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ORIGINAL

From Amino Acids Profile to Protein Identification: Searching for Differences in Roe Deer Papilloma

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Abstract Papillomaviruses, small non-enveloped DNA viruses, are considered as the cause of a number of cancers, such as a cervical, skin, anal, and penile cancers. Roe deer papillomavirus infection can be easily characterized by typical warts on the skin. The aim of this study was to characterize differences of amino acid and protein composition in healthy and tumour tissue of roe deer using methods including ion-exchange chromatography, SDS-PAGE, two-dimensional gel electrophoresis and MALDI-TOF MS. The obtained data were statistically evaluated. Cluster analysis of all variables showed biggest differences in proline in control and papilloma samples. Further, we aimed at electrophoretic analysis of the samples. Discriminant analysis projections to latent structures (Projection to the latent structure Discrimination analysis, syn. Discrimination Partial least squares analysis, PLS DA) was used to evaluate the obtained results. Examining model PLS DA, there was found that the greatest effect on whether the individual is classified as healthy, or with papilloma, is the latent variable that best correlates with the molecular mass from 108.8 to 128.1 kDa and from 231.2 to 290.4 kDa in the original signal. Finally, using mass spectrometry three proteins, pyridoxal kinase, myoblast determination protein and leucine zipper transcription factor-like protein 1, most likely related to cancer were found.

Keywords Electrophoresis · Ion-exchange chromatography · Papillomavirus · Roe deer · Protein fractions · Amino acids profile

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Introduction

Papillomaviruses (PVs) are a diverse, epitheliotropic group of viruses, which can induce lesions by infecting keratinocytes in the basal layer of stratified squamous epithelia [1–3]. All papillomaviruses contain a double-stranded, circular DNA genome ranging from 7.4 to 8.6 kb pairs [3, 4]. Size of PV virion is ~55 nm in diameter [3]. Moreover, papillomaviruses can cause benign tumours in their natural host and occasionally in related species, but sometimes also cause malignancies [2, 3]. Solid tumours typically contain 20–100 protein-encoding mutated genes [5]. To date, it is known that 29 genera formed by almost 200 papillomavirus types isolated from humans (120 types), non-human mammals, birds and reptiles (64, 3 and 2 types, respectively) [6], from those more than 90 human PV (HPV) types have been characterized genetically, and 100 new types have been identified and partially sequenced [6].

Wide range of infected species suggests the long-term evolution of papillomavirus family [1]. HPVs can be classified as cutaneous or mucosotropic type according to a tissue where they are located. The cutaneous type is widely spread in population and cause common warts, while the mucosotropic type is usually responsible for more severe health problems and can be divided into "low-risk" and "high-risk" groups [7]. Benign warts of the genital or oral mucosa are often caused by "low-risk" HPVs such as HPV6 and HPV11 [8]. "High-risk" human papillomavirus (HPV-16, HPV-18 and HPV-31) can progress to the development of malignant lesions [3]. Long-term infections by the "high-risk" HPV serotypes cause dysplasia that can progress to cancer. Life-threatening HPV infections can arise when the virus is rendered abnormal by integration of its DNA into the human host cell genome or when the host is rendered abnormal by immunosuppression. The cutaneous HPVs infect their hosts through cuts, usually on the feet or fingers and the genital HPVs enter through microfissures during sexual intercourse [9]. Except cervix, anogenital sites, skin and oral cavity, there are other sites, where HPV can cause tumours [10]. For instance, after smoking HPV is the second most common cause of lung cancer [11]. Pharynx, larynx and oesophagus can be affected due to their histological similarities to oral cavity, too. Sites like colon, breast, ovary and prostate can be also affected by HPV, but their presence is rare and data are controversial [10].

Ruminants are, next to humans, common hosts of papillomaviruses causing warts and tumours. Roe deer, red deer, reindeer, white-tailed deer and European elk are some among the species which suffers from this infection [4]. First description of roe deer fibropapillomatosis comes from 1960s from Hungary. Recently, the prevalence of the infection was between 0.2 and 1.1 % in endemic areas of Europe [12]. The aim of this study was to assess the differences in amino acid and protein composition in skin from healthy roe deer and roe deer suffering from fibropapillomatosis.

Experimental Section

Chemicals and pH Measurement

All chemicals and water in ACS (American Chemical Society) purity were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The deionized water was prepared using reverse osmosis equipment Aqual 25 (Aqual s.r.o., Brno, Czech Republic). The deionized water was further purified using apparatus MilliQ Direct QUV equipped with the UV lamp from Millipore (Billerica, MA, USA). The conductance was 55.5 nS. The pH was measured using pH metre WTW inoLab (Weilheim, Germany).

Animal Model

A sample of fibropapillomatous tumour was collected from a 6-year old male roe deer (*Capreolus capreolus*) originating from South Moravia (Czech Republic) in 2013. As a control sample, the healthy skin was collected from a male roe deer younger than 1 year. The size of skin lesions was app. 5 cm in diameter. The hair on the skin was removed and samples were cut into small pieces.

