus leading to a clear understanding of metal-associated diseases [1].

Nickel is an essential trace element for each animal species [8]. Nickel is involved in the key processes in the animal body — it interacts with haeme iron and helps in oxygen transport, stimulates the metabolism and is regarded as a key metal element in many plants and animal enzyme systems. As it was shown before, Ni^{2+} ions are necessary for the metabolism of sugars [18] as well as in the transmission of the genetic code (DNA, RNA) [29].

The essential role of nickel ions consists of the action or formation of cGMP, a signalling agent that regulates various physiological processes including blood pressure control, sperm physiology, sodium metabolism and cardiovascular health. Nickel is consistently present in RNA and is bound to several biological substances such as proteins (keratin, insulin), amino acids and serum albumin [39]. In animals, nickel deficiency inhibits growth, reduces reproductive rates, and alters glucose and lipid metabolism that are associated with anemia, hemoglobin reduction, alternations of metal ion contents, and reduced activity of several enzymes [31].

It has been shown by many studies that aminoacids such as histidine, aspartate, glutamate, tyrosine, methionine, trypthophan and cysteine mediate strong interactions with the metal ions [14, 16, 21, 23, 27]. This property was used in the immobilized metal ion affinity chromatography (IMAC) [30] as well as in the other affinity chromatography techniques providing separation and purification of various metal-binding proteins.

Wang et al. [38] used metal affinity chromatography to enrich a fraction of human serum proteins on immobilized columns loaded with cadmium, nickel, zinc, copper or lead in Bis-Tris saline and these proteins were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). They identified tens of nickel-binding proteins, e.g. complement C3, α_2-macroglobulin, serum albumin, apolipoprotein B-100, complement component 4B preproprotein and histidine-rich glycoprotein. A similar approach was used in the work of She et al. [33] where they examined the Cu- and Zn-binding proteins from the human hepatoma using the IMAC and mass spectrometry. Nickel-binding proteins are relatively well-known in microorganisms [22, 32, 36].

In this study, we analysed the nickel-binding proteins in the sera of omnivores (humans, pigs, wild-boars), carnivores (brown bears), polygastric (cattle, sheep, red deer, mouflon, fallow deer) and monogastric (horses) herbivores and a bird (pheasants).

MATERIALS AND METHODS

Animals and blood sampling

Samples of venous blood from four to twenty individual animals (humans, cattle, sheep, red deer, mouflon, fallow deer, horses, pigs, wildboars, brown bears and a pheasant) were collected into sterile glass tubes.

Serum preparation

After blood coagulation (12 h, 18 °C) and centrifugation (25 min, 2500 r. p. m., 4 °C) serum samples were filtered through 0.22 μm syringe filters (Minisoft), pooled, aliquoted and stored at –80 °C until use.

Albumin depletion

The sera were then incubated in 0.1 M NaCl (Sigma-Aldrich, USA) and cold ethanol (Sigma-Aldrich, USA) with gentle rotation at 4 °C for 60 min. The samples were centrifuged at 16,000 × g for 45 min at 4 °C. The supernatants were transferred into fresh tubes and the pellet (No. 1) was retained for further processing. The pH of the supernatants was lowered to 5.7 with cold Na-acetate (pH 4) (Sigma-Aldrich, USA) and mixed with gentle rotation at 4 °C for 60 min. After centrifugation, supernatants containing albumins were removed and the pellet (No. 2) was resuspended in RNA free water (Invitrogen, USA) and mixed with the first pellet. This pellet contained the albumin depleted fraction of proteins.

Metal affinity binding chromatography and protein fractionation

The albumin depleted proteins (approx. 200 µg) were incubated with metal affinity Ni-NTA beads (Nickel-Nitrilotriacetic acid) under the native conditions as per the manufacturer's instructions (Jena Bioscience, Germany). The metal affinity beads were washed in spin-column with native wash buffer (0.05 M NaH_2PO_4, 0.3 M NaCl, 10 % glycerol, 0.1 % Tween) (Sigma-Aldrich, USA).

Proteins were then loaded onto the column with the beads, and mixed gently at 4 °C overnight. Beads were then washed with native wash buffer at least five times and the bound proteins were eluted in NuPAGE® Lithium dodecyl sulfate Sample Buffer (NuPAGE®LDS Sample Buffer) (Invitrogen, USA). The detailed protocol of this pull-down assay is described in the publication by Mlynarcik et al. [24]. Proteins were separated by SDS-PAGE on 10 % polyacrylamide gel and visualised using Coomassie Brilliant Blue staining.

