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Received August 27, 2008
Revised November 21, 2008
Accepted November 23, 2008

Research Article

SELDI-TOF as a method for biomarker discovery in the urine of aristolochic-acid-treated mice

Aristolochic acids (AAs) present in *Aristolochia* plants are substances responsible for Chinese herbs nephropathy. Recently, strong indications have also been presented, which dietary poisoning with AA is responsible for endemic (Balkan) nephropathy (EN), an enigmatic renal disease that affects rural population living in some countries in Southeastern Europe. A mouse model was applied to follow the effects of two forms of AA, AAI and AAII. SDS-PAGE and SELDI-TOF mass spectrometry with normal phase chips were used to evaluate changes in the urine of treated animals. These two methods are demonstrated to be comparable. The use of SELDI-TOF MS for rapid analysis of a large number of samples and the combination of this method with nano-LC-ESI MS/MS for protein identification were demonstrated. Biomarker discovery after analysis of large cohort of EN patients will be the final aim of these investigations.

Keywords:
Aristolochic acid / Chinese herbs nephropathy / Endemic nephropathy / SELDI-TOF MS / Urinary biomarkers
DOI 10.1002/elps.200800548

1 Introduction

Aristolochic acids (AAs) present in *Aristolochia* plants are the toxins responsible for Chinese herbs nephropathy (CHN). This type of rapidly progressive interstitial renal fibrosis is reported in a group of Belgian women after the introduction of Chinese herbs, as a part of slimming regimen [1]. The disease is characterized by early, severe anemia and mild tubular proteinuria, and renal interstitial fibrosis. Urothelial malignancy of the upper urinary tract develops subsequently in almost half of the patients. Exposure to AA was confirmed by the detection of AA–DNA adducts in the kidney tissue samples from CHN patients [2]. Recently, Grollman *et al.* [3] presented strong indications that dietary poisoning by AA is also responsible for endemic (Balkan) nephropathy (EN) and its associated urothelial cancer. This renal disease affects rural population living in Southeastern Europe, in Bosnia and Herzegovina, Bulgaria, Croatia, Serbia and Romania [4]. The main features of human CHN, renal interstitial fibrosis and urothelial malignancy have also been reproduced in rats [5]. Sato and coworkers [6] reported that mice belonging to the sensitive C3H/He strain when treated with AA rapidly developed nephropathy. AA is a mixture of structurally related nitrophenanthrene carboxylic acids, containing two major components – AAI and AAII, additionally also the less common AAIVa and AAVa and aristolactam (AL) (see [7] and Fig. 1). Investigations using purified AAI and AAII revealed that AAI induced strong nephrotoxicity in mice and that AAII resulted in mild nephrotoxicity. The other investigated components, AAIVa and AL, proved as non-toxic [6].

In the present work, AA-sensitive CH3/He mice were used to compare the effects of two forms of AA, AAI and AAII. SDS-PAGE and SELDI-TOF mass spectrometry were used to evaluate changes in the urine of treated animals. After electrophoretic separation, urinary proteins were identified by LC-ESI MS/MS.

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Abbreviations: AA, aristolochic acid; **AL,** aristolactam; **CHN,** Chinese herbs nephropathy; **EN,** endemic nephropathy; **MUP,** mouse urinary protein

2 Materials and methods

2.1 Animal model

Mouse model, introduced by Sato *et al.* [6] was used to compare the effects of two forms of AA: AAI and AAII. XPF mice [8] were maintained on a mixed 129:C57Bl/6 genetic background by intercrossing $XPF^{+/-}$ mice. $XPF^{-/-}$ mice die at or shortly after birth. Eight-week-old male $XPF^{+/-}$ or $XPF^{+/+}$ littermate controls were used. The genotype of each mouse was determined by PCR of genomic DNA prepared from tail biopsies. The animals were treated with AAI or

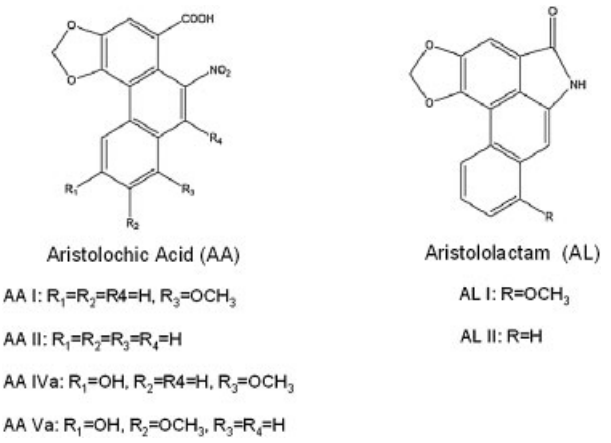

Figure 1. Structure of AAs and ALs.

