Chem 460 Laboratory—Fall 2008
Experiment 5: Purification of Hen Egg White Lysozyme

Before Lab

- Read the appropriate section in your biochemistry text to learn about the in vivo function of lysozyme.
- Search the web to determine the ~5 major proteins in egg white. Obtain molecular weights and pl’s for each. You will find the following links useful: http://www.ncbi.nlm.nih.gov and http://www.scripps.edu/~cdputnam/protcalc.html
- Create a written plan for your purification. Know which column you plan to use and show how you plan to make the solutions you need. You will hand this plan (copy pages) in at the beginning of lab.

Introduction

In this three week lab you will purify lysozyme from hen egg white. (You've already observed the three-dimensional structure of this enzyme using Deep View last week. Lysozyme was actually the first enzyme whose structure was determined by x-ray crystallography.) You will purify lysozyme using ion exchange chromatography and you will assess the purification process by determining 1) the level of enzyme activity using an assay based on hydrolysis of bacterial cell walls, 2) the total protein concentration using the BCA assay, 3) the level of purity using SDS-PAGE and 4) the molecular weight by LCMS. The first week will be spent on ion exchange chromatography and enzyme assays. The second week will be devoted to the protein assay and preparing your samples for the third week when you will carry out gel electrophoresis and mass spectrometry. Information for carrying out Week 1 is given below. Information for Weeks 2 and 3 will be given to you in lab. Please keep a good lab notebook (readable with section headings).

Week 1: Purification of Lysozyme

Be sure to keep track in your notebook the volumes of ALL solutions along the way. In addition, be sure to save aliquots of pertinent solutions for later analysis.

Lysozyme has an unusually high pl, suggesting that ion exchange chromatography might be a good strategy for purifying it. (Why?) We have two different types of ion exchange resin available for you to use: HiTrap Q HP (1 mL) and HiTrap SP HP (1mL). What types of functional groups are on these resins? Which one is a cation exchanger and which one is an anion exchanger? (Information about these resins can be found at the Amersham Biosciences website by searching for these items and then looking at the PDF in the Instruction/Protocol link.)

We will use fresh eggs as our source of lysozyme. Each group will start with one egg. Begin by separating the yolk from the egg. (If you don't know how, talk to your favorite chef.) Carefully filter the egg white through a few layers of cheesecloth. Don't squish it
through—we want a clear, thin liquid. It will be impossible to get all of it through the cloth. Dilute a portion of it (10 mL) into an appropriate buffer solution (10 fold dilution works well, giving you 100 mL of solution) and filter again, this time through a kimwipe. You want a free flowing liquid; if there are solids or clumps in solution, you should filter it again so that you don't clog your chromatography column. The resulting solution is your "egg-white extract".

There are typically four steps to a chromatography experiment: 1) **Pre-wash** (or **equilibrate**), 2) **Load**, 3) **Wash** and 4) **Elute**. Start by using a syringe to equilibrate the column with 10 column volumes of buffer. Next, note that the amount of protein in the egg extract would (way) overload the column; therefore, **only load 1 mL** of your egg extract onto the column. As the extract passes through the column, proteins that don’t stick to the column will pass through (these proteins are in what is called the **FlowThrough**, or **FT**). Next, a wash is carried out to ensure that all non-sticking proteins have passed completely through the column. Now you need to elute the proteins that stuck to the column. One way to do this is to use a salt solution whose ions will compete with the protein for binding to the resin, causing the protein to release from the column. Ideally, you want to use as little salt as possible for removing your protein from the column (because salt interferes with other techniques such as PAGE and MS analysis), therefore it is best to use either **stepwise** or **gradient** elution which both involve adding increasing concentrations of salt to the column. In this lab, you will be using stepwise elution. To do this, pass 1 mL aliquots of each 100, 200 and 300 mM NaCl (in buffer) through the column in stepwise fashion, collecting each eluant (or “fraction”) in a separate **and labeled** microfuge tube.

**Enzyme Assay**

To determine which fractions from the column contain lysozyme and to determine how active the enzyme is, you will employ an enzymatic assay. The traditional assay for lysozyme activity is the hydrolysis of a suspension of *Micrococcus leisodeikticus* cell walls. In this assay, the cell walls get cleaved by lysozyme causing the turbidity of the solution to decrease. Note that **one unit** of lysozyme activity is defined as the quantity of enzyme that produces a decrease in turbidity of 0.001 per minute at 450 nm measured at pH 7.0 (25 °C) using a 0.3 mg/mL suspension of *Micrococcus leisodeikticus* cells as substrate (Biochim Biophys Acta 8, 302). As you carry out this assay, remember that you need to shake the solutions immediately prior to use, since the cell walls settle over time. As we did with fumarase, you will need to determine the right amount of enzyme to add so that the reaction is linear over the time scale that you follow (i.e. 1 minute).

**Purification Table**

The table below was taken from Kendrew et al., in *Biochemistry* 38, 4796. It is typical of the type of table you see in journal articles presenting the purification of a protein. For your report for this lab, you will be setting up a **similar** table, though instead of giving
data for the most active sample in multiple steps (as is done below), you will provide data for the load, flowthrough, wash and eluant fractions from your single purification step. Before leaving lab the first week, create a table for the **volume** (in mL), **protein concentration** (in mg/mL), **activity** (in units/mL), **specific activity** (in units/mg) and **purification fold** for each sample/fraction and fill in the table with the information you have so far.

<table>
<thead>
<tr>
<th>step</th>
<th>protein (mg)</th>
<th>units (µmol/min)</th>
<th>yield (%)</th>
<th>specific activity (µmol min⁻¹ mg⁻¹)</th>
<th>purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell-free extract</td>
<td>531</td>
<td>1210</td>
<td>100</td>
<td>2.29</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td>267</td>
<td>810</td>
<td>66.9</td>
<td>3.03</td>
<td>1.32</td>
</tr>
<tr>
<td>MonoQ HR10/10</td>
<td>68.3</td>
<td>510</td>
<td>42.1</td>
<td>7.47</td>
<td>3.26</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>44.3</td>
<td>420</td>
<td>33.9</td>
<td>9.48</td>
<td>4.14</td>
</tr>
</tbody>
</table>