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Expression And Purification Of HER2 Extracellular Domain Proteins In Schneider2 Insect Cells

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Abstract

Overexpression of human epidermal growth factor receptor 2 (HER2/ErbB2/Neu) results in ligand independent activation of kinase signaling and is found in about 30% of human breast cancers, and is correlated with a more aggressive tumor phenotype. The HER2 extracellular domain (ECD) consists of four domains – I, II, III and IV. Although the role of each domain in the dimerization and activation of the receptor has been extensively studied, the role of domain IV (DIV) is not clearly understood yet. In our previous studies, we reported peptidomimetic molecules inhibit HER2:HER3 heterodimerization. In order to study the binding interactions of peptidomimetics with HER2 DIV in detail, properly folded recombinant HER2 protein in pure form is important. We have expressed and purified HER2 ECD and DIV proteins in the *Drosophila melanogaster* Schneider2 (S2) cell line. Using the commercial *Drosophila* expression system (DES), we transfected S2 cells with plasmids designed to direct the expression of secreted recombinant HER2 ECD and DIV proteins. The secreted proteins were purified from the conditioned medium by filtration, ultrafiltration, dialysis and nickel affinity chromatography techniques. The purified

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Authors' contributions

Shanthi P. Kanthala: Carried out transfection, expression, purification, western blot, and circular dichroism experiments; contributed to writing.

Christopher Mill: Constructed the vector for HER2 ECD domain, transfected the S2 cells with the vector, and contributed to writing.

David Riese: Directed the construction of the HER2 ECD vector in his lab, provided the transfected S2 cells to Dr. Jois, and contributed to writing.

Mihir Jaiswal: Carried out the analysis of the mass spectrometry data to arrive at the sequence of the proteins and contributed to writing the mass spectrometry analysis portion of the manuscript.

Seetharama D. Jois: Corresponding author and project PI. Planned the experiments and analyzed the CD data and contributed to writing and editing the manuscript.

HER2 proteins were then analyzed using western blot, mass spectrometry and circular dichroism (CD) spectroscopy.

Abstract

Keywords

Schneider2 insect cells; HER2 protein; HER2 domain IV; secretion of protein; Human epidermal growth factor receptor 2; S2 insect cell lines; protein sequencing; mass spectrometry; protein secretion; disulfide loops

Introduction

Human epidermal growth factor receptor 2 (HER2/ErbB2/Neu) is involved in regulating cell growth, proliferation and survival. It is overexpressed in about 30% of human breast cancers and this expression is correlated with aggressive tumor phenotypes. This overexpression results in constitutive HER2 dimerization and signaling, typified by phosphorylation of HER2 cytoplasmic tyrosine residues (1-4). Major clinical milestones in the treatment of cancers that overexpress HER2 are the FDA approval of trastuzumab and pertuzumab, humanized monoclonal antibodies that target HER2 (5-7). Therefore, there is significant interest in the mechanisms of HER2 signal transduction and in strategies for disrupting HER2 signaling.

HER2 is a canonical receptor tyrosine kinase with several distinct functional motifs. HER2 consists of an extensive (~600 amino acid residues) extracellular domain; a hydrophobic, single pass transmembrane domain; a cytoplasmic tyrosine kinase domain; and a series of cytoplasmic tyrosine residues that serve as sites of phosphorylation. The HER2 extracellular domain (ECD) can be divided further into four functional domains (I, II, III, and IV) (8). Domain IV (DIV) is near the transmembrane domain and stabilizes the protein-protein interaction between HER2 and its dimerization partner. Mutations in DIV impair phosphorylation (9) and signaling by heterodimers containing HER2. Nonetheless, the information regarding the role of the C-terminal region of DIV in heterodimerization is controversial (10). Consequently, we have designed peptidomimetics that can bind to domain IV of HER2 and modulate HER2 signaling (11-16). These peptidomimetics inhibit

the protein-protein interaction of EGFR:HER2 and HER2:HER3 heterodimers (14). In order to investigate the binding mode of such molecules on domain IV of HER2, structural elucidation of the HER2 extracellular domain-peptidomimetic complex is important.

