Characterization of the Low Molecular Weight Serum Proteome

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Introduction

Serum is attracting increasing interest in proteomics, which is currently striving to broadly characterize its protein constituents. The expectation is that the characterization of the thousands of individual serum proteins/peptides will enable the discovery of an increasing number of reliable disease biomarkers. At first glance, serum presents many beneficial attributes for proteomic investigation since it has a high protein content (i.e. 60-80 mg/mL) with many of these proteins being secreted and shed from cells and tissues. Unfortunately serum proteins are present across an extraordinary dynamic range of concentration that is likely to span more than 10 orders of magnitude, which separates albumin from the rarest proteins now measured clinically. This large dynamic range exceeds the analytical capabilities of traditional proteomic methods making the detection of lower abundance serum proteins extremely challenging.

Affinity methods (e.g., anti-human serum albumin antibody columns, protein A/G) have been developed to remove abundant proteins such as albumin and immunoglobulins from serum prior to mass spectrometric analysis. One of the fundamental oversights of serum protein depletion methodologies, however, is that many important low molecular weight (LMW) proteins or peptides can be concomitantly removed by this sample preparation process as well. An ideal fractionation/depletion method would completely remove highly abundant proteins but leave remaining those peptides and proteins bound to them.

We have developed a simple method for the removal of high molecular weight species from serum without the concomitant loss of LMW components. This method employs centrifugal ultrafiltration using solvent conditions that serve to disrupt protein-protein interactions so that LMW components that may be bound to larger species are released and are free to pass through the molecular weight cutoff membrane. The LMW serum proteome was digested with trypsin and the peptide mixture was initially fractionated by strong cation exchange (SCX) chromatography. Each of these fractions was further analyzed by microcapillary reversed-phase liquid chromatography coupled on-line with electrospray ionization tandem mass spectrometry (μLC-MS/MS).

Methods

Standard serum, obtained from the National Institute of Standards and Technology (NIST), was diluted 1:5 with 25 mM ammonium bicarbonate (NH_4HCO_3) pH 8.4, 20% acetonitrile and subjected to ultrafiltration through a 30 kDa cutoff (Centriplus 30) membrane by centrifugation at 5,000 x g. A duplicate analysis was done, however, the serum was diluted 1:5 with 25 mM NH_4HCO_3 pH 8.4, without the addition of acetonitrile. The filtrates were dried down and resuspended in 25 mM NH_4HCO_3, pH 8.4, reduced and alkylated, and digested with trypsin. The sample was fractionated using strong cation exchange chromatography (SCX). Five minute fractions were pooled and analyzed by reversed-phase mLC-ESI-MS/MS using a quadrupole ion-trap mass spectrometer operating in a data-dependent MS/MS mode. The MS/MS spectra were searched using the SEQUEST algorithm against the non-redundant human genomic database.

Results

Analysis of the NIST serum by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis prior to and after ultrafiltration using a 30 kDa cutoff membrane showed a significant depletion of high molecular weight components. The LMW serum component was separated by SCX into several fractions that were then analyzed by reversed-phase μLC-ESI-MS/MS. While the LMW fraction was digested with trypsin, serum is also known to contain several proteases that result in peptides comprised on non-tryptic termini. Therefore, the resultant MS/MS spectra were analyzed by SEQUEST using both tryptic and non-tryptic constraints. Complete analysis of the resulting MS/MS spectra resulted

in the confident identification more than 300 human serum proteins. No peptides originating from human serum albumin were identified in any of the fractions analyzed. The large number of proteins identified demonstrates the efficacy of this HMW depletion method combined with multi-dimensional fractionation and ESI-MS/MS analysis for the characterization of the LMW fraction of serum. This method provides a rapid and robust method to enrich for those components that have been shown to be discriminating factors in the diagnosis of such diseases as ovarian cancer in the analysis of proteomic patterns.