AAII (1.8 mg/kg/day, i.p.) for 11 days. Urine was collected for 4 h each day and subsequently pooled. After centrifugation to remove debris urine was stored at −80°C. Groups A and B: $XPF^{+/-}$ mice, groups C and D: $XPF^{+/+}$ mice. AAI was given to groups A and C, and AAII was given to groups B and D. Control group was injected only by vehicle (PBS). Each group contained three animals.

2.2 AA

AAI and AAII were purified from a mixture as described previously [9]. Briefly, a mixture of AAI and AAII (40:60) was purchased from Fisher Scientific (Fairlawn, NJ, USA), and the two acids were separated by preparative reverse-phase high-performance liquid chromatography on an X-Terra MS C18 (5 μm with 19 × 50 mm) column (Waters, Milford, MA, USA) and eluted with a gradient of ACN (16–25%) in 0.1 M triethylamine–acetic acid buffer at pH 7.5.

2.3 SELDI-TOF mass spectrometry

Normal-phase arrays (Ciphergen-BioRad, Richmond, CA, USA) were used for protein profiling. Sample preparation was performed by using the Biomek 2000 laboratory automation workstation (Beckman Coulter, Fullerton, CA, USA). Arrays were placed in shaker containing buffer chambers called ''bioprocessor'' and equilibrated with the corresponding buffer for 5 min twice. After the equilibration buffer was removed, 90 μL of 100 mM Tris–HCl, pH 8.5, (dilution/wash buffer) and 10 μL of mouse urine were added to each well and incubated with shaking for 60 min at room temperature in a humid chamber. Samples were removed by aspiration and the arrays were washed three times with a washing buffer and rinsed twice with ''nano-pure'' water for 5 min each. Arrays were removed from the bioprocessor and air-dried. Aliquots containing 1 μL of sinapinic acid in ACN and 1% trifluoroacetic acid were added twice *per* spot and dried.

Arrays were read in a ProteinChip System 4000 (Ciphergen-BioRad). The spectra of samples and protein calibrates were generated in ''auto'' mode using the appropriate laser power for low and high mass ranges. Analyses of these spectra were accomplished using the CiphergenExpress software (version 3.0) with total ion current normalization.

2.4 Protein determination

Protein content in urine was determined by using the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's procedure.

2.5 SDS-PAGE

Protein samples were solubilized in NuPAGE LDS sample buffer and heated at 100°C for 5 min. SDS-PAGE was performed with precast NuPAGE 4–12% or 12% Bis-Tris gels in an XCell Sure Lock Mini-Cell (Invitrogen, Carlsbad, CA, USA) according to the manufacturer. The gels were stained with GelCode Blue (Pierce) and scanned with a VersaDoc Imaging System (BioRad) before excising the bands of interest for in-gel digestion.

2.6 In-gel digestion

Gel bands of interest were excised by extracting 6–10 gel particles with clean glass Pasteur pipettes and digested with trypsin as described preciously [10, 11]. The specific procedure is briefly described as follows.

After excision, gel particles were washed twice with analytical-grade water and 1:1 v/v of 0.1 M NH_4HCO_3 for 15 min with agitation. The washing solution was then removed completely and enough ACN was added to cover the gel particles. All the solvent volumes used in the washing steps should roughly equal twice the gel volume. After the gel particles shrunk and stuck together, the ACN was removed and the gel particles were rehydrated in 0.1 M NH_4HCO_3 for 10 min. An equal volume of ACN was then added to finally get 1:1 v/v of 0.1 M NH_4HCO_3/ACN. After 10 min incubation, removing all liquid, and drying down in a vacuum centrifuge, gel particles were treated with 10 mM dithiothreitol and with 55 mM iodoacetamide in 0.1 M NH_4HCO_3 in order to reduce and alkylate enclosed proteins. After this treatment, the gel particles were washed as described above. Following tryptic digestion for 24 h at 37°C, the peptides were recovered and sequentially extracted from the gel particles by addition of a 10 μL of 25 mM NH_4HCO_3 and 5% formic acid and ACN (5 μL of each). Pooling and drying down all the extracts, the tryptic peptides were dried and redissolved in formic acid:water:ACN:trifluoroacetic

acid mixture (0.1:95:5:0.01) in preparation for the LC-MS/MS analysis.

