Enzyme Kinetics:

$$v = \frac{\frac{V_{MAX}}{\alpha'}[S]}{\frac{\alpha}{\alpha'}K_M + [S]} \qquad \alpha = 1 + \left(\frac{[I]}{K_I}\right)$$

α' = 1 for competitive inhibition

α' > 1 for non-competitive (mixed) inhibition

α: ratio of slopes α': ratio of y-intercept

$V_{max} = k_{cat}[E_T]$ $K_M = (k_{off} + k_{cat})/k_{on}$

Double reciprocal plot: $\frac{1}{v} = \frac{K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}}$

Thermodynamics:

$\Delta G = \Delta G^o + RT\ln[Products]/[Reactants]$

$\Delta G^o = -RT\ln K_{eq}$

$\Delta G^o = \Delta H^o - T\Delta S^o$

$\mu = \mu^O + RT\ln[X]$

$S = R\ln W$

For A↔B: $f_A = 1/(1+K_{EQ})$ $f_b = K_{EQ}/(1+K_{EQ})$

Amino Acid Names:

Alanine: Ala	Arginine: Arg	Asparagine: Asn
Aspartic Acid:Asp	Cysteine: Cys	Glycine: Gly
Histidine: His	Isoleucine: Ile	Lysine: Lys
Leucine: Leu	Methionine; Met	Phenylalanine:Phe
Proline: Pro	Serine: Ser	Threonine: Thr
Tryptophan: Trp	Tyrosine: Tyr	Valine: Val
Glutamine: Gln	Glutamic Acid: Glu	

Acid-Base Chemistry:

$pH = pK_a + \log([A^-]/[HA])$

$pH = -\log[H^+]$

$[HA] = [A_T]/(1+R)$

$[A^-] = [A_T]\,R/(1+R)$

$R = [A^-]/[HA]$

Ligand Binding (L is the Ligand)

$Y = K_{EQ}[L]/(1+K_{EQ}[L]) = [L]/([L]+K_D)$

$Y = [ML]/([M]+[ML])$

Hill Plot: $\log(Y/(1-Y))$ versus $\log[L]$

Hill Equation: $\text{Log}(Y/(1-Y)) = -\log K_\pi + n_h\log[L]$

Miscellaneous Formula & Constants:

$A = \varepsilon C l$

R = 8.3 J/mol-deg RT = 2.5 kJ/mol @ 300K

log2 = 0.3 ln 2 = 0.69 $\ln X = 2.3\log_{10} X$

To convert from °C to K, add 273.

This exam contains 100 points on 7 pages. Use the back of a page if you need additional space.

1. (2 pts) Equilibrium dialysis was used to measure ligand binding. The *total* protein concentration inside the dialysis bag is *X* (e.g. 1 μM). The free ligand concentration outside the bag is equal to the K_D for this particular protein-ligand pair (e.g. 10 μM). Which of the following expressions gives the ***total*** ligand concentration inside the bag, L_{TOT}, at this particular ligand concentration ([L] = K_D), circle the correct answer:

