Biochemistry Lab II Midterm

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Possible Purification Schemes for Yeast Alcohol Dehydrogenase (YADH)

Although one of the primary goals in designing purification techniques is the elimination of cellular contaminants, achieving a high yield of target protein is often desirable, especially if one wishes to conduct multiple experiments with the purified enzyme. Minimizing the number of steps in a purification scheme can be useful in ensuring a maximum yield. Additionally, using fewer steps increases the efficiency of the procedure in the lab. With expanded bed absorption (EBA) systems, purified products can be obtained directly from unclarified cell homogenates. Unlike with columns, particles in an EBA system are in a fluidized state and expand in accordance with adjustments made in the flow rate of the system. Therefore, there is enough room between particles for cell debris to pass through without clogging the system. As with traditional column chromatography methods, the particles are designed to attract and specifically bind the protein of interest. Elution is achieved with specific buffers and a reverse flow.

Purification Scheme Utilizing EBA System

1. Synthesize EBA matrix: For EBA systems, a high-density matrix is desirable because the functionality of the procedure is dependent upon the elutriation of the differentially sized particles making up the matrix. Lower density in the material making up the particles corresponds with a lower maximum flow rate for the system. For this purification scheme, we will coat alumina particles with agarose and mineral oil in order to obtain matrix particles with a sufficiently high density and stability in alkaline environments.
2. Introduction of Cibacron Blue 3GA and iminodiacetic acid/zinc ions to the matrix: Because yeast alcohol dehydrogenase requires NAD^+ as a cofactor, it has been shown to specifically bind Cibacron Blue 3GA, which mimics the structure of NAD^+. Additionally, alcohol dehydrogenase is a metalloenzyme that utilizes zinc for its mechanism of action. Chelating zinc ions to iminodiacetic acid molecules within the matrix can thus be used to attract the target protein. Coupling zinc with Cibacron Blue 3GA will hopefully maximize purification.
3. Equilibration with imidazole: A buffer containing imidazole is applied before the sample is introduced. This step is a safeguard to prevent the

matrix from absorbing contaminant proteins. Imidazole binds to the immobilized zinc ions, ensuring that only proteins with strong affinity to zinc, such as alcohol dehydrogenase, are able to bind to the column.

4. Disruption of over-expressed *E. Coli* cells: A rotating plunger or glass beads is used to homogenize the cells.
5. Introduction of sample into the system: The sample will be allowed to flow through the EBA platform.
6. Elution: Elution must occur in two steps in order to ensure that a maximum number of the contaminants are eliminated. Imidazole is able to displace protein functional groups in order to interact with the zinc ions bound to the matrix. The weaker the protein's affinity for zinc, the lower the concentration of imidazole necessary to displace the protein. Because alcohol dehydrogenase binds strongly to zinc ions, an eluent buffer containing a low concentration of imidazole can be applied first to remove weakly-bound contaminant proteins. This can be followed by a higher concentration of imidazole so that the alcohol dehydrogenase can be eluted and collected in small fractions to be assayed.

Because an EBA platform might not be readily available in many labs and it may not be necessary to obtain large quantities of the protein of interest, it is possible to adapt some of the principles utilized above to fit into a traditional purification scheme, as described below.

Purification Scheme Utilizing a Traditional Approach:

1. Disruption of the *E. Coli* cells: Similar to the procedure above, we can use a rotating plunger or glass beads to homogenize the cells. Then, after subjecting the homogenate to centrifugation, we will use the supernatant as the sample. Yeast has at least two alcohol dehydrogenase isoenzymes in the cytoplasm, so we will be attempting to purify these. It is important to set aside a small volume of the sample to take the protein concentration and enzyme activity.
2. Immobilized metal ion affinity chromatography column (IMAC): As YADH is known to bind to zinc ions, the first purification step might involve the utilization of an IMAC column. Iminodiacetic acid will serve as the chelating group on agarose beads, causing the column to retain YADH. Similar to the EBA procedure, an imidazole buffer solution can be used to equilibrate the column. The sample can then be applied, followed by a buffer of around pH 8.6-9.0 to ensure that the column does not dry up. After this, the elution

buffer of high imadazole and sodium chloride concentration is added to disrupt the relationship between the proteins and zinc ions. Small fractions will be taken, each of which are assayed to determine which fraction contains most of the enzyme. If there is more than one fraction with substantial enzyme activity, they can be combined. It is important to take the enzyme activity and protein concentration of the combined fraction.