2.7 Protein identification by nano-LC-ESI-MS/MS

Peptides extracted from the gels after SDS-PAGE separation and trypsin digestion were separated with a reversed-phase column (C-18 PepMap 100, LC Packings/Dionex, Synnyvale, CA, USA) as previously described [11, 12]. Briefly, the column eluate was introduced directly onto a QSTAR XL mass spectrometer (Applied Biosystems and Sciex, Concord, Ontario, Canada) *via* ESI. Half-second MS scans (300–1500 Thompson, Thompson (Th) = Da/z, z = ion charge) were used to identify candidates for fragmentation during MS/MS scans. Up to five 1.5 s MS/MS scans (65–1500 Th) were collected after each scan. An ion had to be assigned a charge of +2 to +4. The dynamic exclusion was 40. Protein identifications were completed with ProteinPilot (Applied Biosystems and Sciex), setting with 1.5 Da mass tolerance for both MS and MS/MS and using ''RefSeq databases (mouse) from NCBI (http://www.ncbi.nlm.nih.-gov/RafSeq/). ProteinPilot is the successor to ProID and ProGroup, and uses the same peptide and protein scoring method. Scores above 2.0 require that at least two sequence-independent peptides be identified [12].

3 Results

3.1 Changes in protein concentration during AAI and AAII treatment

As shown in Fig. 2, treatment with AAI results in heavy proteinuria in both groups of mice (groups A and C), whereas the treatment with AAII did not result in significant increase in urine protein concentration (groups B and D). Control group treated only with vehicle (PBS) also did not show significant change in protein concentration during the 11-day treatment.

3.2 Changes in protein profile determined with SDS-PAGE

After two (group A) and three (group C) days, the protein pattern in the urine of AAI-treated mice drastically changed. As shown in Fig. 3, the amount of protein in the main band (between 18 and 20 kDa apparent molecular weight in SDS-PAGE) was reduced, and the amount of proteins with higher molecular weight significantly increased. In contrast, the protein pattern in AAII-treated mice (groups B and D) did not change – especially the intensity of both major bands with apparent molecular weight of 18 and about 21 kDa did not change significantly. In the urine of group B animals, the appearance of some proteins with higher molecular weight could be registered after fourth day of

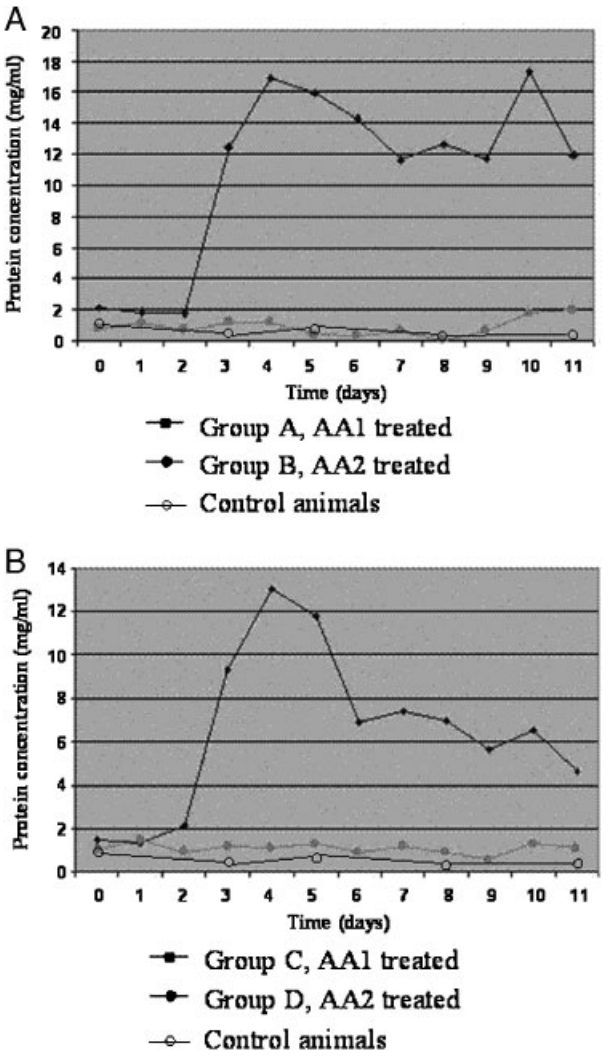

Figure 2. Protein concentration in the urine of mice after treatment with AAI, AAII and control mice. Groups A and B: $XPF^{+/-}$; groups C and D: $XPF^{+/+}$, control group: $XPF^{+/-}$. See also Section 2.

treatment. In group D, only one band with apparent molecular weight of about 95 kDa was detected after 9 days of treatment. In control group, no change in protein pattern was observed (see Fig. 3).

3.3 Identification of mouse urinary proteins with nano-LC-ESI-MS/MS

Low- and high-molecular-weight proteins were excised from the gels, digested and identified with nano-LC-ESI-MS/MS (see Fig. 4). To separate maximal number of protein bands in both high- and low-molecular-weight range, 4–12% or 12% Bis-Tris gels were used. The list of proteins identified in the urine of mice treated with AAI is given in Table 1, and the proteins found in the urine of the animals treated with AAII are listed in Table 2.

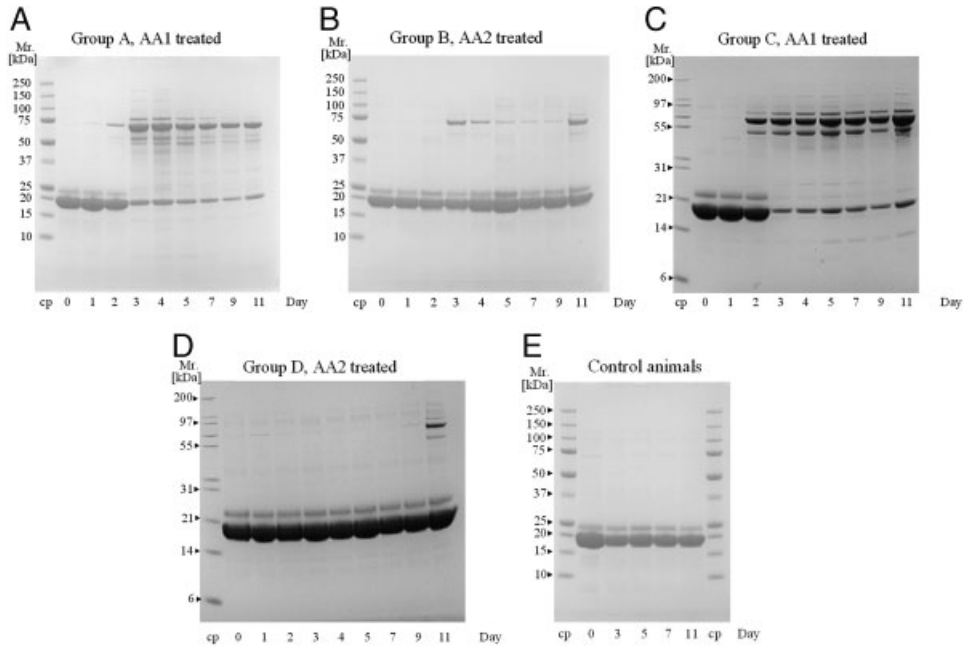

Figure 3. SDS-PAGE of urine samples of mice during treatment with AAI, AAII and control mice.

Table 1. Proteins identification in the urine of mice treated with AA1

Band no.	Protein	Molecular weight
1	Complement C3	186 366
2	Cerruloplasmin	126 920
3	Inter-α-inhibitor, heavy chain 4	104 900
4	Transferrin	76 730
5	Mouse serum albumin	68 648
	Hemopexin	51 350
6	Murinoglobulin	165 196
	Alpha-2-macroglobulin	165 034
7	Cp protein	121 074
8	Transferrin	76 648
	Prot. Inhib. Clade A member 3K	46 822
9	Mouse serum albumin	68 648
	Clade A Member 3K	46 833
	Contrapsin	46 850
10	Alpha-1-antitrypsin	45 946
	Serpin 1a protein	45 868
11	Apolipoprotein A-I	30 569
	Serum amyloid P-comp.	26 230
12	Transthyretin	15 766
	Alpha-2μ-globulin	20 230
18	Fetuin	37 460
	Kininogen 1	47 910
19	Apolipoprotein A-IV	45 040
20	Haptoglobin	38 750
	Alpha-1-antitrypsin 1–6	45 794
21	Apolipoprotein A-IV	45 455
	Compliment C3, fragment	186 366
22	Complement C3, isoform CRA	188 470
23	Cerrulaplasmin	126 920
24	Orosomucoid 1	23 870

Table 2. Proteins identification in the urine of animals treated with AA2

Band no.	Protein	Molecular weight
13	Meprin A alpha subunit	85 000
	Uromodulin	8 992
14	Clade A, member 3K	46 822
	Serum albumin	68 648
15	EGF	150 000
16, 17	Mouse urinary protein 1	18 694
	Mouse urinary protein 2	18 709
	Mouse urinary protein 3	18 817
	Alpha-2μ-globulin	20 230

3.4 Profiling of urinary proteins with SELDI-TOF MS

The protein profiles determined by SELDI-TOF MS in the urine of the mice from group A (AAI treated) are shown in Fig. 5A and B. Figure 5A shows the profiles in the low-molecular-weight range, and Fig. 5B shows the changes in protein profiles during the 11-day treatment in the high-molecular-weight range. Figure 6 shows "gel image" of proteins in the high-molecular-weight range in rat urine after 9 days treatment with AAI. This profile is directly compared with the corresponding MS profile and SDS-PAGE of the same sample.

4 Discussion

Because of its representative protein and peptide content and non-invasive collection method, urine is an obvious

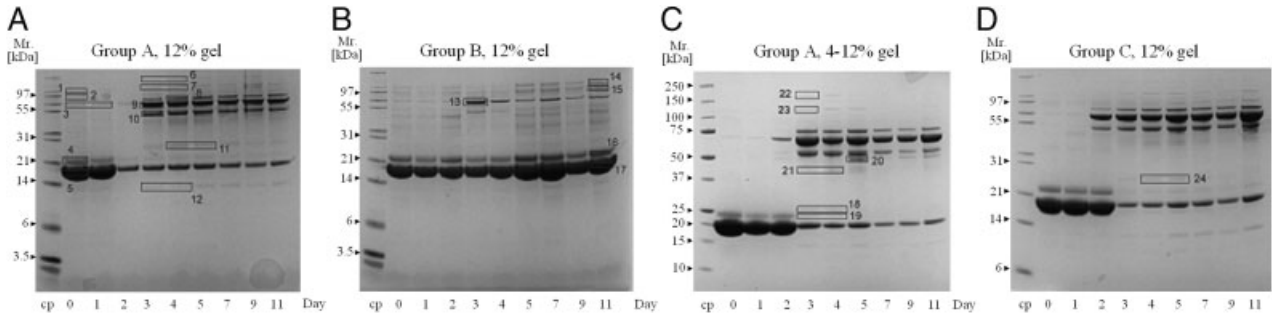

Figure 4. Identification of urinary proteins by nano-LC-ESI MS/MS. After SDS-PAGE, bands of interest were excised and proteins were identified after proteolyitic digestion and extraction (see Section 2). To achieve separation in both high- and low-molecular-weight range, 12% and gradient gels (4–12%) were used. Identified proteins are listed in Tables 1 and 2.

choice of sample for both discovering biomarkers and for use in screening trials for different diseases.

After its promising development a couple of years ago [13, 14], SELDI-TOF mass spectrometry has recently been less frequently used as a method of choice for biomarker discovery. One of the key problems that occur when this technology is applied is the further MS/MS identification of peaks, which are after comprehensive data analysis [15] identified as possible biomarker candidates. The main obstacle is that the SELDI-TOF instrument still requires a relatively complicated procedure for interfacing with the MS/MS instrument for protein identification. An additional problem is the less-effective ionization in the high molecular range resulting with apparently lower sensitivity of the instrument for large proteins. After improvement of both the hardware and software, and thorough investigations of sample storage and preparation, SELDI-TOF MS is again being more frequently used [16–19]. The big advantage of the SELDI-TOF technology is the simple automation of sample preparation and the measurement process that enables its use for high-throughput screening, which is necessary when dealing with large sample numbers during the biomarker discovery process. Corresponding software has been developed for rapid statistical data analysis and reliable identification of possible biomarker candidates [13, 15, 19].

