Changes of Nuclear Matrix Proteins Following the Differentiation of Human Osteosarcoma MG-63 Cells

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Human osteosarcoma MG-63 cells were induced into differentiation by 5 mmol/L hexamethylene bisacetamide (HMBA). Their nuclear matrix proteins (NMPs) were selectively extracted and subjected to two-dimensional gel electrophoresis analysis. The results of protein patterns were analyzed by Melanie software. The spots of differentially expressed NMPs were excised and subjected to *in situ* digestion with trypsin. The maps of peptide mass fingerprinting were obtained by MALDI-TOF-MS analysis, and were submitted for NCBI database searches by Mascot tool. There were twelve spots changed remarkably during the differentiation induced by HMBA, nine of which were identified. The roles of the regulated proteins during the MG-63 differentiation were analyzed. This study suggests that the induced differentiation of cancer cells is accompanied by the changes of NMPs, and confirms the presence of some specific NMPs related to the cancer cell proliferation and differentiation. The changed NMPs are potential markers for cancer diagnosis or targets for cancer therapy.

Key words: nuclear matrix proteins, cell differentiation, human osteosarcoma, HMBA

Introduction

Nuclear matrix (nuclear skeleton) is the filamentous protein framework in eukaryotic cell nucleus. It plays an important role in life activities, such as cell morphology maintaining, dimensional localization, DNA replication and transcription; thereby it is closely associated with cell proliferation and differentiation, as well as carcinogenesis (1, 2).

The role of nuclear matrix in cell activities has drawn increasingly attentions recently. Most of its components, besides the fibrins, are proteins and/or enzymes in DNA replication, transcription, and gene expression (3–5). The nuclear matrix in cancer cells is not only abnormal in morphology but also apparently different in its composition.

Previously, we have found that the morphology of nuclear matrix in human gastric cancer cells showed similar characteristics to that in normal cells when induced by hexamethylene bisacetamide (HMBA) and retinoic acid (RA) (6, 7). It implies that further study on the differential expression of nuclear matrix proteins (NMPs) in response to differentiation reagents

will be able to reveal the mechanism of carcinogenesis and cancer differentiation.

This study was designed to identify specific NMPs associated with the proliferation and differentiation of carcinoma cells for further analysis. The human osteosarcoma MG-63 cells, which have apparent characteristics of terminal differentiation, were selected to undergo the terminal differentiation induced by HMBA. When the differentiation of MG-63 cells was confirmed, their NMPs were selectively extracted and subjected to two-dimensional (2D) gel electrophoresis. The changed NMPs during the differentiation process were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis combined with database mining.

Results

Changes of NMPs

The human osteosarcoma MG-63 cells were cultured in the presence or absence of HMBA for seven days. Samples of NMPs were selectively extracted and sub-

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jected to 2D gel electrophoresis. The criteria for differentially expressed proteins were based on an evaluation of at least two gels per experimental condition. Most of the NMPs were concentrated in the area of pI 4–7 with molecular weight (MW) 10–70 kDa. There were 199 spots in the map of NMPs from control MG-63 cells, whereas there were 197 spots in that from HMBA-treated MG-63 cells. The expression of 12 spots was changed remarkably by the addition of HMBA. The changed proteins were named as special NMPs (SNMPs or S). Among them, six spots (S1–S6) were up-regulated, four (S7–S10) were downregulated, while the remaining two (S11 and S12) were emerged as new spots in the nuclear matrix maps of the differentiated MG-63 cells (Figures 1–3). The relative expression levels of the altered proteins were analyzed in the differentiated and control cells (Figure 4).

Peptide mass spectra of the changed proteins

In the twelve changed proteins, ten proteins (S1–S7 and S10–S12) obtained ideal peptide mass fingerprinting maps. The representative mass spectra (S6, S7, and S12) are shown in Figure 5.

Identification of the changed proteins

Nine differentially expressed proteins (S1–S4, S6, S7, S10–S12) were identified by peptide mass fingerprinting combined with database searching. The identified proteins and their associated parameters are shown in Table 1. Among the identified proteins, S1 was the major histocompatibility complex (MHC) class II antigen; S2 was the interferonstimulated gene factor 3 alpha 91/84 kDa protein, which is associated with the activation of interferonstimulated genes; S3 was the hypothetical protein DKFZp434M2221.1, an unknown function protein; S4 was the 8-hydroxy-guanine glycosylase homolog ogg1, an enzyme in nucleotide metabolism; S6 and S10 were identified as vimentin and actin, respectively, both are common NMPs; S7 was the hnRNP A2/B1, which is associated with mRNA biogenesis; S11 was similar to the 60S ribosomal protein L21; and S12 was identified as the ST2 protein, a member of the interleukin receptor I family. In addition, S5 was matched with two proteins, which needs further identification.

