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A novel chromatographic purification method for high pure CA 15-3

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Abstract

Carcinoma antigen 15-3 (CA 15-3) is the most widely used tumor marker for breast Cancer (Schmidt-Rhode et al. 1987). It is derived from Mucin1 (MUC1) protein. CA 15-3 is a glycosylated protein of molecular weight around 400 KDa (Steger et al. 1989). It is secreted by the surface epithelia of cancer tissue and shed in to the blood stream. Free floating CA 15-3 at high level is detected in the blood of breast cancer patient (Ogawa et al. 1986; Gang et al. 1985). Plethora of literature is available to support the CA 15-3 as a diagnostic biomarker for follow up of breast cancer (Duffy et al. 2010; Al-azawi et al. 2006; Duffy et al. 2004; Duffy et al. 2000; Duffy 1999; Coveney et al. 1995; Geraghty et al. 1992). In recent past, increased demand of diagnostic kit for early detection of breast cancer has in turn raised the demand for high pure CA 15-3 antigen in the diagnostic field. So far not a single method has been published detailing the purification of CA 15-3. Here, we report a novel and concise protocol for large scale purification of high pure CA15-3 from ascites of cancer patient's.

Background

Breast cancer is one of the most prevalent forms of cancer in women. These tumors frequently produce mucinous antigens that are high molecular weight glycoproteins with O-linked oligosaccharide chains (Hakomori, Cummings 2012). Tumor-associated antigens encoded by human MUC-1 gene are known by different names viz, cancer antigen CA 27.29 and CA 15-3(Horm et al. 2012;Horm, Schroeder 2013).

CA15-3 protein is a member of the family of proteins known as mucins. Its normal function is cell protection and lubrication. It plays a role in reducing cell adhesion and is found throughout the body. Elevated levels of this antigen are found mainly in breast cancer where it appears to be involved in metastasis (Gang et al. 1985). CA 15-3 is believed to be the first independent prognostic serum marker in breast cancer (Duffy et al. 2000). In various clinical studies high preoperative levels of CA 15-3 (>30.4 U/mL) have been shown to be associated with breast cancer.

According to the literature available, CA 15-3 can be identified by various techniques such as CLIA, ELISA and western Blotting. The qualitative analysis of CA 15-3 by western Blotting and quantitative analysis by ELISA needs specific antibodies against CA 15-3. Production of specific antibodies relies on high pure CA 15-3 as an antigen. Developing method of production for high pure CA 15-3 would be a great contribution to the field of diagnostics.

Our objective for this study was to optimize and develop a novel protocol for obtaining a high pure antigen from well established source of CA 15-3 i.e. ascitic fluid from the breast cancer patient. In this study we report a concise and simple protocol for high pure CA 15-3.

Materials and Methods

Various ELISA kits were purchased from Calbiotech CA 15-3 (Cat: CA153T), CA 19-9, Calbiotech (Cat: CA199T), CA 72-4, DRG (Cat: EIA-5019) CA 125 Calbiotech (CA 125T). Other chemical reagents such as Per chloric Acid, ($HClO_4$) Sodium Hydroxide (NaOH), Sodium phosphate monobasic ($NaH_2PO_4.2H_2O$), Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4), Sodium Chloride (NaCl), Hydrochloric acid (HCl), Ethanol, Sodium Azide (NaN_3), Tris base, SDS ($C_{12}H_{25}SO_4Na$), Glycerol ($C_3H_8O_3$), Beta Mercapto Ethanol ($C_2H_6O_S$), Glycine ($C_2H_5NO_2$) were purchased from Sigma. DM water (H_2O) was made at Yashraj Biotechnology. 2µ and 0.22µ Syringe filter were purchased from Pall life sciences. Ultrafiltration Cassette (Millipore), Tubing, Sephacryl-100(S-100) and Sephacryl-400(S-400) were from GE. AKTA prime system from GE, all other reagents were either from Thermo Fisher or Sigma Aldrich.

