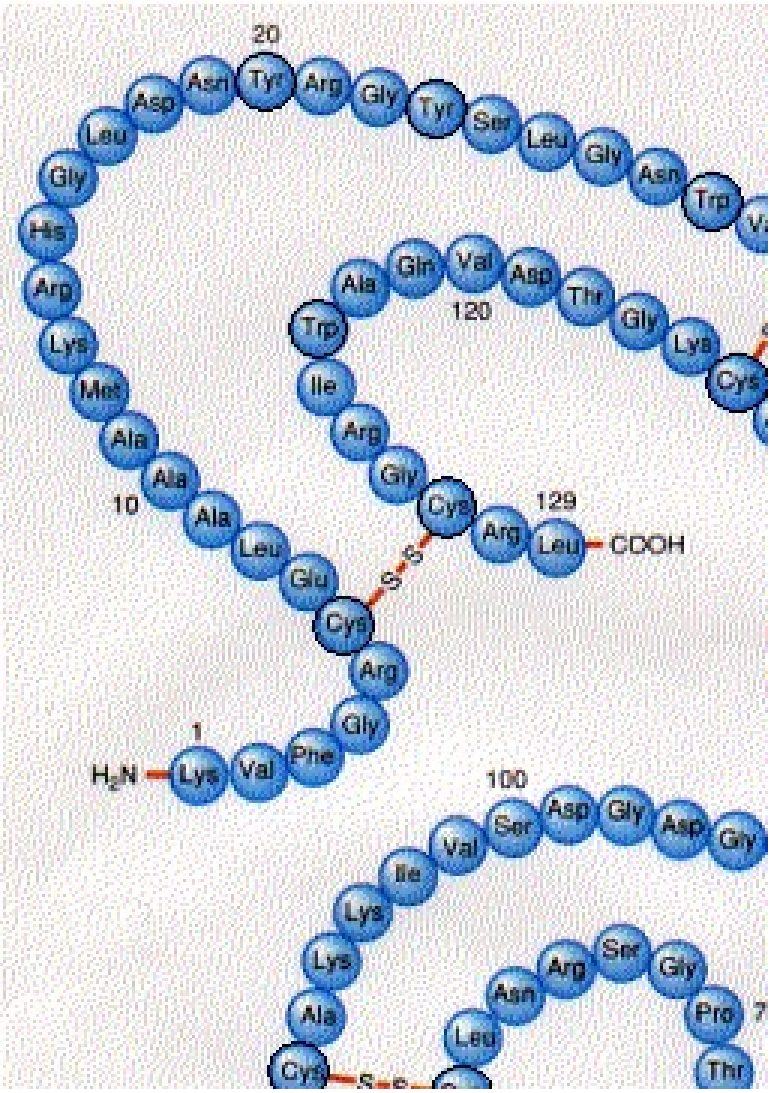
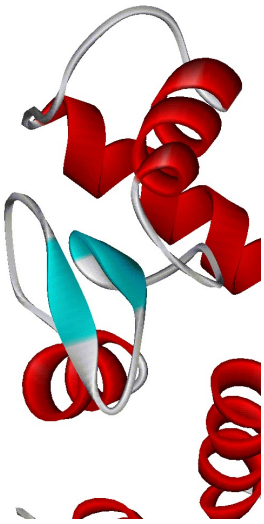
Experiment 1:
Crystallization of Chicken Egg White Lysozyme





The protein lysozyme is an enzyme that disrupts the bacterial wall hydrolyzing the glycoside β(1→4) 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 residue long (about 14 kDa).

Additional information: UniProt entry P00698 (LYSC_CHICK).

