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Purification process development for HER1 extracellular domain as a potential therapeutic vaccine

Dania León[a,1], Yadira Prieto[a,*,1], Eutimio G. Fernández[b], Noemí Pérez[a], José A. Montero[c], Julio Palacios[a], Dubhe Bulté[d], Kathya R. de la Luz[a], Vladimir Peña[a], Williams Ferro[c], Belinda Sánchez[e], Rodolfo Valdés[c], Adolfo Castillo[a]

[a] Process Development Department, Center for Molecular Immunology, 216th Street to 15th, P.O. Box 16040, Havana 11600, Cuba
[b] Inorganic Chemistry Department, Center for Engineering and Chemical Researches, F Street, # 115 be/Ave 5th and Calzada, Havana 10400, Cuba
[c] Monoclonal Antibody Production Department, Center for Genetic Engineering and Biotechnology, Ave 31 be/158 and 190, P.O. Box 6162, Havana 10600, Cuba
[d] Technologies Development Department, Center for Molecular Immunology, 216th Street to 15th, P.O. Box 16040, Havana 11600, Cuba
[e] Vaccine Department, Center for Molecular Immunology, 216th Street to 15th, P.O. Box 16040, Havana 11600, Cuba

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ABSTRACT

HER1 is a tumor associated antigen emerging as an attractive target for cancer therapy. In the present study we demonstrated for first time that HER1 extracellular domain can be purified by a downstream process at pilot scale based on immunoaffinity chromatography from bioreactor supernatant of HEK 293 transfectomes. Filtered supernatant was applied to CNBr-activated Sepharose CL-4B with monoclonal antibody anti-human EGF immobilized, followed by three additional chromatographic polishing steps. HER1 extracellular domain was obtained with high purity (>95%), low DNA content, and biological activity.

1. Introduction

Epidermal growth factor receptor (EGFR or HER) family, also known as the Type I receptor tyrosine kinase family, comprises 4 distinct transmembrane receptors: EGFR or HER1/erbB1, first to be molecularly cloned, HER2/erbB2, HER3/erbB3, and HER4/erbB4 [1]. EGFR over expression and deregulation is well documented in epithelial tumors, including breast, bladder, pancreas, colon, head and neck, ovary, kidney, non-small cell lung cancer and gliomas [1].

Wild-type HER receptors are divided into 3 regions: the extracellular ligand binding region, the intracellular region containing the locus responsible for tyrosine kinase activity and regulatory functions, and transmembrane region that anchors the receptor to the cell [2].

Nowadays several passive EGFR-directed immunotherapy but not active specific approaches have already been included in medical oncology practice. However, some preclinical results have demonstrated the value of active immunotherapy based on EGFR.

Recently, vaccine preparations based on the extracellular domain of HER1 (HER1 ECD) have demonstrated, *in vitro* and *in vivo*, a potent antimetastatic effect on EGFR + Lewis lung carcinoma model, while associated side effects were absent [3]. Thus, a vaccine using HER1 ECD as active ingredient could be used for patients with EGFR/tumors.

Several papers related with the expression and purification of EGFR family have been published but most of them deal only with the specific receptor, HER2 ECD. The EGFR have been expressed and/or extracted from different hosts such as: *Escherichia coli*, rat astrocytes, breast cancer cell line SKBR3 and Chinese hamster ovary cells. However, HEK 293 cell line has not been used before for obtaining this kind of molecules [4–7].

Mammalian cell culture is widely used to produce valuable biotherapeutics including monoclonal antibodies, vaccines and growth factors. Cell lines such as Chinese hamster ovary, myelomas, baby hamster kidney and human embryonic kidney cells have found extended use on industrial scale for therapeutic protein production [8,9].

Thus, the aim of this work was to establish a downstream process at pilot scale based on immunoaffinity chromatography to obtain the HER1 ECD from culture supernatant of HEK 293 transfectomes with high purity and biological activity.

* Corresponding authors. Tel.: +53 7 271 6867; fax: +53 7 272 0644/273 3509.
E-mail address: yadirap@cim.sld.cu (Y. Prieto).
[1] Both authors had the same contribution to the work.