Discussion

Differentiation therapy is a novel and potentially less toxic form of cancer treatment involving the use of reagents that modify the differentiation and growth of cancer cells ($8, 9$). Information on the differentiation of carcinoma cells had been very limited until recently. It was reported that the polymorphism of nuclear matrix changed remarkably in differentiated gastric carcinoma cells ($6, 7$), while changes of NMPs were unexplored. It is of significance to identify specific NMPs associated with cell proliferation and differentiation, as the identified NMPs are potentially markers for the differentiation of carcinoma cells or targets for cancer therapy.

HMBA is one of the most commonly used differentiation-inducing reagents (10). It is reported that HMBA can induce various cancer cells into differentiation. In this study, the human osteosarcoma MG-63 cells were induced by HMBA as a differentiation model. Characteristics of differentiated MG-63 cells were identified in previous studies (11). In brief, divisions of the MG-63 cells were largely inhibited, and the ratio of cells in the G_0/G_1 phase increased remarkably after being treated with HMBA. The proteins specific for osteoblast cells, such as collagen I, osteonectin, and osteocalcin, increased in HMBA-treated MG-63 cells. It was deduced that MG-63 cells in the presence of HMBA were induced into differentiation as it showed characteristics of the osteoblast cells.

In our experiments, twelve spots changed remarkably on the 2D maps of NMPs from differentiated MG-63 cells, and nine spots were identified. According to their expression levels in MG-63 cells, the nine proteins were divided into three classes: (1) upregulated proteins: S1–S4, S6; (2) down-regulated proteins: S7 and S10; (3) newly emerged proteins: S11 and S12. Among them, six proteins, including S1, S6, S7, S10–S12, were reported to be closely associated with cell growth and differentiation ($12–16$).

