UNIVERSITY OF YORK
BSc Stage 3 Degree Examinations 2017-18

Department:
BIOLOGY

Title of Exam:
Biocatalysis

Time allowed: 2 hours
Total marks available for this paper: 100

This paper has two parts:

Section A: Short Answer / Problem / Experimental Design questions (50 marks)
- Answer all questions in the spaces provided on the examination paper

Section B: Essay question (marked out of 100, weighted 50 marks)
- Answer one question from question A, question B or question C
- Write your answer on the separate paper provided and attach it to the back of the question paper using the treasury tag provided

- The marks available for each question are indicated on the paper
- A calculator will be provided

For marker use only:

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Total as %

DO NOT WRITE ON THIS BOOKLET BEFORE THE EXAM BEGINS
DO NOT TURN OVER THIS PAGE UNTIL INSTRUCTED TO DO SO BY AN INVIGILATOR
SECTION A: Short Answer / Problem / Experimental Design questions

Answer all questions in the spaces provided

Mark total for this section: 50

1. a) What advantages do enzymes offer the chemical industry compared to chemocatalysts? (2 marks)

Enzymes are highly efficient catalysts that work under mild conditions (low temperatures, low pressure and mild pH) and are enantiospecific (1 mark). They can catalyse reactions that are not currently possible with chemocatalysts and being non-toxic and biodegradable they are environmentally friendly (1 mark).

b) Biosensors comprise a biological recognition component (usually an enzyme or antibody) and a transducer that converts a biological signal into a signal that can be quantified or processed. Describe why enzymes make excellent recognition components and why the involvement of a cofactor like NAD(P) is desirable? (2 marks)

Enzymes provide specificity and selectivity for a target analyte and turnover of substrate at the active site provides the opportunity for real-time monitoring (1 mark).

Cofactors, such as NAD, can be coupled to a variety of transducers such as an electrode to generate a current that can then be measured (1 mark).

c) You have been asked by HM Customs & Excise to develop an enzyme based biosensor to detect illicit heroin and cocaine; however, there are no suitable enzymes commercially available. Describe a strategy to find a new microbial enzyme that is active towards heroin (diacetylmorphine) and suitable for use in a biosensor. (5 marks)

A successful and proven strategy is to isolate culturable microorganisms that have the ability to metabolise heroin and identify the enzyme(s) mediating its degradation. This is done by selective enrichment where samples are taken from an environment where microorganisms have been exposed to opiates for long periods of time, such as rhizosphere soil from the root zone of poppy.
plants or samples pharmaceutical industry waste streams, and use these samples to inoculate media containing heroin as the sole carbon. By using heroin as the sole carbon and/or nitrogen source in the medium, only those organisms capable of metabolising heroin will grow (3 marks). The cells then need to be harvested, lysed and the subcellular fraction assayed for activity towards heroin (2 mark). Note - it may be necessary to add cofactors to identify the activity in the subcellular fraction. If the enzyme cannot be identified through activity assays, then ‘omics technologies such as transcriptomics and proteomics could be adopted.

d) You identify two enzymes, a heroin esterase and a NADP⁺-dependent morphine dehydrogenase. Show on the scheme below how these two enzymes can be coupled to bioluminescence to detect heroin? (5 marks)

Answer:

The formation of NADPH in the presence of heroin can be linked to bacterial
luciferase and used as the basis of a biosensor (2 mark). An NADPH specific NADP:FMN oxidoreductase generates FMNH$_2$, the luciferin for bacterial luciferase (1 mark), which in the presence of a long chain aldehyde and oxygen generates light (2 marks).

e) Why are members of the Old Yellow Enzyme family of flavoenzymes proving to be important biocatalysts? (3 marks)

Enzymes of the OYE family are stable, they have an open active site which allows them to accommodate a broad range of substrates of varying size (e.g. steroids, opiates, nitroaromatics, RDX, oxidized lipids) and they catalyse a wide range of reactions (2 marks). Examples of reactions include: reduction of a large number of $\alpha,\beta$-unsaturated aldehydes and ketones, reductive denitration of nitrate esters, reduction of nitroaromatics to hydroxyl amino derivatives and formation of hydride meisenheimer complexes with TNT (1 mark).

f) Why is Old Yellow Enzyme such a well characterized enzyme? (3 marks)

OYE was one of the first flavoproteins to be identified (1 mark). The large spectral differences between various flavin oxidation states, means it is possible to monitor the events occurring in OYE mediated catalysis using the flavin itself as a reporter (2 marks).

Module-level learning outcome being addressed:

- Students will be able to describe, through the use of examples the role of coenzymes in enzyme catalysis.
- An appreciation from studying specific examples of the use of enzymes for biotechnological applications.