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2. Materials and methods

2.1. Immunoadsorbent preparation

2.1.1. Matrix activation

The Sepharose CL-4B (Amersham-Biosciences, Uppsala, Sweden) was activated with CNBr according to a modified procedure reported by Axen et al. [10]. Briefly, several initial washings to remove the preservation solution were done with water according to the manufacturer's instructions. Subsequently, the CNBr was dissolved in nitrile acetate (1 g of CNBr/mL of nitrile acetate) in a glass flask and mixed with an aqueous matrix suspension in a 5 L reaction bowl. The pH of reaction was controlled dropping 4 M NaOH, in a range of 10.5–11 at 18–21 °C.

The reaction was then completed in 15 min. Continuous washings with 4 mL of water per milliliter of Sepharose CL-4B, 2 mL of 0.1 M acetic acid per milliliter of Sepharose CL-4B, 5 mL of water per milliliter of Sepharose CL-4B and 2 mL of acetone 40% (v/v) per milliliter of Sepharose CL-4B were performed. Each activation reaction was carried out with 2.4 L of Sepharose CL-4B and 0.09 g of CNBr per milliliter of the inert matrix. Intense agitation was reached using an anchor type impellent at 400 rpm and the concentration of cyanate esters was determined by a modified König reaction [11].

2.1.2. Immunosorbent manufacturing

The monoclonal antibody anti hEGFR (supplied by the Center for Molecular Immunology) immobilization was developed according to the previously described procedure [12]. Coupling efficiency (ξ) for 5 mg/mL ligand density was determined by an indirect method, following the formula:

$$\xi(\%) = \frac{\phi}{\alpha} \times 100$$

where φ is the amount of coupled protein determined as the difference between the initial amount of ligand and α is the amount of antibody detected in the filtration and washing fractions after coupling.

The excess of the reactive groups was blocked by adding 0.1 M glycine; pH 8.0 and immunosorbents were washed with 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0 and 0.1 M $NaHCO_3$–0.5 M NaCl; pH 8.3 to eliminate no covalent bounded monoclonal antibodies.

Finally, immunosorbents were kept in 150 mM phosphate buffered saline solution, pH 7.2 until the moment of use.

2.2. Downstream process

The DEC-HER1 is secreted by the HEK 293 transfectomes from fermentation process. It is true that the Master Cell Bank of HEK 293 cell line used in this study is free of virus, the tests *in vitro* and *in vivo* and electronic microscopy were performed by the Department of Quality Control at Center for Molecular Immunology (data not shown).

2.2.1. Immunoaffinity chromatography

After fermentation, the supernatants containing cells and HER1 ECD were filtered through a 0.22 μm filter. Then, immunoaffinity chromatography (IAC) was performed in XK-50/20 column (Amersham-Biosciences, Uppsala, Sweden) packed with 80 mL of matrix. Immunosorbents were equilibrated with 5 column volume of 150 mM phosphate buffered saline solution (3 mM KCl, 140 mM NaCl, 4 mM Na_2HPO_4, 1 mM KH_2PO_4); pH 7.0. Columns were loaded with 80 mg HER1 ECD from the filtered supernatant. After washing with 150 mM phosphate buffered saline solution, HER1 ECD was eluted with 200 mM glycine/HCl; pH 2.8, and monitored at 280 nm. All steps were performed at 18 cm/h flow rate.

2.2.2. Size exclusion chromatography

We performed two size exclusion chromatography steps, the first after the immunoaffinity step and the second at the end of the process. Both using Sephadex G-25 (Amersham-Biosciences, Uppsala, Sweden) in XK-26/40 columns (Amersham-Biosciences, Uppsala, Sweden) packed with 100 mL and 180 mL of matrix. The linear flow rate during all steps of the size exclusion chromatography was 100 cm/h. In the first SEC step, the equilibrium was done with 5 column volumes of 50 mM Tris, pH 8.4 and the volume of the samples was 50 mL. In the second SEC step the equilibrium was done with 5 column volume of 50 mM Tris, NaCl 150 mM; pH 8.3 and the volume of the samples was 21 mL.