Changes of vimentin and actin are common in the differentiated carcinoma cells (15). In this study, vimentin increased while actin decreased in the differentiated MG-63 cells. In the human hepatocellular carcinoma Hep 3B cells, TPA can induce the increased expression of both vimentin and actin, while RA can increase the expression of actin and decrease that of vimentin. The roles that vimentin and actin play in cell differentiation and their effects on the polymorphism of differentiated cells currently remain unknown.
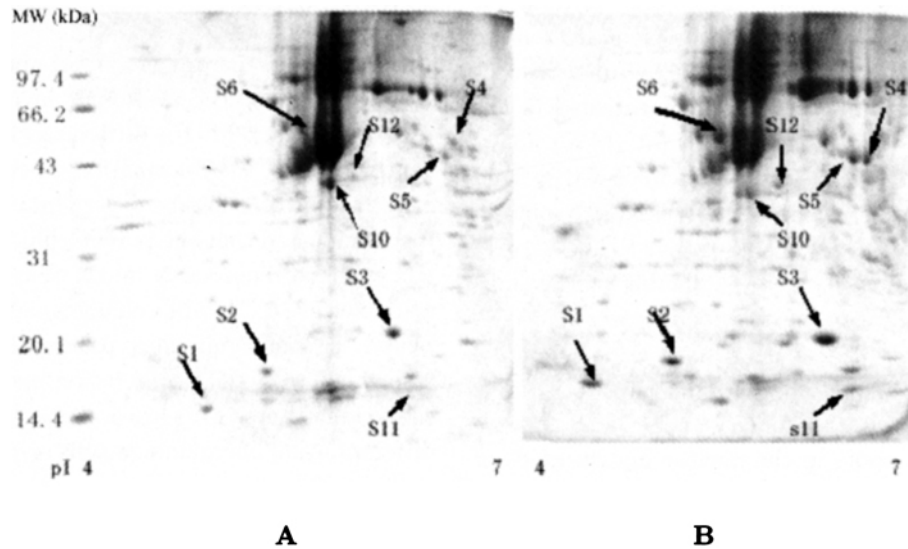
Fig. 1 The 2D maps of NMPs from MG-63 cells (stained with coomassie blue R250). **A**. Control; **B**. MG-63 cells treated with HMBA. Arrows point to differentially expressed proteins. S1–S6: up-regulated; S10: down-regulated; S11 and S12: newly emerged proteins.
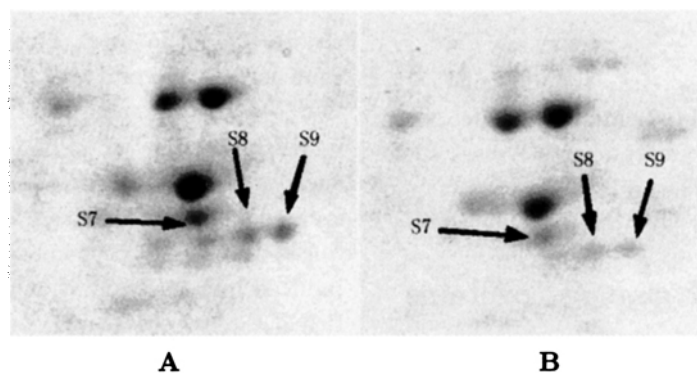
Fig. 2 The 2D maps of NMPs from MG-63 cells (alkaline terminal, pI 8–10, stained with coomassie blue R250). **A**. Control; **B**. MG-63 cells treated with HMBA. Arrows point to differentially expressed proteins. S7–S9: down-regulated.
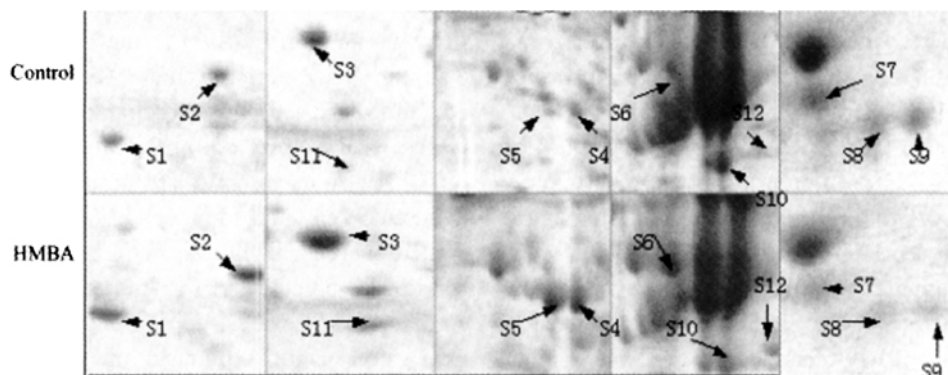
Fig. 3 Enlarged maps of the changed NMPs. Arrows point to the changed NMPs.
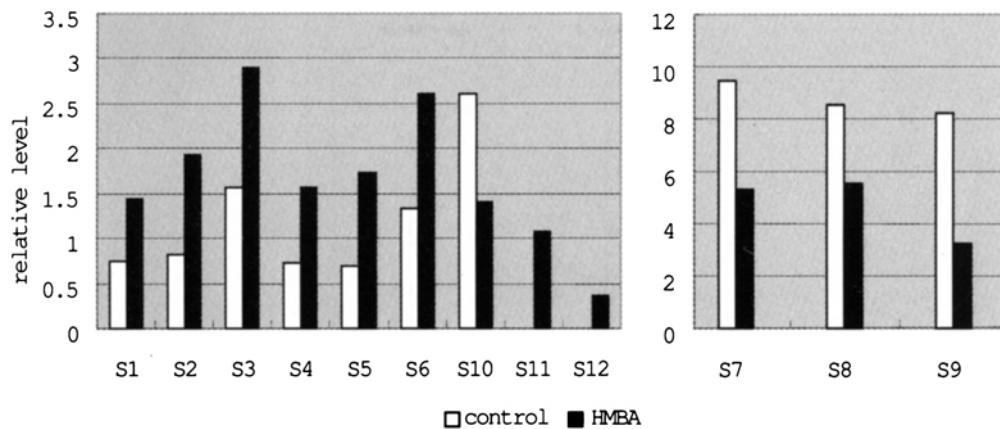
Fig. 4 Relative expression levels of the changed NMPs. The expression levels of S1–S6 increased, whereas those of S7–S10 decreased in the NMPs from HMBA-treated MG-63 cells; the expression levels of S11 and S12 were close to the background in the control cells.

