Effect of sodium dodecyl sulfate on protein separation by hollow fiber flow field-flow fractionation†

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Effects of protein denaturation and formation of protein–sodium dodecyl sulfate (SDS) complexes on protein separation and identification were investigated using hollow fiber flow field-flow fractionation (HF5) and nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS-MS). Denaturation and formation of protein–SDS complexes prior to HF5 separation resulted an increase in the retention of few protein standards due to unfolding of the protein structures and complexation, yielding ~30% increase in hydrodynamic diameter. In addition, low molecular weight proteins which could be lost from the HF membrane due to the pore size limitation showed an increase of peak recovery about 2–6 folds for cytochrome C and carbonic anhydrase. In the case of proteins composed of a number of subunits, denaturation resulted in a decrease in retention due to dissociation of protein subunits. A serum proteome sample, denatured with dithiothreitol and SDS, was fractionated by HF5, and the eluting protein fractions after tryptic digestion were analyzed for protein identification using nLC-ESI-MS-MS. The resulting pools of identified proteins were found to depend on whether the serum sample was treated with or without denaturation prior to the HF5 run due to differences in the aqueous solubility of the proteins. The enhancement of protein solubility by SDS also increased the number of identified membrane proteins (54 *vs.* 31).

1. Introduction

Flow field-flow fractionation (FlFFF) is a class of separation techniques that has been utilized for the separation and characterization of macromolecular species including nanoparticles, water soluble polymers, proteins, DNAs, subcellular species, and cells.[1–3] Separation of macromolecules by FlFFF is carried out in an unobstructed channel space with the use of two orthogonal flow streams which results in a size based elution of sample species. The channel space can be either a flat conduit with a rectangular cross-section (typical FlFFF) or a hollow fiber (HF) membrane with a circular cross-section (HF FlFFF or HF5),[4–6] and with either type, the channels do not contain packing media. In the FlFFF channel, one flow stream called the migration flow moves along the channel axis to elute the sample species, and the other flow stream (cross-flow or radial flow) exits through one wall (normally a semi-permeable membrane layered above the wall) of the rectangular channel or through the fiber wall of an HF and drives sample components toward the wall of the channel. Simultaneously, sample species diffuse against the channel wall depending on their hydrodynamic size, and they reach equilibrium positions at certain heights against the channel wall due to the balance of the two counterdirecting forces (crossflow *vs.* diffusion activity). When a migration flow is applied to sample materials at different equilibrium heights, the result is a size based elution of the sample in which smaller species elute earlier than larger species.

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FlFFF has been increasingly utilized in the field of bioanalysis since the FlFFF channel is free of packing material which often causes possible surface interactions in the chromatographic system and it operates with biocompatible buffer solutions which are conducive to maintaining the native conditions of biomaterials. In addition, with FlFFF, theoretical diffusion coefficients from the retention times of sample components can be calculated. Recently, FlFFF has been applied as a pre-fractionation device for proteomics research[3] in the analysis of bacterial cells in combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS),[7] mitochondria,[8,9] exosomes,[10] urinary proteins,[11] and membrane proteins[12] in combination with nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS-MS). HF5 has also been utilized for proteomics analysis since the separation performance of HF5 is compatible to the conventional FlFFF system while offering cost effective disposability which can reduce run-to-run carry-over problems in bioanalysis. Applications of HF5 to proteomics have been reported for the characterization of whole bacteria with MALDI-MS,[13] for online HF5-ESI-MS of intact proteins,[14] and for the *C. glutamicum* proteome[15] with nLC-ESI-MS-MS. In a recent report, HF5 was on-line coupled with capillary isoelectric focusing (CIEF) as CIEF-HF5[16] for the two-dimensional protein fractionation (isoelectric point: pI, and hydrodynamic diameter: d_s) of a urinary proteome sample followed by an off-line shotgun proteomic analysis for protein identification, a method which does not require gels. While HF5 performs well for the pre-fractionation of proteome samples, there are technical shortcomings since currently available HF membrane materials have limited pore sizes typically larger than 30 kDa and therefore, proteins smaller than the cut-off size exit through the HF wall during separations. In earlier studies[15,16] it was found that most

proteins smaller than ~30 kDa were not successfully characterized from the subsequent shotgun analysis of collected protein fractions.

