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Methods for preparation of low abundance glycoproteins from mammalian cell supernatants

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Received 24 November 2005; received in revised form 20 May 2006; accepted 24 May 2006

Available online 2 June 2006

Abstract

Proteins secreted to mammalian cell supernatants are usually in a low concentration and purity, due to the limitation of the expression systems or the presence of a large amount of contaminant proteins from the cell medium. So, initial protein recovery from cell supernatants requires of a highly specific chromatography step. We compared several purification methods based on affinity chromatography for purification of proteins from cell culture supernatants: metal chelate affinity, strep-tag and immunopurification with a monoclonal antibody. Soluble receptor glycoproteins were engineered with the corresponding peptide tag at their C-terminal end. The proteins were expressed in 293T cells and secreted to the cell supernatant, as monitored by sandwich ELISA. Supernatants were run through the different chromatography columns and several purification-related parameters determined. While all column-retained proteins were easily eluted, the chelating and immunopurification chromatography gave the highest yield and the latest method provided a sample with the highest purity. So, in spite of its cost, immunopurification chromatography gave optimal results for purification of a low abundance protein from a cell supernatant. Finally, we applied a protein expression system together with immunopurification chromatography for preparation of a glycoprotein for crystallization.

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Keywords: Glycoproteins; Affinity chromatography; Crystallography

1. Introduction

Glycoproteins from cellular and viral membranes are usually composed of cytosolic, transmembrane and extracellular regions. In many cases the extracellular regions are heavily glycosylated in the process of synthesis, which is required for proper protein folding and function. Therefore, the production of biologically functional glycoproteins requires of an expression system that assures a correct glycosylation. Structural studies of glycoproteins must be then carried with glycosylated sample proteins.

The complexity of the glycosylation linked to a protein can prevent its crystallization and the subsequent determination of the protein structure at high resolution. This problem can be reduced by decreasing both glycan complexity and heterogeneity. The preparation of glycoproteins suited for crystallization can be achieved by the use of expression cell lines with restricted glycosylation pattern, as the mutant CHO (chinese hamster ovary) Lec 3.2.8.1 (CHO-Lec) [1–3]. In this cellular line glycosylation pattern is simplified in high-mannose chains, composed by two N-acetylgalactosamines and five mannose residues, which can be fully eliminated by treatment with the endoglycosidase-H enzyme (endo-H). This enzyme hydrolyzes the chemical bond between the N-acetylgalactosamines residues located next to Asn of the glycoprotein, leaving only one residue attached to the glycoprotein. Once the CHO-Lec cell line is stably transfected, cells clones can be selected with good levels of native protein secretion to the cellular medium [4]. However, the glycoprotein of interest represents a low percentage of the total proteins in the cell supernatant and has low concentration, so that its purification requires of a highly specific and efficient initial step.

We have compared several affinity purification methods for isolation of low abundance protein from cell supernatants, monitoring the following parameters: yield, purity of sample, protein elution from the resins and costs. We found that immunopu-
2. Materials and methods

2.1. Cloning and transient expression of ICAM-1 variants in 293T cells

Two recombinant cDNAs coding for the N-terminal two domains (residues 1–190) of intercellular adhesion molecule-1 [5] (ICAM-1 or IC1) followed by 6 His residues (IC1-His) or the strep-tag (IC1-stII) epitopes were engineered by PCR using Phusion polymerase. The IC1-His cDNA was prepared using the primers T7 (AAATACGACTCACTATAG) and IC1-His (CTCTGGTGCCCCGTGGATCCCATCATCATCATCACATTCGACCGCGGCG), while for IC1-stII we used T7 and the IC1-stII primer (CTCGAGACTCACTATAG) and IC1-His (CTCTGGTGCCCCGTGGATCCCATCATCATCATCACATTCGACCGCGGCG), both fragments were cloned into unique Sal I and Not I sites engineered in the vector pEF-BOS (Amersham Biosciences). The fidelity of the constructs was determined by sequencing. The recombinant DNA vectors were transfected into 293T cells using the calcium phosphate method [7]. Presence of soluble glycosylated IC1 proteins was monitored by sandwich ELISA about 3 days post-transfection. The size of the IC1 protein was determined by immunoblot and the ECL detection system (Amersham Biosciences).

