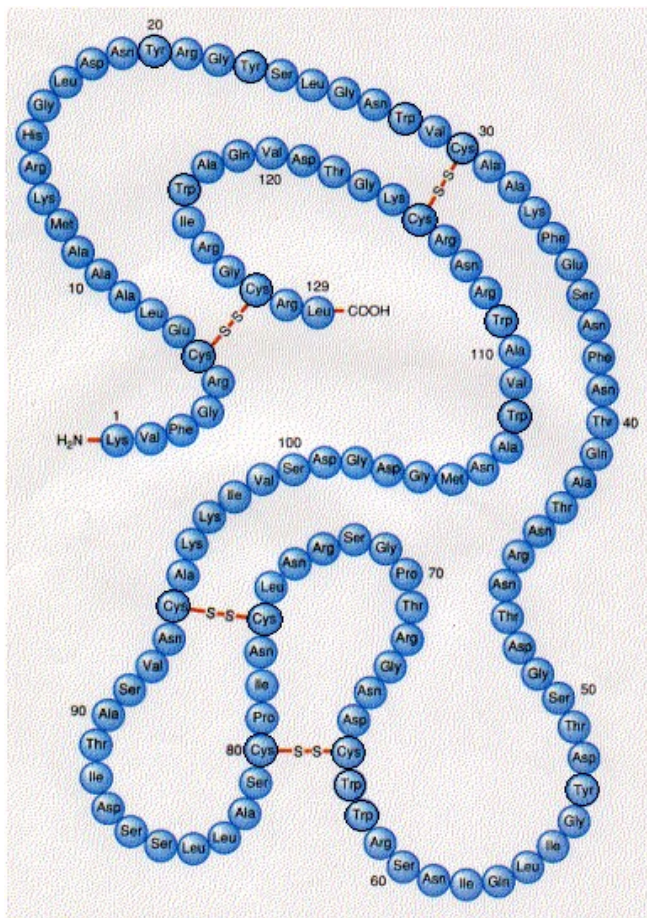


Experiment 1: Crystallization of Chicken Egg White Lysozyme



The protein lysozyme is an enzyme that disrupts the bacterial wall hydrolyzing the glycoside $\beta(1\rightarrow4)$ linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan. Lysozyme is a small enzyme and it was the first enzyme whose structure was determined by X-ray diffraction on single crystal (J Mol Biol 82 p. 371 (1974), PDB entry: 2LYZ). In its mature form, the protein is 129 residues long (about 14 kDa) and contains 4 disulfide bridges.

Additional information: UniProt entry P00698 (LYSC_CHICK).

Sequence:

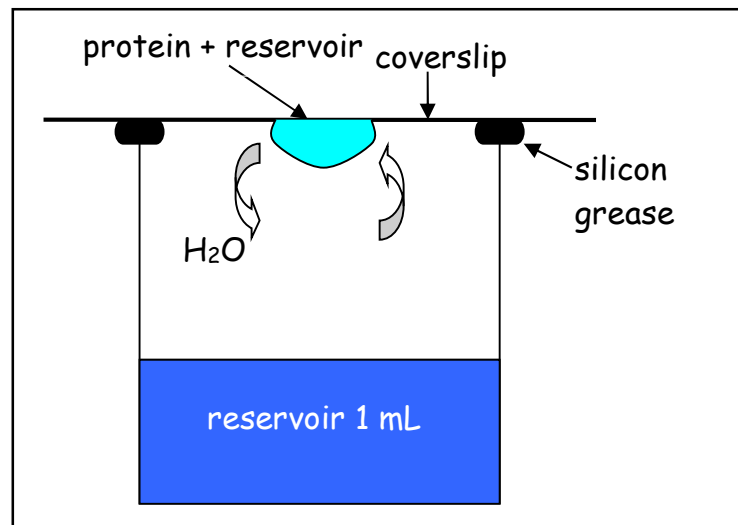
10	20	30	40	50
KVFGRCELAA	AMKRHGLDNY	RGYSLGNWVC	AAKFESNFNT	QATNRNTDGS
60	70	80	90	100
TDYGILQINS	RWWCNDGRTP	GSRLNCNIPC	SALLSSDITA	SVNCAKKIVS
110	120			
DGNGMNAWVA	WRNRCKGTDV	QAWIRGCRL		

Crystallization by vapour-diffusion, hanging-drop technique.

1. Prepare 50 mL of a buffer solution of sodium acetate 50 mM at pH 4.5.

2. Prepare 20 mL of a solution 2 M of NaCl, in acetate buffer.

3. Prepare a solution of lysozyme in acetate buffer: weight about 7 mg of solid, lyophilized lysozyme and dissolve it in 70 μL of buffer solution. Measure the concentration through a spectrophotometric experiment (point 4) and through a BCA assay (point 5). Then, dilute the enzyme solution to the desired concentration.