CA 15-3 purification

Ascitic fluids from breast cancer patient's were collected in accordance with the ethical committee of Yashraj Biotechnology Limited and stored at-70°C. Frozen cancer fluid was thawed at room temperature and centrifuged at 10000 g in a Beckman JA-20 rotor for 15 minutes at 4°C. Initial screening was done to check for the presence of CA15-3. Samples showing high level of CA 15-3 were subjected to further steps of purification. All the ELISA studies were performed with diagnostically approved kit. Screened samples, were pooled

and subjected to 0.6 Molar perchloric acid (PCA) precipitation for 10 minutes at room temperature. The precipitation mix was centrifuged at 10000 g at 4°C.

The cleared supernatant from PCA precipitation step was filtered through 2µ filter paper and pH was adjusted to 7.4 using 2.5M Tris solution (Slow addition). The material was concentrated by Ultrafiltration and Diapermeation with phosphate buffer (50mM Phosphate Buffer, 0.15M Sod. Chloride, 0.1% Sod. Azide, pH 7.4). In order to remove the contaminating human proteins, sample was treated with 100mM β-ME and 1.5% SDS and loaded on pre-equilibrated S-400 gel column (130 X 2.5 cm) with buffer (50mM Sodium phosphate, 0.15M NaCl, 0.1% azide pH 7.4). Suitable fractions based on the chromatogram were subjected to ELISA, after determining the presence of activity and CA 15-3 band on SDS PAGE, fractions were pooled and concentrated using 30 KDa TFF Cassette and concentrate was loaded on the pre-equilibrated S-100 gel column (130 X 2.5 cm) with buffer (50mM Sodium phosphate, 0.15M NaCl, 0.1% azide pH 7.4). Suitable fractions based on the chromatogram were subjected to the ELISA, after determining the presence of activity and CA 15-3 band on the SDS-PAGE, fractions were pooled and re-concentrated using 30 KDa TFF Cassette. Again the concentrate was subjected to SDS-PAGE and western blotting was performed using the specific antibodies against CA 15-3. To measure the efficiency of our protocol, we calculated the relative abundance of cross contaminants in the preparation of CA 15-3, using specific ELISA kits.

Results and discussion

ELISA screening determining the abundance of CA 15-3 and contaminants

Initial screening was done to check the presence of CA 15-3 and other different contaminating cancer antigen; here we screened the samples for CA15-3, CA125, CA 19-9 and CA72-4. Percentage contamination was determined for each antigen using the specific ELISA kits. Activity of CA 15-3 was found to be >50 IU/ml in starting material.

Perchloroic acid (PCA) precipitation

In order to precipitate the contaminating protein, we performed the perchloric acid (PCA) precipitation. Precipitates were removed by centrifugation and supernatant was ultrafiltered and diapermeated against the Phosphate buffer (PB). Activity was checked by ELISA in pre and post dialysis fraction.

β-ME /SDS treatment

Before loading on the S-400 column, most of the cross contaminating human proteins were removed by treating the sample with, 100mM β-ME and 1.5% SDS and boiling for 5 minutes at 95°C.

S 400 gel filtration chromatography

PCA precipitated, concentrated and β-ME/SDS treated samples were loaded on the S-400 gel and different fractions were collected. Chromatogram was recorded in the prime view. CA 15-3 activity was measured in different fraction and depending on the CA 15-3 activity. CA 15-3 positive fractions were pooled Fig1.

