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Profiling of rat plasma by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, a novel tool for biomarker discovery in nutrition research

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Abstract

The recent development of high-throughput proteomic technologies has given us new methods to analyze how an organism responds to changes in its nutritional environment. The analysis of plasma samples by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI–TOF–MS) was investigated as a novel approach to the identification of new biomarkers of nutrient status. Pre-fractionation of rat plasma by anion-exchange chromatography in 96-well filter plates markedly increased the total number of unique peptides and proteins that could be observed in SELDI–TOF mass spectra. Replicate fractionations generated nearly identical pH fractions, not only in terms of peptide and protein composition but also in respect to the ion signal intensity of replicate SELDI–TOF mass spectra. The feasibility of this approach was tested with samples from retinol-sufficient and retinol-deficient rats. The comparative analysis revealed reduced levels of three proteins with molecular masses between 10 000 and 20 000 in plasma of retinol-deficient rats. These results demonstrate that plasma profiling by anion-exchange fractionation and SELDI–TOF–MS may be a promising surveillance tool to detect changes in nutritional status and whole body physiology.

Keywords: Surface-enhanced laser desorption ionization mass spectrometry; Mass spectrometry; Albumin; Proteins; Retinol; Vitamins

1. Introduction

The Human Genome Project has catalyzed the development of novel technologies that have made it possible to analyze large sets of mRNA and/or proteins expressed in a biological sample. High-throughput applied genomics technologies like transcriptomics, proteomics and metabolomics are now available to nutrition researchers. These new screening tools are likely to play a major role in the selection of new and accurate biomarkers that are urgently needed for predicting the outcomes of food-based interventions, given the importance being placed on ingredients in foods contributing to disease prevention and optimal health promotion.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI–TOF–MS) is a novel approach to biomarker discovery that combines specially designed protein chip arrays with surface ionization mass spectrometry [1]. At the core of this technology is the on-chip affinity purification of peptides and proteins from complex mixtures thus greatly reducing signal suppression effects often encountered in the analysis of biological samples by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS). Further advantages are that SELDI–TOF–MS is fast and readily amendable to high-throughput automation. In combination with advanced data mining methodologies, this technology has recently been used successfully in the field of diagnostic proteomics to detect several disease-associated proteins and protein expression patterns in a variety of biological tissues and body fluids [2–4].

As a result of the success of these initial studies, the rapid generation of protein expression profiles from plasma and serum samples has become of special clinical interest [5]. Plasma is the most sampled proteome and contains many other tissue proteomes as subsets. In addition to the classical

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plasma proteins secreted by liver and intestines, the plasma proteome also contains peptides and proteins that are secreted into plasma from other normal tissues, from tumors or diseased tissues, or as the result of tissue damage or cell death. The proteomic profile of plasma might serve as an indicator of the pathophysiological state of internal organs. The plasma proteome is therefore considered to be the most important proteome for the early detection of disease and could possibly also be used for therapeutic monitoring [6]. However, several factors complicate the analysis of plasma and make the detection of low abundance peptides and proteins difficult: (1) the high concentration of albumin and γ-globulins, (2) the wide dynamic concentration range of other peptides and proteins, and (3) the tremendous heterogeneity of the glycoconjugates of plasma proteins. As a result, methods to reduce the complexity of samples by removing highly abundant proteins are rapidly becoming an essential first step in many proteomic analysis schemes. Several different strategies to effect this goal have been recently described for human plasma and serum samples including ultrafiltration [7], multi-component immunoaffinity subtraction chromatography [8]and liquid-phase isoelectric focusing electrophoresis [9].

The aim of this investigation was to determine whether changes in the plasma proteome that can be detected by SELDI–TOF–MS can be related to changes in nutrient status. For the purpose of this investigation we chose to study the vitamin A-deficient rat model. Dietary restriction of vitamin A and β-carotene in rats leads to rapid depletion of hepatic vitamin A stores, followed by the onset of clinical vitamin A deficiency after approximately 35 days [10]. In the first part of this investigation we used sera from retinol-sufficient rats to test different strategies to reduce albumin content and sample complexity. We subsequently evaluated the reproducibility of the optimized fractionation protocol with different rats before applying the methodology to analyze plasma samples from retinol-sufficient and retinol-deficient rats for differential protein expression.

