Published in final edited form as:

Methods Mol Biol. 2012 ; 803: 157–165. doi:10.1007/978-1-61779-364-6_11.

Affinity-Based Purification of Dehydrogenase Subproteomes

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Summary

The high cost of drug discovery and development requires more efficient approaches to the identification and inhibition of tractable protein targets. One strategy is to pursue families of proteins that already possess affinity for a drug lead scaffold, where that scaffold plays the dual role of serving: (a) when tethered to a resin, as a ligand to purify a subproteome of interest, and (b) as a lead molecule that has the potential for optimization for a given member of the subproteome. Here, we describe the former application, the purification of a subproteome using a scaffold tailored to the dehydrogenase family of enzymes. Combined with modern LC-MS/MS and subsequent searching of proteome databases, such affinity chromatography strategies can be used to purify and identify any proteins with affinity for the scaffold molecule. The method is exemplified using the CRAA (Catechol Rhodanine Acetic Acid) privileged scaffold, which is tailored to dehydrogenases. CRAA affinity column chromatography, combined with LC-MS/MS, is described as a method for profiling dehydrogenase subproteomes.

Keywords

Dehydrogenase; oxidoreductase; catechol rhodanine; chemical proteomics; subproteome; affinity chromatography; tandem mass spectrometry; drug discovery

1. Introduction

Chemical proteomics aims to develop and apply technologies for the characterization of protein function on a global, proteome-wide scale (1). The completion of the human genome-sequencing project has provided significant information on complex biological systems, and laid the foundation for a comprehensive analysis of protein function *via* chemical proteomics (2). Chemical proteomics can be used to distill this flood of genomic information, to provide useful information about basic cell function, as well as new approaches to disease treatment (3). This approach makes use of small molecule protein ligands that can be used to identify proteins, which might be pursued as drug targets. The strategy of profiling drug targets, by using affinity chromatography coupled to subsequent high-resolution MS and bioinformatic analyses, is becoming increasingly popular as a post-genomic application of chemical proteomics (4). This method allows rapid biochemical analysis and small-molecule screening of drug targets and off-targets (undesired targets), thereby accelerating the target validation process in drug discovery (5–9).

Dehydrogenases comprise ~5% of most proteomes (10, 11), many of which could be important tractable (“druggable”) targets. For example, Isoniazid (INH) binds to multiple dehydrogenases in *Mycobacterium tuberculosis* (12), Epalrestat targets aldose reductase for the treatment of diabetic neuropathy (13, 14), and the statin drugs inhibit HMG-CoA

reductase. Using an NAD(P)-INH affinity column, Argyrou found that Isoniazid, a widely used drug for treating tuberculosis, does not bind to only one enzyme target, but rather binds to multiple dehydrogenases. In fact, this may well be why isoniazid is effective at killing *Mycobacterium tuberculosis* (12). This was effectively a chemical proteomic approach to profiling the isoniazid drug, as a covalent adduct with NADP⁺. While the general strategy of profiling dehydrogenases using cofactor-based affinity chromatography has been pursued for >30 years (15), the ability to readily identify eluted proteins using tandem mass spectrometry, and the application to profiling drugs, is relatively recent. But, the use of NAD(P) as a ligand, either as a scaffold for building a drug or as part of an affinity matrix, is not ideal because of its instability and poor bioavailability, which is why we developed the use of the catechol rhodanine ligand (9, 16, 17). Most recently, Kim *et al.* used a Cibacron Blue F3GA dye affinity column to ligand-specifically elute and identify aldehyde dehydrogenases from *Mycobacterium tuberculosis* (18). These approaches demonstrate that dehydrogenase subproteomes can be purified and analyzed using affinity chromatography (19).

Affinity column chromatography combined tandem mass spectroscopy (MS) provides an especially useful approach to characterizing subproteomes, based on the affinity of the purified proteins for the ligand that is covalently attached to the resin (12, 20). We have developed this method for dehydrogenase subproteome studies using the recently reported CRAA ligand (17). CRAA was designed to be a privileged scaffold for dehydrogenases (20). Using CRAA affinity chromatography, dehydrogenase protein targets can be purified from the larger proteome, based on affinity for the CRAA probe which binds in the NAD(P) (H) binding site. Then, higher affinity and specificity bi-ligand variants of the CRAA scaffold can be constructed, which selectively bind to the desired dehydrogenase drug target(s) (17).

2. Materials

2.1 Chemical synthesis procedures

2.1.1 Preparation of Catechol Rhodanine Acetic Acid

1. Mixture of 9.6 g 3-rhodanine acetic acid and 7.6 g 3,4-dihydroxybenzaldehyde (1.0:1.1)
2. 8.2 g sodium acetate
3. 150 mL acetic acid.

2. Preparation of the NHS (N-hydroxysuccinimide) Active Ester of CRAA (21)

1. 6.22 g of CRAA from the previous step
2. 5.75 g of N-hydroxysuccinimide
3. 20.6 g of N,N'-Dicyclohexylcarbodiimide (DCC)
4. 50 mL of DMSO
5. (0.1–0.2 g of DMAP (4-Dimethylaminopyridine) catalyst.