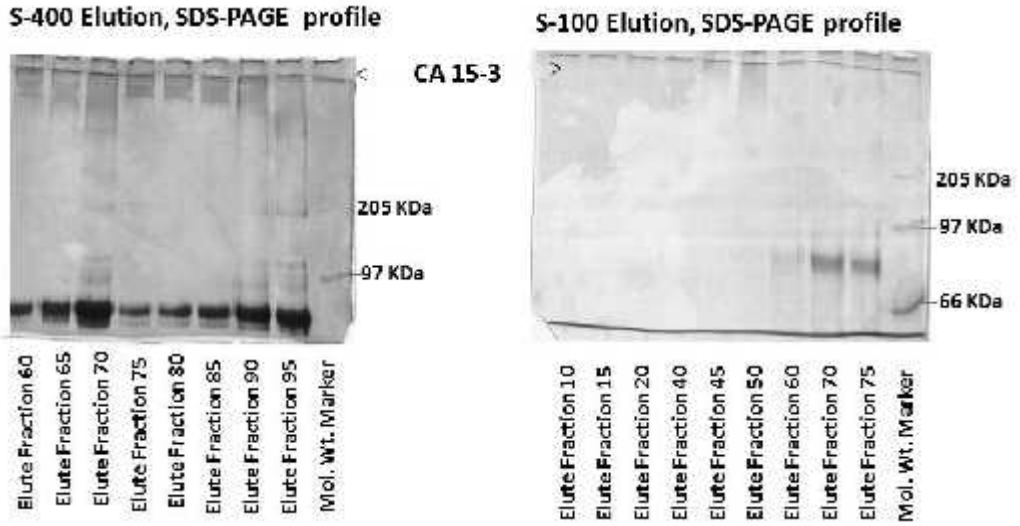

Fig 1- SDS-PAGE profile of S-400 and S-100 Protein was loaded on the S-400 column, various fractions were loaded on the 7% gel and activity was confirmed with CA 15-3 specific ELISA kit, again fractions showing higher activity were pooled, concentrated and loaded on the S-100 column. Various fractions obtained from S-100 column were subjected to SDS-PAGE electrophoresis and silver staining. CA 15-3 was observed at around 400 KDa molecular weight.

S100 gel filtration chromatography

CA 15-3 positive fractions were pooled from S 400 elution and concentrated using TFF cassettes, and loaded on S100 column. Elute from the S100 column was checked on 7% silver stained SDS-PAGE gel Fig 1.

Final concentration and filtration

CA 15-3 positive fractions were subjected for the ultrafiltration using 30 KDa TFF Cassette. The concentrate was filtered through 0.2 μ filters. After

Concentration and filtration steps, protein was checked on 7% SDS-PAGE gel.

Western Blotting

CA15-3 specificity was also confirmed with western blot analysis using specific antibodies against CA 15-3. Western blot analysis gave single band at around 400 KDa with a trailing end, suggesting the glycosylation of the protein. Fig 2

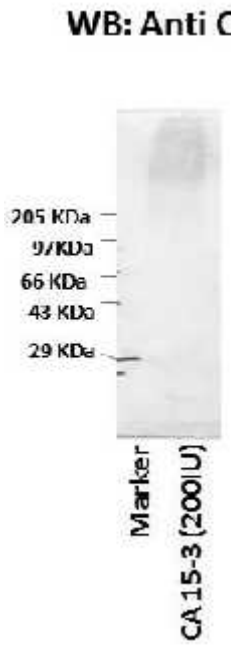

Fig 2. Western blot analysis of high pure CA 15-3 western blot analysis of High pure CA 15-3 (200 IU) was performed with specific primary antibodies against CA 15-3 on 12% gel. Primary antibodies were used at 1:5000 dilution. Glycosylated CA 15-3 was detected around 400 KDa Mol Wt., as a trailing band, representing the glycosylation of the protein.

Determination of percentage purity

Final concentration of the purified CA 15-3 was determined using the ELISA kit. Here we calculated the amount of the protein obtained through purification processes. Final concentration of the protein was found to be 96% indicating high purity of the protein.

Table 1. Relative abundance of the cross contaminants in the purified protein. Relative abundance of the cross contaminants were quantified with specific ELISA kits.

Percentage purity data

Relative abundance of cancer marker contaminants in purified CA 15-3		
	ELISA values	**% Contamination**
CA 15-3	**0.972**	**96.620**
CA 19-9	0.012	**1.193**
CA 72-4	0.010	**0.994**
CA 125	0.012	**1.193**

Conclusion

Till date most of the protocols available for the purification of CA15-3, have been very descriptive and shown to be yielding part pure CA 15-3. All protocols have yielded part pure CA 15-3 along with other cancer antigen e.g. CA125, CA 19-9, CA 72-4. To our knowledge; here we report the first detailed protocol for purification of high pure CA15-3 from cancer patients. Our study is a great improvisation of the existing protocol and will be a valuable contribution for native purification of CA 15-3; a diagnostically important biomarker.

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