2. Phenylalanine Dehydrogenase (PheDH) catalyses the interconversion of L-phenylalanine 1 (below), and a ketoacid 2 (structure not shown) with both water and an oxidised nicotinamide cofactor (NAD$^+$) required for the reaction in the oxidative direction.
a) Complete the reaction Scheme above to show the structure of the ketoacid product 2 in the box provided, and identify co-products 3 and 4. (3 marks)

3 is ammonia (NH$_3$); 4 is NADH (+ H$^+$). (1, 1, 1)

Few fully correct answers although most recognised that NADH is co-product 4.

A representation of the active site of PheDH, in complex with L-phenylalanine is shown below, with selected residues numbered. A table with the genetic code is presented alongside.
b) The structure suggests that aspartate residue D119 makes an interaction (black dashed lines) with the nitrogen atom of L-Phe. Name this type of interaction, and suggest a mutation that would enable you to study the role of this residue.

It is an electrostatic interaction or 'salt-bridge' (1); Mutation of the carboxylate to an Asn (isosteric, but not a carboxylate) would enable one to assess the role of this interaction in enzyme activity (1). Mutation to Ala also acceptable.

Most answers suggested ‘H-bond’ although the structure of 1 suggests an ionic interaction. Almost all answers gave ‘Ala’ or Gly as the answer to the second part, which was acceptable.

The coding sequence for PheDH is shown below:

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1    atgagcattgatagcgcgctgaactgggatggcgaaatgaccgtgacccgctttgatgcg
61   atgaccggcgcgcattttgtgattcgcctggatagcacccagctgggcccggcgggc
121  ggcaccggggcgccgcagctatagcagcacctggggagttcgtggcgcggggcaactctg
181  ggcggccggcgtacacttctgaacattctgggtgagctgctgaccgatgcgctg
241  gcggggcgccggctgaaaatggcggtgagcaacctgccgatgggcggcggcaaaagc
301  gtcgtgctgccggcgccggtgcgctggtgggctgccggcgccgctgctg
361  cgcctggaacgcccccccagcgcgatggcgtggcgcatcgcggcctgggcagcctggatggcctgaccgtgctg
421  gctggcggcgtgcctggccgctggccggtggcgtgtttgaa
481  gcgcgccgggccgtgctggccggtggccgctgctggccgctggccggtggccgctgctggccgctgctggccgctgct
541  ggcggccgggcttgctgggcctggccggtgctgctggccgctgctggccgctgctggccgctgct
601  cggctgcttgctggccgctggccgctggccgctgctggccgctgctggccgctgct
661  atgggcggctggccgctggccgctggccgctgctggccgctgctggccgctgct
721  atgggcggctggccgctggccgctggccgctgctggccgctgctggccgctgct
781  ggcggccggccgccgctggccgctggccgctgctggccgctgctggccgctgct
841  cggctgctggccgctggccgctggccgctgctggccgctgctggccgctgct
901  ggcggccggccgccgctggccgctggccgctgctggccgctgctggccgctgct
961  ctggccggccgccgctggccgctggccgctgctggccgctgctggccgctgct
1021 ctggccggccgccgctggccgctggccgctgctggccgctgctggccgctgct
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c) Showing your working, design a forward 5'-3' primer for a site-directed mutagenesis experiment that would generate this mutant.  

(3 marks)

D119 codon starts at (3 x 118) + 1 bp. ‘gat’. (1)

tat tgg acc ggc ccg gat gtg aac acc aac agc

Replace D with codon for N; ‘aac’ (1) and

primer, with 15 bp either side of the mutation site is:

tat tgg acc ggc ccg gct gtg aac acc aac agc (1)

Lots of good answers here and almost all attempted it. Wrong answers usually due to errors in counting the bases, although even these were given credit for choosing an Asp codon etc.

c) Describe an experiment to investigate the role of D119 in the recognition of the amino acid nitrogen atom using the mutant you have designed, and outline the expected effects on your measurements.

(3 marks)

Kinetic constants of wild-type and D119N (or A) should be determined (1), using UV spectrophotometry, at increasing concentrations of 1 and $K_m$ and $k_{cat}$ (or $V_{max}$) values should be determined (1). For the disrupted electrostatic interaction, one would expect the $K_m$ to increase and the $V_{max}$ would quite probably be reduced (1).

(3 marks)

About one third gave very good answers that recognised a UV assay for NAD reduction, and also the necessity to measure $k_{cat}$ and $K_m$. Others mentioned or suggested at least an ‘assay’ and said that ‘activity’ would be reduced.
d) The structure suggests that lysine K67 and asparagine N263 interact with the carboxylate of L-Phenylanine. Focusing on these residues, suggest TWO protein engineering strategies that may enable you to create an enzyme competent for the interconversion of 5 and 6 below. (5 marks)

\[ \text{Phe-DH mutant} \]

K66 and N262 appear to be primarily responsible for carboxylate recognition, so one would mutate these residues, preferably to hydrophobic ones that would form more favourable interactions with the methyl groups in 5 and 6 (1). One could mutate each to alanine K66A and N262A (1), and also make the double mutant K66A/N262A (1). However, a more comprehensive approach would be to create saturation libraries at each position (to test the effects of mutating in each position) (1), but also a library in which each position was randomised in combination with the other (approx. 400 variants) (1).