a) $L_{TOT} = K_D$

b) $L_{TOT} = K_D + X$

c) $L_{TOT} = K_D - X$

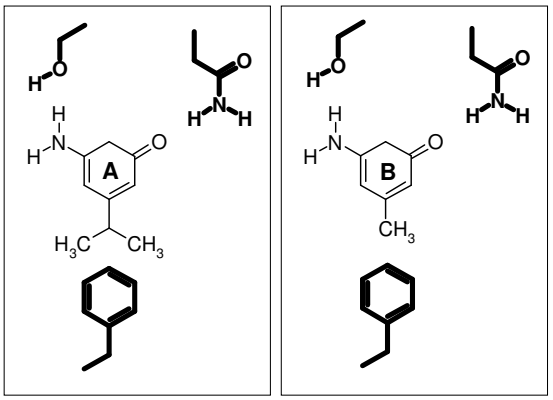
d) $L_{TOT} = \frac{K_D + X}{2}$

e) $L_{TOT} = K_D + \frac{X}{2}$

f) $L_{TOT} = K_D - \frac{X}{2}$

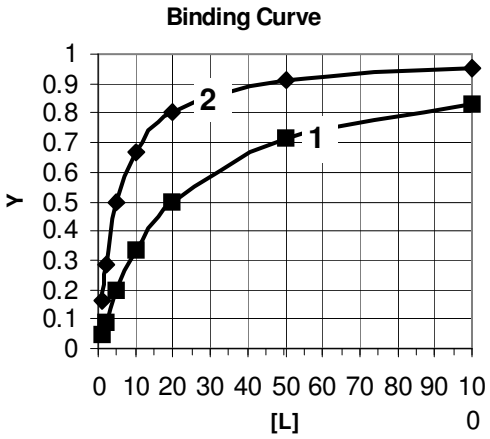
Choice **e)** is the correct answer. The ligand concentration inside the bag is the sum of the free ligand (K_D) + the amount bound to the macromolecule. Since the ligand concentration equals K_D the protein is ½ saturated with ligand and the concentration of (ML) is X/2, giving a total ligand concentration inside the bag as K_d + X/2.

2. (12 pts) Two ligands (A and B) bind to the same protein. The structure of the protein–ligand complexes are shown on the right. The amino acid side chains are shown in bold. Binding curves were measured for each ligand and these are also shown on the right.



i) (8 pts) Determine the K_D values for both binding curves (1 & 2) and *assign* each of your K_D values to one ligand or the other. (*e.g. Ligand A binds with a K_D= xx.x, which was obtained from curve 1.*) Briefly justify your answer with reference to the structure of the ligand and the protein.

K_D values – obtained from the binding curve when Y=0.5



Curve 1: K_D = 20
Curve 2: K_D = 5
(+2 pts)

Curve 2 shows the lower K_D, or higher affinity – indicating stronger interactions. The stronger interaction for ligand A is due to the isopropyl group versus the methyl group, giving increased van der waals and the hydrophobic effect. (+4 pts) therefore:

Ligand A binds with a K_D = 5, which was obtained from curve 2.(+1 pt)

Ligand B binds with a K_D = 20, which was obtained from curve 1.(+1 pt)

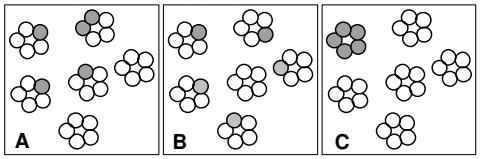
ii) (2 pts) Which ligand binding reaction is likely to show a *larger* increase in entropy (ΔS) on binding? A or B? Why? [Consider the direction of the reaction as: M + L → ML].

Ligand A, since it has a large non-polar group (isopropyl versus methyl). Therefore more ordered water (hydrophobic effect) would be released when it binds. (+2 pts)

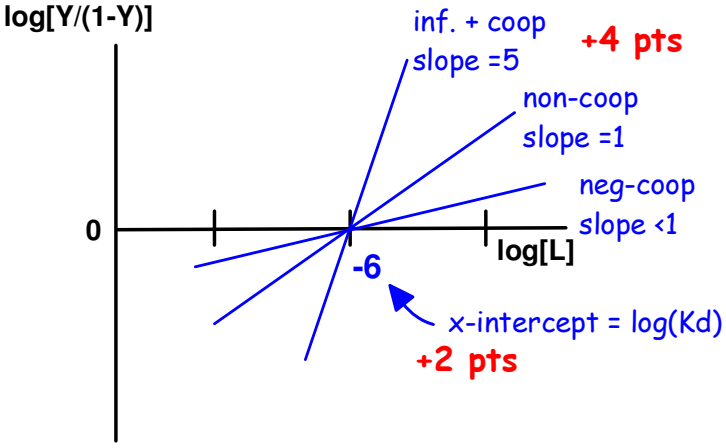
iii) (2 pts) Now assume that A and B are substrates, and that the protein is an enzyme. Which substrate would have the lower K_M, A or B? Why?

Ligand A, because K_M is similar to K_D and ligand A has a smaller K_D. (+2 pts)

3. (10 pts) The following diagram illustrates the distribution of ligands on three different pentameric proteins. Panel A represents a non-cooperative system while panels B and C represent cooperative systems. Sketch the central part of the Hill plot that you would obtain for each of these systems. You can assume that the K_D for all proteins is 10^{-6} M. *Justify your answer on the back of the preceding page.*



Non-cooperative



Panel B: Because there is only a single ligand bound to each protein the binding shows negative cooperativity – once one ligand binds, the binding affinity is reduced. The Hill coefficient (slope of line) will be <1.