2.2.3. Ion exchange chromatography

Ion exchange chromatography using Q-Sepharose (Amersham-Biosciences, Uppsala, Sweden) was performed in a Hitrap column (Amersham-Biosciences, Uppsala, Sweden) packed with 5 mL of matrix. The chromatographic gel was initially equilibrated with 50 mM Tris; pH 8.4 at 3 mL/min flow rate. The column loaded with HER1 ECD was performed at 2 mL/min flow rate, followed by a washing step with 50 mM Tris; pH 8.4 at 3 mL/min flow rate. The desorption was completed with 50 mM Tris, 500 mM NaCl; pH 8.4 at 3 mL/min. The matrix regeneration was achieved with 50 mM Tris, 3 M NaCl at 3 mL/min.

2.3. Analytical procedures

2.3.1. Protein determination

Protein concentration was determined by absorption in spectrophotometer (Amersham Bioscience, USA) at 280 nm following the formula:

$$C = \frac{A}{\xi l}$$

where A is absorbance (sometimes referred to as optical density or OD), ξ is the extinction coefficient ($\xi = 0.549$, it was calculated from the HER1 EDC amino acid composition by General Protein/Mass Analysis for Windows program (Denmark)) and l = path length in cm. The path length is the thickness of the sample through which the light beam passes. For most standard measurements this is constant at 1 cm.

2.3.2. Analytical SDS-PAGE

Samples were analyzed by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels as described by Laemmli [13]. Samples were dissolved in 4× non-reduced SDS-sample buffer and briefly spanned in a microfuge to remove insoluble particles prior to loading on gels (5 μg per lane). The electrophoresis gels were stained with Coomassie brilliant blue. The gel images were captured with calibrated Powerlook III prepress colour scanner (Amersham Biosciences, USA). Gel densitometry analysis was done with Total Lab120 program (nonlinear, UK).

2.3.3. Preparative SDS-PAGE

The same conditions of analytical SDS-PAGE were performed, but sample concentration was increased 5-fold. Proteins were visualized by silver staining compatible with MS analysis [14].

2.3.4. In gel digestion

Protein band corresponding to the final product was excised from silver stained gels. After excision, the band was destained and digested as detailed previously [15].

2.3.5. Liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS)

Following gel digestion, the sample was resuspended in 10 μL 0.1% (v/v) formic acid and desalted using C-18 Zip Tips (Millipore, USA).

Peptide identification by LC–ESI-MS–MS was performed using a QTOF (Micromass, UK), coupled on-line to an Ultimate/Switches/Famos nanoflow LC system (Dionex, UK). Typical gradients were 0–60% B (95%, v/v, ACN; 0.05%, v/v, formic acid) (buffer A: 2%, v/v, MeCN; 0.05%, v/v, formic acid) over 55 min, at a flow rate of 180 nL/min.

2.3.5.1. Protein identification using database searching algorithms. Data were searched against human SwissProt database using the MASCOT algorithm [16]. Search parameters including modifications, such as cysteine propionamidation and methionine oxidation were considered. The tolerance in precursor ion m/z was ±200 ppm, and the tolerance in the product ion m/z was ±200 mTh. One missed cleavage site was allowed in the protein identification.

LC–ESI-MS–MS spectra were manually inspected and considered as reliable identifications when four or more consecutive C-terminal y_n ions were assigned to intense signals, usually substantiated by the presence of one or more *b* ions.

2.3.6. Isoelectrofocusing

Isoelectrofocusing of the purified HER1 ECD was performed on the precast isoelectric focusing gels (pH 3–9). The sample concentration was 0.5 mg/mL. Sample preparation and isoelectrofocusing were performed following the instructions provided by Pharmacia LKB and using the Phast System (Pharmacia). Finally, the gels were stained following the silver staining protocol reported previously [14].