Preparation of Samples for Determination of Protein Fractions and Amino Acids

Two sets of samples were prepared. The first set was used for direct mineralization and determination of amino acids and contained 5 mg of each sample weighted in three repetitions in digestion vials. The second set was used for determination of protein fractions and amino acids after extraction in different solutions and contained 100 mg of each sample weighted three times into three Eppendorf microvials. Samples were homogenized and to each microvial a different solution [MilliQ water, 0.2 % NaOH (0.05 mol L^{-1}) and 5 % NaCl (0.86 mol L^{-1})] in the volume of 0.5 mL was added. Then the samples were vortexed for 24 h at a fridge temperature and centrifuged. The obtained supernatant was used for all measurements.

To the digestion vials with prepared samples from the first set, 0.5 mL of 6 M HCl was added and samples were mineralized in a microwave Anton Paar (Graz, Austria). Parameters for the hydrolysis were: power 80 W, ramp 15 min, hold 90 min, maximal temperature 120 °C, maximal pressure 25 bar. After digestion, samples were diluted in ratio of 1:9 with dilution buffer in sodium cycle (100 µL of sample and 900 µL of sample buffer). Subsequently, samples were centrifuged for 10 min at 25,000 × *g* at 4 °C. After centrifugation, samples were diluted 1:1 with a neutralizing solution (400 µL of sample + 400 µL of 0.6 M NaOH). Prepared samples were analysed by ion-exchange liquid chromatography.

Supernatant (150 µL) of each fraction from the second set was evaporated on a nitrogen evaporator. After evaporation, 300 µL of 6 M HCl was added and after transfer to the digestion vials samples were mineralized. This step was followed by evaporation on a nitrogen evaporator and by the dilution in neutralizing solution. Prepared samples were analysed by ion-exchange liquid chromatography.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

Each sample's supernatant was diluted five times and then was mixed with protein loading buffer (PLB) (under reducing conditions PLB with mercaptoethanol) in a ratio of 1:1

and placed in the wells of the 12.5 % polyacrylamide gel (w/w) prepared from 30 % acrylamide/bis-acrylamide solution (37.5:1). Electrophoresis ran in 1 × tris–glycin–SDS running buffer (3.02 g of Tris, 14.4 g of Glycin, 1 g of SDS, ddH_2O to a final volume of 1 L) for 90 min at a voltage of 120 V in the electrophoretic bath (Bio-Rad, CA, USA). After that, gels were visualized by silver staining.

2D Electrophoresis

The volume of supernatant, corresponding to 200 μg of protein, was added to rehydration buffer (Bio-Rad, CA, USA) into a final volume of 125 μL and the resulting solution was used for the 12-h rehydration of 7-cm IPG strips. Rehydrated IPG strips were focused on Protean® IEF Cell (Bio-Rad, CA, USA) at 20 °C in three steps. In the first step, the electric voltage was increased linearly over 20 min from 0 to 250 V; in the second step, the voltage was increased to 4,000 V, and this value was maintained for 2 h; and in the third step, the electrical parameters were set so that the total value reached 10 kVh. The electric current was limited to the value of 50 mA/strip. After isoelectric focusing, strips were incubated 10 min at room temperature on a rocker shaker with I. equilibration buffer (Bio-Rad, CA, USA). Thereafter, the solution was removed and the strips were incubated with II. equilibration buffer (Bio-Rad, CA, USA) for 10 min. Prepared strips were washed with 1 × tris–glycine–SDS running buffer. The strips were placed on the back of the flatbed electrophoretic glass plate. Subsequently, the agarose was poured between the plates, where the strip was inserted. After solidification of agarose, the plates were inserted in the electrophoretic bath with 1 × tris–glycine–SDS running buffer. Electrophoresis was set to 70 min at a voltage of 200 V. After that, gels were stained with Coomassie Brilliant Blue.

Determination of Total Proteins

The total proteins were determined by standard Biuret method using an automated chemical analyser BS-400 (Mindray, China). In the cuvette, 150 μL of Biuret reagent (Greiner, Germany; 100 mM sodium tartrate, potassium, 100 mM NaOH, 15 mM KI, 6 mM $CuSO_4$) and subsequently 3 μL of a sample were pipetted. The absorbance was measured after 10 min incubation at 37 °C and at a wavelength $\lambda = 546$ nm. Absorbance values of reagent and values after 10 min of sample incubation were used for calculation.

Ion-Exchange Chromatography

For determination of amino acids, an ion-exchange liquid chromatography (Model AAA-400, Ingos, Czech Republic) with post-column derivatization by ninhydrin and absorbance detector in visible light range (VIS) was used. Glass column with inner diameter of 3.7 and 350 mm length was filled manually with strong cation exchanger (Ostion LG ANB, Ingos, Prague, Czech Republic) in sodium cycle with particles of average size of 12 μm and a netting of 8 %. The column was thermostated at 60 °C. Double channel VIS detector with inner cell of 5 μL was set to two wavelengths: 440 and 570 nm. Prepared solution of ninhydrin was stored under nitrogen atmosphere in dark at 4 °C. Elution of amino acids was done by buffer containing 10.0 g of citric acid, 5.6 g of sodium citrate, and 8.36 g of sodium chloride per litre of solution and pH was 3.0. Flow rate was 0.25 mL min^{-1}. Reactor temperature was set on 120 °C.

