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A novel approach for the chromatographic purification and peptide mass fingerprinting of urinary free light chains

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ABSTRACT

We describe a chromatographic approach for the purification of urinary free light chains (FLCs) *viz.*, lambda free light chains (λ-FLCs) and kappa free light chains (κ-FLCs). Isolated urinary FLCs were analyzed by SDS-PAGE, immunoblotting and mass spectrometry (MS). The relative molecular masses of λ-FLC and κ-FLC are 22,933.397 and 23,544.336 Da respectively. Moreover, dimer forms of each FLC were also detected in mass spectrum which corresponds to 45,737.747 and 47,348.028 Da respectively for λ-FLCs and κ-FLCs. Peptide mass fingerprint analysis of the purified λ-FLCs and κ-FLCs has yielded peptides that partially match with known light chain sequences *viz.*, gi|218783338 and gi|48475432 respectively. The tryptic digestion profile of isolated FLCs infers the exclusive nature of them and they may be additive molecules in the dictionary of urinary proteins. This is the first report of characterization and validation of FLCs from large volume samples by peptide sequencing. This simple and cost-effective approach to purification of FLCs, together with the easy availability of urine samples make the large-scale production of FLCs possible, allowing exploration of various bioclinical as well as biodiagnostic applications.

1. Introduction

The basic immunoglobulin subunit consists of two heavy and two light chains held together by covalent disulfide bonds. Plasma cells normally produce more light chains than heavy chains of which kappa light chains are predominant compared with lambda light chains. These excess light chains are not attached with heavy chains and rather they circulate in the blood as free light chains [1]. In most cases of myeloma and Waldenstrom's disease there is an excess synthesis of light chains over heavy chains, resulting in Bence Jones (BJ) proteinemia and proteinuria. Furthermore, in some cases (light chain disease), there is absence of detectable heavy chain synthesis, and the only abnormal proteins detected are BJ light chains [2].

BJ proteins are homogeneous populations of kappa and lambda free light chain molecules that are produced by malignant clones of B cells. The light chains of BJ proteins may be present as monomers, dimers, and larger polymers [3]. Monomers and non-covalently linked dimers have usually been found among kappa-BJ proteins and disulfide-linked dimers among lambda-BJ proteins [4,5]. The plasma cells secrete about 1 g per day of kappa and lambda immunoglobulin free light chains into the extracellular fluids. These free light chains are cleared from the blood by glomerular filtration with a half-life of 2 to 6 h [6]. Measurement of kappa and lambda free immunoglobulin light chains in serum has been shown to be valuable key index in the diagnosis and management of a variety of diseases, especially plasma cell disorders such as multiple myeloma, Waldenström's macroglobulinemia, Amyloid light-chain (AL) amyloidosis, monoclonal gammopathies, and light chain deposition diseases [7]. BJ proteins are used as cancer markers for identifying and monitoring patients with B-cell lineage tumors (*e.g.*, multiple myeloma) [8].

Most of the available knowledge on the characteristics of free light chain has been derived from BJ proteins or similar proteins extracted from amyloid deposits [9]. However, the properties of free light chain from these sources may not correspond to those of the circulating ones, which are less well characterized due to the complexity of their isolation from blood. For more than 150 years, the presence of BJ protein in the urine has been the vital indicator of monoclonal free light chain production [10]. A neoplastic clone of plasma cells must secrete up to 20 g of free light chain per day to saturate free light chain absorption in the proximal renal tubules of healthy kidneys and thus become detectable in urine [11].

Recently, Lavatelli et al. reported a method to purify free light chains from serum by using immunopurification approach. The major obstacles in this work are related to the usually low concen-

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tration of light chains as compared to intact antibodies and to the shared epitopes between bound and free light chains. This prevents the purification of the unbound fraction with most high-affinity commercial antibodies [9]. This process needs the polyclonal antibodies (anti-human free kappa and lambda light chains), which could be expensive and may not be achievable for purification of free light chains from urine due to difficulty of loading large volume of urine, cost of antibodies and unsatisfactory yield of light chains. In addition, immunoglobulin FLCs abnormalities are common in patients with aforementioned disorders and the kidneys are the most affected organs [12]. The number of new cases of single myeloma disease was 6.5 per 100,000 individuals per year whereas the number of deaths was 3.3 per 100,000 individuals per year. Around 0.8 percent of men and women were diagnosed with myeloma [13]. Thus, the rate of diagnosis is negligible as compared with risk of new cancer cases per year because only few FDA (Food and Drug Administration)-approved immunoassays are available which detects abnormalities of FLCs. This immunoassay consists of 2 separate measurements: one quantifies λ FLCs and to the other quantifies κ FLCs. In addition to measuring λ and κ FLCs, the assay determines the FLC κ:λ ratio (*e.g.*, Freelite Human Lambda/Kappa Free ELISA Kit, The Binding Site Ltd., UK). As a result, λ and κ FLCs based-immunoassay is an important diagnostic tool of various renal states and or kidney related disorders. Thus, globally we require large scale production of 'high pure λ as well as κ FLCs' for the development/improvement of newer version of existing immunoassays, calibrators, as well as for the use in clinical trials as a reference form of FLCs. Briefly, additional well-designed large-scale prospective studies are needed to further delineate the diagnostic utility of the FLCs κ:λ ratio [12]. Keeping all these in mind, we aimed to develop an innovative and cost effective method for the large scale purification of free light chain molecules from urine samples of renal failure patients. Furthermore, we describe a novel technology platform to isolate and characterize the urinary free light chains. Our objective for this study was to use an advanced generic downstream purification process of free light chains from easily available bio-medical waste *i.e.* urine and characterize it by using peptide mass finger printing.