In this study, the effect of protein denaturation with dithiothreitol (DTT) and SDS on the HF5 separation of proteins was investigated in relation to the efficiency of protein identification from the analysis of HF5 fractions of eluted proteins by a shotgun proteomic method. In earlier studies,[17,18] it was reported that cytochrome C formed complexes with SDS at concentrations greater than 120 μM resulting in an increase in the hydrodynamic diameter since protein unfolding was predominantly induced by interactions of the hydrophobic protein cavity with dodecyl chains. This work investigated the influence of SDS on the complex formation of proteins by comparing the elution patterns of a few standard protein samples in HF5. Comparisons were made with or without denaturation of the protein prior to HF5 analysis utilizing SDS solution as a carrier liquid. Finally, a serum proteome sample denatured with DTT and SDS was tested with HF5. The protein fractions were collected during HF5 runs for protein identification using nLC-ESI-MS-MS, and identified proteins from the serum sample with or without denaturation were compared.

2. Experimental

2.1. Materials and reagents

Protein standards utilized in this study were, cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), and apoferritin (443 kDa), purchased from Sigma (St Louis, MO, USA). Serum samples were obtained from a healthy adult volunteer (male, 30 years old). NH_4HCO_3, NaN_3, DTT and 10% SDS solution, for both sample preparation and carrier solution, were purchased from Sigma. Urea, cysteine, iodoacetamide, *N*-α-*para*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), sodium phosphate monobasic and dibasic (from Sigma) and sequential grade modified trypsin from Promega Corp. (Madison, WI, USA) were used in the tryptic digestion process. For nanoflow LC-ESI-MS-MS experiments, HPLC grade water and acetonitrile from J.T.Baker (NJ, USA) and formic acid from Sigma were used in the mobile phase. The packing material for nanoflow LC columns was 100 Å to 5 μm Magic C18AQ resin from Michrom BioResources (Auburn, CA, USA), and the same material with a 200 Å pore size was used to prepare the trapping column. The tubing and fittings for the HF5 module were purchased from Upchurch Scientific (Oak Harbor, WA, USA) and 360 μm o.d., 50–200 μm i.d. fused silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) were used for connections and for preparing capillary LC columns.

2.2. Sample preparation

Denaturation of proteins followed the general protocol of SDS-PAGE. All proteins were dissolved in 0.1 M tris buffer at pH 7.4 at a concentration of 1.0 mg mL^{-1}. SDS solution (10%) and 0.1 M DTT in tris buffer were added to the protein solution to make final concentrations of 0.5 mg mL^{-1} protein, 2% SDS, and 10 mM DTT in 0.1 M tris buffer. The solution mixture in a 1.5 mL centrifuge tube was incubated in a 100 °C water bath for 5 minutes. The serum sample was treated using a depletion spin column (from Sigma) to remove albumin and IgG prior to denaturation. 100 μL of diluted serum sample with the equilibration buffer was loaded on the spin column that had been equilibrated prior to use, and the column was incubated for 10 minutes. Then, proteins depleted of albumin and IgG were collected by centrifugation (5000*g*, 1 min). The eluate was reapplied to the spin column for 10 minutes for optimal depletion. The final eluate was injected into the HF5 with or without denaturation.

Protein fractions collected during the HF5 run were concentrated with filtration using a Millipore Amicon-4 centrifugal filter (MWCO 3000) (Bedford, MA, USA) to 100 μL and were then dried by SpeedVac from Thermo Savant (Holbrook, NY, USA). The dried samples were diluted with 100 μL of 8 M urea and 10 mM DTT in 0.1 M phosphate buffer. These solutions were incubated for 2 hours at 37 °C for denaturation and reduction of disulfide bonds. After denaturation, iodoacetamide solution was added to give a 20 mM solution, and the mixture was incubated at 4 °C for 2 hours in the dark for alkylation followed by the addition of cysteine to give a 40 mM solution to stop the alkylation. These solutions were diluted with PBS to a final concentration of 1 M urea and then digested with trypsin for 18 hours at 37 °C, followed by deactivation of trypsin with the addition of TLCK at a concentration 10 times greater than that of the added trypsin. Desalting of the peptide solution was carried out using an Oasis HLB cartridge from Waters (Milford, MA, USA) followed by drying with a SpeedVac and the addition of 0.1% formic acid for storage.