2. Experimental

2.1. Materials

Male Lewis rats with their dams were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA). AIN-93G vitamin A-free diet was obtained from Dyets (Bethlehem, PA, USA). The ProteinChip Reader system (BPS II), Q Ceramic HyperDF anion-exchange resin and WCX2 protein array chips were purchased from Ciphergen (Fremont, CA, USA). The SwellGel Blue Albumin Removal kit was obtained from Pierce (Rockford, IL, USA). LBP Durapore filterplates were obtained from Millipore (Bedford, MA, USA). The ProteoMass Peptide & Protein MALDI–MS Calibration kit, α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid an all other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Animal procedures

Dams were fed the AIN-93G vitamin A-free diet from their time of arrival. Young rats ($n = 3$/group) were weaned after 25 days and split into two groups. The vitamin A-deficient rats continued to receive the AIN-93G vitamin A-free diet whereas the vitamin A-sufficient rats were fed the AIN-93G vitamin A-free diet supplemented with retinyl palmitate. After approximately 90 days rats were euthanized and blood was collected into heparinized syringes from the abdominal vena cava. Plasma was prepared by centrifugation at 800 × g for 20 min at 4 °C and stored at −80 °C.

2.3. Albumin depletion of rat plasma with Cibacron Blue agarose

Twenty microliters of rat plasma were diluted with 30 μl urea–3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS)-Tris–HCl buffer (9 M urea, 2% CHAPS, 25 mM Tris–HCl, pH 7.5, 25 mM NaCl) and incubated for 30 min at 10 °C. Rat plasma sample were then diluted with 50 μl buffer 2 (1 M urea, 0.22% CHAPS, 25 mM Tris–HCl, pH 7.5, 25 mM NaCl) and incubated for an additional 5 min at 10 °C. Diluted samples were applied to a SwellGel Blue spin column, incubated for 2 min at 25 °C and centrifuged at 10 000 rpm for 1 min in a tabletop centrifuge. The flow-through was collected and reapplied to the column. Incubation and elution were performed as described above. Bound peptides and proteins were eluted with 1.5 M KCl (25 mM Tris–HCl, pH 7.5, 1.5 M KCl).

2.4. Anion-exchange fractionation of rat plasma

Twenty microliter of plasma were mixed with 30 μl denaturing buffer A [9 M urea, 2% (w/v) CHAPS, 50 mM Tris–HCl, pH 9] for 30 min at 10 °C. The sample was then diluted with 50 μl of denaturing buffer B [1 M urea, 0.22% (w/v) CHAPS, 50 mM Tris–HCl, pH 9] and incubated for 5 min at 10 °C. A 180 μl volume of a 50% suspension of Q Ceramic HyperDF resin in Tris–HCl buffer (50 mM Tris–HCl, pH 9) was added to each well of a Millipore LPB Durapore filterplate (pore size: 0.65 μm) and washed three times with same buffer. One hundred microliters of the diluted plasma sample were applied to each well and incubated for 30 min at 4°C with shaking at 800 rpm on a MS 1 minishaker (IKA, Germany). The flow-through was collected by vacuum filtration into receiver plates. The anion-exchange resin was incubated with an additional 100 μl of Tris–HCl buffer [50 mM Tris–HCl, pH 9, 0.1% (w/v) OG] for 10 min at 25 °C with shaking. The wash was collected by vacuum filtration. This procedure was repeated two times with 100 μl each of appropriate buffers with decreasing pH. The final wash was performed with an

organic wash buffer containing 33% (v/v) isopropanol and 16% (v/v) acetonitrile in 0.1% trifluoroacetic acid (TFA). Fractionated samples were stored at −80 °C until analysis.

2.5. Sample preparation for SELDI–TOF profiling experiments on WCX2 chips

Unfractionated plasma samples were denatured as described in Section 2.2 and brought to a final dilution of 1:50 with NaAc binding buffer (100 mM NaAc, pH 4, 0.1% OG). Collected fractions were diluted 1:10 with NaAc binding buffer (100 mM NaAc, pH 4, 0.1% OG). One hundred microliters of the diluted samples were incubated in a bioprocessor with a WCX2 chip for 60 min at 25 °C with shaking at 800 rpm. The chip was washed twice with binding buffer for 5 min, washed twice with water for 1 min and air dried.