Previous findings suggest that the ECD of EGFR family members can be expressed successfully in a number of host systems (17-22). The aim of this project is to express and purify the HER2 ECD to study its binding to various antagonists. We used recombinant S2 (Schneider2) cells for this purpose. S2 cells are derived from primary cultures of late stage embryonic cells of *Drosophila melanogaster* (23, 24). The rationale for using S2 cells for obtaining our protein is that these cells are easy to culture and grow as a semi-adherent monolayer at room temperature without CO₂. Moreover, S2 cells can be directed to secrete recombinant proteins into the culture medium in their native form, making for easy recovery and purification without using denaturing conditions. In contrast, recombinant proteins expressed in *E. coli* are typically recovered following lysis of the *E. coli* under denaturing conditions, which necessitates refolding of the recombinant proteins following recovery. In addition, unlike recombinant proteins expressed in *E. coli*, recombinant proteins expressed in S2 cells will form intramolecular disulfide bonds. This is particularly important for the HER2 DIV, which contains intramolecular disulfide bridges and is structurally divided into seven modules (25-27) (**Figure 1**) that are difficult to reconstitute from a recombinant protein expressed by *E. coli*. As a result of these advantages, S2 cells are being used for a variety of purposes, including the production of vaccine antigens and other heterologous proteins. It is noteworthy that the S2 system may allow for higher yields of these proteins than bacterial and yeast expression systems (28, 29).

Materials and Methods

Reagents

Drosophila Schneider2 (S2) cells, the *Drosophila* Expression System-inducible/secreted kit, calcium phosphate transfection kit, hygromycin, fungizone, L-glutamine, probond nickel-chelating resin and purification columns were purchased from Life Technologies (Grand Island, NY). The Amicon 8400-stirred ultrafiltration apparatus was obtained from Millipore Corp. (Bellerica, MA). Fetal bovine serum (FBS) was obtained from ATCC (Manassas, VA). Schneider insect cell medium powder and copper (II) sulfate pentahydrate were from Sigma-Aldrich (St. Louis, MO). The pMT/BiP/V5-His B expression vector plasmid was from Invitrogen (Life Technologies). HER2 ECD and HER2 DIV genes were custom synthesized by Genscript (Piscataway, NJ). Dialysis cassettes were from Thermo Fisher Scientific (Rockford, IL). Penicillin/streptomycin was bought from Mediatech Inc. (Manassas, VA). Nalgene 0.2 µm rapid-flow bottle top filters were from VWR (Bridgeport, NJ). Antibodies for western (immunoblot) analysis were from Abcam, Inc. (Cambridge, MA) and Santa Cruz Biotechnology, Inc. (Dallas, TX). Novex® 4-20 % tris-glycine gels were obtained from Life Technologies (Grand Island, NY).

Maintenance of S2 cells

S2 cells were grown in Schneider2 insect medium with 10% heat inactivated FBS at 22° C without CO₂. Initially, cells were grown at 28° C. However, after transfection experiments,

cell viability at 28° C was low and at 22° C cell viability of transfected cells was optimum hence, cell culture was carried out at 22° C. When a density of 6 to 20×10⁶ cells/ml was reached, they were subcultured at a 1:3 dilution as described by the supplier (Life Technologies).

Construction of expression vectors

Lyophilized pMT/BiP/V5-His A, B, C vectors were obtained from Life Technologies as a part of Drosophila expression system or (DES®) inducible/secreted kit. The complete extracellular domain of HER2 gene was subcloned into the pMT/BiP/V5-His vector using strategies described in earlier publications [17,22]. HER2 ECD/DIV was custom synthesized and subcloned it into the pUC57 plasmid by Genscript (Piscataway, NJ). Following amplification of the plasmid, the HER2 ECD/DIV gene was subcloned into pMT/BiP/V5-His vector to generate an expression construct suitable for expression in S2 cells.