2.3. HF FlFFF (HF5)

The HF used in this study was polysulfone microbore fiber supplied by Kolon Central Research Institute (Yongin, Korea) with a molecular weight cut off (MWCO) of 30 kDa and dimensions of 450 μm i.d., 720 μm o.d., and 21 cm in length giving a fiber volume of 40 μL. The HF5 channel module was assembled in our laboratory by inserting the hollow fiber into two pieces of glass tubing with a 3.2 mm o.d. and a 1.6 mm i.d. connected with a tee. Both ends of the glass tubing were connected to the silica capillaries by unions; details of the plumbing can be found in our earlier work.[19] The remaining outlet of the tee connector was connected with Teflon tubing for the radial flow to exit the module. The carrier solution used for HF5 separation was 10 mM NH_4HCO_3 buffer solution prepared with ultrapure water (>18 MΩ) and 0.02% NaN_3 to inhibit bacterial growth. The SDS solution concentration was set at 0.02% in order to avoid forming micelles above the critical micelle concentration. All solutions were filtered with membrane filters with a 0.22 μm pore size (Millipore, Carrigtwohill, Co. Cork, Ireland). The HF5 module was connected to a Waters model 590 solvent delivery pump (Milford, MA, USA) and a Young-Lin Instrument model M720 UV detector (Seoul, Korea). A Rheodyne model 7125 loop injector with a 20 μL loop (Cotati, CA, USA) was utilized for sample injection. Samples were injected in the focusing/relaxation mode during which flow streams from both the fiber inlet and outlet were focused at a position 1/10 of the way down the fiber and all flows exited through the fiber wall as radial flow. During focusing/relaxation mode, sample was accumulated at the focusing point by seeking equilibrium status

between diffusion and radial flow force. After a period of time required for sample delivery and for relaxation, flow was set to deliver only to the fiber inlet, and separation began. The control of flow rates (outflow and radial flow) was accomplished by applying back pressure, which was induced by a 75 μm capillary tube at the end of the flow stream. Monitoring of eluted proteins was determined at 280 nm, and detector signals were recorded by AutochroWin software from Young-Lin. For serum samples, fractionated proteins were collected at a time interval of 4–8 min. Collected protein fractions were digested with trypsin for protein identification using nLC-ESI-MS-MS.

2.4. nLC-ESI-MS-MS

Peptides from the human serum sample were identified using nanoflow LC-ESI-MS-MS. The nanoflow LC system was composed of an Agilent Technologies model 1200 capillary LC system (Waldbronn, Germany) with a home-made capillary pulled tip column (17 cm × 360 μm o.d., 75 μm i.d.) and a trapping column (2 cm × 360 μm o.d., 100 μm i.d.) for desalting. The column was packed in methanol with C18AQ resin using 1000 psi of He gas, and a sol–gel frit was fit to an end of the trapping column to avoid leakage of resin. A binary gradient elution was applied for LC separation with compositions of 98/2 water/acetonitrile for mobile phase A and 95/5 acetonitrile/water for mobile phase B, with each phase containing 0.1% formic acid for protonation. The peptide mixture samples were injected onto the trapping column from an autosampler in amounts of 0.5–1 μg, including 5% of mobile phase B, for 10 min. After peptide loading, gradient elution was begun with an increase to 15% B for 1 min and then ramping up to 32% B for 70 min. For rinsing the analytical column, the gradient was increased to 80% B for 3 min, maintained at 80% B for 10 min, and then returned to 5% B for 3 min where it was kept for 20 min for column reconditioning.

The pulled tip capillary column was interfaced with a Thermo Finnigan LCQ Deca XP MAX ion trap mass spectrometer (San Jose, CA, USA) with electrospray ionization (ESI). The nLC flow rate was set at about 200 nL min^{-1}, and eluting peptides were sprayed toward the MS *via* ESI at 2 kV. MS/MS scans were carried out from selected ions in data dependent mode with 28% collision energy.