2.6. SELDI–TOF–MS instrumentation, sample preparation and analysis

SELDI–TOF mass spectra were acquired on a ProteinChip Reader system in the linear ion mode using a nitrogen laser (337 nm). SELDI–TOF mass spectra were collected in the positive ion mode using an accelerating voltage of 20 kV and a delay time of 600 ns. Low-molecular-mass SELDI–TOF mass spectra (*m*/*z*, 0–30 000**)** of peptides and proteins were acquired with α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. Each spot of a WCX2 protein chip array was overlaid with 2 × 0.5 μL of a saturated solution of CHCA in 50% (v/v) acetonitrile and 0.1% (v/v) TFA and allowed to air-dry at room temperature. SELDI–TOF mass spectra of proteins larger than *m*/*z* 25 000 were acquired with sinnapinic acid (SA) as matrix. Each mass spectrum shown represents the sum of a total of 150 laser shots collected at 30 different positions within each spot. Before each SELDI–TOF–MS analysis the instrument was calibrated with the ProteoMass Peptide & Protein MALDI–MS Calibration kit using the oxidized beta-chain of insulin, insulin, cytochrome *c* and apomyoglobin as mass standards.

All SELDI–TOF mass spectra shown were corrected by baseline subtraction. For biomarker discovery, the spectra of vitamin A-sufficient and vitamin A-deficient animals were normalized to the total ion current starting at *m*/*z*, 1500 and analyzed for differential protein expression with the Biomarker Wizard software package (Ciphergen). Only peaks with a S/N ratio equal to or greater than 5 were considered for biomarker analysis.

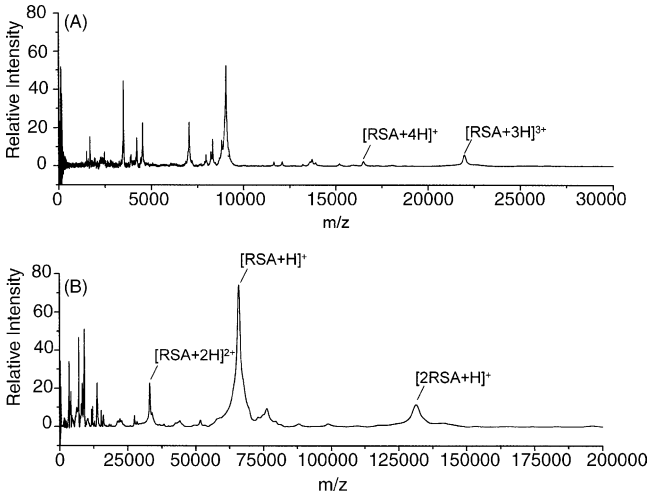

Fig. 1. SELDI–TOF mass spectra of unfractionated rat plasma on a weak cation-exchange (WCX2) chip. Plasma samples were prepared and diluted as described in Section 2. Peptide and protein ion signals in the *m*/*z* range of (A) 0–30 000 and (B) 0–200 000 are shown. RSA, rat serum albumin.

3. Results and discussion

3.1. Analysis of neat rat plasma by SELDI–TOF–MS

The analysis of the rat plasma proteome has mainly been carried out using electrophoretic separation techniques in connection with drug testing and toxicology studies [11]. However, a major drawback of the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) approach is that many low molecular weight peptides and proteins cannot be effectively visualized by current staining methods and are therefore lost from the final analysis. We therefore investigated whether we could apply SELDI–TOF–MS to the analysis of rat plasma with a special focus on low molecular weight peptides and proteins. Fig. 1A shows a representative SELDI–TOF mass spectrum of a diluted rat plasma sample in the low molecular weight range up to a *m*/*z* values of 30 000. Fig. 1B shows a representative SELDI–TOF mass spectrum of the same sample in the high-molecular-mass range up to a *m*/*z* values of 200 000. In the weight range where both mass spectra coincide, differences in the protein expression profiles are due to the different matrices and laser desorption energies that are used to acquire the low-and high-molecular-mass spectra. A total of approximately 50 peptide and protein signals with a S/N ratio > 5 were consistently detected in both spectra (Table 1). Not sur-