3. Dialysis: The combined fractions from the second step will be poured into a dialysis tube in order to remove the access imidazole and sodium chloride as well as to concentrate the protein. The tied dialysis tubing will be placed in a buffer of appropriate concentration, depending on the concentrations of the elution buffer in step 2. This set-up can then be left for several days.
4. Cibacron Blue 3GA affinity column: To finish the purification, the sample obtained from the dialysis procedure can be applied to a Cibacron Blue F 3GA column, which is first activated by urea and equilibrated by the isolation buffer. Again, this chosen buffer should have a pH at around 8.6-9.0, to ensure that the enzyme is fully functional. YADH should bind to the column, as Cibacron Blue 3GA mimics NAD^+, one of its natural substrates. The elution buffer will contain a high concentration of NAD^+, thus removing the target protein from the column. Fractions will be taken and assayed as in the second step. The fraction(s) with the most enzyme activity can be combined. This represents the purified sample, which can be further concentrated via dialysis or further diluted. It depends on the needs of the researcher as to how the sample is stored.

EBA Procedure Supplies:

1. RHOBUST Flex Chromatography System: http://www.dsm.com/en_US/html/dpp/Rhobust_Flex_Chromatography_System.htm
2. Agarose: Sigma Aldrich product A9539-10G. http://www.sigmaaldrich.com/catalog/product/sigma/A9539?lang=en&region=US
3. Alumina (activated for chromatography in basic environment): Sigma Aldrich product 06290. http://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=8671573
4. Mineral oil: Sigma Aldrich product M5904. http://www.sigmaaldrich.com/catalog/product/sigma/m5904?lang=en&region=US

5. Iminodiacetic acid: Sigma-Aldrich product 220000. http://www.sigmaaldrich.com/catalog/product/aldrich/220000?lang=en&region=US
6. Cibracron blue 3GA: Sigma-Aldrich product B1064. http://www.sigmaaldrich.com/catalog/product/sigma/b1064?lang=en&region=US
7. Zinc chloride solution: Sigma-Aldrich product 39059. http://www.sigmaaldrich.com/catalog/product/sigma/39059?lang=en&region=US
8. Imidazole: Sigma-Aldrich product I5513. http://www.sigmaaldrich.com/catalog/product/sigma/i5513?lang=en&region=US
9. BedBug microtube homogenizer: Sigma-Aldrich product Z763705. http://www.sigmaaldrich.com/catalog/product/sigma/z763705?lang=en&region=US

Traditional Approach Procedure Supplies:

1. BedBug microtube homogenizer: Sigma-Aldrich product Z763705. http://www.sigmaaldrich.com/catalog/product/sigma/z763705?lang=en&region=US
2. Centrifuge: Sigma-Aldrich product Z601039. http://www.sigmaaldrich.com/catalog/product/sigma/z601039?lang=en&region=US
3. Zn-IMAC 3 mL Column: bioWORLD product 20170016-1. http://www.bio-world.com/productinfo/2_18_843/126528/Zn-IMAC-mL-Columns.html
4. Imidazole: Sigma-Aldrich product I5513. http://www.sigmaaldrich.com/catalog/product/sigma/i5513?lang=en&region=US
5. Dialysis tubing: Sigma-Aldrich product D9277. http://www.sigmaaldrich.com/catalog/product/sigma/d9277?lang=en&region=US
6. Cibacron Blue 3GA Agarose: Sigma-Aldrich product C1535. http://www.sigmaaldrich.com/catalog/product/sigma/c1535?lang=en&region=US
7. NAD^+: Sigma-Aldrich product N8285. http://www.sigmaaldrich.com/catalog/product/sigma/n8285?lang=en&regi

on=US

Kinetics Supplies:

The only additional material we will need for the kinetics part of the experiment is the alcohol substrate and the spectrophotometer. Everything else was already obtained for the procedures above.