Sequence:

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         10          20          30          40          50
KVFGRCELAA AMKRHGLDNY RGYSLGNWVC AAKFESNFNT QATNRNTDGS
         60          70          80          90         100
TDYGILQINS RWWCNDGRTP GSRNLCNIPC SALLSSDITA SVNCAKKIVS
        110         120
DGNGMNAWVA WRNRCKGTDV QAWIRGCRL
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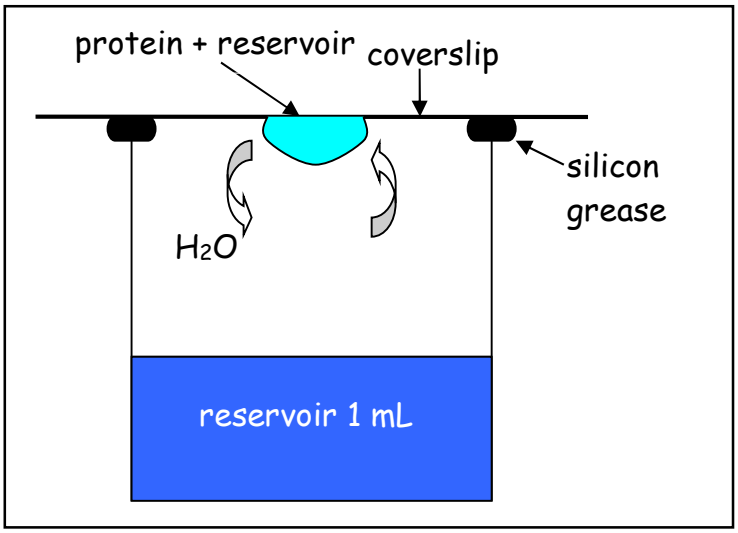
Crystallization by vapour-diffusion, hanging-drop technique.



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

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

3. Prepare a solution of lysozyme 40mg/ml (3.43 mM) 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 law (A=εbc) measuring the absorbance A of the protein solution at 280 nm (optical path b=1 cm). For the spectrophotometric measurement, a diluted solution is obtained from 2 μl of the starting protein solution to 1 mL, using the buffer solution. The molar absorption coefficient at 280 nm is calculated considering the primary sequence of the protein (see Figure and sequence) according to the following equation:
$\varepsilon_{280}(M^{-1}\ cm^{-1}) = (5500 \cdot \#Trp) + (1490 \cdot \#Tyr) + (125 \cdot \#disulfide\ bonds)$
(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. Cu^{+} ions produced in the reaction form a complex with the bicinconinic acid present in the reaction mixture, with a specific absorbance peak in the visible spectrum at 562 nm. The method is useful to determine protein concentrations between 0.5 μg/mL and 1.5 mg/mL. The concentration of the lysozyme sample can be calculated after determining a standardization line, 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 μg/mL, 100 μg/mL, 200 μg/mL, 300 μg/mL, 400 μg/mL, 500 μg/mL, 600 μg/mL.
Using the previously prepared solution of lysozyme, prepare 20 μL of diluted lysozyme solution having a concentration of about 400 μg/mL.
Prepare the reaction mixture of the BCA assay in a 15 mL tube: 3.1 mL of bicinconinic acid solution (reagent A of the BCA kit), plus 62 μL of copper sulfate (reagent B of the BCA kit). Add 380 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. Linear regression of the standard values allows to obtain the standardization line used to determine the sample (lysozyme solution) 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 row 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 µl drop of the protein sample and add 4 µl 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 20° C and observe changes in the drops for a week.

Scheme of Linbro box

	Stock solutions	1	2	3	4	5	6
A	*NaCl 2M* *Acetate buffer*	150µl 850µl	300µl 700µl	450µl 550µl	600µl 400µl	750µl 250µl	900µl 100µl
B	*NaCl 2M* *Acetate buffer*	150µl 850µl	300µl 700µl	450µl 550µl	600µl 400µl	750µl 250µl	900µl 100µl
C	*NaCl 2M* *Acetate buffer*	150µl 850µl	300µl 700µl	450µl 550µl	600µl 400µl	750µl 250µl	900µl 100µl
D	*NaCl 2M* *Acetate buffer*	150µl 850µl	300µl 700µl	450µl 550µl	600µl 400µl	750µl 250µl	900µl 100µl