Table 1
Effect of sample preparation on the number of unique peaks founds in rat plasma samples at S/N ≥ 3 and S/N ≥ 5

Molecular mass range (*m*/*z*)	Total plasma ($n = 5$)		Albumin-depleted plasma ($n = 3$)		Fractionated plasma ($n = 5$)	
	S/N ≥ 3	S/N ≥ 5	S/N ≥ 3	S/N ≥ 5	S/N ≥ 3	S/N ≥ 5
2000–30 000	58 ± 8	25 ± 6	51 ± 9	48 ± 9	178 ± 8	101 ± 9
30 000–200 000	53 ± 8	26 ± 5	28 ± 7	24 ± 7	64 ± 7	41 ± 7

S/N: signal-to-noise ratio.

prisingly, rat serum albumin (RSA) was the most dominant peak at approximately *m/z*, 66 000 (Fig. 1B). However, several additional peaks could also be attributed either to the formation of singly-charged albumin dimers or to multiply-charged albumin molecules (Fig. 1B and A).

3.2. SELDI–TOF–MS analysis of albumin-depleted rat plasma

The high concentration of RSA in plasma significantly decreases the capacity of the protein chip arrays to bind low-abundance proteins and peptides from plasma for subsequent detection by SELDI–TOF–MS. We therefore tested two different strategies for albumin removal and plasma sample fractionation. Albumin removal from rat plasma can be achieved either by dye ligand chromatography with Cibacron Blue immobilized onto agarose or by immunoaffinity chromatography with specific anti-rat albumin antibodies bound to protein G. Since Cibacron Blue columns have a higher loading capacity for plasma than antibody-based columns, we tested how effectively the SwellGel Blue Albumin Removal kit (Pierce) can deplete albumin from rat plasma and whether albumin depletion increases the number of observable peptide and protein signals. Sodium dodecyl sulfate (SDS)–PAGE analysis of the flow-through fraction (Fig. 2, lane 3) clearly shows that a large proportion of albumin is removed from the rat plasma sample. SELDI–TOF–MS analysis of the flow-through fraction yielded an increased number of peaks in the low molecular weight region (*m/z* value up to 30 000) and also improved the S/N ratio for the observed peaks since most of the peptide and protein signals had a S/N ratio greater than 5. In contrast, albumin removal did not improve the number of observable proteins in the high molecular weight region and even led to a loss of some of the less intense protein signals when compared to neat plasma (Table 1). It is well known that Cibacron Blue not only binds albumin, but also plasma proteins that bind ATP and other nucleotides.

3.3. SELDI–TOF–MS analysis of fractionated rat plasma

In order to minimize the risk of losing potentially important proteins and peptides from the plasma sample in the course of the sample preparation process, we investigated anion-exchange fractionation of plasma as an alternative to albumin depletion by dye ligand chromatography. Rat plasma samples were incubated with Q Ceramic HyperD F anion-exchange resin in low protein binding filter plates. Peptides and proteins were eluted by a declining pH gradient run as a step gradient using six different aqueous buffers, followed by a final elution step with an organic wash buffer. Stepwise elution was preferred for this application because it is technically simpler and more reproducible than continuous-gradient elution and can be used with liquid handling workstations. Fig. 3 shows the molecular mass profile of the different fractions in comparison to the total unfractionated plasma sample. Fig. 3 illustrates that several peptides and proteins appear in the flow through fraction (pH, 9 fraction) as well as in multiple fractions. However, a significant number of unique peaks are observed in each fraction that are not apparent in the spectrum of the unfractionated plasma sample. A similar result was also obtained from the fractionated spectra in the high-molecular-mass region (spectra not shown). Table 1 summarizes the total number of unique peaks that were found in the SELDI–TOF–MS spectra of the fractionated samples. Inspection of Table 1 clearly shows that fractionation greatly increases the number of peptide and protein ion signals that could be observed by SELDI–TOF–MS, both in comparison to unfractionated plasma as well as albumin-depleted plasma. Similar