Panel C: Since there are only completely empty, or completely full pentamers, the binding is infinitely positive in cooperativity. Once one binds, the remaining sites on the pentamer become high affinity and bind ligand. The Hill coefficient will be equal to the number of binding sites, 5.) (+4 pts for complete justification, +1.5 pts for a simpler discussion of slope versus type of cooperativity.)

4. (6 pts) List the key properties of a protein that are required for cooperative binding behavior. Feel free to us a diagram to answer this question. [Hint; Just relax and answer the question.]
   - Protein must exist in two states, tense and relaxed (+1 pt)
     - Tense state is less active (lower binding affinity) (+1/2 pt)
     - Relaxed state is more active (higher binding affinity) (+1/2 pt)
   - The two states must interconvert. (+2 pts)
   - Ligand binding must affect the relative ratio of the two states. (+1 pt)
   - There must be more than one ligand binding site (+1 pt)

5. (8 pts) Discuss the role of allosteric behavior in any ***one*** of the following processes. Feel free to use a diagram to answer this question.

Choice A: Oxygen transport to the tissues.
   - Oxygen binding to hemoglobin shows positive cooperativity.
   - High oxygen concentration in lungs favors high affinity state causing complete saturation.
   - Lower oxygen concentration in tissues leads to a reduction in affinity and favors the release of oxygen.

Choice B: Adaptation of oxygen delivery at high altitude.
   - Elevated levels of BPG occur during adaptation Bisphosphoglycerate (BPG) stabilizes the tense form of Hb, cause lower affinity.
   - This reduces the overall affinity, causing the binding curve to shift to the right.
   - Although less O_2 is bound in the lungs, a change in the shape of the binding curve cause more O_2 to be released in the tissue.

Choice C: Adaptation of oxygen delivery during vigorous exercise.
   - Acidification of the tissues in active muscle accomplishes the same result as BPG.
   - The tense state is stabilized, leading to enhanced oxygen release.

6. (14 pts) Using transition state theory, explain why enzymes catalyze reactions. Recall that there are two distinct methods by which this is accomplished, one common to *all* enzymes and one displayed by serine proteases. Briefly describe how *either* of these methods serves to increase the catalytic rate.

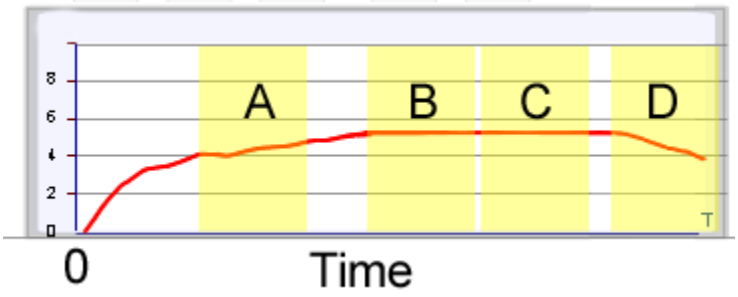
- The rate of the reaction is proportional to the concentration of the transition state (+2 pts)
- The activation energy, or energy difference between the reactants and products, affects the concentration of the transition state (Equivalent to saying the reactants and transition state are in equilibrium) (+2 pts) .
- Enzymes lower the activation energy, generating more transition state and therefore a faster rate (+6 pts) .

Only one of the following points needs to be discussed:

- This can be accomplished by preordering the active site residues in the enzyme so that there is no entropy cost to go to the transition state (all enzymes). (+4pts)

- In some enzyme, interactions that are specific for the transition state also lower its energy, i.e. h-bonds in the oxyanion hole in serine proteases.(+ 4pts)

7. (5 pts) The image to the right shows the concentration of a species in an enzymatic reaction as a function of time, beginning with mixing the enzyme and substrate at t=0. Please answer the following two questions:



i) (2 pts) Which species in the reaction is represented by this curve? (circle your choice):

a) free enzyme, (E).
b) substrate (S)
c) enzyme-substrate complex (ES) (+2 pts)
d) product (P)

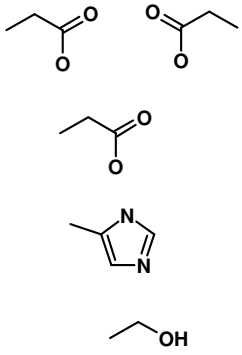
ii) (1 pt) Which of the following periods, A, B, C, or D would be more suitable for measuring the initial velocity of the enzyme catalyzed reaction (circle your answer):

