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

Shoaib Haidar, Paresh B. Bhanushali, Kunal K. Shukla, Deepak Modi, Chander P. Puri, Shamkant B. Badgujar, Manoj Chugh

A Laboratory of Cell Culture and Native Proteins, Research and Development Division, Yashraj Biotechnology Ltd., Navi Mumbai 400705, Maharashtra, India
b Department of Medical Biotechnology, MGM Institute of Medical Health Sciences, Kamothe, Navi Mumbai 410209, Maharashtra, India
c Department of Molecular-Cellular Biology and Biochemistry, National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai 400012, Maharashtra, India

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 theranostic 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-
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 (P28030A01) and XL Ultrafiltration Modules (PX8030A50) 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% CO2, 70–80% humidity) in tissue culture grade CO2 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 × 10⁶ cells/75 cm² 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, 000g 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 polyether-sulphone (PES) membrane with a MWCO of 30 kDa (Fig. 1). The retentate of this TFF was then subjected for dialfiltration using buffer-B. Then, the resultant retentate was applied onto Sephacryl
S-400 high-resolution gel filtration column (2.5 × 135 cm asymmetry range: 0.8–1.2 and HETP > 2000 plates/meter at 20 °C at a flow rate of 1 mL min⁻¹, equilibrated with buffer-A) using ÄKTAPrime plus (GE Healthcare, UK) system. The CA15-3 was fractionated with buffer-B at a flow rate of 1 mL min⁻¹. Fractions positive for CA15-3 (analyzed by ELISA) were subjected to final concentration by using PES membrane (stage-II) with MWCO of 30 kDa (Fig. 1). The resultant protein concentrate acts as a potent and purified form of cell derived CA15-3 protein.

2.9. Gradient gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing and non-reducing conditions on 4–8% gradient polyacrylamide gels and protein(s) were detected by using silver staining method [28]. Electrophoretic mobility of targeted protein (CA15-3) was assessed by comparing relative mobility of marker proteins.

2.10. Western blot analysis

100 IU of purified CA15-3 was subjected to gradient SDS-PAGE as above and the proteins were transferred onto nitrocellulose membrane (GE Healthcare, UK) overnight at 20 °C with constant current of 100 mA in presence of gel transfer buffer (buffer-C). Next day, the membrane was washed using PBST, nonspecific sites were blocked using buffer-D and incubated overnight at 4 °C with primary anti CA15-3 monoclonal antibody (Fitzgerald Industries International Inc., USA). After washing with PBST, membrane was incubated for 1 h at 20 °C with secondary antibody, HRP-conjugated Goat anti-Mouse IgG (Bangalore Genei, India). Detection was performed using TMB substrate as a chromogen [29].

2.11. Estimation of contaminants in purified CA15-3

To measure the efficiency of aforementioned methodology used for purification of CA15-3 was further tested for the availability of contaminants i.e. several kinds of MUC derived antigen(s) and other glycoproteins viz., CA19-9, CA72-4, CA-125, CEA (carcinoembryonic antigen), ferritin, and AFP (alpha fetoprotein). The quantification of these contaminant antigens from the purified CA15-3 preparation were performed by commercially available reference immunoassays i.e. ELISA assays as per manufacturer’s instructions.

2.12. Fourier-transform infrared spectroscopy (FTIR) analysis

FTIR measurements of purified cell derived CA15-3 and commercially available reference CA15-3 proteins were performed using FTIR Model-3000 Hyperion Microscope with Vertex 80 FTIR System (Bruker FTIR Spectroscopy, Germany). Each spectrum of CA15-3 was recorded in step scan mode at 16 cm² resolution.

3. Results

3.1. Cell line related studies

The secretion property of CA15-3 in culture supernatants of T47-D BC cell line was examined under in-vitro culture condition. Culture supernatants from T47-D BC cell line exhibited biological activity of suspended CA15-3 up to 20 IU/mL (commercial CA15-3 specific immunoassay). The calculated doubling time was 36.9 h and the cells attained 90% confluence within 96 h (Fig. 2). Our results of doubling time of T47-D cell line (i.e. 36.9 h) are trustworthy with the previous study of doubling time of breast cancer cell lines viz., LM-MCF-7 cells and MCF-7 cells. Both LM-MCF-7 and MCF-7 cells exhibits 34.3 h and 40.0 h of doubling time respectively [30]. Furthermore, these results are also consistent with earlier findings of doubling time estimation of human cancer cell lines MKN1 and MKN7 i.e. 35.9 h and 37.4 h respectively [31].
secretion
CA15-3
on
Fig.
rates
does
D
tries
culture.
the
achieved
the
oestrogen
mouse
CA15-3
antigen
observed
performed
CA15-3
antibodies
(Fitzgerald Industries International Inc., USA) by immunofluorescence staining. The
CA15-3
secretion.

3 secreting ability of T47-D cell was detected during idiophase of culture. However, the exponential growth of CA 15-3 secretion was observed after 60 h of cell seeding. Whereas, the secretion profile achieved a steady state after 96 h of cells seeding (Fig. 2). The localization of CA15-3 antigen onto the surface of experimental cell line was performed using CA15-3 specific antibodies (Fitzgerald Industries International Inc., USA) by immunofluorescence staining. The
CA15-3
secretion.

