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Simplified approach for *in-vitro* production and purification of cell derived Cancer Antigen 15-3

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ABSTRACT

Cancer antigen 15-3 (CA15-3) is a key biomarker, currently used for understanding the onset and prognosis of breast cancer. In present investigation, CA15-3 has been purified from the culture supernatant of breast cancer T47-D cell line with 76% yield and 3350 fold purification. Isolated CA15-3 was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting (western blotting), chemiluminescence immunoassay (CLIA) and Fourier-transform infrared spectroscopy (FTIR). CA15-3 is a monomeric protein with an apparent molecular mass in between ~250–350 kDa. The FTIR spectroscopy revealed similar profiles of T47-D derived CA15-3 and commercially available CA15-3 protein. With the easy availability of T47-D cell line and a simple purification approach described here will support for the large scale production of CA15-3 to be used for various clinical and diagnostic applications.

1. Introduction

Breast cancer (BC) is one of the common types of cancer among women and its incidence is increasing. Among males and females BC is expected to cross the figure of 100,000 individuals by the year 2020 [1]. The Indian Council of Medical Research (ICMR) predicts that till 2020, new cases of BC in the Indian population will be more than 1,730,000 (http://icmr.nic.in/icmrsql/archive/2016/7.pdf) [2]. The mortality rate by BC was notably reduced since one score year in many developed countries like USA and Canada. This reduction was achieved by modern screening technologies and remarkable theronostic approaches against BC [3–5]. CA15-3 also known as Mucin 1 (MUC1), a transmembrane glycoprotein with a varying molecular weight from 250 to 350 kDa produced by the epithelial cells [6]. The excess concentration of CA15-3 in serum has been associated with benign breast tumors and cancers of lung, ovary, pancreas and intestine [7–11]. The elevated levels of CA15-3 in the serum are thought to reflect the disruption of the basement membrane leading to metastasis [12]. Based on extensive clinical observations the American Society of Clinical Oncology has approved the use of serum CA15-3 levels for early monitoring of BC and also as a prognostic marker to monitor the patients with BC [13–15]. The current strategies for detection of CA15-3 include electrochemical method [16] and immunoassays (*e.g.* fluoroimmunoassay and enzyme linked immunosorbent assay) [17,18]. One of the major problems is that many immunoassays for testing of CA15-3 exhibit both false-positive as well as false-negative results [19]. Thus, there is an instant necessity to improve the sensitivity of the existing immunoassays, reduce both false-positive and false-negative clinical outcomes as well as inter and intra-

Abbreviations: AFP, alpha fetoprotein; ATCC, american type culture collection; BC, breast cancer; BSA, bovine serum albumin; CA, cancer antigen; CLIA, chemiluminescence immunoassay; DMEM, dulbecco's modified eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FTIR, fourier-transform infrared; h, hour; HETP, height equivalent to a theoretical plate; HRP, horseradish peroxidase; ICMR, Indian Council of Medical Research; Ig, immunoglobulin; IL-6, interleukin-6; IU/mL, international units per milliliter; kDa, kilodalton; MUC1, mucin 1; MWCO, molecular weight cut off; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PBST, PBS with 0.05% tween 20; PC-1, chemically defined serum-free medium; PES, polyethersulphone; pH, Power of hydrogen; SDS, Sodium dodecyl sulphate, T47-D: human breast cancer cells; TCA, trichloroacetic acid; TFF, tangential flow filtration; TMB, 3,3′,5,5′-tetramethylbenzidine.

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assay differences. Several authors have reported the purification and characterization of CA15-3 from various biological sources such as serum of BC patient [20,21], saliva [22], breast tissues [23], and ovarian cancer fluid [24] by a myriad of methods. It appears that there are might be tissue/cell specific isoforms of MUC1 gene which exhibit a distinct band pattern seen with analytical methods [25]. These processes involve immunoaffinity column chromatography, which is expensive and may not be commercially viable for large scale production of CA15-3. Keeping all these in mind, we herein describe the remarkable, straightforward and simplified cost effective approach for the *in-vitro* production and purification of CA15-3 from culture supernatant of T47-D cell line.

2. Materials and methods

2.1. Materials

All reagents employed in this study were of the highest grade of purity. Dulbecco's Modified Eagle's Medium (DMEM), glutamax, non-essential amino acids solution, oestrogen, progesterone, trypsin, interleukin-6, D-glucose, dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, USA). The PC-1, a chemically defined serum free medium was procured from Lonza (Walkersville, USA). Gibco's fetal bovine serum (FBS) was procured from Life Technologies (New York, USA). The reference antigens CA-125, CA15-3, CA19-9, and CA72-4 were purchased from Meridian Life Science (Memphis, USA). The reference antibodies against aforementioned antigens were purchased from Fitzgerald Industries International Inc. (North Acton, USA). The CA15-3, CA19-9 and CA-125 specific immunoassay ELISA kit were purchased from Cal Biotech (Spring Valley, USA), whereas CA72-4 specific immunoassay ELISA kit was procured from DRG Instruments (Marburg, Germany). Sephacryl S-400 high-resolution gel filtration medium was procured from GE Healthcare (Uppsala, Sweden). Pellicon 2 Mini (P2B030A01) and XL Ultrafiltration Modules (PXB030A50) were purchased from EMD Millipore Corporation, USA. Prestained Protein Marker and Unstained protein marker were purchased from Thermo Fisher scientific (Vilnius, Lithuania).

