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B.M.S. College of Engineering, Bengaluru-560019

Autonomous Institute Affiliated to VTU

July 2023 Semester End Main Examinations

Program: B.E. **Semester: VI**
Branch: Biotechnology **Duration: 3 hrs.**
Course Code: 19BT6DCETK **Max Marks: 100**
Course: Enzyme Technology & Kinetics **Date: 10.07.2023**

Instructions: 1. Answer any FIVE full questions, choosing one full question from each unit.
2. Missing data, if any, may be suitably assumed.

		UNIT – I	*CO*	*PO*	Marks
1	a)	Suppose you have a mixture of five proteins listed in the table below	*CO1*	*PO1 PO2*	**10**

	Protein	pI	Mol.Weight (kDa)
A	Ovalbumin	4.6	45
B	Myoglobin	7.0	16.7
C	Serum albumin	4.9	68.5
D	Ubiquitin	6.4	8.5
E	Cytochrome c	10.6	13

i. Indicate the order in which these proteins will elute from a gel-filtration column (starting with the one that elutes first). You can use letters A-E (see table) for simplicity.
ii. You load this mixture on a cation exchange column. The buffer you use for this column is acetate buffer, pH 4.76. List proteins that will appear in the flow-through.
iii. In order to elute those proteins that are immobilized on the column, you then apply a linear salt gradient, with NaCl concentration gradually increasing from 0 to 1 M. Indicate the order in which the proteins bound to the column will elute as the salt concentration increases.
iv. You repeat the same procedure as in (ii) but now you use anion exchange column and the buffer is TRIS (pH 8.0). List proteins that will appear in the flow-through.
v. You are interested in further studies of cytochrome c. Based on the results of your experiments above, devise a reliable procedure for its purification from this mixture of proteins.

		b)	You have a crude lysate sample (CL) containing a mixture of six proteins (1, 2, 3, 4, 5, β-galactosidase), and your goal is to obtain purified β-galactosidase. Some characteristics of these proteins are shown in the table below:	*CO1*	*PO1* *PO2*	**10**

Protein	Concentration of ammonium sulfate (AS) required for precipitation	Molecular Weight (kDa)	Isoelectric point (pI)
1	45%	38	3.7
2	80%	22	4.8
3	65%	4	5.3
4	20%	75	6.8
5	30%	55	9.50
β-galactosidase	45%	115	5.3

You begin your purification by performing an ammonium sulfate (AS) precipitation. You add the appropriate concentration of AS to your CL sample, incubate overnight at 4˚C, then centrifuge to generate a supernatant (AS-S) and pellet (AS-P).

i. What concentration of AS will you use to precipitate β-galactosidase?

ii. After addition of that concentration of AS and centrifugation, which protein(s) will be in the supernatant (AS-S)?

iii. Which protein(s) will be in the pellet (AS-P)?

iv. After resuspending the AS-P in column buffer, which method you use to desalt the protein sample.

One way to purify β-galactosidase away from any contaminating proteins in the AS-P sample would be to separate them based on their molecular weight.

v. What type of column separates on this basis?

vi. Which protein (from your AS-P) would elute first from this type of column?

Instead, you decide to use ion exchange chromatography to further purify β-galactosidase away from other proteins in your AS-P sample. You first run an anion exchange column equilibrated using column buffer with a pH of 5.0.

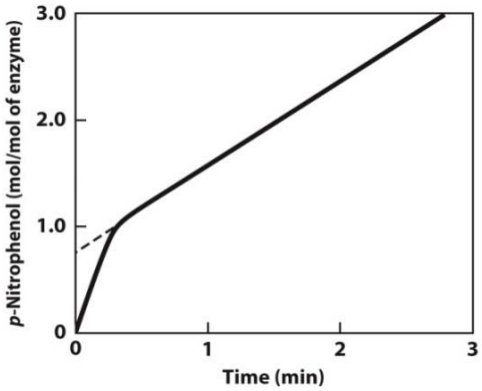
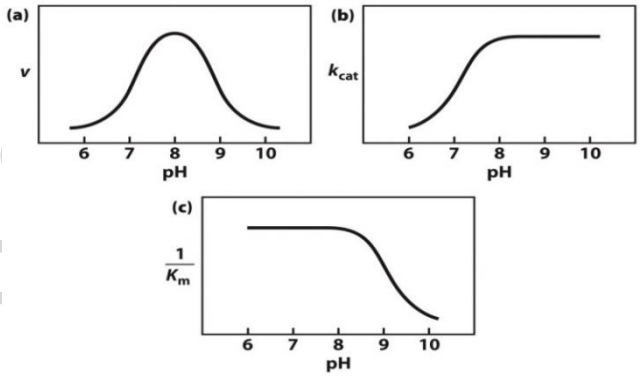
vii. What charge does the matrix of an anion exchange column have?

