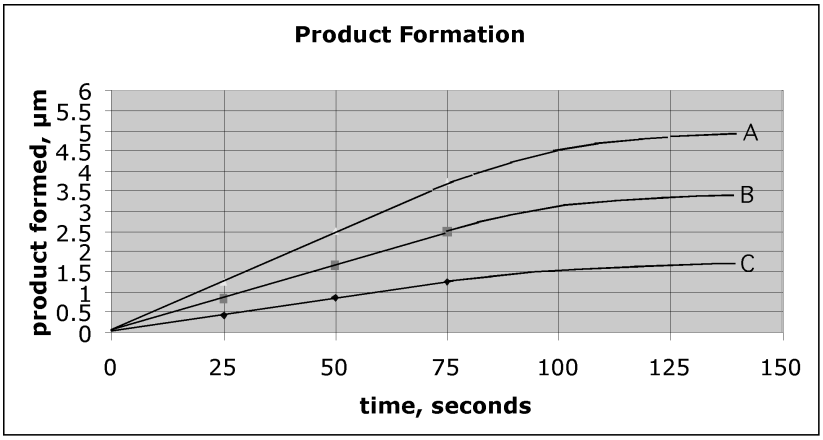
Problem Set 5 Answer Key

1. (20 pts) The plot below shows the amount of product formed (μmoles) as a function of time (sec) in an enzyme reaction. The substrate concentrations were 1, 2.5 and 5 mM.

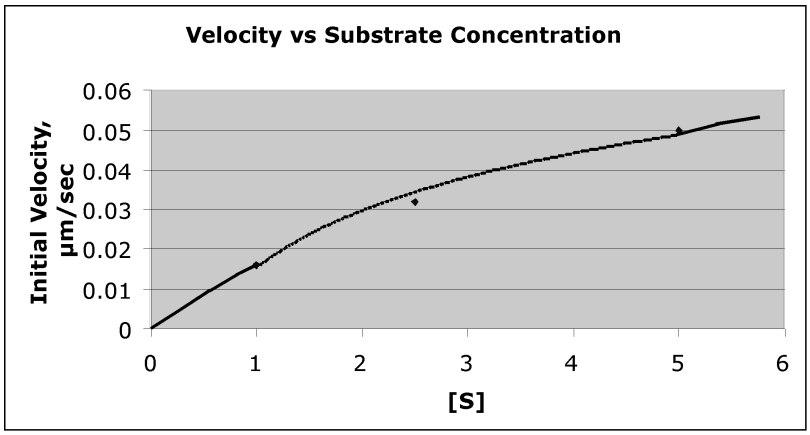


i) Indicate which curve is associated with which substrate concentration. **(1 pt)** **A corresponds to 5 mM, B to 2.5 mM and C to 1 mM**.

ii) Determine the *initial* velocity, in units of μmoles/sec, of product formation for each substrate concentration.
The initial velocity at each substrate concentration is obtained by determining $\Delta product / \Delta t$ at early time points:
1 mM: initial velocity = 0.8 μm/sec / 50 sec = **.016 μm/sec (1 pt)**
2.5 mM: initial velocity = 1.6 μm/sec / 50 sec = **.032 μm/sec (1 pt)**
5 mM: initial velocity = 2.5 μm/sec / 50 sec = **.05 μm/sec (1 pt)**

iii) Suggest an explanation for why the rate of product formation decreases towards the end of the experiment (e.g. t > 100 sec).
Either of the two possible explanations is conceivable: 1) **the rate of product formation decreases as the substrate gets converted to product and becomes limiting** *OR* 2) **the enzyme is inhibited by its product** *OR* **3) the enzyme is inactivated during the course of the reaction**. (**2 pts** for any of these explanations).