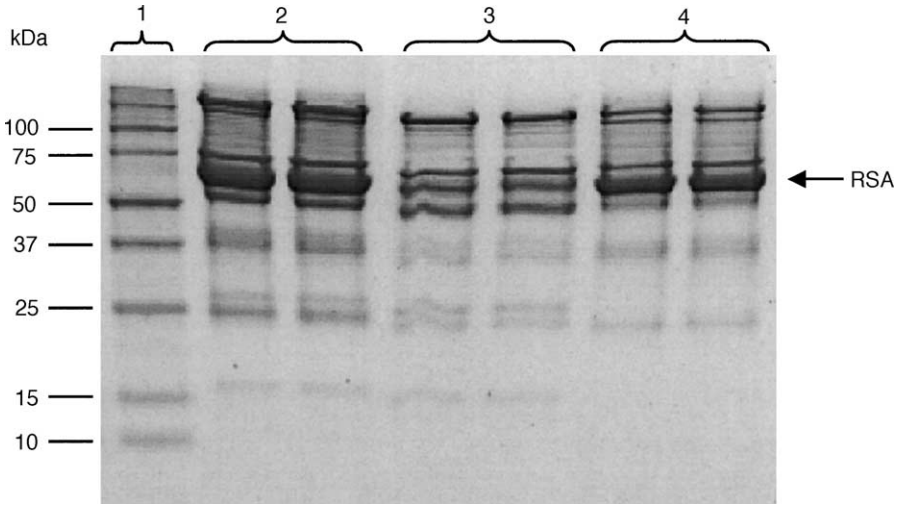

Fig. 2. Albumin depletion of rat plasma with the SwellGel Blue albumin removal kit (Pierce). Lanes: 1–standards; 2–crude serum; 3–depleted serum; 4–bound protein eluted from SwellGel Blue spin column with 1.5 M KCl. Samples (6–10 μg protein per lane) were loaded onto a 10% Tris–Tricine SDS–PAGE gel under denaturing conditions and visualized by staining with colloidal Coomassie Blue G-250. kDa: kilodaltons.

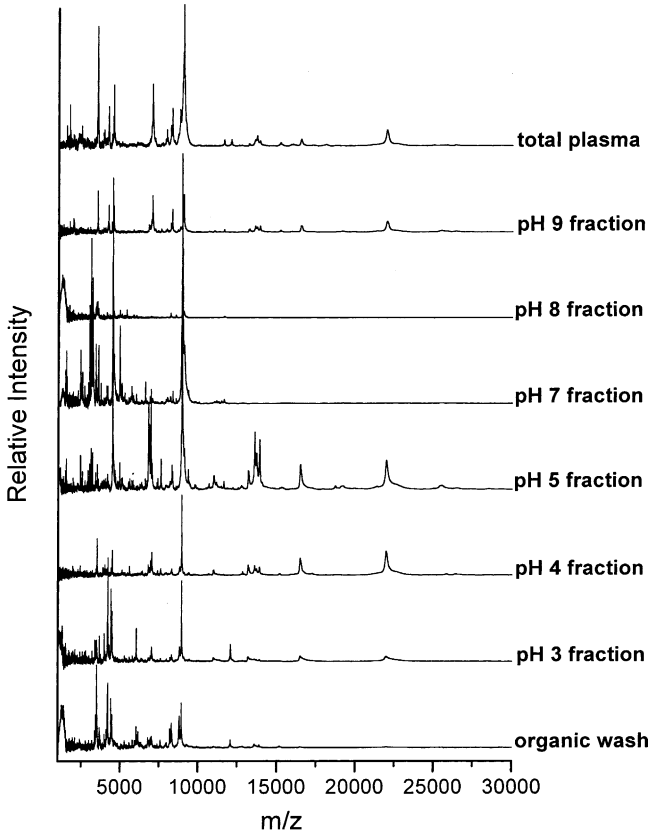

Fig. 3. Anion-exchange fractionation of rat plasma. Rat plasma peptides and proteins were fractioned as described in Section 2. Diluted fractions were allowed to bind to the surface of a WCX2 chip. SELDI–TOF mass spectra of peptide and protein ion signals in the *m*/*z* range 0–30 000 are shown.