In-gel Digestion

From 2D gel of supernatant of fibropapillomatous tumour, three different pieces were cut using an EXQuest™ Spot-Cutter (Bio-Rad, CA, USA) and transferred to 1.5-mL Eppendorf microvials. Then an in-gel digestion with trypsin was performed according to a protocol of Shevchenko et al. [13]. Digested proteins were used for peptide mass fingerprinting.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry for Peptide Fingerprinting

Matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) experiments were performed on a MALDI-TOF/TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Germany). As a matrix 2,5-dihydroxybenzoic acid was used. The saturated matrix solution was prepared in 30 % acetonitrile and 0.1 % trifluoroacetic acid (both v/v). Mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic, Germany) for 2 min at 50 % of intensity at room temperature. Sample preparation method for MALDI-TOF was dried-droplet method (DD), i.e. solutions of digested proteins for analysis were mixed with matrix solution in a volume ratio of 1:1. After obtaining a homogeneous solution, 2 μL was applied on the MTP 384 polished steel target plate and dried under atmospheric pressure at room temperature. A mixture of peptide calibration standards was used to externally calibrate the instrument. All measurements were performed in the reflector positive mode in the *m/z* range 800–6,000 Da. The MS spectra were typically acquired by averaging 500 sub spectra from a total of 500 shots of the laser (Smartbeam 2) with laser power set to 60–75 %.

Peptide mass fingerprinting was done using MASCOT server (Matrix Science, MA, USA) for comparing mass

spectra (excluding peaks of digested trypsin) with SwissProt database. For database searching, the following parameters were used: a trypsin was used as enzyme, 1 missed cleavage was allowed, taxonomy was set to *mammalia*, oxidation of methionine was added as variable modification, peptide tolerance was set to ±0.5 Da, mass values were set as MH^+ and obtained from monoisotopic peaks. As results, there were chosen compounds with best statistical score, which was ranging between 70 and 100 score points.

Statistics

The procedure described in [14] was used for preprocessing of electropherograms images. Signals extracted from different electropherograms were interpolated to the same values by spline interpolation. Signals were discretized using discrete wavelet transform to reduce the dimensionality of the data. Discriminant analysis projections to latent structures (Projection to the latent structure Discrimination analysis, syn. Discrimination Partial least squares analysis, PLS DA) was used for discrimination. PLS DA model coefficients were estimated using the standard method of least squares. All calculations were made in MATLAB.

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA 12 (StatSoft, USA). Results are expressed as mean ± standard deviation (SD) unless noted otherwise (EXCEL®). Statistical significances of the differences were determined using STATISTICA 12. Differences with $P < 0.05$ were considered significant and were determined using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison.

Results and Discussion

There are many differences between healthy and cancerous tissue, both at the level of physiological and biochemical

