Long Assignment 2A: Blue-Plate Assay Analysis

This semester in PEBL, we aim to investigate the impact of the K63E/E67K mutation on the enzymatic activity and structural stability of SNase (a model enzyme) to tackle the overarching questions of the course: how does the chemical composition of a protein relate to its structure and properties? —how does the environment of a protein change its structure and properties? —and how do the structure and properties of a protein relate to its function? In this first experiment, we compare the enzymatic activity of mutant and wild-type SNase by blue-plate assay, assessing the impact of the mutation on function. The blue-plate assay produces a quantitative measure of SNase activity using DNA (the substrate of SNase) in agar. Toluidine blue-O, the intercalating dye present in the matrix, inserts itself between nucleotides, producing a blue color, and is released following cleavage of the DNA by SNase, producing a pink color. By measuring the diameter of the resulting pink circle, we quantify the activity of SNase.

The blue-plate assay tested two SNase solutions: mutant SNase and wild-type SNase and three controls: unfolded SNase, folded/inactivated SNase, and water, in four conditions: 20 mM Ca++ at pH 9; EDTA at pH 9; 20 mM Mg++ at pH 9; and 20 mM Ca++ at pH 7. The water control was chosen as a baseline for zero SNase activity, the unfolded control as a baseline for denatured SNase (which would be the result if the mutation were sufficiently destabilizing), and the folded/inactive control as a baseline for damaged active site (to demonstrate the necessity of active site conservation for proper function). Ca++ is a necessary cofactor of SNase, and SNase is optimally active at pH 9. The environmental conditions were chosen to demonstrate the necessity of SNase cofactor (EDTA chelates Ca++, so the EDTA condition acts as a negative control for Ca++ presence), the specificity of cofactor (the Mg++ condition confirms the required cofactor identity), and the impact of pH on activity.

The collected data is presented in Tables 1-4 and Figures 1-4. Several general trends are visible. The mutant produced similar, yet consistently lower activity to the wild-type in all experimental conditions except for the EDTA condition, where all samples produced zero activity. The unfolded sample produced substantially lower, though non-zero activity in all but the EDTA condition, and the water sample produced zero activity in every condition. Finally, the folded/inactive sample produced zero activity in all but the Mg++ condition, which seems to be an outlier based on the expected trends. We expect an enzyme with destroyed active site to be completely nonfunctional, so this was likely an experimental error. All enzyme samples produced their highest activity in the optimal conditions of 20 mM Ca++ at pH 9 (excluding the mentioned outlier), and their lowest activity in the EDTA condition, as expected. Furthermore, the pH 7 trial displayed a sizable reduction in activity for the mutant, wild-type, and unfolded samples, confirming the pH dependence of SNase activity. Interestingly, the non-zero activity of the unfolded sample (reaching a maximum of 53% that of the wild-type) in all but the EDTA condition indicates a sizable level of refolding in solution, suggesting that favorable interactions towards enzyme activity (including cofactor and substrate binding) can, to some degree, reverse the denaturation of enzymes. Overall, the data suggests that the K63E/E67K mutation mildly reduces the functional efficacy of SNase, though not significantly to the point of denaturation.

Initially, it was hypothesized that the proposed mutation would reduce the activity of SNase by decreasing the structural stability of the protein, since this mutation increases the distance of the 63-67 Coulombic interaction. The data collected supports the hypothesis though not to the extent expected. In optimal conditions, the end diameter for the mutant is only 6% less than that of the wild-type. In comparison, the distance of the Coulombic interaction is increased by 33% in the mutant. The physical basis likely holds; the stability of the protein is reduced by the stretched Coulombic interaction. This shifts the equilibrium slightly more towards the unfolded state, and so a slightly larger percentage of proteins are unfolded in the mutant compared to the wild-type, producing a slight decrease in enzymatic activity.

Table 1: Ca++ at pH 9

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
42	8:20	4	0	3	0	2
92	9:10	4.5	0	4	0	2.5
138	9:56	8	0	8.5	0	4.5

Figure 1:

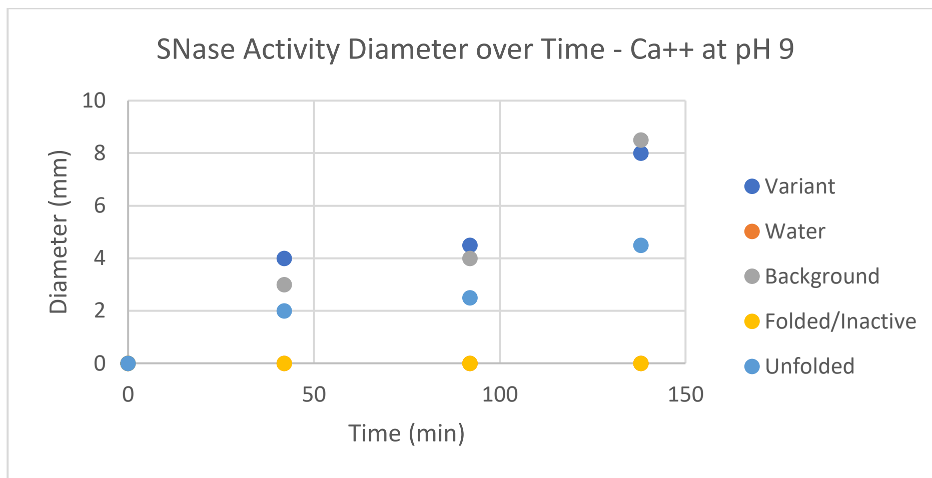

Table 2: EDTA at pH 9

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
44	8:22	0	0	0	0	0
91	9:09	0	0	0	0	0
138	9:56	0	0	0	0	0

Figure 2:

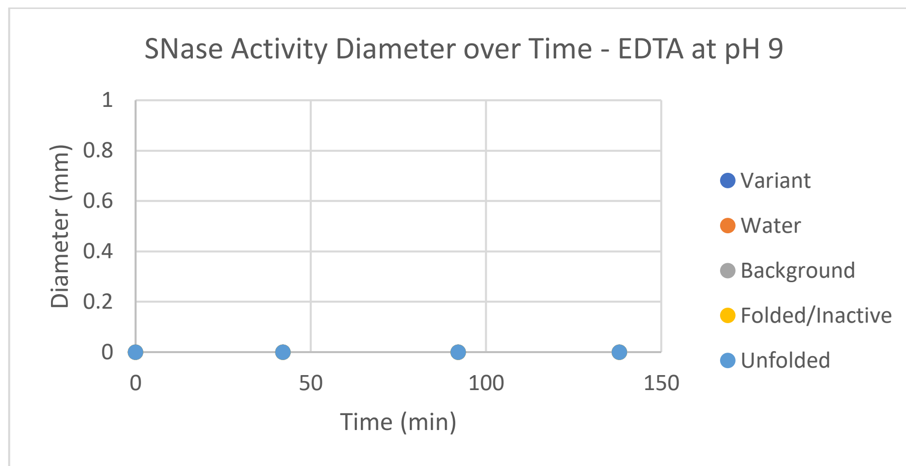

Table 3: Mg++ at pH 9

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
45	8:23	3	0	3	3	2
91	9:09	3	0	3	4	2
138	9:56	3	0	4	4	2

Figure 3:

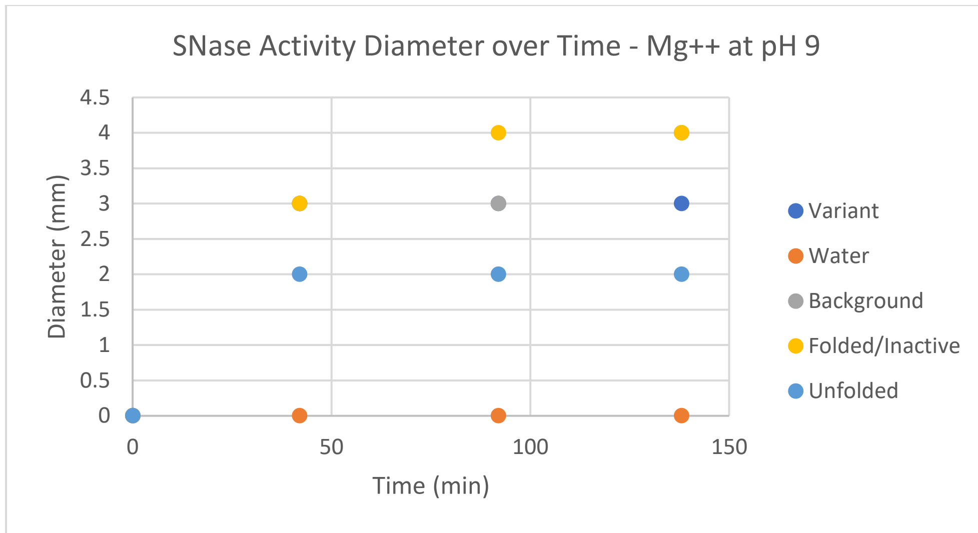

Table 4: Ca++ at pH 7

Total Minutes	Time	Measured Diameter (mm)				
		Variant	Water	Background	Folded/ Inactive	Unfolded
0	7:38	0	0	0	0	0
47	8:25	5	0	5	0	2
90	9:08	6	0	6	0	2
138	9:56	6.5	0	7.5	0	3.5

Figure 4: