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Protein profile of exhaled breath condensate determined by high resolution mass spectrometry

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ABSTRACT

A method based on liquid chromatography/high resolution tandem mass spectrometry coupled with electrophoretic separation, for determination and relative quantification of the protein composition of exhaled breath condensate (EBC), was developed. Application of the procedure to a sample of EBC, pooled from nine healthy subjects, resulted in the identification of 167 unique gene products, 113 of which not previously reported in EBC samples. The abundance of the protein identified was estimated by means of the exponentially modified protein abundance index protocol (emPAI). Cytokeratins were by far the most abundant proteins in EBC samples. Many of the identified proteins were associated with multiple cellular location with cytoplasm constituting the largest group. Cytosol, nucleus, membrane, cytoskeleton and extracellular were other abundantly represented locations. No amylase was detected, suggesting the absence of saliva protein contamination. The profile obtained represents the most comprehensive protein characterization of EBC so far reported and demonstrates that this approach provides a powerful tool for investigating the protein profile of EBC samples. Compared with analogous investigations, this study also shows that the protein profile of EBC is strongly affected by the sampling method adopted.

1. Introduction

Conventional methods of collecting secretions from the lower respiratory tract include sputum collection, sputum induction, and bronchoscopy with bronchoalveolar lavage (BAL). Bronchoscopy with BAL is currently the method of choice for investigation of the lower respiratory tract lining fluid (RTLF). However, the invasive nature of this method, which prevents its application as a screening method or for repeated sampling, has led to search for different approaches. Exhaled breath condensate (EBC), obtained by freezing exhaled air under conditions of spontaneous breathing, is a biological fluid consisting of water vapor and aerosol droplets containing volatile molecules (such as nitric oxide, carbon monoxide, and hydrocarbons), non-volatile salts, phospholipids (approximately 90%), oxidants, nucleotides, proteins (about 10%), and other components of the RTLF [1,2]. The presence of these molecules can be influenced by oxidative stress or inflammation of the airways, caused by various air pollutants, allergens, pathogens, and microbes [3,4].

EBC composition reflects the physiological state of the lung and consequently, in principle, it can be used to identify and monitor several pathologies, including asthma, chronic obstructive pulmonary disease, bronchiectasis, cystic fibrosis, acute respiratory distress syndrome [5–14], infectious [15], and neoplastic lung diseases [16].

Mass spectrometry has been applied both to the analysis of exhaled volatile substances and non-volatile ones, exhaled in the form of aerosol [5,17–19]. Also, NMR spectroscopy has been applied to EBC analysis [20,21] and molecular breath prints have been obtained with electronic noses [22]. However, to our knowledge, all the studies performed so far report the presence of few proteins in addition of keratins.

The aim of our work was to set up a high resolution mass spectrometry based method for obtaining a comprehensive identification of the proteins contained EBC. The accurate mass determination obtained by LTQ-Orbitrap allows the use of substantially lower tolerance thresholds during a database search, which greatly improves the selectivity of protein identification. The result of this study shows that this feature, combined with the high sensitivity of

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LTQ-Orbitrap analysis, substantially increases the number of proteins that can be unequivocally identified in EBC. An estimation of the amount of the identified gene products, based on the exponentially modified protein abundance index (emPAI) was also obtained.

2. Experimental

2.1. Reagents and materials

Formic acid (FA), trifluoroacetic acid (TFA), ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide were obtained from Sigma–Aldrich (Milan, Italy). Modified porcine trypsin was purchased from Promega (Madison, WI, USA). HPLC-grade H_2O and acetonitrile (CH_3CN) were provided by Carlo Erba (Milan, Italy). SDS-PAGE precast gels (8.6 cm × 6.7 cm × 0.1 cm), and Mini-PROTEAN electrophoresis cell for one-dimensional electrophoresis were purchased from Bio-Rad (Milan, Italy).

2.2. Sample collection

EBC samples were collected with a Turbo DECCS 09 (MEDIVAC, Parma, Italia) from 9 healthy non-smoking volunteers (3 male, 6 females, aged 25–27 years). The collection of EBC was performed by breathing for about 10–15 min through a mouthpiece connected to a condenser. Each subject participated in two sampling sessions. Pooled samples from healthy individuals were collected for a 65 mL total volume of exhaled air. A nose clip allowed to exclude pharyngeal tract from the sampling. Once collected, the sample was immediately frozen at −70 C. It was recommended to the participants to abstain from food and drink for at least 2–3 h before the collection. Total sample was lyophilized and reconstituted in SDS-PAGE.

2.3. Total protein content

The total protein content of EBC was measured by a Qubit™ fluorometer (Invitrogen Molecular Probes, USA) using the kit Qubit-it™ Protein Assay Kit 100 Assays 0.25–5 μg.

2.4. 1D-SDS-PAGE analysis

To reduce sample complexity, the pooled EBC samples were separated by one-dimensional SDS-PAGE using a Mini-PROTEAN electrophoresis cell (Bio-Rad, Milano, Italia). 50 μg of total protein content was reconstituted in 70 μL of Tris–HCl (pH 6.8; 50 mM); 2% SDS; 10% glycerol; 0.49 ml DTT; 0.02% bromophenol blue. SDS-PAGE Molecular Weight Standards (Bio-Rad) were loaded onto the first SDS-PAGE well of a 12% precast polyacrylamide gel according to instructions supplied by the manufacturer (Bio-Rad). The protein sample was divided into two aliquots of 35 μL and loaded onto two wells of the precast gel. The running buffer consisted of Tris–Base (pH 8.5; 2.5 mM); 19.2 mM glycine and 0.01% SDS. At the end of the electrophoretic run, the gel was divided into two portions. The portion of the gel containing a well loaded with the EBC sample was directly in-gel digested, and the second portion, containing both the EBC sample and the protein molecular weight markers, was stained by Coomassie Blue, in order to visualize the efficiency of protein separation.

2.5. In-gel trypsin digestion of 1D-SDS-PAGE slices, liquid chromatography and tandem mass spectrometry (LC–MS/MS)

After SDS-PAGE separation of proteins, the gel-lane was manually cut in 5 slices of about 1.4 cm each along the migration path. Excised bands were cut in small pieces, transferred to 1.5 mL microcentrifuge tubes and directly reduced by incubation in 50 μL of ammonium bicarbonate (pH 8.3; 0.1 M); 10 mM DTT for 30 min at 56 °C. Subsequently, alkylation was performed by addition of 50 μL of 55 mM iodoacetamide in ammonium bicarbonate (pH 8.3; 0.1 M) and the reaction was carried out for 30 min in the dark at room temperature. Gel pieces were shrunk in CH_3CN and dried under vacuum. Finally, alkylated proteins were subjected to in-gel digestion using modified porcine trypsin. 15 μL of 10 ng/μL trypsin in ammonium bicarbonate (pH 8.3; 50 mM) was added to the dry gel pieces. After soaking trypsin into the gel pieces, the supernatant containing an excess of enzyme was discarded and the gel pieces were covered with 50 μL of ammonium bicarbonate (pH 8.3; 50 mM). Digestion was allowed to proceed overnight at 37 °C. After in-gel digestion, the digested solution was transferred into a clean 0.5 mL tube. The peptides were extracted from gel pieces with 0.1% aqueous TFA and subsequently with CH_3CN. This extraction procedure was repeated three times. The total extracts were pooled with the first supernatant and lyophilized.

Mass spectrometry data were acquired on a Thermo Fisher Scientific Orbitrap-Elite (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer. Liquid chromatography was carried out using a Dionex UltiMate 3000 system (Sunnyvale, CA) with an Acclaim® Nano Trap C18 Column (100 μm × 2 cm, 5 μm, 100 Å) and a PepMap100 C18 column (75 μm × 50 cm, 3 μm particle size, 100 Å) at a flow rate of 300 nL/m at 40 °C. Samples were reconstituted in 30 μL of 1% FA aqueous solution and 10 μL were loaded onto the trapping column. After washing the trapping column with solvent A (H_2O + 0.1% FA) for 5 min, the peptides were eluted from the trapping column onto the C18 column with a gradient of solvent B (H_2O—CH_3CN + 0.1% FA, 20:80, v/v) in A from 3% to 40% over 35 min, total run time was 65 min. Eluted peptides were ionized by a nanospray (Nanospray Flex Ion Source), achieved using a distally coated fused silica emitter (New Objective, Cambridge, MA) (o.d., 360 μm; i.d. 20 μm, tip i.d. 10 μm). Full MS scans in the m/z range 400–2000 were acquired with MS resolution of 120,000 (at m/z 400) in the Orbitrap and MS/MS scans for the 20 most intense precursor ions in each MS spectrum were selected for collision induced dissociation (CID) MS/MS in the LTQ ion trap at a normalized collision energy of 35% at 15,000 resolution. Other parameters for LTQ-Orbitrap-Elite were as follows: capillary temp. = 250 °C; source voltage = 2.1 kV; isolation width = 2. Charge state screening and rejection were enabled so that charge states of the precursor ion +2, +3 were accepted. Injection waveforms were enabled. Dynamic exclusion was also enabled for the maximum list size of 500 for a duration of 30 s.

2.6. Protein identification

LC–MS/MS data were processed using Proteome Discoverer v1.4 (Thermo Scientific). Data were searched against the human UniProt database (UniProt/SwissProt release May 2013, containing 540,052 entries) using MASCOT algorithm. The search was performed against *Homo sapiens* sequences database (241,977 sequences). Full tryptic peptides with a maximum of 2 missed cleavage sites were subjected to bioinformatic search. Cysteine carboxyamidomethylation was set as fixed modification, whereas oxidation of methionine, and transformation of N-terminal glutamine and N-terminal glutamic acid residue in the pyroglutamic acid form were included as variable modifications. The precursor mass tolerance threshold was 5 ppm and the max. fragment mass error was set to 0.6 Da. Percolator node was used for peptide validation and 1% false discovery rate cut off was used to filter the data.

A protein was considered identified with: minimum of 2 peptides with a MASCOT score >20 and expect <0.05; sequence coverage >5%. Doubtful and low MASCOT scoring MS/MS spectra were also subjected to manual interpretation assisted by the PepNovo