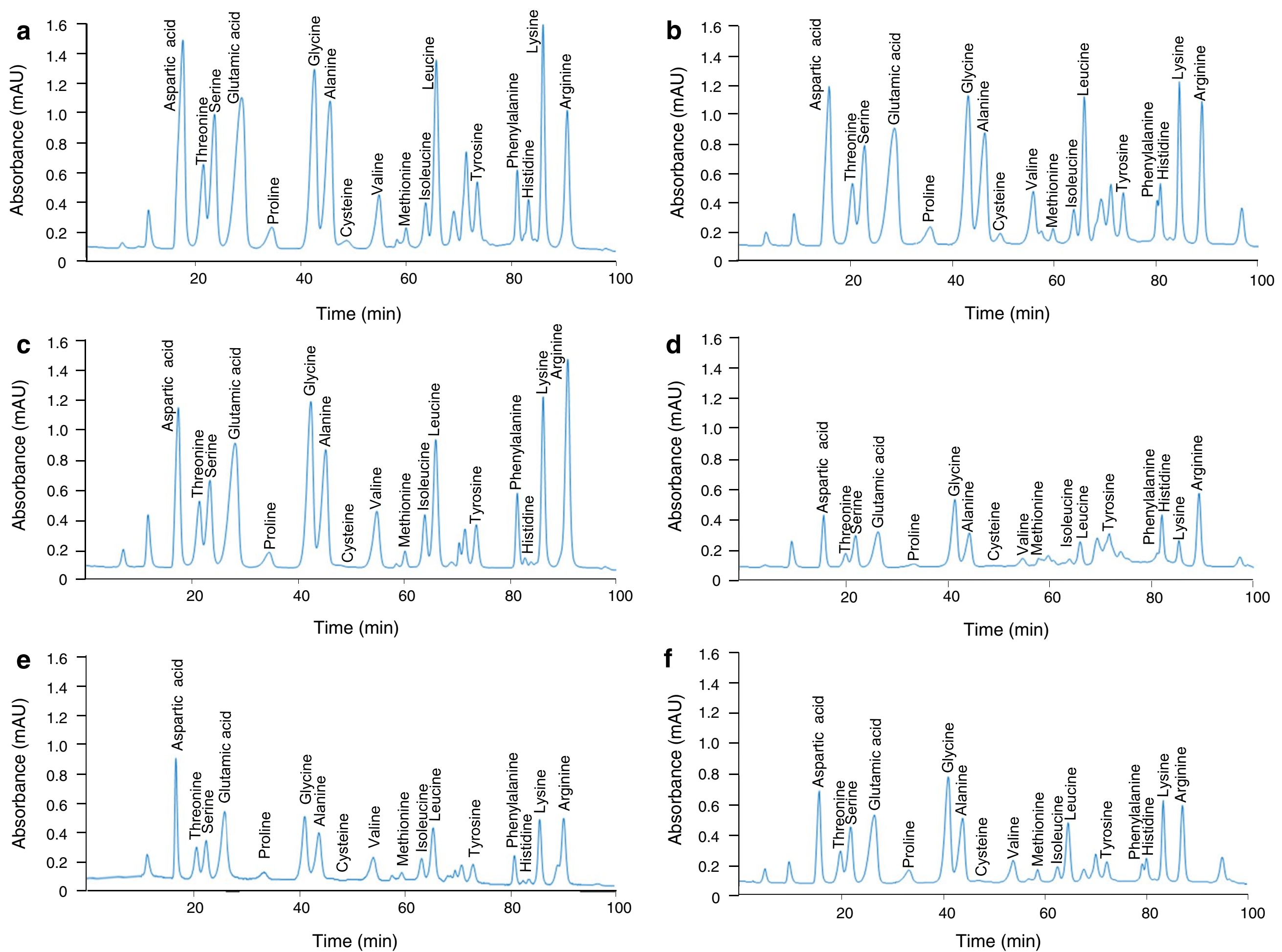
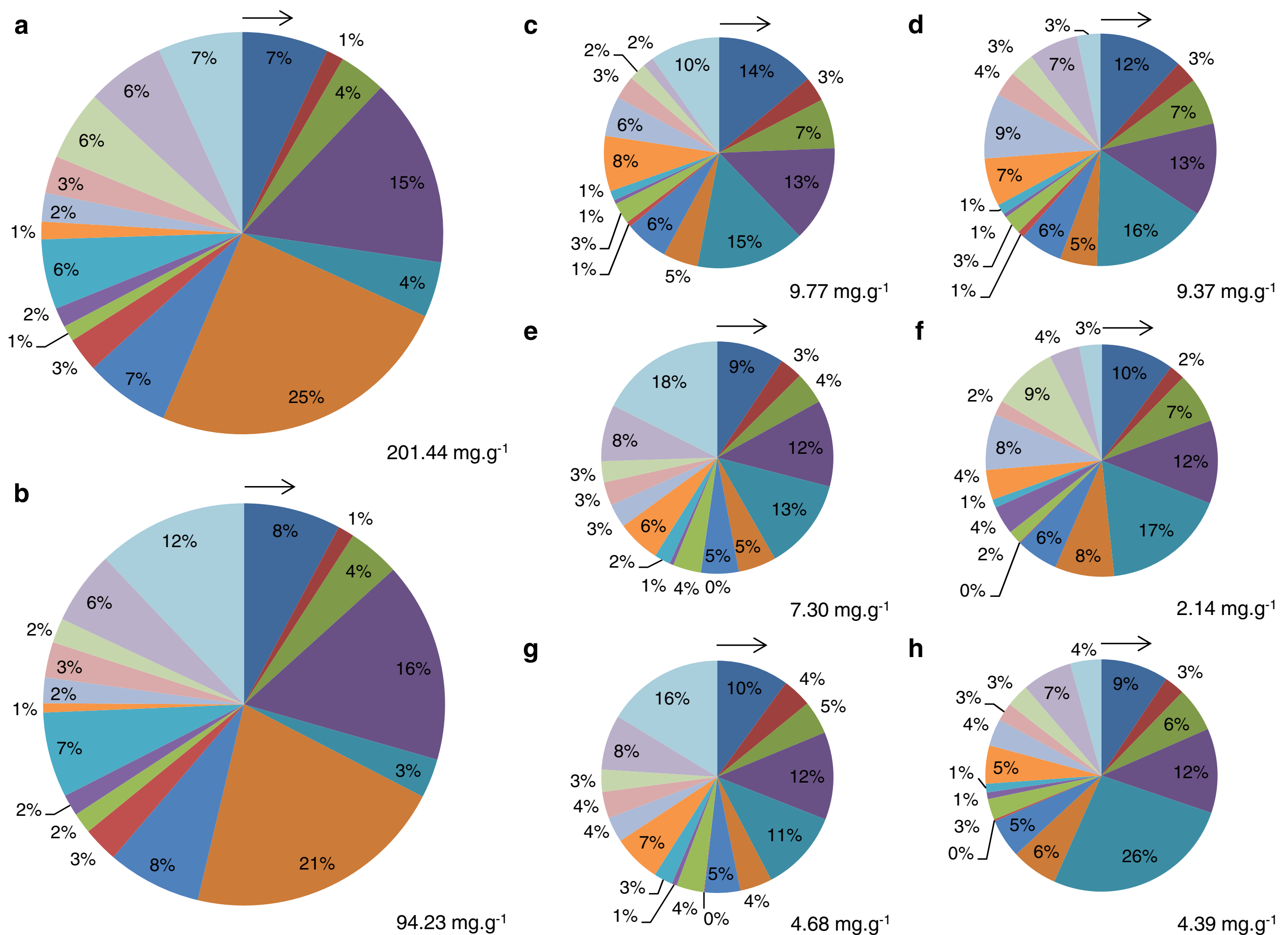


Fig. 1 Ion-liquid chromatograms of amino acid content in samples of (**a**, **c**, **e**) roe deer skin of healthy individual and (**b**, **d**, **f**) roe deer papilloma, in different fractions obtained from AAA 400 instrument. Individual fractions were prepared from (**a**, **b**) water; (**c**, **d**) 0.2 % NaOH and (**e**, **f**) 5 % NaCl

properties; amongst other reasons, it's because of a different metabolism of these tissues [15]. Therefore, it can be expected that the protein and thus the amino acid composition of healthy and tumour tissue will be also different. In this study, differences in amino acid and protein composition in healthy individuals and individuals with papilloma were investigated.