3.2. Purification of CA15-3

Trichloroacetic acid (5% TCA) treated supernatant was assessed for CA15-3 recovery using CA15-3 specific immunoassay kit (Cal Biotech, USA). About 245 IU/mL of CA15-3 was estimated in 5% TCA treated supernatant with a few contaminant protein bands, as observed on 4–8% gradient polyacrylamide gel under non reducing conditions (Supplementary Fig. 1). The acid precipitated supernatant was then subjected to TFF membrane with MWCO of 30 kDa (Stage-1) to achieve buffer exchange and reducing the volume by 40 folds. The retentate of this TFF membrane exhibited high content of active CA15-3 (CA15-3 specific ELISA) i.e. 8806.93 IU/mL. The resultant retentate was then subjected to gel filtration using Sephacryl S-400 column chromatography.

The elution profile of Sephacryl S-400 column constituted three peaks denoted as peak-A, peak-B and peak-C (Supplementary Fig. 2). One set was formed by mixing 10 fractions together; in the same way 10 sets were prepared from the fractionated fractions of Sephacryl S-400 column chromatography. These sets were estimated for recovery of CA15-3 antigen. The sets containing fractions of peak-A were positive for CA15-3. The sets of latter peaks viz., peak-B and peak-C did not show detectable positivity for CA15-3 (Fig. 6A). The sets of containing notable quantity of CA15-3 (peak-
Table 1  
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Purification step</th>
<th>TP (mg)*</th>
<th>CA15–3 (IU)*</th>
<th>CA15–3/TP</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial (culture supernatant)</td>
<td>15100</td>
<td>295.50</td>
<td>0.01996</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Final purified form of CA15–3</td>
<td>3.45</td>
<td>226.21</td>
<td>65.5862</td>
<td>76.55</td>
<td>3350.52</td>
</tr>
</tbody>
</table>

* Total protein (TP) content was estimated by Bradford Protein Assay using bovine serum albumin as the standard protein.  
* CA15–3 content was measured by using CA15–3 specific immunoassay ELISA Kit (Cal Biotech, USA).

...purification stage as has been shown by Bhanushali et al., using quantitative immunoassay in their earlier study [34].

3.3. Purity of cell derived and purified form of CA15–3

The purity of cell derived CA15–3, processed as described above was analyzed by electrophoresis and immuno blotting (Fig. 6). Immunologically, only the fractions from 41 to 70 of peak-A of Sephacyrl S–400 column chromatography exhibited significant activity of CA15–3 whereas remaining pools representing peak-B and peak-C did not show CA15–3 at detectable level. Absence of any protein band on polyacrylamide gel under non-reducing condition confirmed the availability of CA15–3 antigen in pooled fractions of peak-A with the absence of contaminant protein(s) (Fig. 6A). As the CA15–3 has molecular mass varying from 250 to 350 kDa, therefore the purified protein (CA15–3) did not entered into the 4–8% gradient gel mixture. The western blotting using commercial anti CA15–3 antibodies (Fitzgerald Industries International Inc., USA) confirmed the presence of purified CA15–3 protein band near to the base of wells (Fig. 6B). Thus, our preparation appears to be practically homogenous. It was also alike with the molecular mass of concentrated form of purified CA15–3 determined and validated by immunoblotting i.e. ~300 kDa under reduced and non-reducing conditions (Fig. 6B). This high molecular mass of cancer antigen CA15–3 is good in agreement with the earlier observations reported by Grzywa et al. [32], Stieber et al. [35] and Osman et al. [36].

3.4. Comparative profile of CA15–3 (FTIR analysis)

FTIR analysis confirmed the similar chemical behavior of cell derived isolated CA15–3 and native form of commercially available reference CA15–3. The confirmation of molecule was done by infrared spectroscopy by overlapping the fingerprint region (600–1500 cm\(^{-1}\)). Each compound produced its own unique pattern of peaks in fingerprint region and hence one can judge the confirmation of compound by overlapping fingerprint region. The bending vibrations of molecule present in the fingerprint region overlapped with the reference CA15–3 which suggests that both the samples having same chemical composition and behaviors. The Fig. 7 elucidates the functional group similarities between two different forms of CA15–3 analyzed. FTIR measurements were taken using Brukers Model-3000. The spectra were recorded in the wave number range of 500 – 4000 cm\(^{-1}\). The absorbance band at 3454 cm\(^{-1}\) was associated with \(\text{–OH}\) group of both CA15–3 (Fig. 7). The peak at 2241 cm\(^{-1}\) might be \(\text{–C=O}\) stretched nitriles. The \(\text{–C=O}\) band was found in both CA15–3 at 3954 cm\(^{-1}\) position. Similarly, \(\text{–C=O}\) pattern of asymmetric carboxylic acid was also observed at 1745 and 1639 cm\(^{-1}\) position. Remarkably, alkynes \(\text{–C≡C=O}\) were observed at 656 cm\(^{-1}\) position in both CA15–3 proteins. The present band patterns in fingerprint region (600–1500) with similar wavelength position indicates, matched conformation of purified form of CA15–3 was precisely matched with respect to commercially available native form of reference CA15–3 (Fig. 7).
4. Discussion