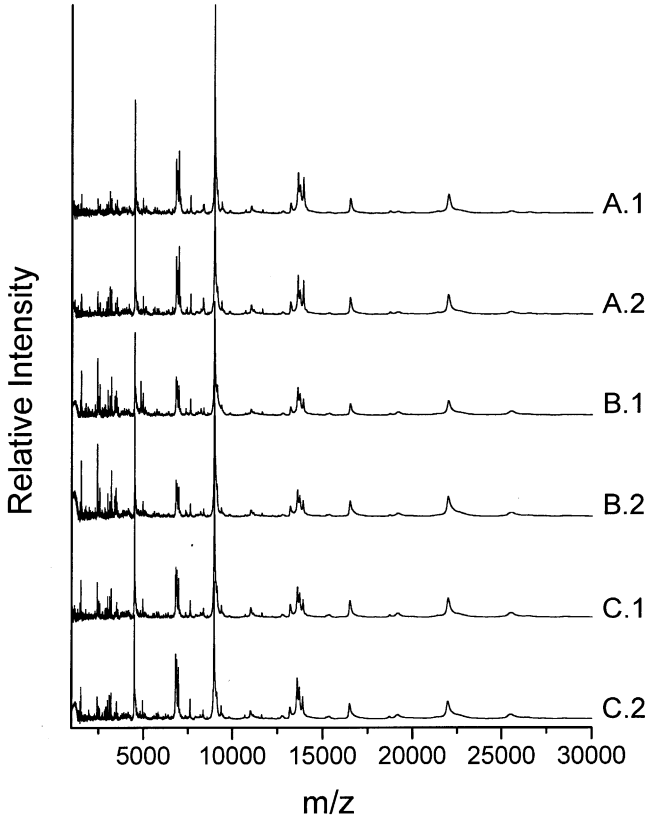

Fig. 4. Replicate SELDI–TOF mass spectra of the pH 5 fraction. Plasma samples from three different rats (A, B, and C) were fractionated on two separate filter plates (l, 2) as described in Section 2. Representative low-molecular-mass SELDI–TOF mass spectra from three different rats and two fractionation experiments are overlaid to indicate the reproducibility of the method.

increases in the total number of unique peptide and protein signals were also obtained for human plasma and serum samples (data not shown). Since it is the goal of any proteomic approach to maximize the number of peptides and proteins under investigation, we investigated the plasma fractionation protocol further in terms of reproducibility and whether it could be successfully applied to the analysis of differential protein expression in plasma of retinol-deficient rats.

3.4. Reproducibility of the anion-exchange fractionation protocol and SELDI–TOF–MS analysis with plasma samples from different rats

Plasma samples from retinol-sufficient rats were fractionated on two separate filterplates. Replicate SELDI–TOF mass spectra of each fraction spotted on the same as well as on different WCX2 chips yielded nearly indistinguishable mass spectra in terms of ion signals and ion signal intensity (data not shown). Furthermore, the fractionation of plasma samples from different rats on separate filterplates also resulted in nearly identical mass spectra. Six representative unnormalized SELDI–TOF mass spectra of the pH 5 fraction are shown in Fig. 4. The intra-animal and inter-animal comparison clearly demonstrates the reproducibility of this protocol. Minor signal differences are detectable at *m*/*z* values < 2000, most likely caused by chemical noise that is inherently present in the lower-molecular-mass range of both conventional MALDI–TOF and SELDI–TOF mass spectra [12]. In contrast, replicate SELDI–TOF mass spectra for *m*/*z* values > 2000 detected the same number of signals with very similar intensities. Fig. 4 also shows that independent fractionations produced fractions with nearly identical peptide and protein composition. Similar results were also obtained for the other pH fractions (data not shown). The reproducibility of this method largely depends on the uniform preparation of the filter plates with the anion-exchange resin and consistent fraction collection by vacuum filtration. Since this fractionation protocol is best carried out in filter plates, it can be easily adapted to automation with robotic sample processors. Using filterplates also had the advantage that sample handling was minimized and that collected fractions could either be analyzed immediately by SELDI–TOF–MS with minimal additional sample handling or frozen directly at −80 °C for storage.

