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An integrated method for the detection of basic proteins in serum-derived proteomes

Shang-Jun Yin[a], Hee Seung Yang[b], Ick-Hyun Cho[b], Qian Wang[a], Yong-Doo Park[a,c,*], Jun-Mo Yang[b,**]

[a] College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo 315100, PR China
[b] Department of Dermatology, Sungkyunkwan University School of Medicine, Samsung Medical Center, Seoul 135-710, Republic of Korea
[c] Zhejiang Provincial Key Laboratory of Applied Enzymology, Yangtze Delta Region Institute of Tsinghua University, 705 Yatai Road, Jiaxing 314006, PR China

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ABSTRACT

Because serum is an optimal source to identify proteins derived from diseased-tissue compartments, serum proteomics has been applied for the discovery and analysis of biomarkers related to human disease. The general 2D-PAGE method is suitable for acidic and neutral proteome separation while highly basic proteins remain unresolved. In this study, we optimized basic proteome fractionation by integrating several methods such as, Micro-Rotofor isoelectric focusing (IEF) fractionation, acetic acid-urea polyacrylamide gel electrophoresis (A-U PAGE) separation, and mass spectrometry analysis for identifying basic proteins in patient sera. The sera samples were obtained from patients with atopic dermatitis (AD) to establish the model method introduced in the present study. We successfully identified the specific presence of a C3 complement component fragment protein and C4A fragment protein in AD patient-derived sera. Highly basic proteins in serum are very difficult to detect due to their separation properties. Thus, the integrated proteomic method approaches described here could be applicable for the detection of basic proteins associated with other diseases.

1. Introduction

The proteome isolated from serum can summarize and reflect factors that are associated with various diseases and different physiological pathways generated by disease-specific proteolytic fragmentation of larger proteins. Therefore, many clinicians and researchers have conducted serum proteomics to identify disease-specific factors for clinical purposes and drug development. In this regard, technical advances and developments in serum proteomics are direct indicatives of new findings in human disease-related factors.

It is generally recognized that serum is the most difficult sample to analyze due to the large proportion of major abundant proteins such as albumin, IgG, transferrin, IgA, α-1-antitrypsin, and haptoglobin, and the removal of these major proteins has been attempted by pre-treatment immunoaffinity column depletion (i.e. MARC) [1–3]. Thus, the general procedure for serum proteomics is usually conducted after abundant protein depletion and followed by general 2D-PAGE. However, this approach is suitable for acidic and neutral proteome separation while highly basic proteins remain unresolved and are therefore not able to be effectively detected using general serum proteomic methods. The classical well-known approach for detecting basic proteins is a continuous acetic acid-urea (A-U) gel system for the separation of highly basic proteins, which was developed based on protein differences in net charge and size variation [4]. Basic proteins generally have a high isoelectric point (pI) with a highly positive charge and these proteins can be discriminated under acidic conditions with specific gel mobility methods during electrophoresis.

AD is a chronic inflammatory skin disease associated with eczema distribution, pruritus, and dry skin in children and in adults, which is generally recognized as a combination of

Abbreviations: AD, atopic dermatitis; ADe, extrinsic type of AD; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; IPG, immobilized pH gradient; MARC, multiple affinity removal column; IEF, isoelectric focusing.

* Corresponding author at: College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo 315100, PR China.
** Corresponding author.
E-mail addresses: parkyd@hotmail.com (Y.-D. Park), junmo.yang@samsung.com (J.-M. Yang).

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environmental and genetic factors as the principal inducers with multiple immunologic and inflammatory responses. The proteomic detection of basic proteins in patient-derived blood samples is expected to reveal the complexity of AD disease and provide an opportunity to find novel clinical biomarkers. In the case of AD, the small size of biopsy tissue samples can be amplified with primary culture methods to fit the requirements of proteome sample amounts [5–7] and the small volume of individual patient-derived sera can be pooled to assure the requested volume. In this study, we detected basic proteins in sera from patients with atopic dermatitis (AD) by integrating methods. The sequential integration MARC pretreatment, Micro-Rotofor isoelectric focusing (IEF) fractionation, A-U PAGE, and LC-ESI-MSMS profiling were used.

We found that C3 complement component fragment protein and C4A protein were specifically detected in AD patient-derived sera. The integrated method used in this study can be applicable for the proteomic studies of other human diseases.

2. Materials and methods

2.1. Materials

IPG strips of pH 3–10 and pH 7–11 (0.5 × 3 × 180 mm), dithiothreitol, urea, CHAPS, Ready Sol IEF 40% solution™ and IPG buffers were purchased from GE Healthcare (Uppsala, Sweden). Sodium dodecyl sulfate, acrylamide, methylene-bisacrylamide, TEMED, Tris, glycine, glycerol, and formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade trypsin was purchased from Roche (Mannheim, Germany).

