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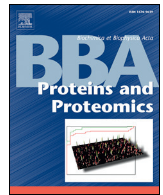

Immuno-proteomics: Development of a novel reagent for separating antibodies from their target proteins ☆

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ABSTRACT

Immunoprecipitation (IP) is a widely used technique for identifying the binding partners of the target proteins of specific antibodies. Putative binding targets and their partners are usually in much lower amounts than the antibodies used to capture these target proteins. Thus antigen identification using proteomics following IP is often confounded by the presence of an overwhelming amount of interfering antibody protein. Even covalently linking antibodies to beads is susceptible to antibody leaching during IP. To circumvent this interference, we describe here a reagent, called Biotin-CDM that reversibly tags all potential target proteins in a cell lysate with biotin. The presence of biotin coupled to the target proteins allows for a secondary separation step in which antibodies are washed away from the reversibly biotinylated target proteins by binding them to an Avidin-coupled matrix. The captured target proteins are released from the Avidin matrix by reversing the Biotin-CDM link, thus releasing a pool of target proteins ready for further proteomic analysis compatible with 2D-electrophoresis. Here, we describe the synthesis and characterization of Biotin-CDM. We also demonstrate Biotin-CDM's use for immunoprecipitation of a known antigen, as well as its use for capturing an array of proteins targeted by the autoantibodies found in the serum a patient suffering from rheumatoid arthritis. The use of this reagent allows one to combine immunoprecipitation and 2D-Difference gel electrophoresis, overcoming the current limitations of Serological Proteome Analysis (SERPA) in discovering autoantigens. This article is part of a Special Issue entitled: Medical Proteomics.

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1. Introduction

Immunoprecipitation (IP) is a ubiquitously used method in biomedical research where antibodies are first bound to a solid matrix, such as Protein-A or Protein-G beads, and then used to capture the antibodies' target proteins from cellular lysates or bodily fluids. An extension of IP is co-immunoprecipitation (co-IP), which is commonly used to capture the binding partners of target proteins via their binding to previously characterized antibodies. Co-IP is primarily used to explore biological processes such as cell signaling and regulation by studying protein-protein interactions. Probing human cell proteome using patient sera referred to as Serological Proteome Analysis (SERPA) is very useful for identifying therapeutic and diagnostic targets [7]. This approach uses 2D-western blots to identify autoantigens in the case of cancer and autoimmune diseases [3]. Sample containing autoantigen is run in three identical 2 dimensional electrophoresis (2DE) gels. Two gels are probed in western blot using healthy and patient anti-sera respectively. Using the western blot results as a map, the corresponding protein spots are cut from the third gel. First of all, there is a considerable amount of gel-to-gel variability between 2DE experiments. Secondly, low-abundant autoantigens suffer from poor identification using a cut-map from a different 2DE gel. Although multiple strategies have been developed [2] to address these issues in identifying autoantigens using SERPA, lack of reproducibility and inability to identify low abundant proteins persist as severe limitations to this approach. Immunoprecipitation enriches low abundant proteins and would be the ideal tool for identifying antigens. Unfortunately, proteomic analysis of immunoprecipitated samples also has very significant limitations.

Proteomic analysis of IP and co-IP target proteins is often complicated by the presence of variable amounts of immunoglobulins and other background proteins derived from the anti-sera or the target-containing cellular extracts. The background proteins are proteins that bind non-specifically to the antibody or anti-sera beads [6]. Addition of competing proteins such as BSA helps reduce non-specific proteins from binding the antibody beads. Increasing the salt concentration, number of washes and adding detergent to the wash steps help in reducing the non-specific proteins from binding [5]. These are simple solutions to the non-specific protein problem.

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However, the larger problem is the release of abundant immunoglobulins from the affinity beads, which can eclipse the detection of low-abundance target proteins. Generally, high concentrations of denaturants, such as urea or SDS, are used to elute proteins bound to antibodies [5]. Under these strong denaturing conditions, antibodies leach from the resin, contaminating the eluted sample [19]. This poses a serious problem for mass spectrometry (MS)-identification of low abundance target proteins. Currently, there is no simple, generic solution to eliminate antibodies from the eluate. Typical solutions to this problem are to either covalently cross-link the antibodies directly to the polymer bead matrix or to covalently cross-link the antibodies to Protein-A/G beads, via protein-protein crosslinking. Directly conjugating antibodies to a resin results in the random orientation of the antibodies on the matrix, potentially reducing the efficiency of the antibody–antigen interaction. Also, only purified antibodies can be directly linked to the resin, limiting one's options when working with patient anti-sera. Cross-linking of the antibodies to Protein-A/G beads can be a tedious process that requires optimization for each serum or antibody used. Over cross-linking risks losing antibody reactivity, while under cross-linking again can lead to variable amounts of antibody leaching from the beads during target elution. Antibodies and anti-sera are usually precious reagents. One is often loath to expend this limited material optimizing the cross-linking protocol. Since antigen affinity is very specific to each antibody, the cross-linking of each anti-sera to be tested needs to be optimized. This is not practical when dealing with patient sera for SERPA.

Seeking a way to overcome this critical limitation of IP, we describe here a method that allows one to efficiently separate antibodies from their target proteins. Since antibodies are derived from a huge array of anti-sera, which are usually limited in supply, we decided to focus on the source of target proteins. Target proteins are typically derived from commonly used, tissue culture cell lines, which can be grown in abundance, or from tissue samples. While tissue samples may not be as abundant as cultured cell lines, they are certainly more easily obtained than specific anti-sera. To discriminate between the target proteins and the antibodies that bind them, one needs to attach a suitable affinity reagent to the target proteins. Ideally, the affinity tag should be able to function properly under the harsh conditions used to elute proteins from antibody beads. Biotin is arguably the best candidate for such an affinity tag since it binds to Avidin and Avidin derivatives, such as NeutrAvidin, with exceptionally high affinity under quite harsh conditions. The ultimate goal of this method is to subject the isolated proteins for proteomic analysis, which may be affected by the presence of the affinity tag. This is particularly true for two-dimensional electrophoresis (2DE) [12], gel-based proteome analysis methods, such as DIGE [15]. The tag is expected to alter the mass and charge of the targets. To avoid this effect, we designed a reversible, biotin-labeling reagent, called Biotin-CDM. We show here: that Biotin-CDM can be used to extensively tag cellular protein lysates, that these tagged, target-proteins can be captured by their cognate antibodies, that the target proteins can be separated from their cognate antibodies and that the tag can be efficiently removed from the target proteins for 2DE, gel-based proteome analysis.

2. Materials and methods

2.1. Materials

The hydroxysuccinimidyl ester of 2-propionic-3-methylmaleic anhydride (CDM-NHS ester) was custom synthesized by GLSynthesis Inc (Worcester, MA). Biotin was purchased from AK Scientific corporation (Union City, CA). 2,2′-(Ethylenedioxy) diethylamine was purchased from Tokyo Chemical Industry Company of America (TCI America, Portland, OR). The target compound was purified on a Buchi Corp. Sepacore (New Castle, DE), MPLC system and further analyzed by UPLC (Waters Corp., Acquity, Milford, MA). ^{1}H-NMR and COSY experiments were performed on a Bruker Corp. 300 MHz instrument (Billerica, MA). ESI-MS analysis was performed on a Finnigan LCQ (Thermo Fisher Scientific, Waltham, MA). Bovine Serum Albumin, Fraction V (BSA) was purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA) and Alcohol dehydrogenase (ADH) was purchased from Sigma Aldrich Corp., St. Louis, MO). Minimal DIGE dyes Cy3 and Cy5 were obtained from GE Healthcare (Pittsburgh, PA). Amicon Ultra 4 ml 10 K NMWL spin filters were purchased from EMD Millipore (Billerica, MA). High capacity NeutrAvidin was purchased from Pierce (Thermo Fisher Scientific, Waltham, MA). Protein-A Sepharose CL-4B was purchased from GE Healthcare (Pittsburgh, PA). 18 cm pH 3-10NL IPG strips were purchased from BIO-RAD Corp. (Hercules, CA).

N-Boc-2,2′-(ethylenedioxy)diethylamine, *BocNH-2p-NH$_2$* was prepared according to the procedure of Lee et al. [9]. The starting material for this reagent, 1,2-Bis(2-aminoethoxy)ethane was purchased from TCI America Inc. Biotin-NHS ester was purchased from Chem-Impex International,Inc. Diethylisopropylamine was purchased from Aldrich Chemical Company, Inc. Ethyl acetate, methanol, acetonitrile and Dimethylformamide (DMF) were purchased from EMD Millipore. Silicagel 60 Å, Premium R_f was purchased from Sorbend Technologies. All the other reagents were purchased from Fisher Scientific. MPLC chromatography was performed on a Buchi Sepacore system using RP-18 (SMT Bulk-C18) manufactured by Separation Methods Technologies.

To prepare N-Boc-2,2′-(ethylenedioxy)diethylamine, a solution of 150 ml of MeOH with cooling at 0 °C, HCl gas (17 g) was added with stirring for 15 min. The mixture was stirred for 15 min at room temperature and was carefully added to N,N′-bis(aminoethyoxyethane) (67 g, 0.466 mol) at 0 °C. The mixture was stirred for 15 min at room temperature before adding 50 ml of H_2O and stirring for another 0.5 h. To the solution $(BOC)_2O$ (101 g, 0.466 mol) in 200 ml of MeOH was added at room temperature for 10 min, and the resultant solution was stirred for 1 h. The mixture was concentrated in vacuo. Unreacted diamine was removed by diethyl ether (300 ml × 2). The residue was treated with 2 N NaOH (500 ml) solution. The product in the organic layer was extracted with CH_2Cl_2 (300 ml × 3). The combined extracts were washed with 300 ml of brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo to yield 64.6 g (87%) of mono-BOC product as a colorless oil with more than 97% purity by HPLC.

2.2. Chemical synthesis

2.2.1. Synthesis of Biotin-2p-NH$_2$ hydrochloride **1** (Fig. 2)

N-Boc-2,2′-(ethylenedioxy)diethylamine *BocNH-2p-NH$_2$* (992 mg, 4 mmol) was added to biotin-NHS ester (1.36 g, 4 mmol) dissolved in 5 ml of dry DMF. Diethylisopropylamine (0.7 ml, 4 mmol) was added and the reaction mixture was stirred at 50 °C for 2 h. The reaction mixture was concentrated under vacuum. The residue was dissolved in ethyl acetate (70 ml) and washed with 1 M citric acid, water and 1 M bicarbonate. The organic phase was dried over sodium sulfate and concentrated to give 1.28 g (67% yield) of an oily residue.

The TLC (silica gel) showed one spot at Rf = 0.2 (eluent: ethyl acetate/20% methanol) that stained positive for biotin.

Biotin-2p-NH-Boc (1 g, 2.1 mmol) was dissolved in methanol (5 ml) and 1 N HCl (5 ml) was added. The reaction was stirred overnight at room temperature. TLC showed the removal of the Boc group. The reaction mixture was concentrated and the resulting solid used as such in the next reaction step. ESI/positive M^+ 375.3 g/mol.

2.2.2. Synthesis of Biotin-CDM **4**

Biotin-2p-NH2 hydrochloride **1** (2.2 g, 5.3 mmol) was dissolved in 1 M triethylammonium bicarbonate buffer pH 8.5 (10 ml). CDM-NHS ester **2** (0.75 g, 2.65 mmol) dissolved in dry dioxane (5 ml) was added dropwise under stirring at room temperature. Stirring was continued for 1 h. The reaction mixture was concentrated to a volume of 1 ml. 2 N HCl (5 ml) was added to hydrolyze **3** and the reaction mixture was stirred for 30 min. The reaction mixture was separated by MPLC chromatography on a Sepacore system on RP-18 with acetonitrile/water/0.1%TFA as

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