1. Genesys 20 spectrophotometer: Sigma-Aldrich product Z376027. http://www.sigmaaldrich.com/catalog/product/sigma/z376027?lang=en&region=US
2. Cuvettes: Sigma-Aldrich product Z276863. http://www.sigmaaldrich.com/catalog/product/aldrich/z276863?lang=en&region=US
3. Propanol: Sigma-Aldrich product 279544. http://www.sigmaaldrich.com/catalog/product/sial/279544?lang=en&region=US

Purification Table:

Because the EBA procedure combines many steps, the table would only consist of two sets of measurements. In essence, it would be a shorter version of the purification table for the traditional approach.

The table below represents a model that could be utilized for taking measurements during the traditional approach procedure. Stage 1 represents the protein concentration and enzyme activity measurements taken directly after the supernatant was obtained from the disrupted, centrifuged *E. Coli* cells. Stage 2 represents the measurements from the combined fractions after subjecting the sample to a Zn-IMAC column. Stage 3 corresponds with the properties of the concentrated enzyme sample, after the dialysis process was complete. Stage 4 is associated with the Cibacron Blue 3GA affinity chromatography step.

Stage	**Volume (mL)**	**ΔA/min**	**unit/mL**	**Total units**	**mg/mL**	**Total mg**	**Specific activity**	**Recovery (%)**	**Fold Purifica**
1									
2									

3									
4									

Assays:

Regardless of the approach taken, it is important to be able to test for enzyme activity and protein concentration in order to track the progress of the purification scheme.

Because YADH-catalyzed reactions consume NAD^+, we consider the rate of NADH activity as a measure of enzyme activity. NADH absorbs strongly at 340 nm, so it is possible to follow its production using a spectrophotometer. For each assay, we will add sodium pyrophosphate buffer, ethanol, and NAD^+, so we can "visualize" the enzyme's oxidation of the alcohol indirectly.

To test protein concentration after each purification step, we will use the Bradford Assay. The Bradford method is esteemed for its simplicity and known accuracy. The technique relies on the binding properties of an aromatic dye called Coomassie Brilliant Blue G-250. This compound enables protein to absorb maximally at 595 nm in acidic solutions through the formation of noncovalent interactions. Thus, the more absorbance detected, the more protein there is.

Kinetics:

As mentioned above, it is possible to measure the rate at which YADH converts NAD^+ into NADH because NADH absorbs strongly at 340 nm. One of the most common ways an enzyme's kinetics is first analyzed is through the application of the principles of the Michaelis-Menten model. Determining Michaelis-Menten constants, such as K_m and V_{max} , not only helps researchers to determine the enzyme mechanism of action, but it also allows for comparison of enzyme qualities over a span of conditions. For instance, because YADH is known to oxidize a variety of alcohols, aldehydes, and ketones, it is interesting to compare how the rates of catalysis differ with each of these substrates.

In order to come to an understanding of how YADH works, I propose that the initial rates of YADH-catalyzed reactions be taken at various concentrations of a given alcohol. Because Dickson & Monger showed that enzyme kinetics decreases with increasing length of alcohol chain and with a transition from primary to secondary alcohol type, it may be best to choose propanol or something similar as

the substrate. This will enable us to see YADH at its best.

Each cuvette ought to contain the following reaction components: YADH (must be the same concentration for each reaction), NAD^+ (must be the same concentration for each reaction; should be present in excess, i.e. more than enough to fully saturate each enzyme), varying concentrations of propanol, and Tris buffer of pH 8.6-9 (enough to bring the volume up to 1 mL).

Exact concentrations of the propanol substrate used will partially depend upon the concentration of the enzyme. The amount will increase incrementally for each reaction, beginning with the smallest quantity necessary to observe activity and ending once the initial rate becomes independent of substrate addition (i.e. the enzymes are fully saturated and the initial rate measurements have leveled off). For instance, one could begin with 0, 15 μM, 30 μM, and continue to double the concentration of propanol until the saturation point. The amount of buffer added to each reaction is adjusted in accordance with how much propanol is present. Essentially, the buffer is used to bring the reaction volume to 1 mL.

After obtaining the initial rate at each concentration, we can construct a Lineweaver-Burk plot, graphing the reciprocal of the substrate concentration versus the reciprocal of the corresponding initial rate. On this plot, we can find K_m and V_{max} by locating the x- and y-intercepts. This information will be useful for further analysis, including the determination of a mechanism of action and the analysis of the functionality of the enzyme under different conditions.

References:

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