Table 1 Nuclear Matrix Proteins Regulated in Differentiated MG-63 Cells Identified by Peptide Mass Fingerprinting

Class	Protein	Accession No.	Name	Mr/pI	Matched peptide	Sequence coverage
Up-regulated	S1	gi|2944406	MHC class II antigen	10,995/5.14	3*	34%
	S2	gi|252585	interferon-stimulated gene factor 3 alpha 91/84 kDa protein (SH2 and SH3 domains)	11,763/5.19	4	40%
	S3	gi|7512660	hypothetical protein DKFZp434M2221.1	25,911/8.72	5	28%
	S4	gi|11281913	8-hydroxy-guanine glycosylase homolog ogg1	39,472/8.75	7	24%
	S5[#]	gi|27544933	solute carrier family 25, member 16	36,542/9.87	7	35%
		gi|29126189	Rho GTPase activating protein 19	35,949/9.90	8	24%
	S6	gi|2119204	vimentin	53,676/5.06	12	29%
Down-regulated	S7	gi|4504447	hnRNP A2	36,041/8.67	8	37%
		gi|14043072	hnRNP B1	37,464/8.97	8	35%
	S8[#]	–	–	–	–	–
	S9[#]	–	–	–	–	–
	S10	gi|15277503	actin	40,536/5.55	11	35%
Newly emerged	S11	gi|41147370	similar to 60S ribosomal protein L21	11,324/10.14	5	59%
	S12	gi|423090	ST2 protein	37,655/8.21	4	21%

*S1: matched peptides below 4; [#]S5, S8, S9: unidentified proteins. S5 was matched with two proteins, which needs further identification.
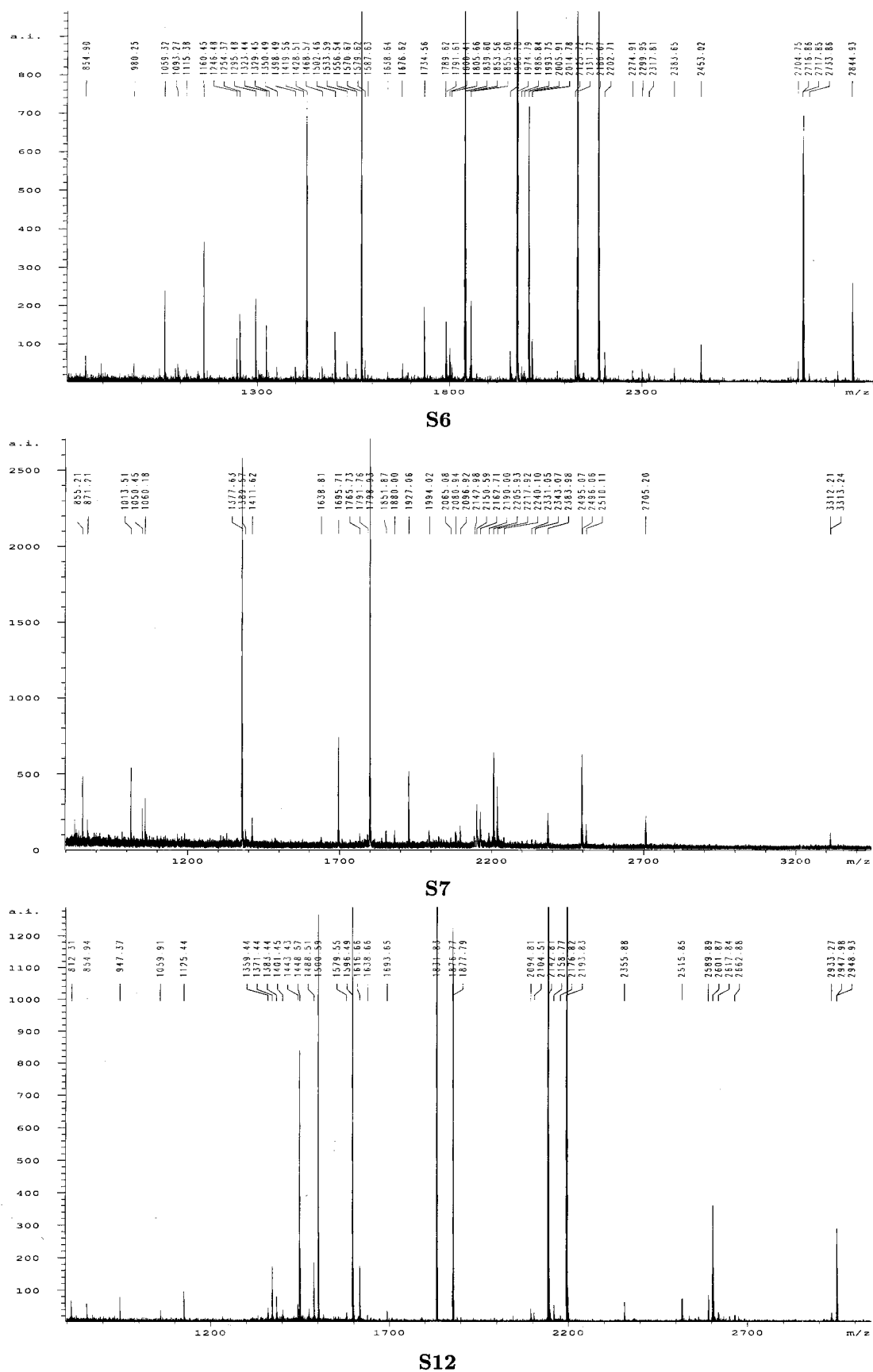
Fig. 5 The MALDI-TOF-MS maps of S6, S7, and S12.