Transfection

The calcium phosphate transfection kit was obtained from Life Technologies and the supplier's protocol was adopted for transfecting S2 insect cells. Cells were cotransfected with the pCoHygro selection vector and selected with hygromycin to establish stable cell lines. An expression vector to selection vector ratio of 19:1 (w/w) was used as per the supplier's protocol. Briefly, 35 mm plates were seeded with 3×10⁶ cells in 3 ml medium and incubated at 22° C. After 12 h, the cells had entered log phase growth and were used for transfection. Solution A of the transfection mix was prepared by mixing 2 M calcium chloride, the pMT/BiP/V5-His (expression) vector, and the selection vector in tissue culture sterile water. Transfection solution B was prepared from 2X HEPES buffered saline (50 mM HEPES, 1.5 mM Na₂HPO₄, 280 mM NaCl, pH 7.1). Solution A was added drop wise slowly to solution B with vigorous mixing and incubated at room temperature for 40 min to allow for the calcium phosphate-DNA precipitates to form. This mixture was added to cells and swirled to evenly distribute the precipitate. After incubating for 24 h, the cells were washed twice with S2 medium to remove the calcium phosphate precipitate. The cells were replated in the same plates in fresh medium and allowed to grow for two days. On day four, copper sulfate (500 µM final concentration) was added to the medium to induce the expression due to pMT (metallothionin promoter) for 24 h. The medium was replaced with medium containing hygromycin at 300 µg/mL. The selection was continued for four weeks to obtain stable transfectants. pMT/BiP/V5-His/GFP was used as a positive control.

Cell expansion and protein expression

S2 stock cultures were maintained in T-25 flasks at 22° C without CO₂. Once the stock culture density reached 1×10⁷ cells/mL, a 250 mL Erlenmeyer flask with 50 mL S2 (cells were grown in Schneider2 insect medium with 10% heat inactivated FBS at 22° C without CO₂) medium was inoculated with 5 mL of the stock culture. From this point forward, the cells in flasks were incubated with continuous shaking at 22° C. When the density of the 50 mL culture reached 1×10⁷ cells/ml, the culture was expanded to 300 mL in a 500 mL Erlenmeyer flask. Similarly, once the density of the 300 mL culture reached 1×10⁷ cells/mL, the culture was expanded to 1000 mL in a 1 L Erlenmeyer flask. Once the density of the 1000 mL culture reached 1×10⁷ cells/mL, the cells were recovered by centrifugation and

resuspended in 700 mL serum free media in a 1 L Erlenmeyer flask. Cells were incubated at 22° C for five days in medium supplemented with copper sulfate to induce protein expression. The cells were recovered by centrifugation of the cell suspension and the conditioned medium supernatant (which contains the secreted recombinant protein) was retained. The supernatant was passed through a 0.2 µm bottle top filter to remove cell debris or other solids.

Ultrafiltration

The conditioned medium was concentrated using stirred cell ultrafiltration with a 5000 MW cutoff membrane to retain the secreted protein in the concentrated retentate. Ultrafiltration was performed at 4° C with continuous stirring under 60 psi N₂. The concentrated protein solution (about 10-15 mL) was collected after approximately 2 h of filtration.

Nickel affinity chromatography

A 1.5×15 cm column was packed with 4 mL of probond nickel-chelating resin and equilibrated with 10 column volumes (40 mL) of 20 mM phosphate buffer pH 7.8 and 500 mM NaCl. The protein solution was loaded onto the column and allowed to bind for 60 min. The column was washed with 10 volumes (40 mL) of 20 mM phosphate buffer pH 7.8, 500 mM NaCl and 40 mM imidazole. The protein was eluted using a solution of 500 mM NaCl and 250 mM imidazole in 20 mM phosphate buffer, pH 7.8.

Dialysis

Slide-A-Lyzer cassettes with a 3500 MW cutoff were used to dialyze the protein sample. The cassettes were hydrated for 5 min in a 2 L plastic beaker filled with PBS. The protein was injected into cassettes using a 10 mL disposable syringe with 20-gauge needle. With the help of a buoy, the cassettes were allowed to float in the beaker atop the PBS. Samples were dialyzed for 24 h at 4° C with continuous stirring and buffer changes every 8 h. The dialyzed protein solution was recovered using a syringe and stored at -80° C for further analyses.

Western blot analysis

The concentrated protein was mixed with 2X protein sample buffer, boiled in a water bath for 5 min and loaded on 4-20 % polyacrylamide gel. Following electrophoresis for 1 h, the protein was electro blotted onto a nitrocellulose membrane. The blot was blocked using 2 % bovine serum albumin in TBST, then probed with an antibody that recognizes the hexahistidine tag of the recombinant HER2 ECD protein. Primary antibody binding was detected using a secondary antibody conjugated with HRP and enhanced chemiluminescence (Pierce, Rockford, IL). The blots were digitized using a C-Digit Blot Scanner (LI-COR Biotechnology, Lincoln, NE).