2.3.7. Western-blot for HER1 ECD detection

Electrophoresed HER1 ECD was transferred by a semi-dry electrophoretic transfer with the transfer buffer (25 mM Tris–192 mM glycine and 20% methanol) at mA value, for 1 h onto a nitrocellulose membrane (Scheiler & Schuell, Daseel, Germany) [17]. After incubating with 5% non-fat milk in 150 mM phosphate buffered saline solution–0.1% Tween 20 for 1 h at 37 °C the membrane was washed three times in 150 mM phosphate buffered saline solution–0.1% Tween 20 and incubated for 1 h at room temperature with 100 μg of monoclonal antibody anti hEGFR. Then, three washes were repeated and finally the membrane was incubated for 1 h with an anti-human immunoglobulin alkaline phosphatase conjugated antibody (Sigma, St. Louis, USA). Bands were visualized by reacting with Fast Red TR/Naphthol AS-MX in enzyme substrate buffer (0.1 M Tris; pH 8.0). The reaction was stopped with deionized water.

2.3.8. High performance liquid chromatography

Size molecular exclusion high performance liquid chromatography was employed to determine the HER1 ECD purity (AKTA Purifier, Amersham Pharmacia Biotech). With this purpose, TSK-Gel 3000SWxL column and 50 mM Tris–150 mM NaCl as the mobile phase were used. Parameters employed were: 0.5 mL/min flow rate, UV detection at 280 nm, column temperature 25 °C, and 100 μL injection volume. Column was calibrated with a molecular weight kit (BIO-RAD).

2.3.9. DNA determination by Dot-Blot hybridization

DNA quantification was performed by Dot-Blot hybridization using Gene Images Random Prime Labelling Module (Amersham Bioscience) in order to obtain labelled probe with fluorescein. The kit used for detection was Gene Images CDP-Star detection module (Amersham Bioscience, USA), which employs an antibody anti-fluorescein alkaline phosphatase conjugate. Chromosomal DNA was purified from host cell (HK293 cells) in order to obtain probe for labelling and DNA curve preparation by protease and phenol treatment. For DNA hybridization in nitrocellulose membranes, DNA was extracted from samples by protease and phenol treatment. Final radiographies were analyzed using Image Scanner System software.

2.3.10. Western-blot for leakage monoclonal antibody anti hEGFR detection

The electrophoresed monoclonal antibody anti hEGFR was transferred by a semi-dry electrophoretic transfer with the transfer buffer (25 mM Tris–192 mM glycine and 20% methanol) at 150 mA, for 1 h onto a nitrocellulose membrane (Scheiler & Schuell, Daseel, Germany) [17]. After incubating with 5% non-fat milk in 150 mM phosphate buffered saline solution–0.1% Tween 20 for 1 h at 37 °C the membrane was washed three times in 150 mM phosphate buffered saline solution–0.1% Tween 20 and incubated for 1 h with an anti-human immunoglobulin alkaline phosphatase conjugated antibody (Sigma, St. Louis, USA). Then, the membrane was washed three times again and the bands were revealed by adding the substrate Fast Red TR/Napthol AS MX dissolved in 0.1 M Tris, pH 8.0. The reaction was stopped with deionized water.

2.3.11. Biologic activity

BALB/c mice ($n = 5$) were immunized with 100 μg of HER1 ECD resuspended in buffer 50 mM Tris–150 mM NaCl, pH 8.4 (100 μL final volume) were mixed with the same volume of Incomplete Freund's Adjuvant. The mix was injected into Balb/c mice and 14 days after immunization the blood from the mice was extracted. Serum was prepared from the blood and was diluted 1/200 in 150 mM phosphate buffered saline solution, pH 7.2. Recognition of EGFR expressed in A431 cell line by sera of immunized mice was measured by flow cytometry.

2.3.12. FACS analysis

Cells were stained with sera from immunized mice (1/200 dilution) followed by FITC-goat anti-mouse IgG (Jackson Immunoresearch laboratories). Up to 10,000 cells were acquired using a FACScan flow cytometer and analyzed using the CellQuest software (Beckton Dickinson, San Jose, CA).

3. Results and discussion

3.1. Downstream process

HEK 293 transfectomes producing soluble HER1 ECD were generated at CIM [3].