Protein identification from the raw MS/MS data was performed using TurboSEQUEST program from Thermo Finnigan and the NCBI database. The mass tolerance for both the precursor peptide ions and the fragment ions was set to 1.0 u between the measured monoisotopic mass and the calculated mass. The parameters of the filter in search results were set as follows: minimum delta-correlation (ΔCn) scores of 0.1 and cross-correlation (Xcorr) values larger than 1.5, 2.0, and 2.5 for single, double, and triple charged ions, respectively.

3. Results and discussion

Interaction of proteins with SDS was examined with a few standard proteins by denaturing the proteins by the protocol described in the experimental section followed by injecting the denatured proteins into the HF5 channel with a carrier solution of 0.02% SDS in 10 mM NH_4HCO_3. Depending on molecular size, the SDS-protein complexes gave different elution profiles from HF5. Fig. 1 shows a comparison of elution profiles of two standard proteins before and after denaturation, obtained at flow rates of inflow/outflow $\dot{V}_{in}/\dot{V}_{out}$ = 0.60/0.06 mL min^{-1}. While intact BSA (66 kDa) molecules shown at the top of Fig.1 appeared to elute at 4.7 min, the denatured BSA sample eluted at an extended retention time (based on peak maximum point) and with a broader distribution. A similar trend was observed in the case of carbonic anhydrase (CA, 29 kDa), shown at the bottom of Fig. 1. The shift to a longer retention time can be explained by two possible reasons: an increase in molecular weight of BSA after complex formation with SDS and a structural change of BSA from globular to linear chain after denaturation. The latter effect would result in an increase in retention in the FlFFF separation due to the increase of hydrodynamic volume.

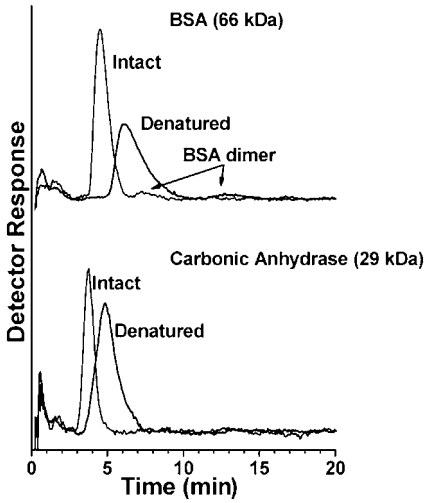

Fig. 1 Effect of protein denaturation on the elution of BSA and carbonic anhydrase by HF5 before and after denaturation. Carrier solution for HF5 was 10 mM NH_4HCO_3 with 0.02% SDS, and experimental flow rates are $\dot{V}_{in}/\dot{V}_{out}$ = 0.60/0.06 in mL min^{-1}.

While these two proteins showed distinct changes in retention in HF5, cytochrome C, shown at the top of Fig. 2, exhibited

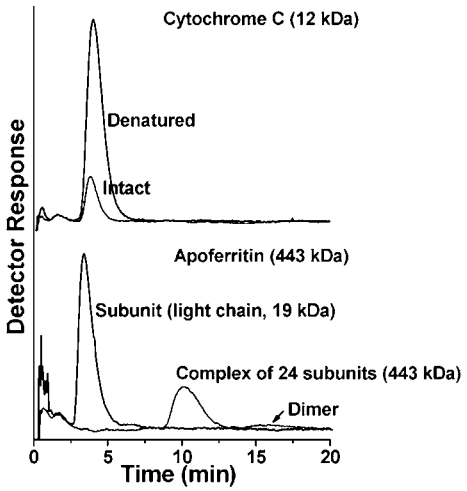

Fig. 2 HF5 fractograms of cytochrome C and apoferritin. Run conditions were the same as those used in Fig. 1.

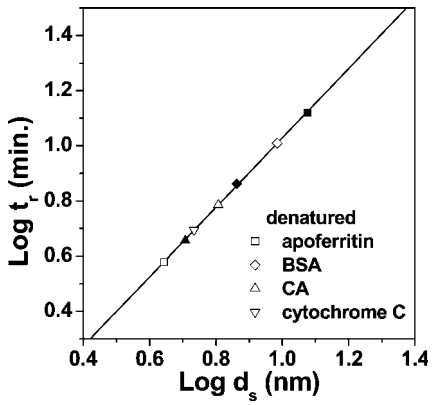

Fig. 3 Regression plot of log t_r *vs.* log d_s for the three intact proteins (marked with filled symbols: carbonic anhydrase, BSA, apoferritin). Open symbols representing the data for denatured proteins were superimposed on the calibration plot.