A (+1/2 pt) The assay should be performed during steady-state, d[ES]/dt=0
B (+1 pt) Either B or C are correct in this regard.
C (+1 pt) B is the best answer since there will be less product to cause product inhibition and the substrate concentration will be closer to the starting value. **(+2 pts)** Steady state has not been reached during "A", any data collected during this period could not be analyzed using v=Vmax[S]/(Km+[S]).
D

*Briefly justify your answer in the above space, or the back of the previous page. (**2 pts**).*

8. (3 pts) Fill in the blanks: At very low substrate concentration the measured velocity in an enzyme catalyzed reaction is essentially linear (proportional to, limited by) (+1) with respect to substrate concentration ([S]), while at high substrate concentrations the velocity is ___independent (+1)___ of substrate concentration, indicating that the enzyme is saturated (+1) with substrate.

9. (10 pts) Please do ***one*** of the following choices (labeled drawings are acceptable)



Choice A: Briefly explain the molecular basis for the substrate specificity of ***either*** trypsin, chymotrypsin, elastase, ***or*** HIV protease. Which kinetic rate-constant, k_{on}, k_{off}, or k_{cat} will be most affected by changing substrates? Why?

Choice B: Using ***either*** serine proteases ***or*** HIV protease as an example, discuss how functional groups on the enzyme lead to cleavage of the peptide bond. The diagrams to the right may be helpful.

Choice C: Explain how the pH dependencies of K_M ***or*** V_{MAX} (k_{CAT}) can be used to identify functional groups that are important for substrate binding ***or*** catalysis. Be sure to illustrate your answer with an example from ***either*** serine proteases ***or*** HIV protease.

Choice A:

Trypsin likes lysine and arginine as substrates because the positive charge on the sidechain of the substrate interacts with the negative charge of an Aspartic acid residue in the specificity pocket (+6 pts). There is also good van der Waals contact between the sidechain and the enzyme (long skinny binding pocket) (+1 pts)

Chymotrypsin likes large aromatic residues, its binding pocket is large and non-polar to optimize the contribution from the hydrophobic effect and van der Waals contacts.

Elastase likes small non-polar residues (e.g. Ala), its binding pocket is small and non-polar to optimize the contribution from the hydrophobic effect and van der Waals contacts.

HIV protease is similar to chymotrypsin in specificity, its binding pocket is large and non-polar to optimize the contribution from the hydrophobic effect and van der Waals contacts.

Rate constant changes: Different substrates are more likely to show differences in k_{off} (+3 pts).

Choice B:

Serine proteases	HIV protease
• Catalytic triad contains Ser, His, and Asp. • Histidine activates serine to become an nucleophil by remove a proton form serine. • This positively charged His is stabilized by neg charge on Asp. • Serine attacks electropositive carbonyl carbon. • Tetrahedral transition state forms. • Decay of transition state leads to bond cleavage, and formation of acyl intermediate. • Cleavage of ester in the acyl intermediate occurs by same mechanism, except H_2O is the nucleophil.	• Two Asp are required for activity, one protonated and one deprotonated. • Water is activated as a nucleophile by removal of a proton by the deprotonated Asp. • Hydroxide attacks electropositive carbon. • Tetrahedral transition state forms. • Decay of transition state leads to bond cleavage. • There is no acyl intermediate since water was the first nucleophil.

Choice C:

The K_M or k_{CAT} will be proportional to f_{HA} if the protonated form is required for substrate binding or catalysis.

The K_M or k_{CAT} will be proportional to f_{A^-} if the deprotonated form is required for substrate binding or catalysis.

The K_M or k_{CAT} will be at half their maximum value when the pH=pKa if a single ionization is involved.

For example: The K_M of for trypsin will be high at low pH since the Asp in the specificity pocket must be deprotonated to bind the positive charge on the substrate. The K_m will drop as the group is deprotonated due to stronger binding.

10. (12 pts) Briefly explain why a competitive inhibitor only affects K_M but not k_{CAT} (V_{MAX}) while a mixed type inhibitor can affect both. Your answer should include a discussion/description of the general molecular structure of each type of inhibitor and its binding site on the enzyme.