2.2. Cloning and stable expression of the S glycoprotein of TGEV in CHO cells

A recombinant cDNA coding for the N-terminal 616 residues of the spike (S) protein of the TGEV- HOL87 coronavirus (CoVSH1) followed by a thrombin recognition site and a C-terminal Flag epitope (DYDDDDK) was prepared by PCR. The fragment was cloned into unique Sal I and BamHI sites of a modified pEF-BOS vector. The cDNA fragment with a translation stop codon at the 3' end was subcloned into the pBIS-GS vector, which has the glutamine synthetase gene [4]. Linearized recombinant vector was used for transfection of the lecit resistant CHO-Lec cells with the calcium phosphate method [7]. Presence of soluble glycosylated IC1 proteins was monitored by sandwich ELISA about 3 days post-transfection. The size of the IC1 protein was determined by immunoblot and the ECL detection system (Amersham Biosciences).

2.3. Protein purification from cell culture supernatants by metal chelate affinity chromatography

The anti-ICAM-1 monoclonal antibody R6.5 coupled to sepharose 4B (1 ml, Amersham Biosciences) was used for immunopurification of soluble IC1 proteins. Cellular supernatants with IC1-stII or IC1-His (50 ml) were run through the streptactin column. Next, the column was washed with 100 ml of W-buffer having 5 ml of W-buffer having 2.5 mM desethylthiobiotin. Finally, the column was regenerated with W-buffer containing 1 mM HABA (2-(4-hydroxyphenyl)benzoic acid).

2.4. Protein purification from cell culture supernatants by immuno-affinity chromatography

The cellular supernatant (4 l) was loaded through a column with the anti-flag M2 antibody (SIGMA) coupled to agarose beads. The column was washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 8.4) and the retained protein was eluted with TEA-saline buffer (50 mM triethylamine and 150 mM NaCl, pH 11.0) and neutralized with 1 M Tris buffer, pH 6.0. The column was equilibrated with Tris-saline buffer pH 7.4 and stored in the presence of 0.025% sodium azide.

2.5. Protein purification from cell culture supernatants by immuno-affinity chromatography

The cellular supernatant (4 l) was loaded through a column with the anti-flag M2 antibody (SIGMA) coupled to agarose beads. The column was washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 8.4) and the retained protein was eluted with TEA-saline buffer (50 mM triethylamine and 150 mM NaCl, pH 11.0) and neutralized with 1 M Tris buffer, pH 6.0. The column was equilibrated with Tris-saline buffer pH 7.4 and stored in the presence of 0.025% sodium azide.

2.6. Purification of the S protein from cell culture supernatants

The cellular supernatant (4 l) was loaded through a column with the anti-flag M2 antibody (SIGMA) coupled to agarose beads. The column was washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 8.4) and the retained protein was eluted with glycine buffer (50 mM glycine, 150 mM NaCl, pH 3.0), neutralized with 1 M Tris buffer, pH 8.0. Protein fractions were concentrated and in some cases treated with endo-H for removal of glycosylation. A second ion exchange purification step was done with a mono Q column (HiTrap, Amersham Biosciences). The protein was eluted by a NaCl concentration gradient and at a salt concentration near 250 mM.

2.7. S protein crystallization

The S protein was concentrated (15–20 mg/ml) and mixed with a precipitant solution in different volume ratios (1:1, 2:1,
Crystallization was carried by the vapor diffusion method using hanging drop protocols.

3. Results and discussion

A soluble protein fragment with the N-terminal two domains of intercellular adhesion molecule-1 (ICAM-1 or IC1) [8] was used for testing the different purification methods. A well established and highly sensitive ELISA assay has been used for monitoring the IC1 protein in solution, using purified protein as standard for determination of the protein concentration. We carried IC1 purification from cellular supernatants with three different methods, all of them based on affinity purification: Chelating, Strep-tag and Immunoaffinity. IC1 proteins having the required tags at the C-terminal end were expressed in 293T cells, so proteins were glycosylated and secreted to the extracellular medium. After 3 days of expression the concentration of the IC1 variants in the cell medium was around 1–2 μg/ml.

3.1. Chelating chromatography purification

The chelating chromatography method is based on the formation of a cationic aminated complex between histidine side chains and divalent cations attached to chelating groups coupled to sepharose beads. We expressed an IC1 protein with six histidine residues at the C-terminal end (IC1-His) in 293T cells. The cell supernatant with the IC1-His protein was loaded onto the chelating column as described in Section 2.

Determination of the protein in the supernatants before and after running through the chelating column showed that about 95% of the protein was retained (Fig. 1A). The column-bound IC1-His protein was eluted by increasing concentrations of imidazol (10, 20, 100, 250 and 500 mM) in PBS. The protein began to elute with 250 mM imidazol, while the peak of the protein elution was at a concentration of 500 mM imidazol (Fig. 1B). The purity of the fractions was determined by SDS-PAGE (Fig. 1C). Fractions corresponding to the protein elution from the column had the IC1-His protein, contaminated with serum proteins present in the cell supernatant. So, even though the chelating chromatography method gave a high yield, the purity of the protein sample was low.