4. Spectrophotometric measurement of protein concentration. Protein concentration can be determined using Lambert-Beer's law ($A = \epsilon bc$) measuring the absorbance A of the protein solution at 280 nm. For the spectrophotometric measurement, 2 μL of the protein solution are diluted in 1 mL of acetate buffer. The molar absorption coefficient at 280 nm is calculated considering the primary sequence of the protein according to the following equation:

$$\epsilon_{280}(\text{M}^{-1} \text{cm}^{-1}) = (5500 \cdot \#\text{Trp}) + (1490 \cdot \#\text{Tyr}) + (125 \cdot \#\text{disulfide bonds})$$

(Protein Science 1995, 4, 2411).

5. Determination of the protein concentration through the BCA assay. This assay is based on the reduction of peptide bonds by Cu^{2+} salts in a basic solution. The Cu^{+} ions produced in the reaction form a complex with the bicinconinic acid added to the reaction mixture, with a specific absorbance peak at 562 nm. The method is useful to determine protein concentrations between 0.5 $\mu\text{g/mL}$ and 1.5 mg/mL. The concentration of the lysozyme sample can be calculated after determining a standard curve, using standard solutions of Bovine Serum Albumine (BSA).

The available BSA standard solution has a concentration of 2 mg/mL and has to be diluted in acetate buffer to obtain 20 μL of solutions with the following concentrations: 0 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, 200 $\mu\text{g/mL}$, 300 $\mu\text{g/mL}$, 400 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$, 600 $\mu\text{g/mL}$.

Using the previously prepared solution of lysozyme, prepare 20 μL of diluted lysozyme solution having a concentration of about 400 $\mu\text{g/mL}$.

Prepare the reaction mixture of the BCA assay in a 15 mL tube: 1.3 mL of bicinconinic acid solution (reagent A of the BCA kit), plus 26 μL of copper sulfate (reagent B of the BCA kit). In a 96-well plate, add 160 μL of the reaction mixture to each of the standard solutions prepared before, and to the diluted sample.

Solutions are incubated at 60°C for 15 minutes to allow reaction to occur. The reaction mixtures are cooled to room temperature, before measuring the absorbance of each solution at 562 nm with a plate reader. Linear regression of the standard values allows to obtain the standardization line used to determine the sample concentration.

6. Lysozyme crystallization. Lysozyme crystallization experiments using the hanging-drop method are performed at different lysozyme concentrations (from 10 mg/mL, row D, to 40 mg/mL, row A) and at different concentrations of sodium chloride in the reservoir solution (from 0.3 M, column 1,

to 1.8 M, column 6). Fill each column of wells in the Linbro box with 1mL of buffer solution, at increasing concentrations of NaCl, from 0.3 to 1.8 M, interval 0.3 M, obtained by mixing suitable volumes of NaCl solution and acetate buffer (see scheme of Linbro box). Put a small layer of silicon grease on the rim of each well. Prepare the first row of protein crystallization experiments (row A): Dilute the lysozyme solution to obtain a concentration of 40 mg/mL. On the cover slip, prepare a 4 μ l drop of the protein sample and add 4 μ l of reservoir. Seal the well with the coverslip using tweezers, so that the drop hangs on the coverslip over the reservoir solution.

7. Prepare the following rows (from B to D): Dilute the protein sample to 30 mg/ml and prepare the drops of row B following the instructions at point 6. Dilute the protein solution to 20 mg/ml and fill row C. Finally, dilute the lysozyme solution to 10 mg/ml and fill row D.

8. Store the crystallization box at 18° C and observe changes in the drops for a week.

Scheme of Linbro box

	<i>Stock solutions</i>	1	2	3	4	5	6
A	<i>NaCl 2M</i> <i>Acetate buffer</i>	150 μ l 850 μ l	300 μ l 700 μ l	450 μ l 550 μ l	600 μ l 400 μ l	750 μ l 250 μ l	900 μ l 100 μ l
B	<i>NaCl 2M</i> <i>Acetate buffer</i>	150 μ l 850 μ l	300 μ l 700 μ l	450 μ l 550 μ l	600 μ l 400 μ l	750 μ l 250 μ l	900 μ l 100 μ l
C	<i>NaCl 2M</i> <i>Acetate buffer</i>	150 μ l 850 μ l	300 μ l 700 μ l	450 μ l 550 μ l	600 μ l 400 μ l	750 μ l 250 μ l	900 μ l 100 μ l
D	<i>NaCl 2M</i> <i>Acetate buffer</i>	150 μ l 850 μ l	300 μ l 700 μ l	450 μ l 550 μ l	600 μ l 400 μ l	750 μ l 250 μ l	900 μ l 100 μ l