The BC cell line T47-D (ATCC-HTB-133) was supplied by American Type Culture Collection Center (Manassas, USA). Cell line was cultured in DMEM media which is further supplemented with 10% FBS, 1% glutamax, and 1% nonessential amino acids solution and maintained at controlled environment (37 °C, 5% CO_2, 70–80% humidity) in tissue culture graded CO_2 incubator.

2.2. Buffers employed

Phosphate buffered saline (buffer A) was 10 mM phosphate buffer with 0.9% sodium chloride (pH 7.4 ± 0.2). Processing buffer (buffer B) was 50 mM phosphate buffer with 0.9% sodium chloride and 0.1% sodium azide (pH 7.4 ± 0.2). Gel transfer buffer (buffer C) was 3.02 g/L tris-buffer, 14.4 g/L glycine and 20% methanol. Blocking buffer used in western blotting (buffer-D) was 20 mM tris-buffer, 150 mM sodium chloride with 5% (w/v) nonfat milk powder and 0.5 mL/L Tween 20 (pH 7.4 ± 0.2).

2.3. Quantification of CA15-3 in cell supernatants

The concentration of CA15-3 was quantified by commercially available CA15-3 ELISA Kit (Cal Biotech, USA) according to the manufacturer's instructions with few alterations. Briefly, the culture supernatant (100 μL) was added to the antibody coated wells and subjected for incubation at 37 °C for 1 h in humidified chamber. The unbound CA15-3 were removed by washing the wells three times and treated with HRP conjugated secondary antibody (100 μL). Then, unbound HRP conjugated antibody was removed by washing the wells three times. Finally, 100 μL of TMB (3,3′,5,5′-Tetramethylbenzidine) was added and plate was incubated at room temperature for 10 min. Then, the reaction was stopped by adding 100 μL of stopping reagent. ELISA plate was accessed at 450 nm using BioRad ELISA reader.

2.4. Growth pattern and doubling time

The doubling time of T47-D cell line and time required to reach 90% confluency was estimated by seeding the cells with 3×10^6 cells/75 cm^2 densities. Briefly, total numbers of cells were counted in 24 h duration after trypsinization process of monolayer using hemocytometer. The cell count was noted down until the culture attained its 90% confluency.

2.5. Immunolocalization of CA15-3

The T47-D cells were grown in 6 well plates until 70% confluency and fixed in 4% paraformaldehyde (AppliChem GmbH, Germany) for 10 min at room temperature. Then, the fixed cells were incubated with 1% BSA for 30 min followed by incubation with 10 μg/mL of anti CA15-3 as a primary antibody for 1 h at room temperature. Unbound primary antibody was removed by washing the plate three times using PBST buffer (buffer-A containing 0.05% Tween 20). Fluorescein isothiocyanate (FITC) labelled secondary antibody was added to each well and the plate was incubated for 1 h. The unbound secondary antibody was removed by washing the plate three times with PBST buffer. Then, FITC labelled monolayer was mounted in antifade medium and observed under Olympus inverted fluorescence microscope [26].

2.6. Low serum adaptation

The cells were adapted for low serum in culture media with decreasing concentration of FBS [27]. The BC T47-D cells (at 90% confluency, grown in 10% FBS) were trypsinized and maintained at 5% FBS for three passages. After three passages, the cells were cultured in 2.5% of FBS to attain maximum confluency. The cells adapted to 2.5% FBS were used in all further experiments of present study.

2.7. Augmentation of CA15-3 synthesis and secretion in cultured T47-D cells

We examined the effect of glucose, IL-6, oestrogen, progesterone, PC-1 and combined form of oestrogen and progesterone on production of CA15-3 in T47-D cell line by adding with 2.5% FBS supplemented media. The level of CA15-3 was quantified in aforementioned culture mediums individually at the end of 96 h using CA15-3 specific immunoassay.

2.8. Purification cell derived CA15-3 protein

The CA15-3 activity and protein content in culture supernatant were determined before 5% TCA precipitation using CA15-3 specific immunoassay and Bradford protein assay (Bangalore Genei, India) respectively. The acid-enriched solution was slowly stirred at 4 °C for 30 min. The supernatant was collected by centrifugation at 10, 000*g* for 15 min at 4 °C in a Kubota-7780 centrifuge (Kubota Corporation, Japan). The resultant supernatant's pH was adjusted up to 7.3 using sodium hydroxide (alkali solution). The supernatant containing significant quantity of CA15-3 was analyzed by ELISA and supernatant was subjected to TFF (stage-I) using polyethersulphone (PES) membrane with a MWCO of 30 kDa (Fig. 1). The retentate of this TFF was then subjected for diafiltration using buffer-B. Then, the resultant retentate was applied onto Sephacryl

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