Amino Acid Composition of Protein Fraction

The skin tissue of wild deer was selected as a model for testing of differences in the composition of normal and tumour tissue. We decided to evaluate the amino acid composition of protein fractions, based on the articles of amino acid changes [16–18], to find changes in the protein composition. Chromatograms from the analyses are shown in Fig. 1. After evaluation of these records, overall representation of amino acids in tissues (Fig. 2a, b) as well as the percentage of amino acids of individual fractions (Fig. 2c–h) was compared. At the same time, the concentration of total protein (numerically expressed in graphs) was determined. This shows a difference in the concentration of amino acids in both samples, where this concentration is doubled in the healthy tissue. Similar trend was observed with values in breast and lung cancer study [19] and colorectal cancer study [20] in humans, where content of amino



Read in the direction of the arrows: ■aspartic acid, ■threonine, ■serine, ■glutamic acid, ■proline, ■glycine, ■alanine, ■cysteine, ■valine, ■methionine, ■isoleucine, ■leucine, ■tyrosine, ■phenylalanine, ■histidine, ■lysine, ■arginine

Fig. 2 The relative representation of individual amino acids in samples of **a**, **c**, **e**, **g** roe deer skin of healthy individual and **b**, **d**, **f**, **h** venison papilloma obtained from AAA 400 instrument. Samples **a**, **b** were digested, **c**, **d** were determined in the water fraction, **e**, **f** in the fraction of 0.2 % NaOH; **g**, **h** in a fraction of 5 % NaCl. *Values* showed in the *lower right corner* of each graph represent the concentration of each sample

Group	Fraction	His	Leu	Tyr	Pro	Lys	Gly	Phe	Thr	Arg	Val	Ile	Met	Cys	Glu	Ala	Ser	Asp
Papiloma	Total	1.6	0.6	2.1	30.1	5.1	16.0	3.2	1.7	12.4	1.5	6.7	1.3	4.7	11.7	7.2	5.4	7.4
Control	Total	10.5	2.7	5.3	95.0	12.2	41.6	7.5	4.0	15.6	2.6	12.2	2.7	10.1	24.7	14.6	10.9	14.7
Papiloma	Fraction 1	0.33	0.63	0.85	1.52	0.65	0.48	0.33	0.29	0.30	0.25	0.13	0.05	0.09	1.21	0.56	0.61	1.1
Control	Fraction 1	0.24	0.76	0.55	1.49	0.15	0.48	0.32	0.34	0.96	0.29	0.13	0.05	0.07	1.13	0.60	0.68	1.36
Papiloma	Fraction 2	0.20	0.09	0.17	0.37	0.09	0.18	0.04	0.05	0.07	0.04	0.02	0.08	0.00	0.25	0.13	0.15	0.22
Papiloma	Fraction 3	0.14	0.23	0.17	1.16	0.31	0.28	0.11	0.13	1.29	0.13	0.05	0.04	0.01	0.52	0.24	0.27	0.41
Control	Fraction 3	0.14	0.31	0.16	0.53	0.36	0.21	0.17	0.19	0.19	0.18	0.12	0.03	0.00	0.57	0.23	0.22	0.47
Control	Fraction 2	0.21	0.44	0.24	0.93	0.57	0.38	0.24	023	0.76	0.28	0.16	0.04	0.00	0.88	0.38	0.32	0.68

Fig. 3 Cluster analysis of all variables. The higher intensity of *red colour*, the lower dependence. The higher intensity of *blue colour*, the higher dependence. *P* level <0.05 was considered significant