3.5. Protein expression profiling of retinol-deficient and retinol-sufficient rats

We wanted to test whether the outlined fractionation protocol could in combination with SELDI–TOF–MS detect differential protein expression in plasma samples of animals with different nutritional status. For this purpose we generated vitamin A-deficient and vitamin A-sufficient rats according to standard protocols [13]. Plasma samples of each group were fractionated in triplicate, spotted on WCX2 protein chips and analyzed by SELDI–TOF–MS. Spectra were baseline subtracted and normalized to total ion current in the mass range *m/z* 1500–30 000. Each pH fraction was then analyzed for peptide and protein expression differences with the Biomarker Wizard software tool. Three different proteins at *m/z* 10 693, 15 203 and 18 720 were detected that were found to be differentially expressed in the pH 9 fraction of plasma samples of retinol-sufficient samples and retinol-deficient rats (representative SELDI–TOF mass spectra are shown in Figs. 5–7). Two of the three signals (*m/z*, 10 693 and 18 720) were not detected in unfractionated plasma samples, probably due to ion signal suppression effects or to the insufficient binding capacity of the WCX2 protein chip arrays in the presence of high concentrations of albumin.

Current research efforts are directed toward the identification of the three differentially expressed proteins. On-chip identification can be achieved by ProteinChip MS–MS analysis of proteolytic digests of proteins [14]. This approach, however, requires that the captured protein of interest is sufficiently pure. Since multiple peptides and proteins from fraction pH 9 were captured on the surface of the WCX2 protein chip array, identification by on-chip tryptic digestion and peptide mapping is not directly possible. Therefore, successful identification will depend largely on whether these three low abundance proteins can be significantly enriched from plasma before they are spotted on protein chip arrays for on-chip analysis.

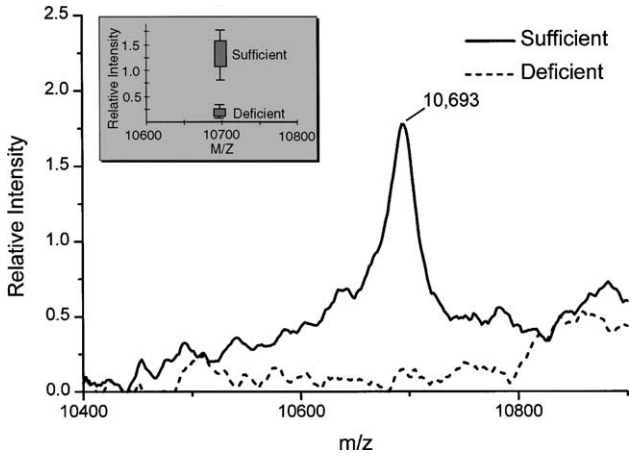

Fig. 5. Differential protein expression in plasma of vitamin A-deficient ($n = 3$) and vitamin A-sufficient ($n = 3$) rats at *m/z*, 10 693. Samples were fractionated two times in triplicates and spotted on at least two different WCX2 protein chip arrays. Two representative SELDI–TOF mass spectra of the pH 9 fraction are shown. Bold line, vitamin A-sufficent M_r profile; dotted line, vitamin A-deficient M_r profile. Insert: box and whiskers plot of the signal intensity distribution of the peak at *m/z* 10 693.