Supernatant from HEK 293 transfectomes was obtained by fed-batch culture performed in 15 L bioreactor (Infors HT, model Techfors S, Bottmingen, Switzerland) with a final working volume of 13 L. The culture was stopped after 15 days when viability falls below 50%, cell concentration higher than 6×10^6 cell/mL and a HER1 ECD concentration of 30 mg/L. Supernatant containing the soluble HER1 ECD protein was filtered prior to the downstream process.

The first step consists in an immunoaffinity chromatography. The CNBr activation method was selected to immobilize the monoclonal antibody anti hEGFR. A matrix with a moderate activation (12 μmol cyanate esters/mL of matrix) was enough to bind efficiently this monoclonal antibody at 5 mg/mL of matrix. The coupling efficiency showed values around 98%.

IAC results demonstrated high recovery, concentration and purification factors for the target protein (Table 1). The recovery

Table 1
HER1 ECD immunoaffinity chromatography step behavior.

	Purity by electrophoresis		Purification factor (fold)	Concentration		Concentration factor (fold)	Recovery (%)
	Initial (%)	Final (%)		Initial (mg/mL)	Final (mg/mL)		
Average ± SD	4.78	98 ± 1	20.5 ± 0.2	0.03	0.41 ± 0.08	13.7 ± 2.7	84.8 ± 10.1

SD: Standard deviation.

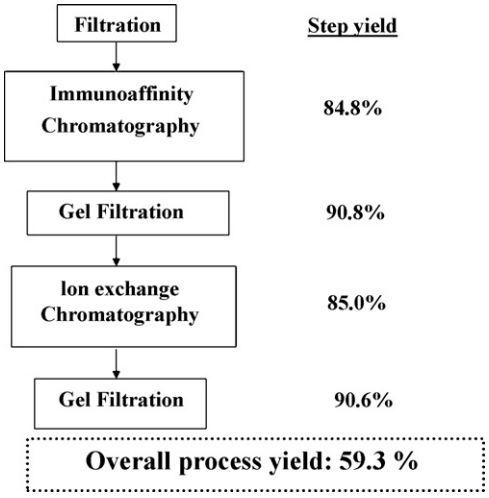

Fig. 1. Flow sheet and recovery of the downstream process for purifying the HER1 ECD molecule from culture supernatant.

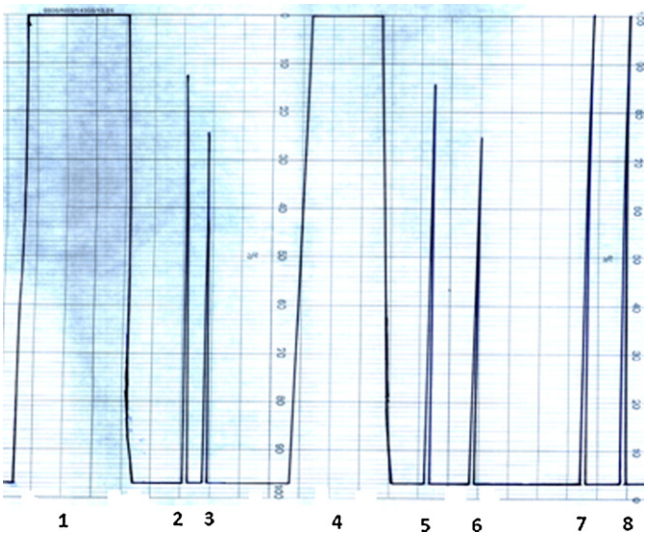

Fig. 2. DEC-HER1 purification process chromatogram: 1,4—Supernatant application, 2,5—Immunoaffinity elution, 3,6—G-25 elution, 7—Q elution, 8—Final G-25 elution.

was higher than 80%, which is an accepted value for loading material from cell culture supernatant when affinity chromatography is used [18]. This recovery together with purification (20.5-fold) and concentration (13.7-fold) factors allowed the extraordinary simplification of further downstream process.