2.2. Subjects

AD sera were collected from non-asthmatic atopic patients according to our previous report [8]. Non-atopic control samples were selected from subjects who had personal/family history or any sign of atopic diseases. The age of both control and AD patients ranged between 19 and 32 years. Informed consent was obtained from all patients and control individuals. The Samsung Biomedical Research Institute Medical Ethics Committee approved this study.

2.3. Cell culture

The primary cultured fibroblasts stored in liquid nitrogen were obtained during previous studies [6,7] and the cells were pelleted after a proper thawing procedure and resuspended in DMEM containing 10% FBS and 1% Antibiotic-Antimycotic (Gibco, Rockville, MD, USA). The cells were grown in a 37 °C incubator under 5% CO_2.

2.4. Depletion of major abundance proteins in serum with immunoaffinity column

Depletion of the six abundant proteins in serum (albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin) was carried out using a multiple affinity removal column such as MARC (Agilent, USA) within chromatographic separation using a mobile phase reagent kit according to the standard LC protocol provided by the manufacturer. Briefly, crude human serum samples were diluted 5-fold with Buffer A containing protease inhibitors (COMPLETE™, Roche), which were then filtered through 0.22-μm spin filters by spinning at 16,000*g* at room temperature for 1 min. The sample was injected, and flow-through fractions were collected and stored at −80 °C until use. To resolve depleted serum proteins on 2D-PAGE, flow-through fractions from MARC were pooled and precipitated according to the TCA precipitation method.

2.5. Sample preparation and 2D-PAGE

Serum proteins were quantified using the Bradford protein assay. IEF was carried out using the commercially available Multiphor II (GE Healthcare) apparatus. IPG strips were used according to the manufacturer's instructions. Samples containing appropriate amounts of protein were diluted to 350 μl with rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 2% (v/v) IPG buffer, trace bromophenol blue) and applied to strips (pH 3–10 and pH 7–11; non-linear) by overnight rehydration in a tray. The rehydrated IPG strips underwent sequential IEF at a total of 80 kVh. The platform temperature was maintained at 18 °C and 0.05 mA current was passed per strip. IPG strips were equilibrated to reduce the disulfide linkages in the IPG strip equilibration tubes (SPL Lifesciences Inc., Korea) containing 10 ml equilibrating solution (6 M urea, 2% SDS, 0.375 M Tris, pH 8.8, 20% glycerol, 2.5% acrylamide, 0.5% isopropanol, and 2.6% tributyl phosphine) with gentle rocking for 30 min. The 2-DE was performed using 8–16% second-dimension gels (200 × 250 × 1.0 mm) in an Iso-DALT DALT apparatus (Hoefer Scientific Instruments, USA) until the tracking dye reached the anode end of the gel.

2.6. Liquid-phase IEF using Micro-Rotofor

Serum samples were diluted to a final concentration of 4% (v/v) in fractionation buffer (3.5 M urea, 1 M thiourea, and 2% CHAPS) and then fractionated in the Micro-Rotofor device (BioRad, USA) equipped with a focusing chamber containing 10 compartments. The anolyte and catholyte were 0.1 M phosphoric acid and 0.1 M sodium hydroxide, respectively. After 3.5 h of focusing with 10 mA, all 10 fractions were collected at once. The concentration of each fraction was measured using the Bradford assay method.

2.7. Protein visualization and image analysis

Gels were stained with commercially available colloidal Coomassie solution. Gels were washed with deionized water and stored in sealed plastic bags at 4 °C. Protein patterns in the gels were digitalized with a high-resolution scanner (GS-700Calibrated Imaging Densitometer, Bio-Rad). Scanned images were analyzed using Melanie III software (GeneBio, Switzerland).

2.8. In-gel digestion, MALDI-TOF analysis and database search

Coomassie stained spots were excised and destained with a solution containing 25 mM ammonium bicarbonate and 50% acetonitrile. The destained gels were dehydrated and rehydrated in 25 mM ammonium bicarbonate with 25 ng/μl sequencing-grade trypsin (Promega, Madison, WI, USA) on ice for 10 min. After excess trypsin was replaced with 25 mM ammonium bicarbonate, digestion was allowed to proceed overnight at 37 °C. Peptides were extracted by sonication after adding extraction buffer (50% acetonitrile and 25 mM ammonium bicarbonate) at the same volume as the gel digestion volume. Peptides were then eluted directly onto a stainless steel target with 1 μl 50% acetonitrile, 0.1% trifluoroacetic acid, and 5 mg/ml α-cyano-4-hydroxycinnamic acid. Protein spots of interest were subjected to MALDI-TOF-MS. Mass analysis was performed on a PerSeptive Biosystem Voyager-DE STR™ MALDI-TOF-MS (Applied Biosystems) in the reflector mode for positive ion detection. The spectrometer was run with the following settings: accelerating voltage, 20 kV; grid voltage, 65%; and DELAY, 100 NS.

Tryptic peptide extracts (0.5 μl) were dispensed on a MALDI sample plate along with 0.5 μl matrix solution consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid and 50% acetonitrile. External peptide calibrants, angiotensin I (monoisotopic mass, 1296.6853), rennin substrate (1758.9331)