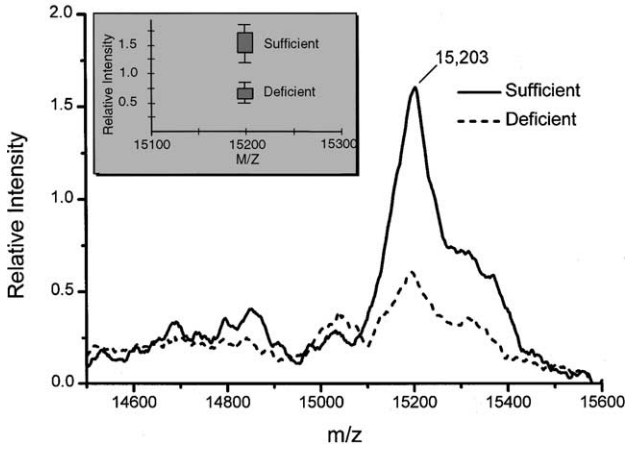

Fig. 6. Protein expression differences in plasma of vitamin A-deficient ($n = 3$) and vitamin A-sufficient ($n = 3$) rats at *m/z*, 15 203. Samples were prepared and spotted as described in Fig. 5. Two representative SELDI–TOF mass spectra of the pH 9 fraction are shown. Bold line, vitamin A-sufficient M_r profile; dotted line, vitamin A-deficient M_r profile. Insert: box and whiskers plot of the signal intensity distribution of the peak at *m/z* 15 203.

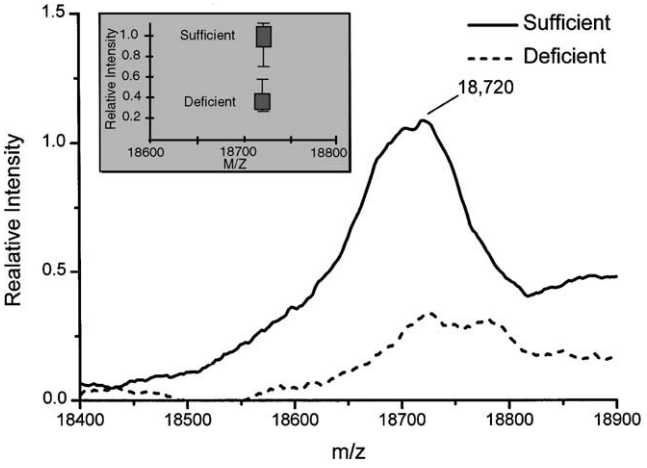

Fig. 7. Differential protein expression in plasma of vitamin A-deficient ($n = 3$) and vitamin A-sufficient ($n = 3$) rats at *m/z* 18 720. Two representative SELDI–TOF mass spectra of the pH, 9 fraction are shown. Bold line, vitamin A-sufficient M_r profile; dotted line, vitamin A-deficient M_r profile. Insert: box and whiskers plot of the signal intensity distribution of the peak at *m/z*, 18 720.

4. Conclusions

SELDI–TOF–MS analysis of crude plasma or serum samples is negatively affected by the high concentration of albumin and γ-globulins and yields only a fraction of the peptide and protein ion signals that are thought to be present. Our

studies show that anion exchange fractionation of plasma samples increases the number of unique peptide and protein peaks detectable by SELDI–TOF–MS more than three-fold when compared to unfractionated plasma samples and more than two-fold when compared to samples that were depleted of albumin by dye ligand chromatography with Cibacron Blue. Most important for differential protein expression studies by mass spectrometry, SELDI–TOF mass spectra of replicate eluted fractions were reproducible in terms of peptide and protein composition. Ion signal intensities of peptide and protein peaks were found to be very similar when constant settings for laser desorption energy, delay times, acceleration and detector voltages were used throughout the SELDI–TOF–MS analysis of the samples. The described fractionation protocol allows the simultaneous processing of large numbers of samples in less than 3 h since all fractionation and elution steps are carried out in 96-well filter and receiver plates. In addition, the entire protocol can be easily adapted to high throughput sample processing with the support of liquid handling workstations.

SELDI–TOF–MS analysis of fractionated plasma samples from retinol-sufficient and retinol-deficient rats revealed three potential biomarkers of vitamin-A deficiency, two of which were not detectable in crude plasma. While SELDI–TOF–MS analysis of unfractionated plasma or serum samples has recently been extensively used for biomarker discovery in cancer research, our results are the first demonstration that plasma fractionation and SELDI–TOF–MS analysis can be successfully applied to the field of nutrition research. This approach quickly identified those peptides and proteins in plasma whose expression changed in response to vitamin A status. Despite the disadvantage that peptides and proteins cannot be easily identified directly from the protein array chips and usually require either further enrichment or proteolytic digestion before SELDI–TOF–MS–MS analysis, this high-throughput approach can quickly identify differentially expressed peptides and proteins and therefore helps to focus time and research effort on the most promising biomarker candidates.

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