2.3. Synthesis of CRAA Agarose Matrix (22)

1. Coupling reaction buffer: 600 mL of 100 mM phosphate buffer, pH 10.0 at 7 °C.
2. Quenching buffer: 1 M Tris-HCl buffer, pH 6.5.

2.4. Affinity Chromatography to Purify the Dehydrogenase Subproteome (23)

1. Human liver proteins (Sigma-Aldrich).
2. *M. tuberculosis* H37Rv whole cell lysate (provide by Colorado State University).
3. Buffer A: 25 mM Tris-HCl, 50 mM NaCl, and 0.1% NaN₃, pH 7.8.
4. Buffer B: same as buffer A, except containing 4 mM CRAA, pH 7.8.
5. Novex gels and buffers and SilverQuest® staining kit for SDS-PAGE (Invitrogen).

2.5. Tandem MS Analysis to Identify Dehydrogenases in the Subproteome

1. Polyacrylamide gel mixture: 100 µL of acrylamide/bis (30% T/2.67% C), 2 µL of 10% ammonium persulfate, and 2 µL of TEMED.
2. Proteolysis mix: 20 mM ammonium bicarbonate, pH 8.0, containing 1 µg of trypsin (Promega).
3. Extraction Buffer: 6 M guanidine-HCl in 5 mM potassium phosphate and 1 mM DTT, pH 6.5.
4. LTQ mass spectrometer (Thermo-Fisher) coupled to a Surveyor HPLC system (Thermo Fisher) equipped with a Finnigan Micro AS autosampler, interfaced with an Aquasil C18 PicoFrit capillary column (75 µm × 10 cm) (New Objective).

3. Methods

3.1. Preparation of Catechol Rhodanine Acetic Acid (CRAA)

The first intermediate in the synthesis is Catechol Rhodanine Acetic Acid (5-[(3,4-Dihydroxyphenyl)methylene]-4-oxo-2-thioxo-3-thiazolidineacetic Acid)

1. The components x and y (see Materials) are combined and the reaction mixture was heated to reflux and stirred for 6 hours.
2. After cooling, yellow crystals form. The solution containing the crystals was poured into 150 mL cold water, filtered, and washed extensively with water.
3. CRAA was then crystallized from acetic acid, yielding 12.7 g (82% yield) product with m.p. 328–329 °C.
4. The CRAA product was dried in an oven at 100 °C for 24 hours, and then was ready to use in next step.

3.2. Preparation of the NHS (N-hydroxysuccinimide) Active Ester of CRAA

The second intermediate in the synthesis is the NHS (N-hydroxysuccinimide) Active Ester of CRAA: (5-[(3,4-Dihydroxyphenyl)methylene]-4-oxo-2-thioxo-3-thiazolidineacetic N-Hydroxysuccinimide Ester) (19)

1. Under a N₂ atmosphere, the 50 mL CRAA/NHS reaction mixture was reacted at room temperature overnight, stirring using a magnetic stir plate.
2. The reaction was monitored by thin layer chromatography (using EMD silica gel 60 F₂₅₄ plates developed with chloroform/methanol/acetic acid, 12:3:1 v/v/v), and visualized using a 254 nm UV light (the *R_f* of CRAA is 0.39 and the product's *R_f* is 0.68).
3. The DCU (dicyclohexylurea) was removed by vacuum-filtration, and the NHS-CRAA DMSO solution was used in the next step without further purification.

3.3. Synthesis of CRAA Agarose Matrix

The final product of the synthesis is the CRAA Agarose Matrix: (5-[(3,4-Dihydroxyphenyl)methylene]-4-oxo-2-thioxo-3-thiazolidineacetic ω-Aminohexylagarose Amide) (20)

1. The 50 mL NHS-CRAA ester DMSO solution was added dropwise into 100 mL of ω-aminohexylagarose suspended in coupling reaction buffer.
2. The pH of the reaction mixture was maintained at 10.0 (pH adjusted with 2 M NaOH), and then the reaction was allowed to progress at 7 °C in a refrigerator overnight, stirring using a magnetic stir plate.
3. The next day, 60 mL of quenching buffer was added to the reaction mixture to stop the reaction.
4. Then, 47.7 g of sodium chloride was added to form a final 0.5 M saline solution. The liquid layer was decanted, and the labeled matrix was washed with a large amount of deionized water.
5. About 10 mL of matrix was packed into a 1 cm × 20 cm column for column chromatography.
6. The CRAA-agarose column was washed with a large amount of deionized water before use.