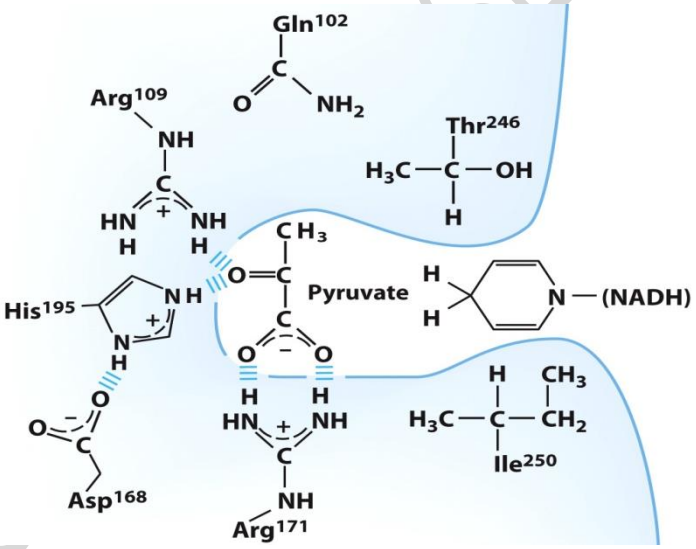
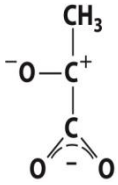
			viii. At pH 5.0, which protein(s) from the AS-P stick to the anion exchange column? ix. State how you would elute a protein bound to an anion exchange column, and explain how this elution method works in one or two sentences.			
			UNIT – II			
	2	a)	You measured the initial velocity of an enzyme in the absence of inhibitor and with inhibitor A or Inhibitor B. In each case the inhibitor is present at 10 µM. The data are shown in the table.	*CO2*	*PO2*	**10**

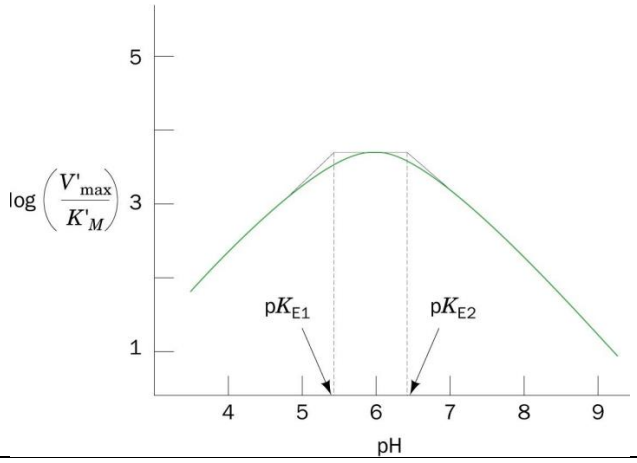
[S], (mM)	Velocity, (M s-1) x 10^7 Uninhibited	Velocity, (M s-1) x 10^7 Inhibitor A	Velocity, (M s-1) x 10^7 Inhibitor B
0.333	1.65	1.05	0.794
0.40	1.86	1.21	0.893
0.50	2.13	1.43	1.02
0.666	2.49	1.74	1.19
1.0	2.99	2.22	1.43
2.0	3.72	3.08	1.79

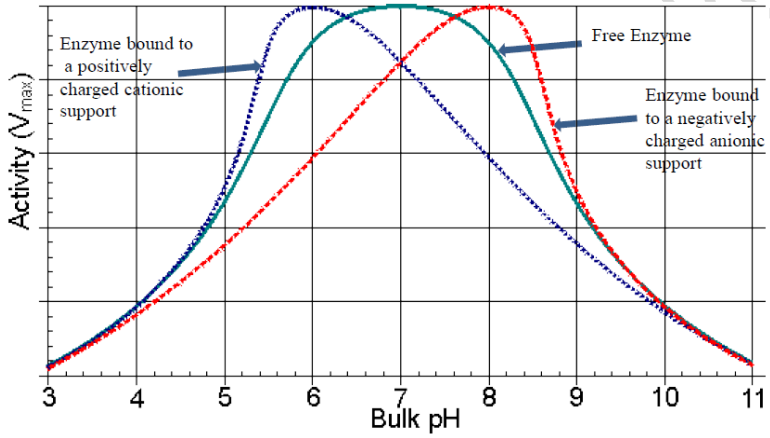
i. Determine the Km and Vmax of the enzyme.
ii. Determine the type of inhibition imposed by inhibitor A and and K_I.
iii. Determine the type of inhibition imposed by inhibitor B and calculate and K_I.