a negligible change in retention because the separation resolution of HF5 for a small change in hydrodynamic volume is limited; however, the recovery was greatly improved. In case of cytochrome C, a significant increase in peak height was observed due to the increase of molecular size by SDS complexation resulting in less pore penetration. Since the molecular weight cut-off of the HF used in this study was 30 kDa, proteins with MWs much smaller than 30 kDa readily penetrate the HF wall pores. Intact cytochrome C under a typical FlFFF carrier solution without SDS (*i.e.* ammonium bicarbonate solution) in the current HF5 channel did not elute at all (data not shown here) due to the loss of proteins through the HF pores. The small peak of intact cytochrome C observed in Fig. 2 supports the idea that a small portion of cytochrome C underwent formation of a complex with SDS during HF5 separation in SDS carrier solution and was eluted from the channel. These observations support the use of SDS on protein separations in HF5 to enhance the recovery of smaller MW proteins, which are abundant in most proteome samples. Denaturation of proteins with SDS may bring about the opposite result in the case of large proteins with a number of subunits because dissociation of the large protein molecules into subunits can occur. Apoferritin (443 kDa) from horse spleen, shown at the bottom of Fig. 2, eluted at ~10 min along with a dimer peak for the intact protein; however, the denatured apoferritin sample appeared as a single peak at 3.4 min, which indicates that dissociation of apoferritin (complex of 24 subunits) occurred. In addition, each subunit (equivalent to 19 kDa) eluted without being lost through the HF membrane wall pores due to the formation of SDS–subunit complexes. Elution of an intact apoferritin peak in Fig. 2 also indicated that even with the SDS containing carrier solution, intact proteins were not likely dissociated into subunits during elution unless a denaturation process was applied.

The size of the SDS–protein complex can be estimated from the relationship of retention time and hydrodynamic diameter value of the intact proteins by HF5. A calibration curve was simply established with the retention time data and the reported diameter information of the three standards[20] in Fig. 3: $\log t_r = 1.26 \times \log d_s - 0.23$ with an R^2 of 0.9992. From the calibration curve, the hydrodynamic diameters of protein–SDS complexes were calculated to be 5.42 nm for cytochrome C, 6.42 nm for CA, and 9.65 nm for BSA as listed in Table 1. These values showed that protein–SDS complexes for CA and BSA resulted in an increase of hydrodynamic diameter about 32%. The diameter for apoferritin subunits was calculated to be 4.40 nm. The hydrodynamic diameter of denatured cytochrome C calculated in this work was to some degree smaller than values reported in the literature: 6.1 nm at pH 8.0 in greater than 0.196 mM SDS solution[18] and 6.3 nm in 0.300 mM SDS solution.[17] It was also found that the hydrodynamic diameter of denatured BSA increased with an increase in urea concentration from 10.24 nm with 3 M urea to 17.30 nm with 6 M urea.[21] Compared to these values, the calculated hydrodynamic diameter of BSA denatured using DTT and 2% SDS (69.4 mM) in HF5 showed an intermediate degree of denaturation.

The effect of protein denaturation on proteomic analysis using healthy human serum was examined by HF5 followed by a shotgun proteomic analysis for the digested peptides of the collected protein fractions. Before the serum proteome sample was denatured, it was treated with a depletion column to remove albumin and IgG. This was essential to reduce the ionization suppression effect from high abundance proteins/peptides for the subsequent nanoflow LC-ESI-MS-MS analysis of digested serum peptides. Fig. 4 shows the HF5 fractograms of the depleted serum proteome sample with or without denaturation. The experimental flow rate conditions were the same as were used for Fig. 1. The denatured serum sample displayed a broader distribution of proteins while the native serum sample after depletion gave a bimodal peak. The diameter scale (d_s) represented at the top of the figure was based on the calibration curve established in Fig. 3. During the HF5 run, protein fractions were collected at three different time intervals as shown in Fig. 4. Since the serum samples with or without denaturation showed different elution patterns, fraction collection intervals were selected before and after the peak maxima. The collected fractions were digested using trypsin and the resulting peptide mixtures in each fraction were analyzed by nLC-ESI-MS-MS for protein identification.