Western-blot analysis of nickel-binding proteins

The eluted proteins from the metal affinity binding chromatography were fractionated by SDS-PAGE (Invitrogen, USA) and electrotransferred onto a nitrocellulose membrane. The membrane was blocked for 45 min in 0.05 % TTBS (Tris Buffered Saline TBS) (25 mM Tris, 150 mM NaCl, pH 7.2) with 0.05 % Tween 20 (Sigma-Aldrich, USA) containing 2 % bovine serum albumin (BSA) (Sigma-Aldrich, USA). After two washings with TTBS, the membrane was incubated with horseradish peroxidase-conjugated Ni-NTA(Ni-HRP conjugate) (Pierce, USA) (1 : 5000 diluted in 0.05 % TTBS) (Pierce, USA) for 1 h. After five washings with TTBS, the membrane was incubated in enhanced chemiluminescence substrate (Pierce, USA) for 5 min and the signal was documented by an imaging system (Li-Core, USA).

Mass spectrometry and database searching

A protein band with an approximate molecular weight of 42 kDa from the brown bear was excised from gel under keratin-free conditions, washed in RNA-free water and digested by trypsin [34]. The digested aliquots were mixed with α-cyano-4-hydroxycinnamic acid (Bruker, Germany) in 33 % aqueous acetonitrile and 0.25 % trifluoroacetic acid (Sigma-Aldrich, USA). This mixture was deposited onto a 600 µm AnchorChip prestructured matrix-assisted laser desorption/ionization (MALDI) probe (Bruker-Daltonics) and allowed to dry. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) data were obtained in an automated analysis loop (Ultraflex, Bruker-Daltonics) equipped with a LIFT-MS/MS device. Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1000 individual spectra were averaged. The automated analysis of the peptide mass data were performed (FlexAnalysis software; Bruker-Daltonics). MALDI-MS and MALDI-MS/MS data were combined (BioTools, Bruker-Daltonics) to search a non-redundant protein database (NCBInr) using Mascot software (Matrix Science) and the results were subjected to the BLAST analysis (http://blast.ncbi.nlm.nih.gov/) compared by NCBI (https://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/) databases.

Functional category analysis

The EggNOG 4.5.1 (Evolutionary Genealogy of Genes: Non-supervised Orthology Groups) was carried out to determinate the orthologous genes and the functional category analysis (http://eggnogdb.embl.de) [11].

RESULTS

Nickel-binding proteins

SDS-PAGErevealed the presence of a few nickel-binding proteins in the serum of each animal species (Fig. 1). These proteins can be classified into several groups: the protein with molecular weight approximately 100 kDa, observed in

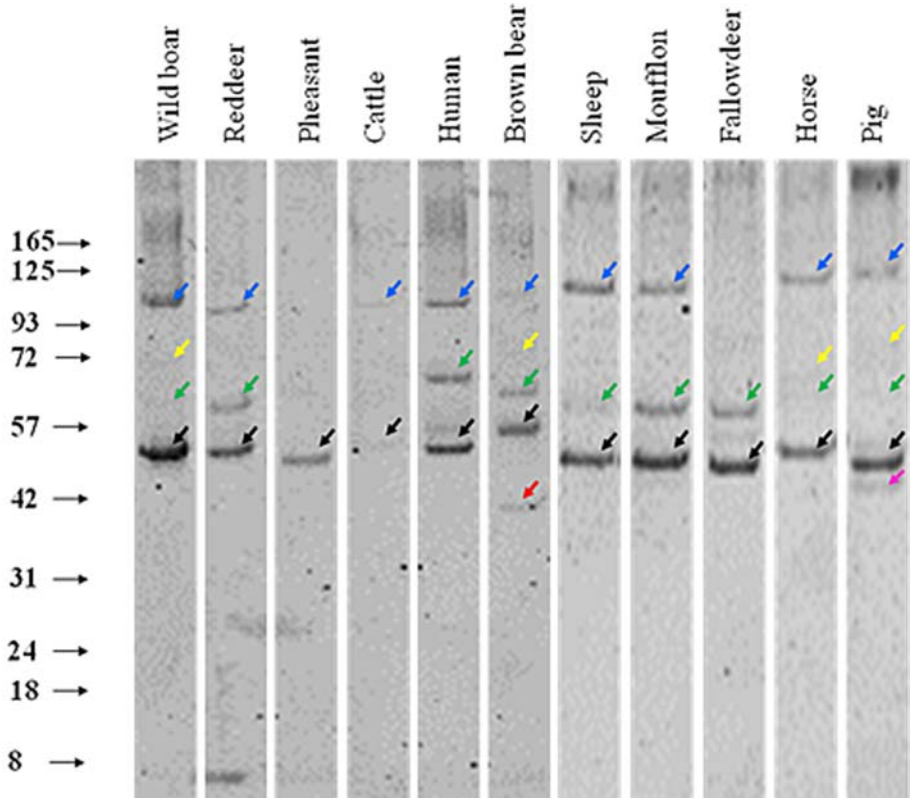

Fig. 1. Assessment of nickel-binding proteins by metal affinity binding chromatography. The nickel-binding proteins from the sera of various animals and humans were bound to metal affinity beads and fractionized by SDS-PAGE

blue arrow — Ni-binding protein with molecular weight of approx. 100 kDa; **yellow arrow** — Ni-binding protein with a molecular weight of between 68—72 kDa; **green arrow** — Ni-binding protein with a molecular weight of approximately 60 kDa; **black arrow** — Ni-binding protein with a molecular weight of approximately 54 kDa; **red arrow** — 42 kDa Ni-binding protein of the bear chosen for identification; **magenta arrow** — 50 kDa Ni-binding protein of a pig

all animal sera (Fig. 1, blue arrows) except the pheasant and fallow deer; the second with a molecular weight of around 60 kDa which were found in the sera of wild boar, red deer, human, brown bear, sheep, mouflon, fallow deer, horse and pig (Fig. 1, green arrows); the third with a molecular weight of approximately 54 kDa, detected in all sera (Fig. 1, black arrow). In the sera of the wild boar, bear, horse and pig, proteins with molecular weight between 68–72 kDa were found (Fig. 1, yellow arrow) and in the case of the pig, the low abundant 50-kDa protein was observed (Fig. 1, arrow with magenta colour). The unique 42-kDa protein was observed only in the serum of the bear (Fig. 1, red arrow). This protein was subjected to further identification by mass spectrometry.

Identification of 42-kDa nickel-binding protein in the bear

To confirm that the 42-kDa protein from the bear serum was a nickel-binding protein, a western blot was performed with a Ni-HRP conjugate (Fig. 2). This protein was further excised from polyacrylamide gel and analysed by MALDI/TOF/MS. The Mascot search revealed that this protein was a haptoglobin-like protein (Accession number–gi|301776456, score 597.6, 7 matched peptides) (Table 1).

EggNOG functional category analysis

In order to find the orthologous genes of the haptoglobin-like protein, the EggNOG analysis was used. Fifteen categories were identified with the score more than 500 (Table 2). The clusters with the highest score were identified as ENOG410CHIE, with the functional annotation amino acid transport and metabolism — haptoglobin, having the orthologs in 3 other species: Felis catus, Canis lupus familiaris and Mustela putorius furo (Fig. 3) [11].

Table 1. Results from the identification of 42 kDa protein of the brown bear from the Mascot server

Rank	Protein	Accession	Mw [kDa]	Score	No. Peptides	SC [%]	RMS90 [ppm]
1	Haptoglobin-like (*Ailuropoda melanoleuca*)	gi|301776456	38.2	597.6 (M: 597.6)	7	25.6	19.56

Mw — molecular weight; SC — sequence coverage

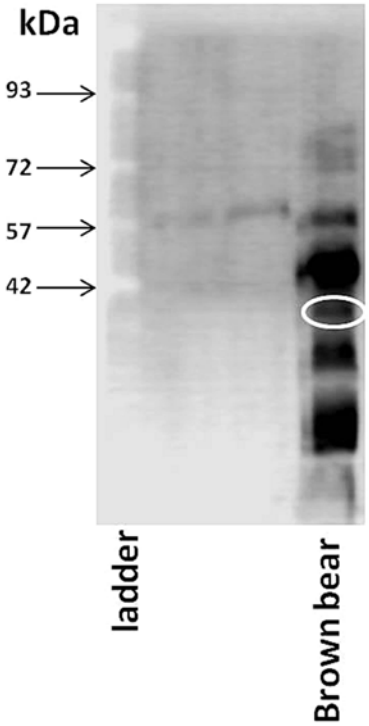

Fig. 2. Validation of nickel binding abilities of 42 kDa serum protein in the bear by the western blot. Albumin-depleted sera of the brown bear were electrotransferred onto a nitrocellulose membrane and the interaction of Ni-HRP conjugate with Ni-binding proteins was visualized. The 42 kDa serum protein in the bear is highlighted in the white ellipse

Table 2. The EggNOG automatic classification and functional analysis of the haptoglobin-like protein orthologs

EggNOG	Score	Function	Nr. of species/ proteins
ENOG410CHIE	807.5	Amino acid transport and metabolism	4/4
ENOG410UTJ0	707.0	Amino acid transport and metabolism	34/34
ENOG410RPEA	683.0	Amino acid transport and metabolism	4/4
ENOG4118TY1	681.4	Extracellular structures	5/5
ENOG411B3ZT	680.2	Amino acid transport and metabolism	17/17
ENOG4116793	652.1	Amino acid transport and metabolism	10/10
ENOG410A494	651.1	Amino acid transport and metabolism	40/42
ENOG410DRTE	651.1	Amino acid transport and metabolism	40/42
ENOG410VF3W	651.1	Amino acid transport and metabolism	40/42
ENOG4113N0R	651.1	Amino acid transport and metabolism	40/42
ENOG411CU98	651.1	Amino acid transport and metabolism	40/42
ENOG410S11U	642.8	Amino acid transport and metabolism	2/2
ENOG410V09D	642.8	Amino acid transport and metabolism	2/2
ENOG4116M9C	642.8	Amino acid transport and metabolism	2/2
ENOG411B98W	642.8	Amino acid transport and metabolism	2/2

Fig. 3. Phylogenetic tree of the orthologs of haptoglobin-like protein (Ailuropoda melanoleuca) with the highest score (EggNOG ENOG410CHIE). The functional annotation from the Pfam database is depicted on the right [28]. The phylogenetic tree was generated by the EggNOG database

DISCUSSION

The results of our study demonstrated that various species, including birds, humans, and other mammals contained a repertoire of the nickel-binding proteins in their blood sera.

Some proteins of this repertoire have approximately similar molecular weights (e. g. 100 kDa, 60 kDa, and 54 kDa), so probably they were the ortholog of the same proteins, thus we focused on the unique, 42-kDa protein that was found in the bear serum only.

This unique protein was identified as a haptoglobin-like protein. This group of proteins is not well-known in mammals, although haptoglobins play a role in the acute phase response and their expression is stimulated by a broad range of the inflammatory stimuli. The haptoglobin-like proteins with the molecular weight around 40 kDa and pI in the range 7.7—8.6 have been observed also in human sera [38], while most studies of haptoglobin-like proteins are associated with the pathology of the female reproductive system [15, 19].

There is also very strong evidence for an essential role of haptoglobin in brown and black bears [9, 25]. Mominoki et al. [25] tested the concentration of haptoglobins in hibernating and non-hibernating bears. They determined a higher concentration of haptoglobins in the hibernating bears during winter than in spring, and they suggested that its concentration in plasma is more associated with the hibernation [25].

The group of nickel-binding proteins is very well-studied with regard to the important bacterial pathogens: Helicobacter pylori, *Escherichia coli, Klebsiella aerogenes* and others [10, 20, 22]. However, the function and utilization of nickel-binding proteins in mammals are poorly understood. Nickel is an essential component of several enzymes and it is involved in several metabolic pathways in both, prokaryotes and eukaryotes [40]. Nickel-binding proteins are usually histidine-rich plasma proteins, which are able to bind metals and other metal-containing proteins, e. g. zinc, copper, cadmium, heparin and haeme [17], but there are only 3 % of histidine residues in the amino acid sequence of the haptoglobin-like proteins of the giant panda (7 peptide match of 42 kDa with haptoglobin-like proteins of the giant panda). Furthermore, in the amino acid sequence there are no His-Pro rich domains or motifs usually responsible for the interaction with divalent cations [26]. On the other hand, the principle of immobilized metal ion affinity chromatography is an interaction of histidine residues of protein with transition metal ions (e. g. nickel or zinc) immobilized on the agarose or metal beads. However, as our identification in the case of the brown bear failed, we can only speculate that the 42-kDa protein found by SDS-PAGE has similar, but not identical sequence as the haptoglobin-like protein of the giant panda identified by the peptide mass fingerprinting analysis. The sequence analysis of the whole coding region of haptoglobin-like protein of brown bear will reveal the nickel binding pockets.

The affinity of serum proteins to Ni^{2+} has not been described in animal species and this study can be the first step toward a deeper characterization of the metabolism and unique features of nickel-binding proteins.

CONCLUSIONS

In the current study we found that several animal sera contained the specific nickel-binding proteins with varied molecular weights. The unique 42-kDa protein of the brown bear was chosen, which showed very strong binding to nickel in the western blot experiments. This protein was not present in any other animals tested or human sera and the identification showed the match with the haptoglobin-like protein of the giant panda. The function of this protein is unknown for us at present. The combination of metal affinity binding chromatography, protein fractionation and MALDI-TOF/MS represents a powerful tool for studying metal-binding proteins in biological tissues.

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