Mass spectrometry

6200-TOF LC-MS (Agilent) was used to detect HER2 ECD protein and MALDI TOF was used to identify the molecular peak for DIV protein. At least 50 scans were summed up to generate mass spectrum. Data was processed using Mass Hunter qualitative software (B. 02.00) at the Mass Spectrometry Facility, LSU, Baton Rouge.

Sequence analysis

Protein samples were resolved by electrophoresis essentially as described earlier. Protein bands were visualized by Coomassie staining using a reagent (Gelcode) that possesses a sensitivity of approximately 25 ng protein. After washing, molecular weight markers resolved on the same gel were used to identify the recombinant protein of interest and the band was excised using a scalpel. The band was stored in a microcentrifuge tube at -80°C until needed.

Each protein gel band was subjected to in-gel trypsin digestion as follows. Gel slices were destained in a solution containing 50 % methanol (Fisher) and 100 mM ammonium bicarbonate (Sigma-Aldrich). The protein was reduced using 10 mM Tris[2-carboxyethyl]phosphine (Pierce) and alkylated using 50 mM iodoacetamide (Sigma-Aldrich). Gel slices were dehydrated in acetonitrile (Fisher), after which the protein was digested at 37°C for 12-16 hours using 100 ng porcine sequencing grade modified trypsin (Promega) in 100 mM ammonium bicarbonate (Sigma-Aldrich). Peptides diffuse out of the gel during the 12-16 hour digestion period. Peptide products were then acidified in 0.1 % formic acid (Pierce) to dissolve the products and the peptide solutions were transferred to a 96-well plate for LC-MS/MS analysis. Tryptic peptides were resolved by reverse phase Jupiter Proteo resin (Phenomenex) on a 100×0.075 mm column using a nanoAcquity UPLC system (Waters). Peptides were eluted under the following conditions. Buffer A = 0.1 % formic acid, 0.5 % acetonitrile; buffer B = 0.1 % formic acid, 75 % acetonitrile. Gradient from 97:3 to 35:65 buffer A:B ratio and duration 40 min. Eluted peptides were analyzed by MS/MS analysis using collision-induced dissociation on an LTQ Orbitrap Velos mass spectrometer (Thermo). MS data were acquired using the FTMS analyzer in profile mode at a resolution of 60,000 over a range of 375 to 1500 m/z. MS/MS data were acquired for the top 15 peaks from each MS scan using the ion trap analyzer in centroid mode and normal mass range with normalized collision energy of 35.0. Proteins were identified by database search using Mascot (Matrix Science). Proteins were also identified by database search using Peaks Server v7.0 (30).

Circular dichroism studies

CD spectroscopic analysis was performed on JASCO J-815 CD spectrometer. The purified protein sample was added to a 1 mm cuvette. Analyses were performed at 190-300 nm wavelengths with eight accumulations per sample. The wavelength vs. CD ellipticity spectra obtained were analyzed using spectral analysis software. For stability studies protein was incubated in CD cuvette at 21°C . Spectra was acquired at 0, 1, 3, 6, 10, 24 and 30 hrs of incubation period. The baseline obtained with solvent has been subtracted. Proteins purchased from Abcam were used as positive controls for comparison.

Results and discussion

Transfection of the expression vectors was confirmed by fluorescence microscopy of the GFP transfected control cells (**Figure 2**). The standard temperature for S2 cells is $27-28^{\circ}\text{C}$ (31). However, at this temperature the S2 cells transfected with HER2 expression vector grew slowly. Therefore, we determined experimentally that 22°C is optimal for cell growth

(data not shown). Enhanced production of proteins using non-standard cell culture conditions, including propagation at 22 °C has been reported in the literature (32). The crude protein secreted into the medium of S2 cells was concentrated by ultrafiltration and purified using ion exchange chromatography followed by dialysis. Immunoblot analyses of the samples using anti-HER2 and anti-hexahistidine antibodies revealed bands at approximately 65 kDa for the HER2 ECD and 20 kDa for the DIV protein (**Figure 3A and B**). For HER2 protein domain IV, SDS-PAGE analysis was performed with different metal beads to evaluate which one of the metal beads is ideal for purification of the protein. Nickel beads yielded products of greater yield and apparent purity and hence were determined to be the preferred bead for purification by protein band intensity.