CA15-3 is a high molecular weight cancer antigen which belongs to a subgroup of polymorphic epithelial mucins [37]. Though the international recommendations vary with respect to the CA15-3 antigen level, many diagnostic guidelines support the application of CA15-3 as the prominent cancer biomarker used for monitoring therapy effectiveness [38]. The rate of diagnosis of various cancers are negligible as compared with risk of new cancer cases per year as expected because only few FDA (Food and Drug Administration) approved immunoassays are available which precisely detects CA15-3 cancer antigen. The estimated new cancer cases are 1,688,780 diagnosed in USA in 2017. Also, the estimated cancer deaths in the USA may be increasing up to 600,920 in 2017 [39]. In order to be ready to face the alarming situation, requirement of accurate and cost effective assays are inevitable. We took the study to optimize large scale, quick production and simplified purification of ‘high pure form of cancer antigen’, with better recovery and getting away with the dependency over biomedical fluid from patients to purify native protein. Briefly, additional, well-designed large-scale prospective studies with contaminant free cancer antigens productions are needed to further delineate the diagnostic utility of the CA15-3.

The production can be driven by specific media designed with commercially available media supplements. Individually neither d-glucose nor progesterone, oestrogen effects on CA15-3 secretion in the culture medium. The fact that in combination oestrogen and progesterone stimulates secretion of CA15-3 in culture medium was corroborated with earlier findings reported by Ozturk et al., [33]. However, it should be noted that the combination of PC-1 with 2.5% supplemented DMEM high glucose media resulted in maximum secretion of CA15-3.

The approach for purification described here has distinct advantage over the previously reported method of purification of native form of CA15-3 [34,40]. It may not be easy to purify CA15-3 biomarker from large volume of native biomedical fluids with several similar contaminants. Also, very low concentration of desired biomolecule, therefore requiring an expensive immunoaffinity technique [34,40]. Hence, the new method adopted for the present investigation could offer a valuable approach and innovative platform for multiple fold purification of cell derived cancer antigen i.e. CA15-3. The steps employed by us are very easy to perform and can be easily scalable. The anti CA15-3 monoclonal antibody recognized the isolated cell derived CA15-3 by immunoblotting confirming positive TMB reactive CA15-3 smear above 260 kDa position. These corroborates with the earlier findings of dot blot analysis reported by Grzywa et al. [32].The bands were observed for reduced as well as non-reduced form of gel electrophoresis. Remarkably, under reduced condition the cell derived CA15-3 didn’t show any extra fragmented part of protein compared to unreduced one, which confirming the monomeric nature of isolated CA15-3 antigen.

The FTIR band pattern of purified cell derived CA15-3 exhibited unique pattern of peaks in the fingerprint region (600–1500 cm$^{-1}$). The FTIR profile of reference form of native CA15-3 suggesting the availability of similar functional groups, conformation and therefore, possibly similar antigenic epitopes present in both form of cancer antigens [41].

5. Conclusion

In summary, the present investigation demonstrates the in-vitro production of CA15-3 with simple as well as single step chromatographic approach for the purification of cancer antigen from TCA treated culture supernatant of T47-D BC cell line. The purified CA15-3 protein was further validated using anti-CA15-3 antibody, ELISA and FTIR profile. Briefly, this study attempted to test the feasibility of chromatographic purification approach of cell derived cancer antigen (CA15-3).

Author contributions

All authors contributed equally in thinking and designing of experiments, performing the experiments and analysis of the data. All authors read and approved the final version manuscript.

Acknowledgements

This study was supported by the Yashraj Biotechnology Limited, Mumbai, India. The authors are grateful to our Chairpersons Arvind K. Bhanushali and Bharat T. Dagha for giving encouragement in carrying out present investigation.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbiomac.2017.10.012.

References

Institutional Ethics Review Committee

To,
Mr. Shoaib Haidar
Department of Biotechnology,
MGM School of Biomedical Sciences,
Kamothe, Navi Mumbai.


Dear Mr. Shoaib,

The meeting of the Institutional Ethics Review Committee was held on 24th April at 10.00 a.m. in the Conference Room of MGMIHS with Dr. Chander Puri as Chairperson. At the said meeting, the members of the IERC committee reviewed the scientific and ethical consideration of your proposal for the clinical study entitled, "Development, Characterization and Clinical Validation of in vitro Diagnostic Method for Diagnosis of Breast Cancer."

The IERC reviewed your proposal and approved it.

It is the policy of IERC that it be informed about any serious adverse event occurring during the course of the study within seven days of the occurrence of the adverse event. The IERC expects to be informed about any changes in the protocol and Informed Consent Document and asks to be provided a copy of the final report. The status of the study (completed / ongoing / terminated) should be reported to the IERC every six months.

Yours sincerely,

[Signature]

Signatures of Member Secretary