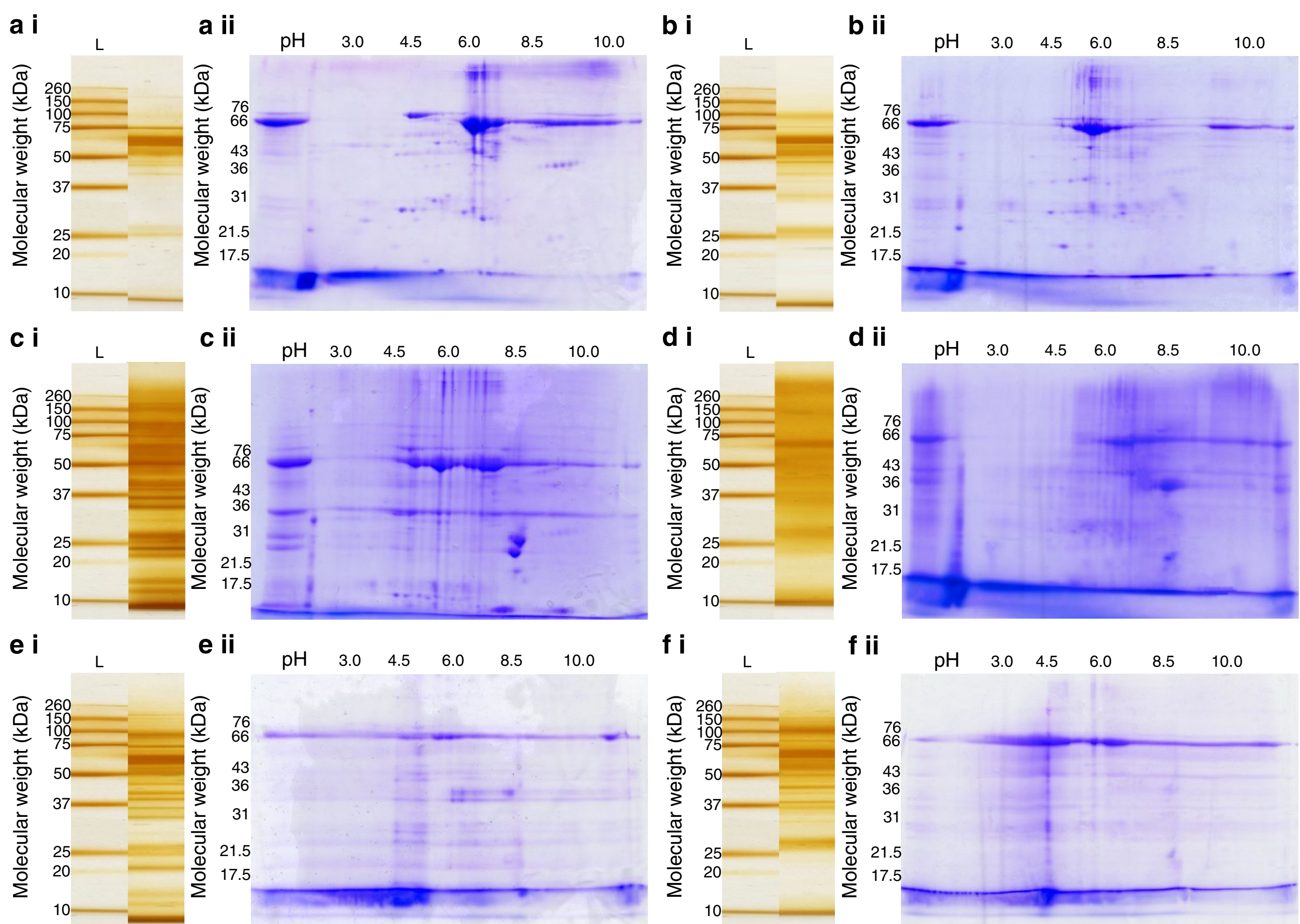


Fig. 4 (**i**) SDS-PAGE and (**ii**) 2D gels of proteins in samples of (**a**, **c**, **e**) roe deer skin from a healthy animal and (**b**, **d**, **f**) roe deer papilloma. Fractions were prepared from (**a**, **b**) water; (**c**, **d**) 0.2 % NaOH and (**e**, **f**) 5 % NaCl. SDS-PAGE gels were stained with silver and 2D gels with Coomassie Blue. Experion protein ladder (L) was used for size comparison of proteins

acids in blood cells, respectively, peptide levels and their amino acids content, was decreased in comparison with controls.

Despite not very significant changes in the composition of total amino acids, there can be seen significant differences in the various protein fractions. For the aqueous fraction, the most pronounced difference is in the ratio of the amino acid in the healthy and tumour tissue in arginine (10–3 %) and lysine (2–7 %), while in 0.2 % NaOH the representation of arginine (18–3 %), but also of histidine (3–9 %), tyrosine (3–8 %), lysine (8–4 %), proline (13–17 %), methionine (1–4 %) and serine (4–7 %) was particularly changed. In the protein fraction obtained from leachate in 5 % NaCl, most pronounced change in proline (11–26 %) and arginine (16–4 %) was found. Apart from these results, it is interesting o note more than threefold reduction of the total concentration of amino acids in the 0.2 % NaOH fraction, whereas in other fractions, this trend was not observed.

Cluster Analysis

The concentrations of individual amino acids were statistically evaluated to confirm the facts above. Cluster analysis of all variables (Fig. 3) showed biggest differences in proline in control and papilloma samples. This is confirmed by the determined concentration of proline in the 5 % NaCl fraction (Fig. 2g, h). There is a high content of proline in collagen. Proline metabolism is distinct from that of primary amino acids and is one of the most abundant amino acids in the cellular microenvironment. Together with hydroxyproline, proline constitutes more than 25 % of residues in collagen, which is the predominant protein (80 %) in the extracellular matrix of the human body. An important source of proline comes from the degradation of collagen in the extracellular matrix by sequential enzymatic catalysis of matrix metalloproteinases and prolidase. According to the cluster analysis, proline is an amino acid that shows large concentration differences between healthy and cancerous tissue. Proline degradation is activated by p53 protein. Proline oxidase/proline dehydrogenase (POX/PRODH) is encoded by p53-induced gene 6 (PIG 6), i.e. POX/PRODH is regulated by p53 protein, but also the apoptotic response to p53 protein is dependent on POX/PRODH. Therefore, POX/PRODH may function as a tumour suppressor [21]. POX/PRODH catalyses the first step in proline degradation and uses proline to generate adenosine triphosphate for survival or reactive oxygen species for programed cell death. POX/PRODH is induced by p53 protein under genotoxic stress and initiates apoptosis by both mitochondrial and death receptor pathways [10].