Deconvoluted mass spectral peaks obtained for each purified protein are shown in **Figure 4A**. A molecular ion peak at ~66 amu (66,431 Da) was obtained for the HER2 ECD protein and a doubly charged ion was observed at 8668 m/z (17,336 Da) for the DIV protein (**Figure 4B**) (33). Complete sequence analysis of each protein was carried out by analyzing PAGE-purified protein bands using tandem mass spectrometry with CID (collision induced dissociation) fragmentation and data dependent acquisition (34). The peptides were identified at 1 % FDR (false discovery rate) using the target-decoy method. The spectra were analyzed using MASCOT and Peaks Server v 7.0. Both search engines identified identical proteins in a particular sample. Therefore, here we report the results only from the Peaks Server. For the 65 kDa putative HER2 ECD protein, the top hit is tyrosine protein-kinase receptor ERBB2 (HER2/Neu; **Uniprot id: P04626**). The ERBB2 sequence was identified with 38 unique spectra, 23 unique peptides and 76 total spectra.

Figure 5 shows representative spectra for this identification. **Figures 5A and 5B** show the spectra representing the first peptide identified from the N-terminal of the protein. **Figure 5C and 5D** show the spectra representing the last peptide identified near the C-terminus of the protein. The spectra representing the N-terminus and the hexahistidine-tagged C-terminus were not observed. The spectra in **Figure 5** are high quality with the majority of high intensity peaks annotated. These peaks identified the 65 kDa putative HER2 ECD protein as being ERBB2 (HER2/Neu) with a protein identification probability of 100 %.

For the 20 kDa putative DIV protein band, three unique peptides, four unique spectra and six total spectra were identified corresponding to those of HER2 domain IV. **Figure 6A** is a spectrum of the first peptide identified from N-terminal, while **Figures 6B and 6C** are spectra of peptides identified near to the C-terminal of the domain IV. CID fragmentation primarily generates b and y ion by breaking the amide bonds in peptides. High coverage of b and y ions in the spectra (**Figures 5 and 6**) and successful annotation of high intensity peaks give high confidence to the identification of the peptides and proteins. The red peaks in the spectra are y ions and the blue peaks are b ions.

The secondary structure of the HER2 ECD protein was confirmed by circular dichroism analysis, which exhibited a positive peak at 195 nm and negative peaks at 208 nm and 222 nm (**Figure 7A**). The peak around 222 nm was smaller in intensity compared to 208 nm, suggesting the small percentage of α -helical structure in the protein and a mixture of sheets and turns in the protein structure (35). The stability of the HER2 ECD secondary structure

was analyzed at room temperature by incubating the protein in the buffer for different time intervals and analyzing the CD spectra of protein at different time points. The overall CD spectra of the HER2 ECD protein did not change upon incubation at room temperature for 30 hrs, as shown by the overlapping spectra obtained at different time points (**Figure 7B**). The CD spectra of the DIV protein had a negative band around 202 nm and a positive band around 190 nm, suggesting the absence of α -helical structure and a structure consisting mostly of β -strand/ β -turn and loop structures (36) (Supporting information). These results are consistent with **Figure 1**, which illustrates that domain IV of HER2 consists of disulfide loops and turns.

The HER2 protein is overexpressed in different cancers such as gastric, breast, ovarian and levels of HER2 correlate with the aggressiveness of cancer. Expression of such protein, which is pathologically relevant, is of tremendous significance. It is possible to study the structural and functional aspects of the protein if it is readily available. This would unveil the mechanistic role of HER2 protein in passing the extracellular signals into the cells.

Most recombinant proteins are expressed in *E. coli* cells because of the greater yield afforded by this strategy. However, this is not an ideal choice for eukaryotic proteins in which posttranslational modifications and secondary structure are important, as prokaryotic cells typically cannot recapitulate the posttranslational and folding machinery present in eukaryotic cells. A new *E.coli* strain has been developed recently to enable abundant expression and authentic folding of proteins that possess intramolecular disulfide bonds (37). Mammalian cells are another option for protein expression. However, mammalian systems are complex and it is difficult to purify the desired protein from a mixture of a thousand other proteins. Moreover the denaturing conditions involved in purification from mammalian cells may alter the secondary structure and function of the recombinant protein. Although the Drosophila S2 cells do not allow all of the posttranslational modifications that occur in mammalian cells (38,39), they are capable of secreting the protein with correct disulfide linkages. Here, the term posttranslational modifications is used in a sense to indicate the correct protein folding achievable with drosophila system analogous to disulfide bonding posttranslational modifications in mammalian cells. Because the HER2 DIV protein is both very flexible protein and comprised of numerous intramolecular disulfide bonds, S2 cells are the ideal choice for expressing and purifying this protein.