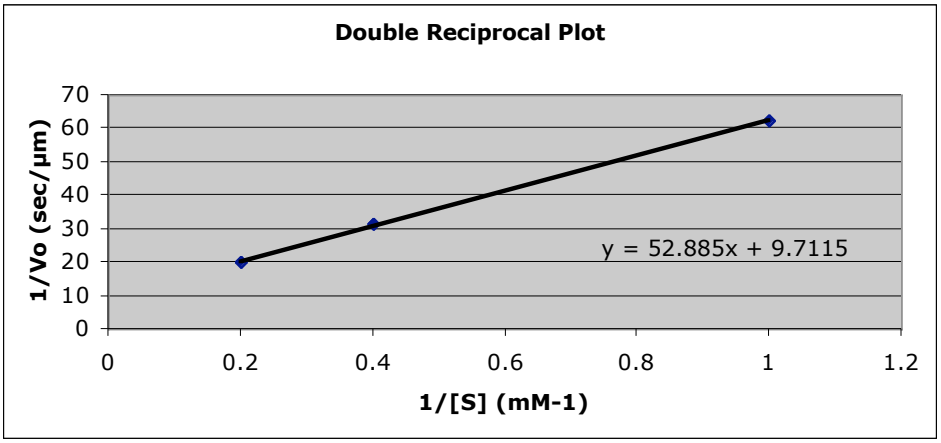
iv) Plot the initial velocity versus substrate concentration for these data. Be sure



to label the axes. **(1 pt for correct x-axis, 1 pt for correct y-axis and 2 pts for hyperbolic curve as shown)**

v) *Without* constructing a double reciprocal plot, estimate K_m and V_{max} from your plot done in part (iv).
From the above plot, it appears as though Vo approaches a maximum of 0.06 μm/sec. Thus V_{MAX} is estimated to be 0.06 μm/sec (1 pt). From the above velocity plot, the K_m , the substrate concentration at which V_o = 1/2 V_{MAX}, appears to be ~ 2 mM. (1 pt)

vi) Now create a double reciprocal plot (1/v versus 1/[S]) to obtain V_{max} and K_m.



The y-intercept on the double reciprocal plot is $1/V_{MAX}$.
Thus V_{MAX} = 1 / 9.7sec/μm = 0.1 μm/sec. (2 pts) The slope of the line is K_M/V_{MAX}. Therefore, K_M = slope*V_{MAX} = 52.9*0.1 = **5.29 mM. (2 pts)**

vii) How close was your estimate in part (v) to the 'true' value? What modification of the enzyme assay would have led to an estimate closer to the 'true' value? **The V_{MAX} estimated from the velocity plot was significantly less than the actual V_{MAX}. As a consequence, the estimated K_M was also significantly lower than the actual value.** (1 pt) What modification of the enzyme assay would have led to an estimate closer to the 'true' value? The estimated values differ from the true values because it was difficult to determine the correct value of V_{max} without extrapolating to high substrate concentration. **If higher substrate concentrations were used initially**, a better estimate of V_{max} would have been obtained. (1 pt)

viii) Assuming that the enzyme concentration was 1 nM and the volume of the reaction was 2 mls, calculate the turnover number, or K_{cat} of this enzyme.
K_{cat}, or turnover number = V_{max}/E_t . $[E_t]$ = 1 nm/L = 2 pm/2 ml. So K_{cat} = 0.1 μm/sec / 2pm = **.05 μmol product/sec/pmol enzyme**. Since we are just interested in the number of products produced/enzyme it is acceptable to simplify the units, even though the numerator refers to moles of product and the denominator refers to moles of enzyme. Therefore, $K_{cat} = 5x10^{-8}$mol-sec$^{-1}/10^{-12}$mol = **$5x10^4$ products/sec. (2 pts for either answer)**

2. (9 pts) The following problem requires that you visualize the active site of Trypsin using Chime. The URL can be found on the problem set page. The normal, wild type, structure is shown as "unmodified". In addition, there are 6 mutant versions, each of which contains a single amino acid change in the active site. Each mutation affects either substrate specificity or the catalytic mechanism, but not both (clearly an oversimplification!). Depending on your last name, select the appropriate mutant enzyme and answer the following questions:

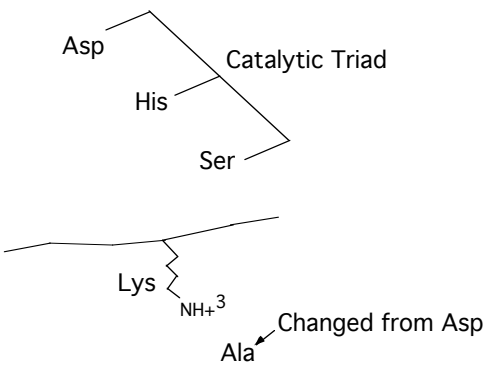
A-C:	Trp 1	M-O:	Trp 4
D-H:	Trp 2	P-S:	Trp 5
I-L:	Trp 3	T-Z:	Trp 6