3.2. Strep-tag chromatography purification

The Strep-tag affinity method is based on the high affinity binding of a mimotope (WSHPQFEK) to a streptavidin mutant called strep-tactin [9]. An IC1 protein with the strep-tactin binding peptide at the C-terminal end (IC1-stII) was expressed in 293T cells and purified by Strep-tag affinity chromatography (see Section 2).

The yield of the strep-tactin column was very low, with only 30% of the total protein present in the cellular supernatant being retained (Fig. 2A), in spite of an expected column capacity of 3 mg of protein. To investigate whether the cause of this low yield could be related to the presence of some binding inhibitor in the cellular supernatant, IC1-stII was first purified on immunoaffinity column (see below), and subsequently loaded onto the strep-tactin column. We observed again a very similar yield, around of 30% (data not shown), confirming the low retention properties of the column. The column-retained protein was easily eluted (Fig. 2B) and fractions containing IC1-stII did not show contaminant proteins from the cellular supernatant.
medium (Fig. 2C). The low yield suggested a slow association of IC1-stII to strep-tactin in spite of its expected high affinity.

3.3. Immunoaffinity chromatography purification

The immunoaffinity chromatography we used was based on the anti-IC1 monoclonal antibody R6.5, which has a high affinity for an epitope covering the N-terminal two domains of the receptor molecule. The antibody provides a very high binding specificity. We coupled the R6.5 monoclonal antibody to Sepharose for immunoaffinity purification of the soluble IC1-His or IC1-StII proteins. Using 1 ml antibody column we were able to retain about the 85% of the IC1 protein present in the cellular supernatant of 293T cells (Fig. 3A). The protein was efficiently eluted from the column with high pH (11.0) buffer (Fig. 3B). The purity of the eluted protein was also very high, as shown by PAGE analysis of the elution fractions (Fig. 3C).

We concluded that immunoaffinity chromatography combined both high yield and purity of the protein sample. So that affinity purification with a monoclonal antibody appears the most suitable methodology for the preparation of low abundance proteins from cell culture supernatants.
3.4. Production, purification and crystallization of a heavily glycosylated viral glycoprotein

We applied an immunopurification methodology for preparation of a viral glycoprotein for crystallization. A fragment of the spike protein of a porcine coronavirus (CoVSH1) with 16 N-linked glycosylation sites was expressed in the lectin resistant CHO-Lec cells using the glutamine synthetase expression system, as described in Section 2. The CoVSH1 has a C-terminal Flag tag recognized by the monoclonal antibody M2 (SIGMA).

Four liters of cell supernatant with the CoVSH1 protein were loaded onto an M2-agarose column (10 ml, SIGMA). About 95% of the protein was retained in the column, as revealed by a sandwich ELISA (not shown). The purity of protein in the elution fractions was tested by PAGE (Fig. 4A). Some protein impurities were detected, but a highly pure CoVSH1 protein was eluted in this first step of purification. Next, fractions were concentrated up to 3 ml and the glycoprotein digested with endo-H (Fig. 4B). The complete removal of the glycans by the enzyme probed it contained just high-mannose carbohydrates and that it was highly homogeneous. The molecular weight of the protein sample after the digestion was 70 kDa, similar to the expected value of 67 kDa. A second ion exchange purification step was sufficient to have a pure protein sample suitable for crystallization (Fig. 4C). The sample (5 mg) was concentrated up to 20 mg/ml for crystallization.

Crystallization trials were carried out by the vapor diffusion method with fully glycosylated and endo-H treated CoVSH1 protein. About 200 commercial crystallization conditions were tested and protein crystals were raised with the endoglycosidase treated sample (Fig. 5A), while the glycosylated protein...
did not crystallize. The crystals had a cube-like shape with size around 0.5 mm × 0.5 mm × 0.5 mm, although they diffracted to low resolution (Fig. 5B).

We can then conclude that mammalian expression in cell lines with restricted glycosylation pattern combined with immunopurification protocols is a highly efficient methodology for preparation of glycoproteins for structural studies.

Acknowledgements

This work was supported by Grants from the Ministerio de Educación y Ciencia of Spain (BIO2002-03281 and BFU2005-05972) to J.M.C. D.O. was supported by fellowships from UAM, CSIC (I3P) and the Ferrer Foundation for Research.

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