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Aggregation analysis of Con A binding proteins of human seminal plasma: A dynamic light scattering study

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ABSTRACT

Concanavalin A (Con A) binding fraction of human seminal plasma is vital as it shows decapacitating activity and contains proteins which have critical roles in fertility related processes. Con A binding proteins were isolated by lectin affinity chromatography. These proteins form high molecular weight aggregates at near physiological pH (7.0) as inferred by gel filtration. Aggregation analysis was performed by dynamic light scattering (DLS). DLS analysis was also performed at different pH values and in presence of various additives including NaCl, EDTA, cholesterol and sugars, such as D-glucose, D-fructose and D-mannose to identify their effect on aggregation size. The results indicate that degree of aggregation was highly reduced in presence of D-fructose, EDTA and at lower and higher pH values as depicted by lowering of hydrodynamic radii. This aggregation behaviour might be decisive for fertility related events with a suggestive role towards inhibition of premature capacitation.

1. Introduction

Protein–protein interactions are critical for complete understanding of a biological process and play important roles in modulation of related processes [1]. Sometimes, mutual interactions among a large array of proteins forming high molecular weight protein aggregates display a characteristic nature leading to a specific function of the participating proteins. Therefore, study of these aggregates is vital for functional characterization of interacting partners. These interactions create a barrier to the separation of interacting proteins using traditional chromatographic approaches and reduction of these interactions, prior to separation, is an essential requirement. Many factors such as temperature, pH, ionic strength and presence of other molecules may affect the degree of aggregation. Generally, particle size analysis is the experimental technique to study aggregation as it leads to a physical change in molecular size. Dynamic light scattering (DLS) is widely applied for detection of the aggregates due to the relationship between particle size and light scattered. It utilizes the Stokes–Einstein equation to calculate the size of a particle:

$$R_H = \frac{k_B T}{6\pi\eta D_0}$$

where k_B is the Boltzmann constant, T is the temperature in Kelvin degrees, D_0 is the diffusion coefficient, η is the viscosity of the solvent and R_H is the particles hydrodynamic radius [2].

Human seminal plasma (SP) contains a large array of proteins of clinical importance which play critical roles in sperm maturation and are essentially required for sperm functions [3]. Furthermore, these proteins are essential to maintain the reproductive physiology of spermatozoa and for successful fertilization through various physiological events including acrosome reaction and sperm capacitation. Specifically, concanavalin A (Con A)-interacting fraction of human SP was reported to have decapacitating activity [4]. Sperm capacitation includes a series of sperm modifications which enable sperms to acquire their fertilization capabilities [5]. It is a pre-requisite for acrosome reaction and an essential process for successful fertilization. Thus, a sperm must undergo a series of modification during capacitation and any decapacitating activity may reduce its fecundity.

In our previous study, we have isolated and identified proteins of Con A-binding fraction of human SP, viz. aminopeptidase N, lactoferrin, prostatic acid phosphatase, human zinc-alpha-2-glycoprotein, prostate specific antigen, progestagen-associated

Abbreviations: Con A, concanavalin A; DLS, dynamic light scattering; R_H, hydrodynamic radius; SP, seminal plasma.

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endometrial protein, kinesin light chain 4, izumo sperm–egg fusion protein 1 and prolactin inducible protein [6]. Present study reports analysis of aggregation/dis-aggregation behaviour of this fraction by DLS. This study will surely help us in better understanding of underlying mechanism of decapacitating activity of this fraction of SP and thus, better understanding of human fertilization.

2. Methodology

2.1. Sample collection and preparation

Human semen samples were collected from Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India following the World Health Organization (WHO) 2010 recommendations and with written consent from donors. The HIV positive samples and/or those contaminated with blood were excluded. Only normozoospermic samples were included in this study and selected using the following criteria – age group of donors – 20–40 years, sperm count > 15 million/mL, sperm motility > 40% and normal sperm morphology. The samples were centrifuged at 10,000 × *g* for 30 min at 4 °C to remove spermatozoa. The supernatants obtained were pooled and stored at −20 °C.

2.2. Lectin affinity chromatography: isolation of concanavalin-A binding glycoproteins from normal seminal plasma

Con A binding proteins of SP were purified as previously described [6]. In brief, Con A–agarose (Sigma–Aldrich, Inc.) column (2.5 cm × 20 cm) was prewashed with wash solution (1 M NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 5 mM $CaCl_2$) and equilibrated with phosphate buffer saline (PBS: 50 mM phosphate buffer, pH 7, 200 mM NaCl). 10 mL SP, diluted 1:4 with PBS, was applied to the column and bound proteins were eluted with 0.5 M methyl-α-D-mannopyranoside. The fraction of bound proteins was desalted and concentrated using ultrafiltration (Millipore, Billerica, MA). Then, it was lyophilized and stored for further use.

