Paper

A Novel Approach to Protein Analysis in Hard Tissues

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This preliminary study is aimed at a new approach in the protein analysis of hard tissues. Analytical methods that have been used for the identification of proteins in bones and teeth always require one critical step – demineralization. This chemical treatment is responsible for loss of proteins and it also negatively influences the possibility of protein quantification. The method of peptide mass mapping described in this paper facilitates a gentle releasing of peptides from the hard tissues without the loss of qualitative and quantitative information. Using this method led to identification of proteins from chicken thighbone, human jawbone and teeth, which proves the feasibility of this method.

1. Introduction

The isolation, identification and characterization of hard tissues proteins are essential for the understanding of a number of physiological processes on a molecular level. Such processes in hard tissues, particularly in bones, are relatively less described and understood because the proteomics of bones still represents an analytical challenge. Usually, the identification of proteins is performed by their specific enzymatic cleavage in a solution, followed by analysis of the resulting characteristic peptide fragments by mass spectrometry. The individual peptide fragments resulting from specific digestion of proteins are sequenced and the sequence data are compared with database derived from DNA sequence. No. of MS/MS peptides means experimentally sequenced peptides which agrees with the protein database. As unambiguously identified protein is usually considered protein identified at least by two MS/MS peptides.

Obviously, proteins from hard tissues cannot be easily solubilized. For their isolation from hard tissues, a complicated and time consuming process of demineralization is required [1]. The process of demineralization also represents various complications of analysis, which can of course have a negative influence on protein identification [2].

Recently we developed a completely new method, which enables identification of proteins even in insoluble materials. The major principle of this method is that intact proteins are not isolated from insoluble materials, which may be almost impossible, but digested in situ, specifically by trypsin. The resulting soluble specific peptide fragments can be easily extracted and subsequently analyzed by mass spectrometry. This method was originally developed for the identification of protein binders in art works [3] and subsequently successfully used for analysis of proteins in insoluble materials like color layers or historical mortars [4-8]. We anticipated that the above-mentioned principle could also be applied to the analysis of proteins in hard tissues. The presented study was performed on chicken thighbone, human jawbone and teeth.

2. Materials and methods

Specific cleavage with trypsin

Approximately 1 mg of each sample (chicken thighbone, human jawbone and human tooth dentin) was washed three times with 1 mL of

distilled water and placed into 20 μL 50 mM NH_4HCO_3. For the disruption of disulfide bonds, the reduction of cysteine residues with DTT (dithiothreitol) (5 mM at 50 °C for 30 min) was used, followed by alkylation with iodoacetamide (25 mM at room temperature in the dark for 30 min). Digestion was carried out in 15 μL of solution of 10 μg/mL sequencing grade trypsin (Promega) in 50 mM NH_4HCO_3 at 37 °C for 4 hours. The solution containing released peptides was desalted using ZipTips packed with reversed phase (C_{18}) resin and vacuum dried.

Mass spectrometry and protein identification

LC-MS/MS was performed using an Acquity UPLC system coupled to an ESI-Q-ToF Premier tandem mass spectrometer (Waters, UK). Prior to the analysis, protein digests were solubilized in 0.1% formic acid and loaded onto a Symmetry C18 trapping column (180 μm i.d. x 20 mm length, particle size 5 μm, reverse phase); with a flow rate of 15 μL/min for 1 minute. Trapping was followed by a reverse phase HPLC with a flow rate of 0.4 μL/min through a BEH 300 C18 analytical column (75 μm i.d. x 150 mm length, particle size 1.7 μm, reverse phase; Waters, UK). A linear gradient (initial 3 % B, 1 min - 40 % B, 60 min) was followed by a cleaning step (85 % B, 62 min; 85 % B, 67 min; 3 % B, 70 min; solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitril). Peptides eluted from the column flowed directly into the ESI source. A collision energy ramp from 15V to 30V was used for peptide fragmentation.

Protein identification was carried out using PLGS 2.3 software (Waters, UK) by searching a species specific, non-redundant Uniprot protein database with the following search parameters: 2 missed cleavages; acetyl N-term, carbamidomethyl C and oxidized M as variable modifications, peptide accuracy 50 ppm and MS/MS fragment mass accuracy 0.2 Da.

3. Results and discussion

The method, which does not require demineralization, was used for analysis of hard tissues. Small parts (about 1 mg) of chicken thighbone, human jawbone and human tooth were directly submitted to specific digestion with trypsin and resulted soluble peptides were subsequently analyzed by LC-MS/MS.