Another significant difference was found in glycine values. Glycine is consumed by rapidly proliferating cells and released by slowly proliferating cells, suggesting that glycine demand may exceed endogenous synthesis capacity in rapidly proliferating cancer cells, whereas in slowly proliferating cells, glycine synthesis may exceed demand. Increasing glycine consumption with faster proliferating rate was observed across 60 tumour cell lines, and was even more pronounced within specific tumour types, including ovarian, colon, and melanoma cells [22].

Proteins on SDS-PAGE and 2D Electrophoresis

Because the fact that the solubility of individual proteins is dependent on the environment [23], we selected three different solutions to extract proteins (MiliQ water, 0.2 % NaOH, 5 % NaCl) and these fractions were assessed by several methods. Firstly, a characterization of the proteins was obtained in the individual fractions by SDS-PAGE and 2D electrophoresis (Fig. 4). These results suggest that there are changes at protein levels in all three variants of protein fractions. In particular, the most significant changes by SDS-PAGE are in the aqueous fraction at a molecular mass of 34 kDa (Fig. 4a, b), while in the 0.2 % NaOH (Fig. 4c, d) obvious changes are in a wide range of molecular masses. In 5 % NaCl solution, there is particularly noticeable difference at 20 kDa (Fig. 4e, f).

Mathematical Evaluation of Changes on SDS-PAGE

The two instances of data were obtained after preprocessing. Data contained no missing values. These instances belonged to one healthy subject and to one subject with papilloma. Because fair use of PLS DA, all variables were centred on zero mean by subtracting the average of values and scaled to

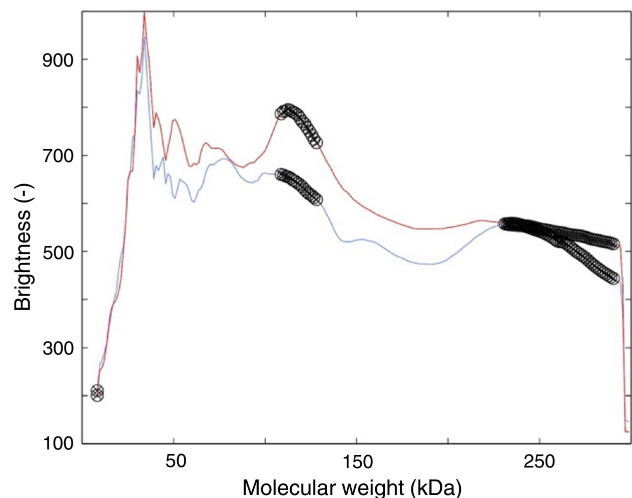


Fig. 5 The diameters of the curves obtained from electropherograms of proteins. Significant parts are marked with a *cross wheel*. The *blue curve* indicates the sample of roe deer skin of healthy individual, *red* indicates the sample of the papilloma. Significant parts of the latent variable are regions of the original signals, corresponding to molecular mass: from 108.8 to 128.1 kDa, 231.2 to 290.4 kDa

unit variance by dividing with the standard deviation. PLS DA model used for discrimination can transform input variables to the new latent variables according to the following:

$$X = T \cdot P^T + E$$

where X is the original matrix of variables, T are estimated latent variables, P is a matrix of transformation between X and T, and E is the residue matrix after the decomposition of matrix X into latent variables T. The correlation between the predictor and response is maximized.

PLS DA was used for construction of a model that performs discrimination between healthy subject and subject with papilloma according to the following:

$$\hat{Y} = T \cdot C$$

where $\hat{Y}$ is the estimate of the classification of healthy/papilloma, T is the matrix of latent variables and C is a vector of estimated coefficients for discrimination.

The ten latent variables were used on the basis of the proportion of variance explained by means of the PLS model in DA data, while using these latent variables explained more than 99 % of the variance in the matrix of variables X.

Correctness of the model was verified by leave-one-out validation, i.e. we have repeatedly removed one instance from the data, which we kept for testing, and on the rest we trained the model. Thus, we gradually proved all instance data. The average success rate of validation is 91.67 % with a standard deviation of 28.87 %.

The coefficients of the model were estimated:

$$\hat{Y} = 0.47 \cdot t_1 + 5.62 \cdot t_2 + 0.83 \cdot t_3 + 1.04 \cdot t_4 + 0.75 \cdot t_5 + 1.15 \cdot t_6 + 0.30 \cdot t_7 + 0.29 \cdot t_8 + 0.33 \cdot t_9 + 0.19 \cdot t_{10}$$

For a better understanding of the PLS DA model, it is appropriate to examine the transformation matrix of variables X, along with the amount of variance, which each latent variable explains in response Y.