Table 1 Experimental retention times and the calculated hydrodynamic diameter values of standard proteins for intact and denatured conditions

	Retention time/min		Hydrodynamic diameter/nm	
Name	Intact	Denatured	Intact[a]	Denatured
Cytochrome C	—	3.9	3.30	5.42
Carbonic anhydrase	3.7	4.9	5.10	6.42
BSA	4.7	6.2	7.28	9.65
Apoferritin	10.0	3.4	11.9	4.40[b]

[a] From ref. 20. [b] Diameter of subunit.

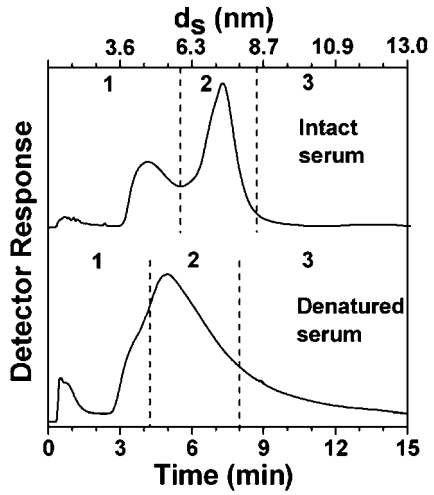

Fig. 4 HF5 fractograms of intact and denatured serum samples. Three fractions were collected for each sample for proteomic analysis using nLC-ESI-MS-MS.

A list of proteins identified from the three sets of experiments (direct digestion of serum samples without using HF5 pre-fractionation and HF5 fractions of serum samples with or without denaturation) can be found in ESI†, Table S1, and the resulting protein numbers are compared in the diagram in Fig. 5. The number of identified proteins from the denatured serum sample with HF5 was compared to those from the intact serum sample with or without fractionation by HF5. Including all three sets of experiments, a total of 729 proteins were identified from 7 nLC-ESI-MS-MS runs (one for the digested serum sample without HF5 and six runs for the three fractions from both HF5 runs). For the three protein fractions of the denatured serum samples, nLC-ESI-MS-MS analysis resulted in the identification of 340 proteins in total, while HF5 fractions of the intact serum sample yielded 291 proteins. Among these proteins, each set of experiments exhibited 217 and 266 unique proteins for the intact and denatured samples, respectively, while only 74 proteins were found to be common to both sets of experiments. This difference in the number of unique proteins identified from the two sets of experiments may originate from differences in the solubility of proteins after denaturation with SDS. Evidence for the improvement of solubility can be found from the number of identified membrane proteins. In case of membrane proteins with poor aqueous solubility, protein solubility can be enhanced by

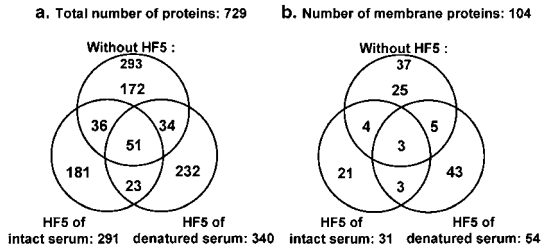

Fig. 5 Comparison of number of proteins identified from the serum sample before and after denaturation prior to size sorting with HF5 and from the direct digestion without using HF5.

denaturation and complex formation with SDS. The number of membrane proteins found was 31 for the intact serum sample and 54 for the denatured sample, with 6 common to both runs. Membrane proteins are marked with bold characters in the ESI†, Table S1.

4. Conclusions

Protein denaturation using DTT and SDS prior to size fractionation showed evidence of protein structure unfolding or dissociation of subunits, which resulted in changes in elution from flow field-flow fractionation. Denaturation of a serum proteome sample followed by size sorting with HF5 showed a significant change in the size distribution (from bimodal to unimodal distribution) of proteins in the serum sample. Subsequent proteomic analysis of collected fractions from the denatured serum proteome by nLC-ESI-MS-MS provided an identification of different protein pools, which are believed to originate from differences in protein solubility as compared to the same analysis for the intact serum sample. This study showed that the characterization of poorly soluble proteins can be facilitated by treating proteome samples with a denaturation process.

Acknowledgements

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