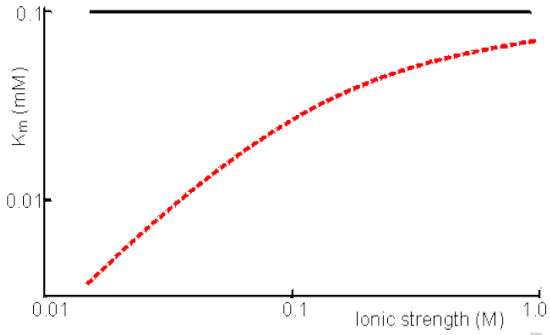
		b)	An enzyme is discovered that catalyses the chemical reaction SAD ⇌ HAPPY A team of motivated researchers sets out to study the enzyme, which they call happyase. They find that the k_{cat} for happyase is 600 s^{-1}. They carry out several experiments. When $[E_t]$ = 20 nm and [SAD] = 40 µM, the reaction velocity, V_0 is 9.6 µM s^{-1}. Calculate K_m for the substrate SAD.	*CO2*	*PO2*	**05**
		c)	For a given enzyme catalysed reaction, the Michaelis constant is 0.6 mM and the substrate concentration is 1.0 mM, what is the fractional saturation of the enzyme under these conditions?	*CO2*	*PO2*	**05**
			UNIT – III			
	3	a)	The pre-steady state kinetic evidence for an acyl-enzyme intermediate was obtained by measuring the amount of p-	*CO3*	*PO1*	**10**

			nitrophenol released during hydrolysis of p-nitrophenyl acetate by chymotrypsin (refer the figure given below).  i. Initially, the reaction releases a rapid burst of p-nitrophenol nearly stoichiometric with the amount of enzyme present. The subsequent rate is slower. Provide a plausible explanation for this evidence. The rates of chymotrypsin-mediated hydrolysis produce a bell shaped pH-rate profile with an optimum at pH 8.0 (refer figure (a) below). The plot can be further dissected by first obtaining the maximum rates and K_m at each pH, and then plotting k_{cat} alone versus pH (refer figure (b) given below) and 1/ K_m versus pH (refer figure (c) given below).  ii. Give your opinion for decline in k_{cat} at lower pH and changes in 1/ K_m at higher pH? iii. Debate the possible role played by the catalytic triad in the chymotyrpsin catalysed reaction mechanism. iv. Predict the structual basis of substate specificity exhibited by the chymotrypsin towards the peptide bond.			
	b)	The thiozolium ring of TPP plays role in many biochemical reactions by acting as a “electron sink”. Elucidate the mechanism with suitable example	*CO3*	*PO1*	**06**

		c)	Identify the vitamin present in each of the following coenzymes. i) $NADP^+$, ii) $FADH_2$, iii) Coenzyme-A and iv) Pyridoxal phosphate	*CO3*	*PO1*	**04**
			OR			
	4	a)	Examining the structure of an enzyme results in hypothesis about the relationship between different amino acids in the protein's structure and the protein's function. One way to test these hypothesis is to use recombinant DNA technology to generate mutant versions of the enzyme and then examine the structure and function of these altered forms. One example of this kind of analysis is the work of Clarke and colleagues on Lactate dehydrogenase (LDH), which catalyses the reduction of pyruvate with NADH to form lactate. A schematic of the enzyme's active site with the pyruvate at the centre is given below.  The reaction mechanism is similar to many NADH reductions. The transtition state involves a stongly polarized carbonyl group of the pyruvate as shown below.  i. A mutant form of LDH in which Arg^{109} is repalced with Gln shows only 5% of the pyruvate binding and 0.07% of	*CO3*	*PO1*	**10**