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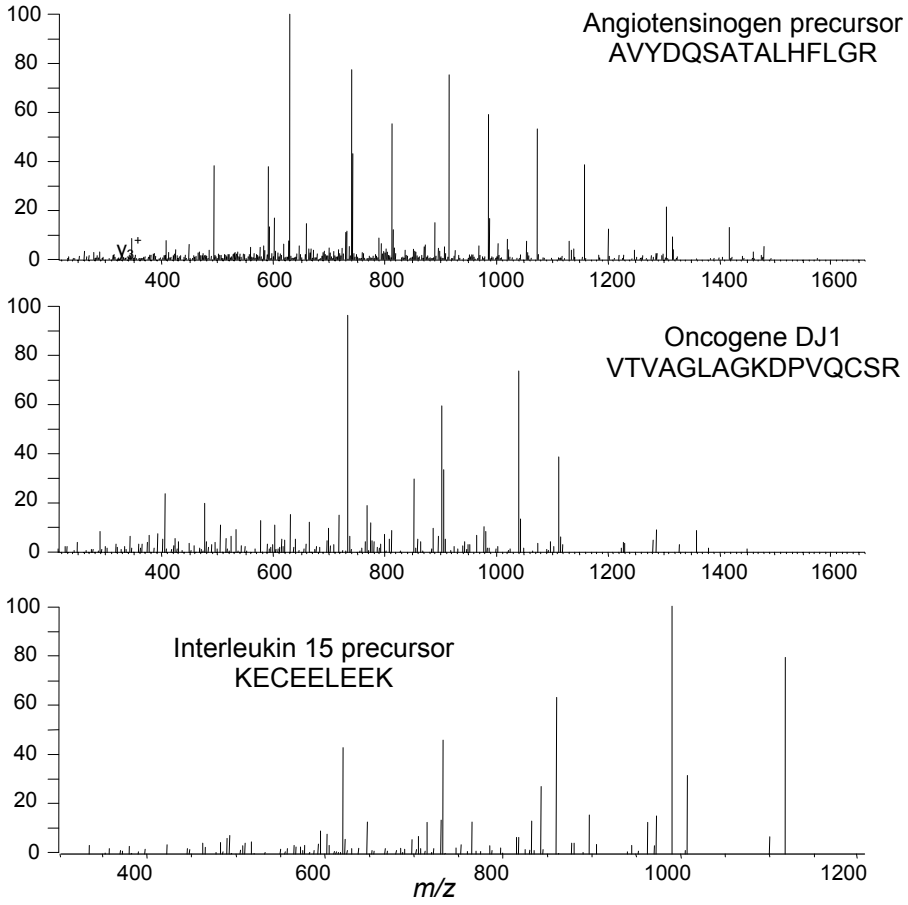

Figure 1. Tandem mass spectrometry spectra of selected peptides identified in the proteome analysis of the low molecular weight serum proteome.

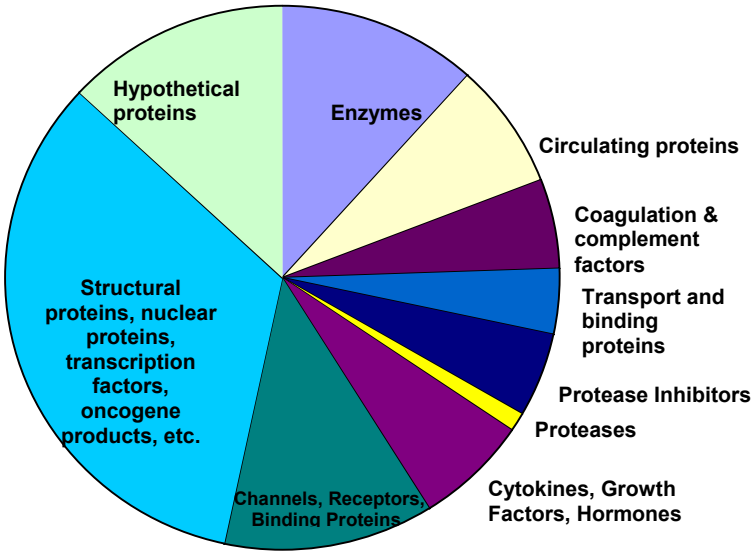

Figure 2. Pie chart representing the relative numbers of proteins identified within the low molecular weight serum proteome.