Interestingly, the HER1 ECD was very unstable in the elution buffer a 200 mM glycine/HCl; pH 2.8. Fragmentation of the molecule was detected after few hours by SDS-PAGE and size exclusion high performance liquid chromatography (data not shown). Thus, it was necessary to include a size exclusion chromatography in order to exchange the elution buffer to 50 mM Tris; pH 8.4 to guarantee HER1 ECD biochemical stability. In the case of DNA impurities, there is a possibility that host-cell DNA contains deleterious DNA fragments, such as viral DNA and oncogenes [19]. This residual host-cell DNA is an undesirable and critical contaminant of therapeutic recombinant protein for human use in terms of biological safety and tolerance. Levels of 100 pg to 10 ng of residual DNA of host-cell origin per dose of a parenterally administered biopharmaceutical is considered to be an acceptably safe level by most of the regulatory agencies [20]. Taking into consideration this safety problem, a cation exchange chromatography with Q-Sepharose was employed to remove host DNA. The HER1 ECD recovery of this chromatography step was 85 ± 5.1%. Finally another size exclusion chromatography was used to preserve the HER1 ECD in appropriate buffer 50 mM Tris; 150 mM NaCl, pH 8.3 according molecule final preparation and formulation. The flow sheet of the downstream process has been represented in Fig. 1. The overall process recovery was 59.3%, and the process chromatogram is shown in Fig. 2.

3.2. Biochemical characterization of HER1 ECD

At the end of the downstream process the biochemical characterization of the purified HER1 ECD molecule was performed by purity (SDS-PAGE, size exclusion-HPLC), identity (Western-blot, isoelectrofocusing) and biological activity techniques.

The SDS-PAGE purity of HER1 ECD was higher than 99%, the electrophoresis profile was characterized by a 100 kDa band compared with a molecular weight kit (BIO-RAD) (Fig. 3A).

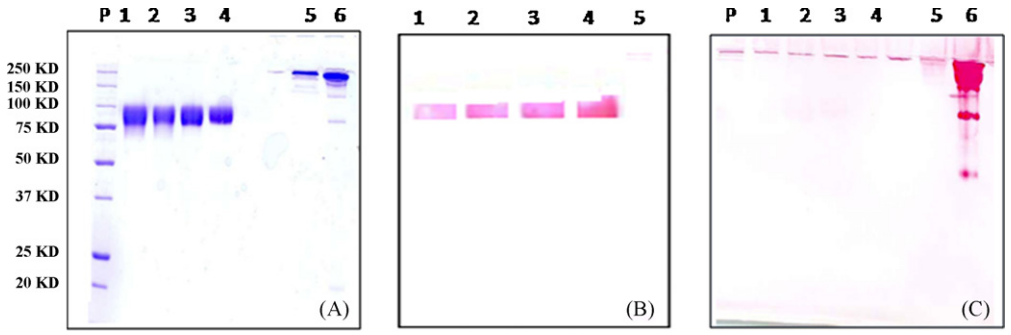

Fig. 3. Purity analysis of purified HER1 ECD molecule. (A) SDS-PAGE of the purified HER1 ECD. (B) Western-blot analysis. (C) hR-3 Western-blot analysis. Line P, Molecular weight marker. Lines 1–3, samples of purified HER1 ECD. Line 4, control. Lines 5–6 monoclonal antibodies IE10 and hR-3 respectively.

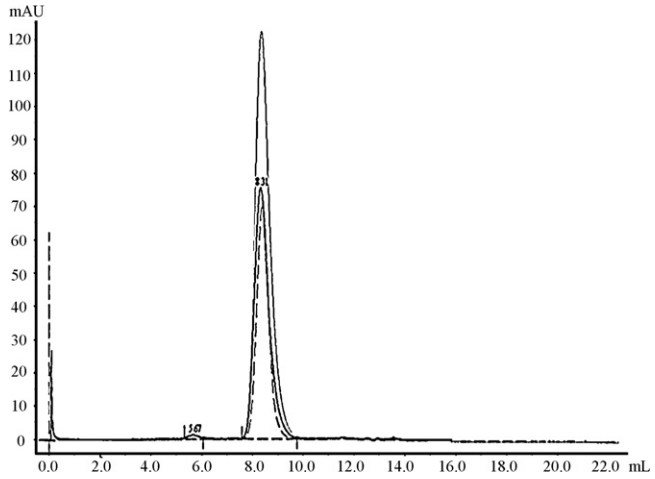

Fig. 4. Size exclusion-HPLC chromatographic profile of the purified HER1 ECD.