SDS-PAGE and 2-D electrophoresis combined with LC-MS/MS are the most frequently used methods for investigations of urinary proteome [20–22]. These methods are suitable for thorough investigation and identification of target proteins. Screening of the large number of patient samples and samples of healthy individuals is the first step of disease biomarker discovery, and these time-consuming analytical methods can hardly be applied at this stage of the process.

The aim of our investigations is to find the SELDI-TOF surface, which in MS measurement has the protein profile that is closest to the corresponding profile in SDS-PAGE. For this purpose, the urine of AAI- and AAII-treated mice was analyzed.

As shown in Fig. 2, treatment with AAI causes a heavy proteinuria after 2–3 days in both groups of mice (A and C). Treatment with AAII did not have this effect in either treated groups (B and D). In AAI-treated mice, the protein pattern in the urine also changed (see Fig. 3). The main band with an apparent molecular weight of about 18–20 kDa that was identified as mouse urinary protein (MUP) (see Fig. 3 and Table 2 and [23]) was significantly reduced, and the amount of high-molecular-weight proteins increased (see Fig. 3). The protein pattern in the urine of mice treated with AAII did not change drastically – only slight increase of protein amount in the high-molecular-weight range could be detected. The relative amount of MUP, the main protein in the urine of healthy mouse, did not change. As shown in Table 1, in the urine of mice treated with AAI, several plasma proteins were identified, among them are serum amyloid and transthyretin. These two proteins are also candidate biomarker for renal injury and cancer, following poisoning with nephrotoxins [24, 25]. Inter-alpha-trypsin inhibitor heavy chain 4, which was also identified, is a plasma glycoprotein that acts as an acute-phase protein in several species. This protein and its cleavage fragments are also candidate biomarkers associated with different malignant diseases [26, 27]. Serum amyloid, transthyretin and inter-alpha-trypsin inhibitor heavy chain 4 were not detected in the urine of AAII-treated mice. In the urine of both, AAI- and AAII-treated groups, serum albumin and alpha-2-microglobulin were identified. Interestingly, higher levels of these two proteins were also detected in the urine of EN patients [28]. Urinary alpha-1-microglobulin is known as a marker for tubular, and serum albumin on the other hand for glomerular proteinuria [25, 28]. In summary, most of the (glyco)proteins identified here are often prominent in both inflammation and cancer [26, 29].

As shown in Fig. 5, protein pattern determined by SELDI-TOF MS with the use of a chip having a normal phase surface chemistry was very similar to the pattern in SDS-PAGE. To make it more illustrative, the pattern in gel-imaging mode is also shown. These two patterns from SELDI-TOF MS were compared with the same sample, analyzed by SDS-PAGE (see Fig. 6). Other surfaces (cation-exchanger CM10 and metal affinity IMAC30) show much higher selectivity, and the protein patterns from