- A competitive inhibitor binds at the active site and is similar in structure to the substrate (+2 pts).
- Since the substrate has to complete with the inhibitor for the binding site, a higher concentration of substrate is required to half-saturate the enzyme – so the observed K_M increases (+3 pts)
- Since all of the inhibitor is displaced at high substrate, it is possible to saturate the enzyme with substrate, so Vmax is the same (+3 pts).
- A mixed inhibitor binds elsewhere and distorts the structure of the activity site, affecting both the substrate binding (Km) and the catalytic step, Vmax (+4 pts).

11. (6 pts) Please answer ***one*** of the following two choices:

Choice A: Provide a definition for specific activity and describe its usefulness in protein purification.

Choice B: Select one method of column chromatography and briefly explain the basis for the separation of proteins by that method.

Choice A: The specific activity is the ratio of the amount of target protein/total mass of the protein. It should increase if a step in the purification has increased the purity.

Choice B:

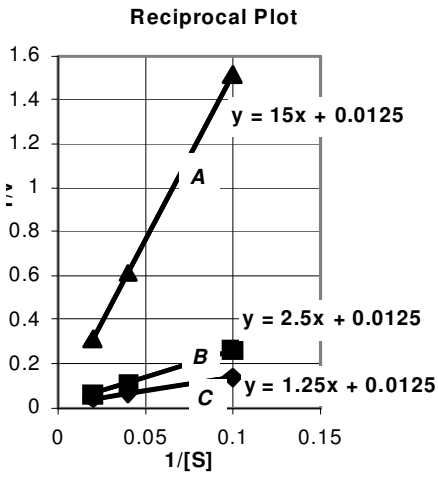
Ion exchange: Proteins stick to opposite charges on beads due to the presence of negative charges on the protein (anion exchange) or positive charges on the protein (cation exchange). Proteins with a higher level of charge will elute later.

Affinity chromatography: The beads have a ligand (or antibody) bound that causes the target protein to stick to the beads, allowing all other proteins to be washed away. The protein is eluted with free ligand, or in the case of antibody columns, a change in the solution conditions.

Gel filtration: The beads are porous and smaller proteins enter the beads while larger proteins go around the beads and elute first. The smaller proteins have spent more time on the column and elute last.

12. (12 pts) Please do ***one*** of the following three choices. Choices B and C can be found on the following page.

Choice A: The ability of a drug to inhibit the wild-type HIV protease and a mutant HIV protease (valine82 → serine) were tested using steady-state enzyme kinetics and the experimental data was plotted on the double reciprocal plot shown to the right. *Either enzyme was equally active against the substrate used in the assay, i.e. the mutation did not affect the steady state kinetics when the drug was absent.*



The structure of the drug-enzyme complexes are shown below. The drug is less active in individuals infected with the mutant virus.

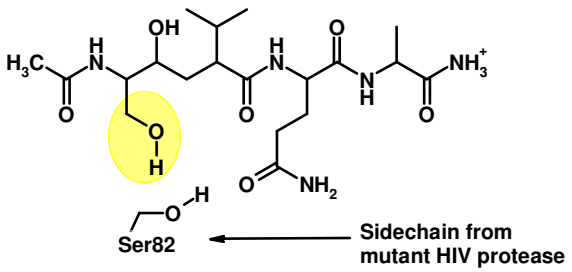
i) (4 pts) Which of the three lines (A, B, or C) corresponds to the data acquired without the inhibitor? *Briefly justify your answer.*

Curve C, because it shows the highest velocity, no inhibitor must be present (+4 pts).

ii) (4 pts) Which of the three lines (A, B, or C) corresponds to the data acquired with the *wild-type* enzyme plus the drug. *Briefly justify your answer.*

Since the drug binds more tightly to the wild type enzyme, it should reduce the velocity more effectively, so **curve A** corresponds to the wild-type enzyme. (Alternatively, the K_I will be lower for the wild-type enzyme, giving a larger α-value or a larger slope on the double recp. Plot.) (+4 pts).

iii) (4 pts) Briefly explain how you would modify the original drug such that it will be more effective against the virus with the mutant HIV protease. A sketch is acceptable, feel free to use the template on the right. *Briefly justify your answer on the back of the previous page*



Replacing the non-polar cyclohexane ring with a polar hydrogen bond donor (or acceptor) would enhance the binding. For example, an alcohol group is shown on the right. (+4 pts).

Choice B: Devise a purification scheme that will separate protein C from the following five proteins (A-E). You may summarize your purification scheme using a flow-chart, be sure to briefly indicate the logic you employed for each step.