It is of significance to make clear the roles that vimentin and actin play in the polymorphism and organization of nuclei during the differentiation of carcinoma cells, since they are commonly expressed in eukaryotic cells.

The hnRNP A2/B1 is involved in various steps of mRNA biogenesis and transport. The overexpression of hnRNP A2/B1 was found in various carcinoma cells (*16*), so hnRNP A2/B1 was believed to play an important role in carcinogenesis and cell differentiation. In this study, the expression of hnRNP A2/B1 decreased remarkably in the nuclear matrix of differentiated MG-63 cells, gaving further evidence that hnRNP A2/B1 was involved in the differentiation of carcinoma cells.

The 8-hydroxy-guanine glycosylase homolog ogg1 is responsible for repairing the 8-hydroxyguanine (oh^8G), a major base lesion produced by reactive oxygen species, thus repairs the mutations that lead to carcinogensis (*17*). The expression of ogg1 increased in the differentiated MG-63 cells, implying that DNA repair enzymes are involved in the HMBA-induced differentiation process.

The results of this study confirm that specific NMPs are related to the proliferation and differentiation of carcinoma cells. Further study on the functions of the changed proteins on the human osteosarcoma cell carcinogenesis and differentiation, as well as their relationship with activities of associated oncogenes and tumor-suppressor genes, is important as it could not only reveal the regulation mechanism on cell proliferation and differentiation, but also shed light on the principle of carcinogenesis and its regression. On the other hand, it will provide novel markers of carcinoma cell differentiation for clinical diagnosis and/or novel targets for cancer therapy.

Materials and Methods

Materials

The human osteosarcoma MG-63 cells were bought from the China Center for Type Culture Collection (Wuhan, China). HMBA (Sigma, St. Louis, USA) was used to induce the differentiation of MG-63 cells. Sequence grade, modified trypsin (Promega, Madison, USA), and iodoacetamide (Sigma) were used in the in-gel digestion. ReadyStrip IPG strips (pH 3–10, 11 cm) and IPG buffer (pH 3–10) were bought from Amersham Biosciences (Pittsburgh, USA). Other

reagents used in 2D gel electrophoresis and the coomassie blue R250 were bought from Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., China.

Cell culture

The MG-63 cells were cultured in the RPMI-1640 medium supplemented with 15% newly-born bovine serum, 100 ku/L penicillin, and 100 mg/L streptomycin at 37°C. The cells were seeded in a constant density overnight, and then treated with 5 mmol/L HMBA for 7 days. Fresh culture media were added to the cells every 48 h. The cells were harvested at subconfluency and stored at −80°C.

Purification of NMPs

NMPs were extracted by a method of Michishita *et al* (*18*). MG-63 cells were washed with PBS and extracted with a cytoskeleton buffer CSK100 (10 mmol/L PIPES pH 6.8, 300 mmol/L sucrose, 100 mmol/L NaCl, 4 mmol/L $MgCl_2$, 1.0 mmol/L PMSF, and 0.5% Triton X-100) at 0°C for 10 min. After being sheered through a 16-gauge needle, the nuclei were subjected to centrifugation for 5 min at 400 *g*. The deposition was washed twice with CSK50 (10 mmol/L PIPES pH6.8, 300 mmol/L sucrose, 50 mmol/L NaCl, 4 mmol/L $MgCl_2$, 1.0 mmol/L PMSF, and 0.5% Triton X-100), then digested for 30 min at 25°C in the same buffer containing 500 u/mL DNase I. A concentration of 1 mol/L ammonium sulfate was added dropwise to a final concentration of 0.25 mol/L. After incubation for 15 min, the NMPs were pelleted by centrifugation at 1,000 *g* for 5 min, and washed once with the CSK50 buffer, then stored at −80°C. In addition, the protein concentrations were determined by the Bradford method.