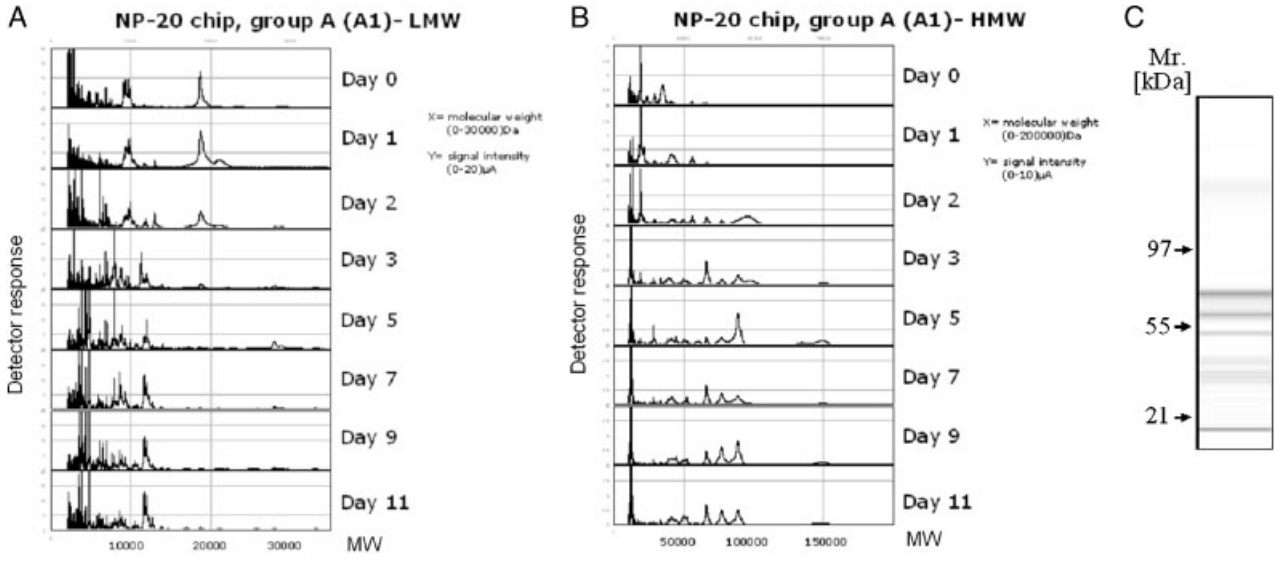

Figure 5. Protein profiles determined in the urine of mice treated with AAI (group A, see Figs. 2 and 3). (A) Profiles in the low-molecular-weight range. (B) Profiles in the high-molecular-weight range. (C) ''Gel image'' of proteins detected in the high-molecular-weight range.

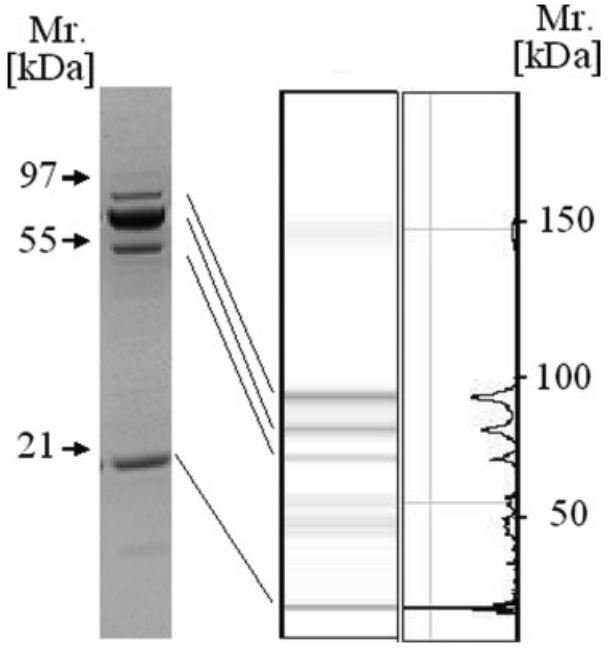

Figure 6. Comparison between SDS-PAGE and profiles obtained by SELDI-TOF in high-molecular-weight range. For comparison, sample after 9 days treatment with AAI was chosen.

MALDI-TOF MS gained by these chips were not comparable with those from SDS-PAGE (data not shown).

After 2 days of treatment with AAI, the peak with a molecular weight of about 18 000 (representing MUPs, see Fig. 3 and Table 2) is decreasing (Fig. 5A). At the same time, the appearance of several peaks in the high-molecular-weight range can be observed (see Fig. 5B). A big advantage of the SELDI-TOF technique is the possibility to follow changes in protein patterns in both high- and low-molecular-weight range (below 8 kDa). Using the system for sample preparation and analysis that were described in these present experiments, high-throughput screening of urine samples is possible. After detection of possible biomarker candidates, they can be identified by subsequent 1-D or 2-D electrophoretic separation, followed by LC-MS/MS. This strategy will be applied in future investigation for biomarkers of EN that is already designed for the endemic region in Northern Croatia [4, 30].

This work was supported by National Institutes of Health, Centers for Biochemical Research Excellence (COBRE), grant no. P20RR017695. The authors thank Dr. Arthur P. Grollman (SUNY, Stony Brook, NY, USA) for valuable comments and great support.

The authors have declared no conflict of interest.

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