Identified proteins from the three mentioned samples can be divided into three groups: the first group contains the proteins resulting from contamination by traces of blood (hemoglobin, globins), the second group contains proteins that can be ascribed either to blood contamination or can originate from hard tissues and finally the third group contains proteins that are undoubtedly proteins from hard tissues. The discussion is focused on the third group (see Table 1) because it proves that proteins in hard tissues can be identified using a simple method without time consuming demineralization and isolation of intact proteins. These mentioned proteins are summarized in Table 1 and shortly discussed below.

Chicken thighbone

Ovocleidin is a protein found in eggshell matrices as well as in avian skeletal tissues [9]. Decorin is known to be a component of connective tissues bound to type I collagen fibrils and to have a role in matrix assembly. This protein is capable of suppressing the growth of various tumor cell lines [10]. Vimentin is a member of intermediate filament proteins and was localized also in tooth germ cells where it seems to play a role in the formation of enamel tubules [11].

Human tooth

Collagen is generally the main protein of connective tissues abundantly occurring also in dentin [12]. Some types of collagens even play a role in tooth enamel formation [13]. Periostin was isolated as an osteoblast-specific factor that functions as a cell adhesion molecule for preosteoblasts [14] and relatively recently was found to be expressed within the developing teeth at the sites of epithelial-mesenchymal interaction [15]. Vimentin, which is known to be found in tooth, was mentioned above in connection with its identification in the chicken thighbone. Lumican is known to be present in tooth cementum [16]. Osteoglycin (known also as mimecan) is a protein that induces ectopic bone formation in conjunction with transforming growth factors beta [17].

Humane jawbone

Both chondroadherin and collagen are cartilage proteins with cell binding properties that are produced by chondrocytes [18].

4. Conclusion

In preliminary experiments it was shown that the proteomics of hard tissues can be performed elegantly without the need for demineralization. Our newly developed method has several advantages. It is very simple, fast and additionally

the proteins are digested directly in original hard tissues, which eliminate the risk of undesirable protein modifications and contamination.

These preliminary results seem promising and thus we plan to continue in investigation of this approach particularly towards the possibility of quantification of proteins in hard tissues under various physiological/pathological conditions, which could contribute to obtain deeper insight to pathological processes.

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Table 1. Hard tissues proteins identified in chicken thighbone, human tooth and human jawbone.

Accession No.	***Protein identified in Chicken thighbone***	**MW kDa**	**pI (pH)**	**No. of MS/MS peptides**
Q9PUT1_CHICK	Ovocleidin 116 OS *Gallus gallus* PE 2 SV 2	77	6.6	14
PGS2_CHICK	Decorin OS *Gallus gallus* GN DCN PE 1 SV 1	40	8.6	11
VIME_CHICK	Vimentin OS *Gallus gallus* GN VIM PE 3 SV 2	53	4.9	10
	Proteins identified in Human tooth			
CO6A3_HUMAN	Collagen alpha 3 VI chain OS *Homo sapiens* GN COL6A3 PE 1 SV 4	34	6.2	20
C0IMJ3_HUMAN	Periostin isoform thy6 OS *Homo sapiens* PE 2 SV 1	87	7.9	22
Q53HU8_HUMAN	Vimentin variant Fragment OS *Homo sapiens* PE 2 SV 1	54	4.9	11
B2R6R5_HUMAN	Lumican OS *Homo sapiens* GN LUM PE 2 SV 1	38	6.2	3
DYH6_HUMAN	Dynein heavy chain 6 axonemal OS *Homo sapiens* GN DNAH6 PE 1 SV 3	48	5.6	21
B7Z6N2_HUMAN	cDNA FLJ56154 highly similar to Gelsolin OS *Homo sapiens* PE 2 SV 1	85	5.5	7
LMNA_HUMAN	Lamin A C OS *Homo sapiens* GN LMNA PE 1 SV 1	74	6.6	4
SVIL_HUM	Supervillin OS *Homo sapiens* GN SVIL PE 1 SV 1	25	6.5	22
Q5TBF5_HUMAN	Osteoglycin Fragment OS *Homo sapiens* GN OGN PE 4 SV 1	30	8.5	3
	Protein identified in Human jawbone			
CHAD_HUMAN	Chondroadherin OS *Homo sapiens* GN CHAD PE 2 SV 2	40	9.7	6
CO1A1_HUMAN	Collagen alpha 1 I chain OS *Homo sapiens* GN COL1A1 PE 1 SV 4	139	5.4	4
CO1A2_HUMAN	Collagen alpha 2 I chain OS *Homo sapiens* GN COL1A2 PE 1 SV 6	129	9.2	4

Note: Accession No. is a characteristic description assigned to each protein in proteomic database.