Protein	[Ammonium Sulfate] that precipitates 50% of protein*	# Residues (Mol Wt)	#Asp (pK_a=4.0)	#His (pK_a=6.0)	#Lys (pK_a=9.0)
A	1.0 M	120 (13,200 Da)	2	0	10
B	1.5 M	120 (13,200 Da)	4	4	12
C	**4.0 M**	**120 (13,200 Da)**	**4**	**8**	**4**
D	4.5 M	125 (13,800 Da)	2	8	4
E	4.0 M	240 (26,400 Da)	2	0	10

*Assume an ammonium sulfate concentration that is 1 M above the 50% ppte concentration will precipitate all of that protein and 1 M below will leave all of the protein in solution; i.e. protein A is completely ppte by 2M ammonium sulfate.

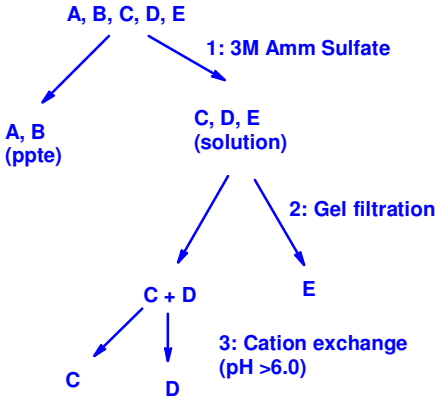
Step 1: Ammonium sulfate @ 3 M will ppte A & B, leaving C, D, and E in solution.

Step 2 : Gels filtration will separate C and D from E due to differences in size.

Step 3 : Cation exchange chromatography will separate C from D.

At pH = 6.0 (All Asp residues will be deprotonated, His ½ protonated, Lys protonated):

q_C = -4 +4 + 4 = +4 q_D = -2 +4 +4 = +6



(+9 pts for reasonable scheme, +3 pts for brief justification.)

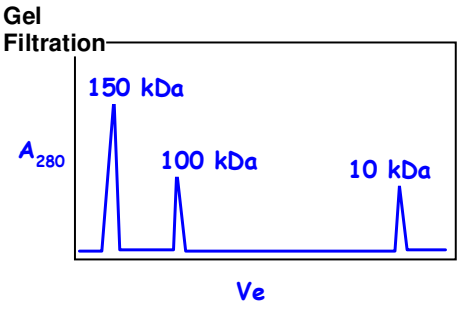
Choice C: You are verifying the quaternary structure of an antibody, which consists of two light chains (25 kDa) and two heavy chains (50 kDa). One light chain is crosslinked to one heavy chain via a disulfide bond. Assume that there are no disulfide bonds between the heavy chains.

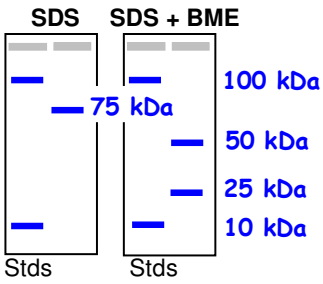
i) (+3 pts) *Sketch* the gel filtration profile you would observe for this protein. Assume that you included size standards of 10 and 100 kDa on the gel filtration column.

The overall native molecular weight of the antibody is 2 x (25+50) = 150 kDa. This would elute before the 100 kDa standard.

ii) (+3 pts) *Sketch* the SDS gel that you would obtain from this sample, again including size standard of 10 and 100 kDa. You should assume that β-mercaptoethanol (BME) was not included. The left lane should be used to indicate the position of the standards (Stds).

The disulfide bond will remain intact, so the light and heavy chain will have a molecular weight of 75 kDa. Since the heavy and light chains are identical in an antibody, a single band will be found at that molecular weight.





Grading note: Some students may have assumed that only one H+L was linked via disulfide bonds while the other was not. This would give three bands on the SDS gel, 25 & 50 & 75 kDa, which should get full credit.

iii) (+3 pts) Sketch the SDS gel that you would obtain form this sample, assuming that BME was included.

This would just show the individual chain molecular weights. 25 & 50 kDa.

iv) (+3 pts) Briefly explain how SDS-PAGE separates proteins by size.

The SDS gives the protein a negative charge. The proteins are then forced to migrate through a gel. The smaller proteins can more easily go through the gel and so migrate further than larger proteins.