2D gel electrophoresis

To solubilize nuclear proteins, the pellets of NMPs were resuspended in a 2D buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, and 50 mmol/L DTT, then were ultrasonicated for 2 min. The supernatant was centrifuged for 30 min at 16,000 *g* at 4°C. Typically 200 μg protein was loaded. IEF was performed in 12×0.2 cm acrylamide rods or ReadyStrip IPG strips, respectively. The acrylamide rods contained 4% acrylamide, 9.5 mol/L urea, 2.0% NP-40, and 0.5% carrier ampholytes of pH 3–10. The rods were subjected to a three-step pre-electrophoresis

with 15 min at 200 V, 30 min at 300 V, and 1 h at 400 V. Focusing was performed for a total of 11,000 V·h. ReadyStrip IPG strips were rehydrated overnight in a reswelling tray with rehydration buffer. IEF was carried out on a Protean IEF Cell (Bio-Rad, Hercules, USA) at 19°C with a maximum current setting of 80 μA/strip. Focusing was performed for a total of 70,000 V·h. Before carrying out the 2D SDS-PAGE, the strips were equilibrated in an equilibration buffer consisting of 10% glycerol, 2% SDS, 60 mmol/L Tris-HCl of pH 6.8, and 5% mercaptoethanol for 20 min at room temperature. The strips were then transferred onto the 1-mm-thick 2D SDS-PAGE gel and sealed in place with 1% agarose. The 2D SDS-PAGE was performed on a 12.5% acrylamide/bisacrylamide gel at 50 V for 30 min followed by 170 V for 8 h. The gel was run in the following electrode buffer: 25 mmol/L Tris, 192 mmol/L glycin, and 0.1% SDS. The 2D SDS-PAGE standards were used for gel calibration. The gel was stained with 0.25% coomassie blue R250 and destained with 5% methanol and 7.5% acetic acid. The 2D maps of NMPs were subjected and analyzed by Melanie software. Protein spots were manually excised for MS analysis.

Protein identification by MALDI-TOF-MS analysis

In-gel protein digestion

Spots were cut into about 1 mm^3 pieces, washed twice with 80 μL of 25 mmol/L NH$_4$HCO$_3$ and 50% acetonitrile for 5 min, then dehydrated with 80 μL of acetonitrile. Reduction was achieved by 1 h treatment with 10 mmol/L DTT in 100 mmol/L NH$_4$HCO$_3$ at 57°C. Alkylation was performed with 25 mmol/L iodoacetamide/100 mmol/L NH$_4$HCO$_3$ for 45 min at 25°C. Finally, gel spots were washed 3 times for 5 min alternatively with 100 mmol/L ammonium carbonate and acetonitrile. Gel pieces were completely dried with a Speed Vac before trypsin digestion. Appropriate volumes of trypsin (12.5 mg/L, freshly diluted in 50 mmol/L NH$_4$HCO$_3$) were added to the dried gel. The digestion was performed at 37°C overnight. After centrifuged for 5 min in a Speed Vac, the gel pieces were incubated in 10 μL of 20 mmol/L NH$_4$HCO$_3$ for 20 min at room temperature, and then incubated in 10 μL of 5% TFA/50% acetonitrile for 5 min at room temperature. The mixture was centrifuged at 6,000 g for 5 min. The supernatant was mixed and then completely dried with a Speed Vac.

MALDI-TOF-MS analysis

For MALDI-TOF-MS analysis, samples were resolved in 2 μL of 0.1% TFA separately. Mass measurements were carried out on a Brucker ULTRAFLEX TOF/TOF mass spectrometer (Bremen, Germany). This instrument was used at a maximum accelerating potential of 20 kV (in positive mode) and was operated in reflector mode. A total of 0.5 μL saturated solution of the α-cyano-4-hydroxy cinnamic acid in 0.1% TFA/30% acetonitrile was mixed with 0.5 μL sample solution, and added to the target. In positive mode, internal calibration was performed with tryptic peptides coming from autodigestion of trypsin (monoisotopic masses at m/z 842.51 and m/z 2211.10). Monoisotopic peptide masses were assigned and used for database searching. The data were searched against an NCBI non-redundant protein sequence database using the Mascot tool from Matrix Science. All proteins presented in the NCBI database were taken into account without any pI or Mr restrictions. One possible missed cleavage for trypsin digestion was selected; the fix modification was carbamidomethyl (C) and the variable modification was oxidation (M).

Acknowledgements

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