			the activity of wild-type enzyme. Provide a plausible explanation for the effects of this mutation. ii. A mutant form of LDH in which Arg^{171} is replaced with Lys shows only 0.05% of the wild-type level of substrate binding. Why is this dramatic effect surprising? iii. A mutant form of LDH in which Ile^{250} is replaced with Gln shows reduced binding of NADH. Provide a plausible explanation for this result. Clarke and colleagues also set out to engineer a mutant version of LDH that would bind and reduce oxaloacetate rather than pyruvate. They made a single sustitution, replacing Gln^{102} with Arg; the resulting enzyme would reduce oxaloacetate to malate and would no longer reduce pyruvate to lactate. They had therefore converted LDH to malate dehydrogenase. iv. Sketch the active site of this mutant LDH with oxaloacetate bound. v. Provide a plausible explanation for why this mutant enzyme now " prefers" oxaloacetate instead of pyruvate.			
	b)	The active site of RNase A contains two amino acid residues essential for catalysis: His^{12} and His^{119}. The pKa values of the imidazole side chains of these residues are 5.4 and 6.4 respectively. i. What is the ionization state (protonated or deprotonated) of each residue at pH 6.0, the pH optimum of RNase A? ii. How can the ionization states of these residues explain the pH-activity profile of RNase A shown below?  	*CO3*	*PO1*	**07**

		c)	Which of the listed effects would be brought by any enzyme catalysing the following simple reaction? $S \underset{k_2}{\overset{k_1}{\rightleftarrows}} P$ where $K'^{0}_{eq} = \frac{[P]}{[S]}$ (i) Decreased K'^{0}_{eq} (ii) Increased k_1 (iii) Increased K'^{0}_{eq} (iv) Increased $\Delta G^{\ddagger}$ (v) Decreased $\Delta G^{\ddagger}$ (vi) More negative $\Delta G'^{\circ}$ (vii) Increased k_2	*CO3*	*PO1*	**03**
			UNIT – IV			
	5	a)	Following is the schematic diagram of the variation in the profiles of activity of an enzyme, immobilized on charged supports, with the pH of the solution.  i. Why there is apparent shifts in the behaviour of the kinetic constants for the immobilized enzyme with respect to the solution pH? ii. How do you produce a pH of 7.0 within the microenvironment?	*CO4*	*PO1* *PO 7*	**08**

		b)	The following diagram dipicts the effect of immobilisation and ionic strength on the K_m of bromelain for its positively-charged substrate, N-α-benzoyl-L-arginine ethyl ester. The support is the negatively-charged poly anionic polymer, carboxymethyl cellulose. (------ for immobilized enzyme and ——— for soluble enzyme).  Justify the fact that K_m increases with increase in the ionic strength.	*CO 4*	*PO1* *PO7*	**04**
		c)	An enzyme with a pH optimum of 7.5 was bound in a positively charged support. Estimate the pH optimum of the immobilized enzyme at 310 K, given that the electronic potential was found to be 0.01 V. Assume that R = 8.314 J K^{-1} mol-1 and F = 96487 J V^{-1}	*CO4*	*PO1* *PO7*	**04**
		d)	For an enzyme-catalysed reaction under specified conditions, K_m = 4.0 mmol l^{-1}, V_{max} = 0.50 mmol l^{-1} min^{-1} and the initial substrate concentration is 20.0 mmol l^{-1}. How long will it take for the product concentration to rise from zero to 5.0 mmol l^{-1}, assuming that the Michaelis-Menten equation is valid throughout?	*CO4*	*PO1* *PO7*	**04**
			UNIT – V			
	6	a)	Another extremely promising area of genetic engineering is protein engineering. Using this technology new enzyme structures may be designed and produced in order to improve on existing enzymes or create new activities. Design and construct any novel enzyme using protein engineering techniques.	*CO5*	*PO1* *PO3*	**10**
		b)	Thermophilic and hyperthermophilic organisms can able to tolerate high temperature. These organisms should have macromolecules (enzymes and other structural proteins, DNA and lipids), which are more stable than their counterparts in	*CO5*	*PO1* *PO3*	**05**

			mesophiles. Appraise about the structural features of these macromolecules, which make these extreme thermophilic organisms to thrive at higher temperature.			
		c)	What is biotransformation? Enumerate key steps in developing a successful biotransformation process.	*CO5*	*PO1 PO3*	**05**
			OR			
	7	a)	Biocatalytic transformations performed in organic media offer certain advantages. One of the solvent systems commonly used for enzyme-catalyzed reactions containing organic media is "Enzyme suspended in a Monophasic Organic Solution". Enumerate the parameters to be taken into consideration in order to tune a biocatalytic reaction in a monophasic organic solvent system.	*CO5*	*PO1 PO3*	**10**
		b)	Metagenomics has proven effective for identification of microbes and isolating novel biocatalysts from the environment as well as to acquire ecological data. Design metagenomic screening methods for the isolation of novel enzymes.	*CO5*	*PO1 PO3*	**10**

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