2.3. Ion exchange chromatography

Con A binding proteins, purified by lectin chromatography, were applied onto a DEAE-Sepharose (GE-Healthcare, Uppsala, Sweden) column (2.5 cm × 20 cm), an anion-exchanger. The column was pre-equilibrated with phosphate buffer (50 mM, pH 7.0). The unbound proteins were washed with equilibration buffer and bound proteins were eluted by 0.5 M NaCl in equilibration buffer.

2.4. Gel filtration

Twenty milligram Con A binding proteins were dissolved in 1 mL of equilibration buffer (50 mM phosphate buffer, pH 7.0) and loaded on Sephadex G-100 (Sigma–Aldrich, Inc.) column (1.5 cm × 125 cm), pre-equilibrated with 0.2 M NaCl in equilibration buffer. Proteins were eluted at a flow rate of 6 mL/h. Fractions of 1 mL were collected and measured at 280 nm for presence of proteins. Protein peaks were pooled and concentrated by ultrafiltration and lyophilized.

2.5. Gel electrophoresis

Protein separation at each step was analysed by gel electrophoresis. SDS-PAGE was carried out using polyacrylamide gel (12% separating and 4% stacking) under reducing conditions at 120 V as previously described [7]. Proteins on gel were visualized by Coomassie Brilliant Blue G-250 staining.

2.6. Dynamic light scattering: aggregation analysis

In solution behaviour of Con A binding proteins was analysed by DLS under various conditions. All reagents were filtered using 0.22 μm filters and protein concentration of 5 mg/mL was used. All DLS measurements were performed using a Spectroscatter 201 (RiNA) with a sampling time of 30 s and a wait time of 1 s. A 659 nm diode laser was used as the source and scattered light was collected at a fixed angle of 90°. The CONTIN program was used to obtain hydrodynamic radius (R_H) distributions by autocorrelation function analysis and data analysis was done by PMgr v3.01 software supplied with the instrument. All the DLS experiments were repeated five times. The conditions in which DLS experiments were performed include: (i) pH from 2.0 to 11.0 (50 mM phosphate buffer), (ii) in presence of (a) NaCl (0.1 M, 0.2 M, 0.5 M), (b) EDTA (1 mM, 2 mM, 5 mM), (c) $CaCl_2$ (1 mM, 2 mM, 5 mM), (d) D-fructose (10 mM, 25 mM, 50 mM), (e) D-glucose (10 mM, 25 mM, 50 mM), (f) D-mannose (10 mM, 25 mM, 50 mM), and (g) cholesterol (1 mM, 2 mM, 5 mM). A hydrodynamic radius at pH 7.0 was used as a reference value in each case.

3. Results

3.1. Chromatography

Con A binding protein fraction of human SP was loaded on DEAE-Sepharose (anion exchange chromatography) and Sephadex G-100 (gel filtration) columns to achieve further separation and separation at each step was analysed by SDS-PAGE. In anion exchange, proteins of the fraction did not bind to the column and all of them were recovered in unbound fraction (Fig. 1). In gel filtration, 1 mL fractions were collected and measured at 280 nm. A single major peak was observed in void volume, indicating towards aggregation or mutual interaction of the proteins present in this fraction (Fig. 2).

3.2. Dynamic light scattering

DLS studies indicate that proteins present in Con A binding fraction make high molecular weight aggregates as depicted by hydrodynamic radii, R_H (27.74 nm at pH 7.0 in phosphate buffer) (Fig. 3). Results of DLS study, highlighting the effect of pH, NaCl, EDTA, cholesterol and sugars on aggregation size (in terms of R_H), are shown in Fig. 4 and Table 1. In brief, degree of aggregation was reduced highly at a pH below or above the physiological range as evident by approx. 3–5 times decreased R_H values (5.60 nm at pH 2.0 and 8.65 nm at pH 11.0). Also, additives such as sugars, NaCl, and EDTA displayed dissociating effect, i.e. reduced R_H. Fructose and EDTA showed the higher reduction (7.81 nm at 50 mM fructose and 8.89 nm at 5 mM EDTA) while NaCl had the least effect on R_H (26.19 nm and 18.72 nm at 0.1 M and 0.5 M NaCl respectively). Glucose and mannose had medium effects. At lower cholesterol concentration (1 mM), increased R_H (32.26 nm) was observed but on further increase of cholesterol concentration, R_H decreased significantly (16.71 nm at 5 mM cholesterol).

4. Discussion

SP proteins are involved in different physiological events related to fertilization including sperm maturation, sperm movement in female reproductive tract, capacitation and acrosome reaction. SP proteins interact with different ligands, i.e. polysaccharides, phospholipids and bivalent metal ions; and other proteins to facilitate successful fertilization. Heparin binding proteins are known to modulate capacitation and acrosome reaction and display aggregation behaviour in solution [8], zinc binding proteins are reported