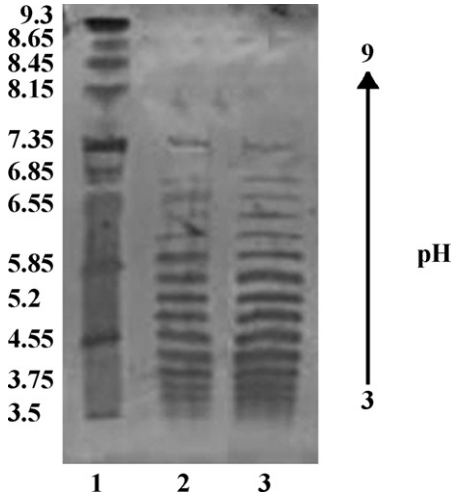

Fig. 5. Determination of isoelectric point. Line 1, isoelectric point standards. Lines 2–3, HER1 ECD samples.

Size exclusion-HPLC corroborates the high purity and molecular homogeneity of the HER1 ECD final preparation (Fig. 4). In every sample, the purity was higher than 98% and neither aggregations nor fragmentations were observed. The theoretical elution volume of the HER1 ECD was in a range from 8.8 to 9.2 mL when compared with standard markers. However, HER1 ECD was eluted at an elution volume of 8.30 mL. This result could be related to the molecular structure of the protein because this molecule presents high percent of carbohydrates [21]. When HER1 ECD was incubated with denaturalizant's agents (8 M urea and 0.5 M EDTA) the elution volume was the same (data not shown), that demonstrated that the decrease in the elution volume of HER1 ECD was not due to aggregation effects.

Bands obtained by SDS-PAGE corresponded with that obtained by Western-blot by using a monoclonal antibody anti hEGFR (Fig. 3B).

Another important parameter to be measured in downstream processes that use immunoaffinity chromatography is the ligand leakage level. The monoclonal antibody released from the matrix could manifest immunogenic capacity or neutralize part of the target protein. In this work, the content of monoclonal antibody anti hEGFR in the HER1 ECD preparation was measured by the SDS-PAGE and Western-blot (Fig. 3C). The amount of monoclonal antibody anti hEGFR present in final product should be less than 1 μg/mg of HER1 ECD. Results evidenced that level of monoclonal antibody anti hEGFR was under the permissible limit. The sensitivity limit of the SDS-PAGE combined with the Western-blot was 3 ng of monoclonal antibody anti hEGFR (data not shown). In addition, due the monoclonal antibody anti hEGFR is a therapeutic humanized antibody used in the therapy of many human epithelial tumors, such as head and neck tumors [23], the immunogenicity of this molecule should be undetectable at this level of ligand leakage.

The HER1 ECD preparation is characterized by a great number of isoforms because this molecule presents 11 sites of N-glycosylation [22]. Isoforms of the HER1 ECD obtained by the present purification process were defined using the isoelectric point technique for this protein (Fig. 5). There were 13 isoforms obtained in a range of 4–7.35 pH.

Mass spectrometry analysis was performed in order to confirm the purity and sequence of final product. The band obtained from SDS-PAGE gels was digested and analyzed by ESI-MS–MS. Four peptides were sequenced and corresponded with 30% of sequence coverage (Fig. 6).

Remanent DNA concentration values were in range of 0.42–0.83 ng DNA/mg HER1 ECD, which meets the range established by most of the medicinal agencies [20].

In the biologic activity analysis it was observed that sera from all immunized mice recognized more than 90% of the EGFR positive A431 tumor cell line (Fig. 7). Then, it was demonstrated that final product obtained is not only highly pure but also with the correct spatial conformation to induce antibodies capable to recognize EGFR positive tumors, which is indispensable to be used in a potential cancer vaccine formulation.