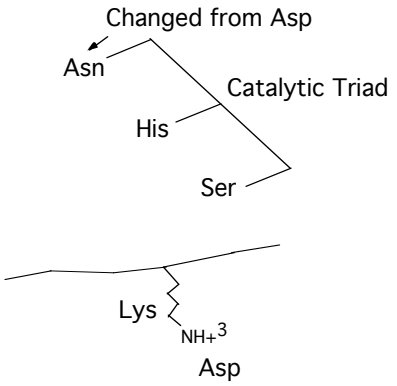
i) State which residue was altered and how (eg, Asp 189 to Phe). **(3 pts)**

First letter of last name	Structure	Residue Altered	Affect on V_{MAX}?	Effect on K_M?
A-C	Trp 1	**Asp 189 to Ala**	**no**	**yes**
D-H	Trp 2	**Asp 189 to Glu**	**no**	**yes**
I-L	Trp 3	**Asp 189 to Lys**	**no**	**yes**
M-O	Trp 4	**Asp 189 to Met**	**no**	**yes**
P-S	Trp 5	**Asp 102 to Asn**	**yes**	**no**
T-Z	Trp 6	**Ser 195 to Ala**	**yes**	**no**

ii) State whether the mutation will affect either the specificity of the enzyme (K_M) or the catalytic properties of the enzyme (V_{MAX}). Briefly justify your answer. **His 57, Asp 102 and Ser 195 are part of the catalytic triad and therefore alterations in these residues are likely to reduce V_{MAX}. (Students P-Z should have this answer. (3 pts) Asp 189 is in the specificity pocket and determines substrate specificity, which for Trypsin is after Lys and Arg. Alterations in Asp 189 will affect the ability to bind the correct substrate and therefore affect K_M. (Students A-O should have this answer. (3 pts)**

iii) Sketch the active site, indicating the location of the amino acid change in your particular structure. (You need only include the residues in the catalytic triad, the main-chain of the substrate, the side-chain of the Arg residue on the substrate, and the residue in the specificity pocket). One example of each type is shown: **(1 pt for showing triad, 1 pt for showing specifity pocket and 1 pt for showing where the change occurred)**



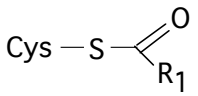


3. (9 pts) Replacement of the Ser 195 residue of the catalytic triad in a serine protease with an Ala residue produces a relatively inactive enzyme. In contrast, replacement of Ser 195 with a Cys residue produces a functional, albeit less active, enzyme.

i) Provide an explanation for why Cys 195, but not Ala 195, can function in the place of Ser 195. **Because it can ionize to form an anion and the sulfur is electronegative, Cys can function as a nucleophile**. **In contrast, Ala cannot**. **(3 pts)**

ii) Provide an explanation for why the mutant enzyme containing Cys 195 has a lower V_{MAX} than the wild type enzyme. **Although Cys can function as a nucleophile, it is not as potent as Ser because sulfur is less electronegative than oxygen**. **(3 pts)**

iii) Sketch the stable enzyme intermediate that would be found with the mutant (S195C) enzyme. **(3 pts)**



4. (9 pts) Alteration of Asp 102 in the catalytic triad of the serine protease trypsin to Asn (D102N) leads to a 10^4 fold reduction of the K_{cat} of the enzyme.

i) Taking advantage of what you know about the catalytic mechanism of serine proteases, explain the observed reduction in K_{cat} (i.e. describe the specific step in the catalytic cycle that is inhibited in the mutant enzyme). **Replacement of Asp 102 with Asn leads to less effective proton extraction by His 57. This is because there is no longer a negative charge that favors His protonation. Less effective proton extraction results in an inability to make Ser195 nucleophilic. (3 pts)**

ii) Analysis of the active site structure of the D102N mutant indicated that the specificity pocket, oxyanion hole and orientation of the nucleophilic Ser were identical to that of the wild type protease. Is the K_m of the D102N mutant likely to be higher, lower, or the same as the wild type protease? Explain. **Since the specificity pocket is unaffected in the D102N mutant, its K_M is likely to be similar to wild type. (3 pts)**

iii) Remarkably, the Kcat of the D102N mutant was restored (to 6% of wild type) simply by raising the pH of the enzyme assay solution to 10.2. Explain this observation. **Actually the precise mechanism by which the D102N mutant functions at pH 10 is not known. The high pH-dependence is suggestive of the deprotonation of a critical functional group, but the identity of the functional group has yet to be determined! Full credit was given for all answers.**