3.4. Affinity Chromatography Purification of a Dehydrogenase Subproteome (21)

1. The CRAA affinity column was equilibrated with buffer A. Washing was done until the eluent was nearly colorless (CRAA is intensely colored).
2. The 0.5 mL protein sample was loaded onto the affinity column and washed with a large amount of buffer A until no protein sample was detected using a Bradford assay (BioRad). The wash buffer volume used was usually 10-fold of the packing volume of the column.
3. The affinity column was eluted with buffer B. The elution buffer volume was 5 times the bed volume of the column.
4. Fractions were collected in 2 mL aliquots, then separated on an SDS-PAGE gel and stained using a SilverQuest[®] kit following the manufacturer's protocol.

5. The entire fraction, or bands extracted from SDS-PAGE gel, can then be subjected to proteolytic digestion with trypsin and MS/MS analysis (see next section).

3.5. Tandem MS Analysis to Identify Dehydrogenases in the Subproteome

1. Pooled fractions, after elution from the CRAA affinity column, were concentrated using a Centricon filter with 10 kDa cutoff (Millipore).
2. Then, 100 μ L of affinity purified protein mixtures were polymerized in the polyacrylamide gel mixture. With this mixture a 15% gel piece was formed.
3. Polymerization was performed in the cap of an Eppendorf tube. The polymerized gel pieces were then transferred to the corresponding Eppendorf tube in 1 mL of 40% methanol and 7% acetic acid and incubated for 30 min.
4. The gel pieces were washed twice in water for 30 min each time while sonicating. Gel pieces were then washed twice in 50% acetonitrile for 30 min each time while sonicating.
5. The gel pieces were then washed twice again, this time in 50% acetonitrile in 50 mM ammonium bicarbonate, pH 8.0. The gel pieces were then dried using a speed vacuum from Savant.
6. To each gel piece was added 200 μ L of the proteolysis mix; this was incubated overnight at 37 °C.
7. Each gel piece with the digested proteins was then extracted twice with 70% acetonitrile in 0.1% formic acid. From this step onward, all water used was MS quality water.
8. Corresponding extracts of each gel were pooled together and dried. To each dried sample was added the Final Extraction Buffer. This was sonicated, and peptides were extracted using a C₁₈ ZipTip from Millipore.
9. Extracted peptides were then collected into an insert in a vial to be used for mass spectrometry and dried in the inserts. To each dried sample was added 5 μ L of 0.1% formic acid in MS water containing 5% acetonitrile. Samples were then ready for mass spectrometry and were injected into the LTQ LC/MS.
10. The MS/MS data were collected and searched against the appropriate subset of the Uniprot database.

4. Notes

- 1 Synthesis of CRAA is a typical Aldol condensation. It is necessary to use flame dried glassware to run the reaction.
- 2 The small excess of 3,4-dihydroxybenzaldehyde can help to convert most rhodanine acetic acid to CRAA and itself can be readily removed by crystallization from acetic acid.

- 3 Synthesis of NHS-CRAA can also be monitored with ^1H -NMR of the reaction of NHS and CRAA in d_6 -DMSO at room temperature; make sure there is at least 30% conversion of CRAA to product.
- 3 The NHS-CRAA active ester can be readily converted to an amide group by reaction with amines. But, the NHS-CRAA ester is moisture sensitive; it should be prepared right before the synthesis of the affinity matrix, and one should keep all glassware dry before it covalently converted to the final affinity matrix.
- 4 Excess NHS-CRAA ester will react with the amine on the Tris buffer, and the product as well as free CRAA can be washed away with a large amount of basic buffer A.
- 5 The CRAA affinity matrix can be stored at 2–8 °C for at least 6 months. The affinity matrix was stored in the 0.5 M saline with 0.02% thimerosal. Avoid using any oxidizing reagents or strong base to rinse column, because CRAA is not stable to these reagents.
- 6 The best pH to incubate and elute dehydrogenases using CRAA is pH 7.8. One needs to balance considerations of stability of proteins at this pH, and the best performance (higher binding affinity) of CRAA at this pH where it is at least partly ionized (the catechol –OH is relatively acidic).
- 7 Depending on the concentration of protein samples, the eluted protein fractions may need to be concentrated before the SDS gel electrophoresis step.
- 8 Affi-Gel 10 (BioRad®) is an NHS activated agarose (or sepharose) bead with 10 carbon spacer, and can be covalently linked to primary amines or hydroxyl groups to form the desired affinity matrix. It is another way to build the affinity column with small molecules which have –NH₂, –OH or –SH group, rather than the –CO₂[–] that is present on the CRAA ligand. Cyanogen bromide activated resins are also useful for attaching ligands that contain amine groups, and resins with epoxide groups can be used for ligands with a range of nucleophilic functional group.

Acknowledgments

We thank Dr. Bassam Wakim for previous assistance with mass spectrometry studies. This work was supported in part by GM085739 (NIH) and shared instrumentation grants S10 RR019012 (NIH) and CHE-0521323 (NSF).

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Fig. 1. Schematic representation of the process whereby a dehydrogenase subproteome is purified using a CRAA-based resin, then characterized using tandem mass spectrometry.

Fig. 2.
Synthesis of the CAA-based affinity resin.