2. Materials and methods

2.1. Materials and reagents

All reagents employed in this study were of the highest grade of purity available with various vendors. Capto L gel, Lambda Fab Select gel and Sephacryl S-100 high resolution gel filtration medium were purchased from the GE Healthcare (Uppsala, Sweden). UNOsphere-Q anion exchanger gel was purchased from Bio-Rad Laboratories (California, USA). Sequencing or proteomics grade trypsin singles and Cellulose membrane tubing for dialysis (avg. flat width 76 mm) were purchased from Sigma-Aldrich (St. Louis, USA). Vivaspin 20 Protein Concentrators (MWCO 3000) was purchased from the GE Healthcare (Little Chalfont, United Kingdom). All other chemicals were of the highest purity and analytical HPLC grade were purchased from SRL Chemicals (Mumbai, India); Qualigen Fine Chemicals (Mumbai, India); Merck Chemicals, India; and Bangalore Genie, India.

Human urine samples of 25 randomly selected patients with advanced renal disease were collected early in the morning with the kind permission of Maharashtra Pollution Control Board, Mumbai, India and Ministry of Health and Family Welfare, (Government of India), New Delhi, India. Furthermore, all human samples were studied after approval by review board and ethics committee of Yashraj Biotechnology, Ltd., Mumbai, India. All urine samples were labeled and stored at 4 °C in a cold room until further use.

2.2. Quantitative procedure

Total lambda and kappa free light chain content of each urine sample and fractions generated during purification process were measured according to microplate enzyme immunoassay using human immunoglobulin free light chains κ and λ ELISA system (BioVendor-Laboratorni Medicína a.s., Czech Republic). In addition, total protein content was estimated by BCA Protein Assay (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as the standard protein.

2.3. Buffers employed

Salt precipitated dialysis and UNO-Q equilibrium buffer (buffer-A) was 10 mM tris acetate, pH 4.5 ± 0.2. Immunoaffinity column equilibrium buffer (buffer-B) was 20 mM phosphate buffer, 145 mM sodium chloride, 5 mM potassium chloride, pH 7.2 ± 0.2. Lambda free light chain immunoaffinity column (*i.e.* Lambda Fab gel) elution buffer (buffer-C) was 100 mM sodium acetate buffer, pH 4.0 ± 0.5. Kappa free light chain immunoaffinity column (*i.e.* Capto-L gel) elution buffer (buffer-D) was 100 mM sodium citrate buffer, pH 4.0 ± 0.5. AKTA buffer (buffer-E) was 50 mM phosphate buffer, 150 mM sodium chloride, pH 7.4 ± 0.2. Gel transfer buffer (buffer-F) was 3.02 g/l Tris-buffer, 14.4 g/l glycine and 20% methanol. Western blotting washing and blocking buffer (buffer-G) was 20 mM tris-buffer, 150 mM sodium chloride, 0.5 ml/l Tween 20, pH 7.4 ± 0.2.

2.4. Chromatographic purification of urinary free light chains

2.4.1. Initial sample

The collected urine was centrifuged at 10,000*g* for 30 min at 4 °C in a Kubota-7780 centrifuge (Kubota Corporation, Japan). The protein content in the supernatant was determined and then adjusted with Milli-Q water before precipitation with ammonium sulfate at 70% saturation. The salt-enriched solution was slowly stirred at 4 °C for 1 h. The precipitate was collected by centrifugation as above, and dissolved using buffer-A. The solution was dialyzed using cellulose membrane tubing with at least 5 changes with buffer-A. The salt free dialysate is referred as the "ASPF" throughout the manuscript and was used for further purification of free light chains.

The ASPF was subjected to negative chromatography using UNOsphere-Q (2.5 × 5 cm equilibrated with buffer-A) column at 20 °C at a flow rate of 8 ml min^{-1}. The flow through of this column along with the wash was pooled. Then the pooled fraction was dialyzed using cellulose membrane tubing with at least 5 changes with buffer-B. The resultant dialysate is referred as "crude light chain fraction", which was used for further processing.

2.4.2. Immunoaffinity column chromatography

2.4.2.1. Lambda free light chains. The crude light chain fraction was applied onto immunoaffinity column of Lambda Fab select gel (2.5 × 5 cm) pre-equilibrated with buffer-B and washed with 200 ml of buffer-B. After that, lambda free light chains were eluted with buffer-C. Fractions containing light chains were recovered at a flow rate of 3 ml min^{-1}.

2.4.2.2. Kappa free light chains. The flow through and washed material of above Lambda Fab gel column was applied onto another immunoaffinity column of Capto-L gel (2.5 × 5 cm) pre-equilibrated with buffer-B and washed with 200 ml of buffer-B. Subsequently kappa free light chains were eluted with buffer-D. Fractions containing light chains were recovered at a flow rate of 3 ml min^{-1}.

2.4.3. Gel filtration: isolation and purification of free light chains

The pH of individual pooled fractions (separately eluted from Lambda Fab gel and Capto-L gel's column) were adjusted to 7.5 ± 0.2