Immunoblotting using an anti-HER2 or anti-hexahistidine antibody detected bands of the expected molecular weight for the HER2 ECD protein or the DIV protein. The intensity of the band increased with the concentration of the DIV protein. Consistent with these results, mass spectrometry results proved the successful expression of HER2 ECD and DIV proteins. CD spectra of the proteins showed a similar pattern compared to that of control protein samples suggesting the secondary structure is intact. In the future, we would like to investigate the effect of our compounds on protein-protein interactions. This involves the use of the recombinant proteins purified in this work in NMR binding studies for a long period of time at room temperature. Hence, establishing the stability of secondary structure of protein at room temperature is very important. The analysis of the stability was done using CD instrument and the results suggest that the sample is stable at room temperature even after 30 h.

Conclusion

In conclusion, the HER2 ECD and DIV proteins were successfully expressed and purified in S2 insect cells. Since the method of purification does not involve protein-denaturing conditions, the secondary structure of the proteins remains in the native form and is stable at room temperature >30 h. Hence, a novel insect cell based method for expression and purification of HER2 ECD and DIV proteins has been developed in this project.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

HER2 protein extracellular domain and domain IV were expressed in S2 insect cells. Proteins were secreted into the medium in fully folded form.

Proteins were purified and stability was analyzed by circular dichroism spectroscopy.

Sequence analysis was done by tandem mass spectrometry.

Sequence information was obtained from collision induced dissociation fragmentation.

Figure 1.

Crystal structure of HER2 extracellular DIV (25). Disulfide bonds are shown as dark sticks. Loops of DIV are stabilized by the disulfide bonds and the structure of these loops in domain IV are structurally divided into seven modules (**PDB ID: 3N85**).

Figure 2.
20 × image of S2 cells transfected with GFP expression construct showing green fluorescence. This indicates a successful transfection of insect cells.

Figure 3.

A) Western blot analysis of concentrated HER2 extracellular domain protein using anti-HER2 antibody (lanes 1 and 2 are the duplicates of the same protein and lane 3 represents protein ladder for molecular weight). Bands were detected at ~65 kDa. **B)** SDS-PAGE analysis showing western blot bands at 15-20 kDa with polyhistidine antibody characteristic of DIV protein. *Lane 1:* Marker/Ladder, *Lane 2:* Cobalt eluted, *Lane 3:* Copper eluted, *Lane 4:* Nickel eluted, *Lane 5:* Zinc eluted, *Lane 6:* Cobalt unbound (wash), *Lane 7:* Copper unbound (wash), *Lane 8:* Nickel unbound (wash), *Lane 9:* Zinc unbound (wash), *Lane 10:* Crude protein.

Figure 4.

Deconvoluted mass spectral peaks for purified proteins. **A)** Mass peak observed at 66,431 amu corresponding to HER2 ECD protein. Sample was analyzed on 6200-TOF LC-MS (Agilent). At least 50 scans were summed up to generate mass spectrum. Data was processed using Mass Hunter qualitative software. **B)** Mass spectrum of protein HER2 DIV without fragmentation. Peak for doubly charged ion peak observed at 8668 amu corresponds to HER2 DIV protein. Sample was analyzed on 6200-TOF LC-MS (Agilent). At least 50 scans were summed up to generate mass spectrum. Data was processed using Mass Hunter qualitative software.

Figure 5. Analysis of the sequence of protein receptor tyrosine kinase ERBB2 (HER2 ECD) by tandem mass spectrometry. Peptide fragments (**A & B**) near N-terminal and peptide fragments near the C-terminal (**C & D**).

Figure 6.

Analysis of the sequence of protein Receptor Tyrosine Kinase ERBB 2 (HER2) domain IV by mass spectrometry. Peptide fragments near N-terminal (**A**) and, peptide fragments near the C-terminal (**B & C**).

Figure 7.

A) CD spectra obtained for the protein samples analyzed after purification indicating the presence of pure HER2 ECD protein; **B)** Stability of HER2 ECD protein at 21° C monitored using CD at different time points. No changes in spectra were observed even after 30 h.