Also a study of stability for the product HER1 ECD where previous techniques were measured in a time interval of one year,

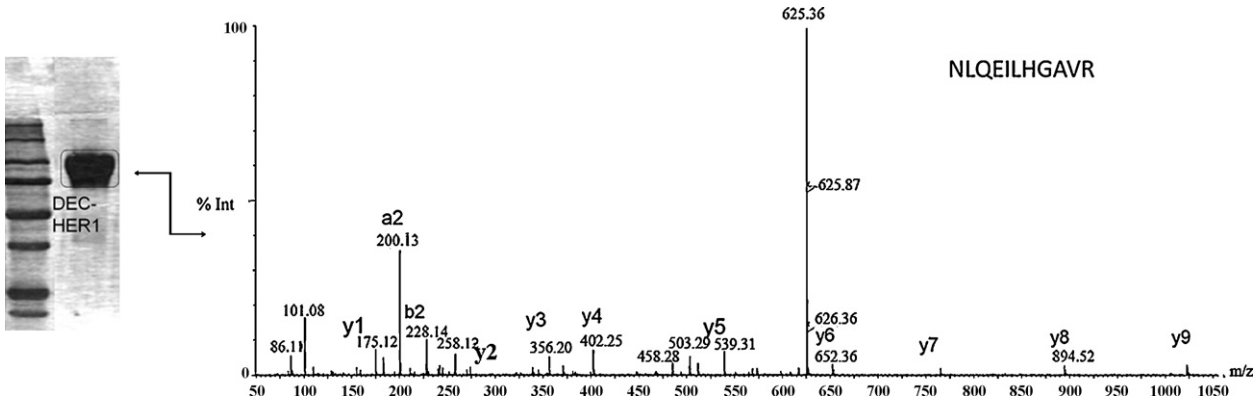

Fig. 6. Production spectrum acquired during, LC–MS/MS analysis, with CAD of m/z 625.36, corresponding to the $[M+2H]^{2+}$ ion derived from the peptide NLQEILHGAVR.

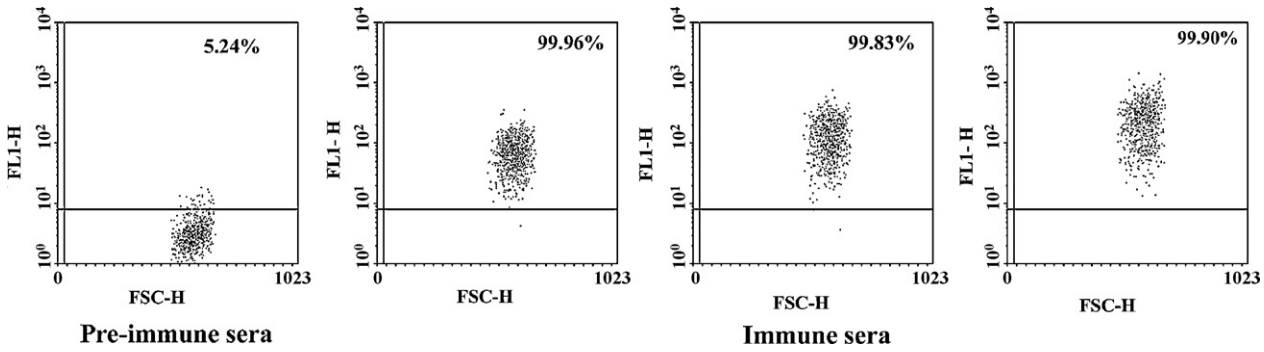

Fig. 7. Immune sera recognition EGFR + tumor cell (A431 cell line). 1/200 sera polyclonal antibodies dilutions. Pre-immune sera diluted 1/200 were used as negative control.

demonstrated that the molecule is stable at this time (data not showed).

4. Conclusion

Established downstream process based on immunoaffinity chromatography at pilot scale allowed to obtain a recovery of 59%.

HER1 ECD protein was obtained with identity, quality, strength, purity (>95%), and biological activity, which allow potential testing of HER1 ECD as a vaccine.

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