Discrimination between healthy individual and individual with papilloma on the basis of symptoms is shown in Fig. 5. There was created a model with latent variables on the basis

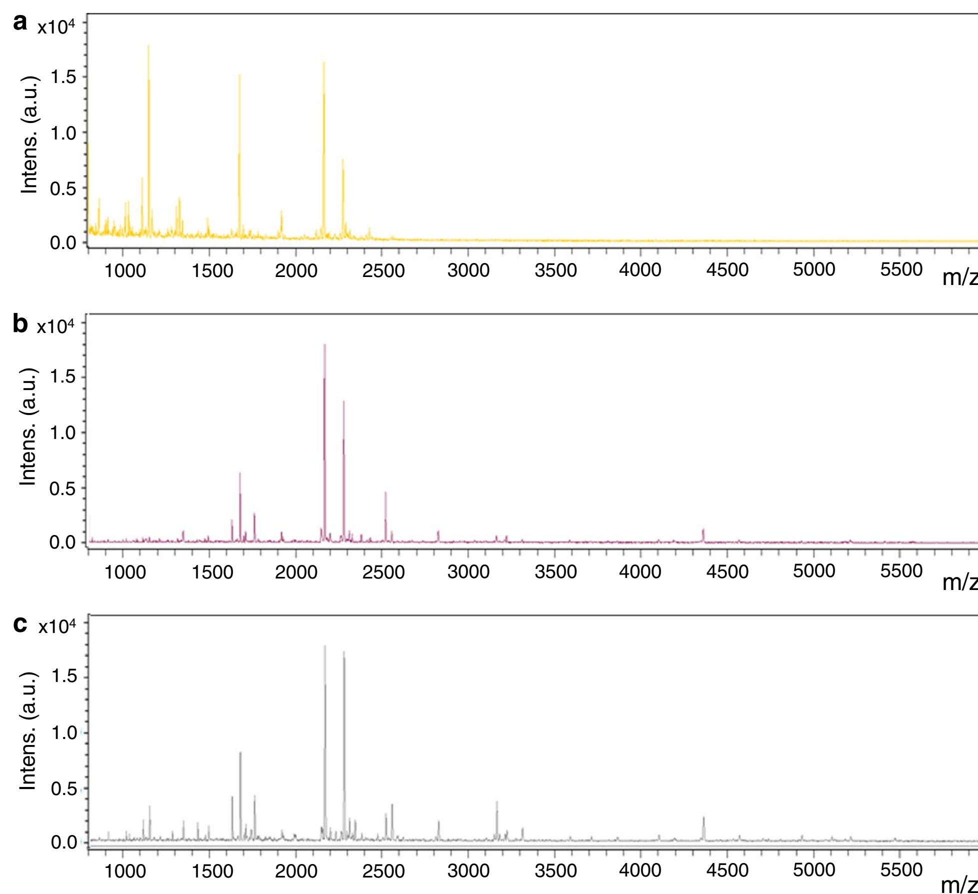


Fig. 6 MALDI-TOF mass spectra of digested proteins. Selected spots from 2D gel electrophoresis were cut and in-gel digestion by trypsin at 45 °C for 2 h was performed. 2,5-dihydroxybenzoic acid (DHB) was used as a matrix. Spectra were acquired in a positive reflector mode, *m/z* range 800–6,000 Da, with laser power set at 70 %. **a** Pyridoxal kinase, **b** Myoblast determination protein 1, **c** leucine zipper transcription factor-like protein 1

of the variables extracted from the electropherograms and using PLS DA which is capable to classify whether there is a healthy individual or individual with papilloma with an average success rate of 91.67 % with a standard deviation of 28.87 %, estimated on the basis of leave-one-out validation. Examining model PLS DA, there was found that the greatest effect on whether the individual is classified as healthy, or with papilloma, is the latent variable that best correlates with a molecular mass ranging from 108.8 to 128.1 kDa and from 231.2 to 290.4 kDa in the original signal.

MALDI-TOF Characterization of Proteins

Three spots on the 2D electropherogram of a tumour tissue (Fig. 4b ii), which differ from healthy tissue, were defined after mathematical analysis. Molecular size of these spots corresponds with the band at the appropriate SDS-PAGE (Fig. 4b i). As a confirmation of the diversity of the proteins contained in the tumour tissue, the spots were excised and purified according to the protocol and digested for subsequent protein analysis on MALDI-TOF MS. Mass spectra of digested proteins obtained on the basis of differences in 2D electropherogram were characterized using MASCOT (Fig. 6). Proteins were identified as pyridoxal kinase, leucine zipper transcription factor-like protein 1, which is a novel gene with unknown biological functions and tumour suppressive factor in gastric cancer and was also found in nerve cells, brain, and a number of other tumours [24, 25]. Another identified protein is a myoblast determination protein 1, which is a marker of cell differentiation [26, 27].

Conclusion

It is known that the tumour tissue differs from healthy tissue in many aspects we attempted to characterize differences in the amino acids and protein levels. As an experimental model a deer tissue with papilloma was used. It was found that the levels of several amino acids were significantly different in skin of healthy roe deer and papilloma of ill subject. Total amino acids concentration was lower in papilloma compared to the control group. The biggest differences were found in proline and glycine values. Significant changes were also detected at the protein level. MALDI-TOF characterized different 2D gel spots, that represent individual proteins, which are identified as associated ones with cancers, pyridoxal kinase (35,591 Da), myoblast determination protein 1 (34,186 Da) and leucine zipper transcription factor-like protein 1 (34,556 Da).

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