Most popular answers featured saturation mutagenesis and chemical modification, each of which were fine but they should also have mentioned straight SDM for replacing K and N with, for example Leu, encouraging a hydrophobic interaction with the new methyl group.

e) Describe a mutational strategy for the creation of a PheDH variant competent for the formation of D-Phenylalanine from 2, dependent on random mutagenesis. (4 marks)

A fully randomised mutagenesis strategy might be adopted for this. Error prone PCR could be used to generate a library of single point variants throughout the length of the PheDH gene (1). This is accomplished by increasing either the magnesium ion or dGTP concentration in a PCR (1).
The library should be assayed for any sign of formation of D-Phenylalanine from 2, ideally using a high-throughput colorimetric assay if possible (1). The best mutant should be used for further rounds of epPCR (1), which will introduce further point mutations. (4 marks)

The key here was to choose a random mutagenesis method i.e. epPCR or gene shuffling, and those answers were given substantial credit. CASTing was not the answer sought here, but answers that gave this were awarded partial marks.

Module-level learning outcome being addressed:

- To be able to explain how site-directed mutagenesis experiments can be used to probe enzyme catalysis and improve/alter enzyme activity and specificity.
- To be able to explain how ‘random mutagenesis’ or ‘in vitro’ evolution experiments can be used to improve or alter the catalytic properties of enzymes.

3. What differentiates a hammerhead ribozyme cleaving in *trans* from most other site-specific nucleolytic ribozymes? (2 marks)

An “in trans” hammerhead ribozyme cleaves another RNA molecule (1 mark), i.e. not itself, thus it is able to catalyse multiple cleavage reactions without itself being modified (1 mark).

Module-level learning outcome being addressed: To be able to explain using specific examples how RNA can act as a catalyst and how ‘in vitro’ evolution can be used to generate novel RNA catalysts.

4. Mg$^{2+}$ is important for ribozyme function. Using examples, briefly describe the two roles it can have in ribozymes. (3 marks)

Mg$^{2+}$ can have structural and catalytic roles (0.5 mark each). Most ribozymes will non-specifically bind Mg$^{2+}$ in a structural role where it neutralises the intrinsic negative charge of the RNA backbone (1 mark). Some ribozymes use a hydrated Mg$^{2+}$ as a catalytic co-factor (general base) or as a means of reaction acceleration through transition state charge stabilisation (answer needs to mention one of these catalytic examples for 1 mark).
Module-level learning outcome being addressed: To be able to explain using specific examples how RNA can act as a catalyst and how ‘in vitro’ evolution can be used to generate novel RNA catalysts.

5a. How are Group I and II self-splicing mechanisms different in the first phosphoryl-transfer reaction step? (2 marks)

Nucleophile is an external hydroxyl group in Group I introns (1 mark), and a distant internal hydroxyl group in Group II introns (1 mark).

5b. What intronic structure is uniquely generated during the Group II self-splicing reaction compared to Group I? (1 mark)

Lariat structure

5c. Is this structure always generated in the Group II self-splicing reaction? Briefly explain your answer. (2 marks)

No (0.5 mark). During hydrolytic cleavage (0.5 mark), the lariat structure does not form because the nucleophile in the first phosphoryl-transfer reaction is water (1 mark), rather than a distal internal hydroxyl group.

Module-level learning outcome being addressed: To be able to explain using specific examples how RNA can act as a catalyst and how ‘in vitro’ evolution can be used to generate novel RNA catalysts.

Feedback should be indented in blue

Suggested rewording for questions should be given in the body of the paper in green.

(Please ensure no wording of questions is included in comments.)
SECTION B: Essay question

Answer one question on the separate paper provided

Remember to write your candidate number at the top of the page and indicate whether you have answered question A or B

Mark total for this section: 50

EITHER

A) Discuss the roles flavins play in enzyme catalysis and their importance in a variety of enzyme-based applications.

An introduction should describe why coenzymes are required for various enzyme catalysed reactions. A short description should be included on the structure of FMN and FAD, the importance of the chemistry of the isoalloxazine ring in redox reactions and a brief description on why the flavoenzymes need to catalyse both a reductive and oxidative half reaction. The importance of flavins to participate in one electron transfers to various metal centres should be covered. The answer should also cover the reactions of reduced flavins with molecular oxygen and the ability of flavins to catalyse two electron dehydrogenations and the importance of this in metabolism. Examples of flavoenzymes covered in the lecture material include oxidases, succinate dehydrogenase, pyruvate dehydrogenase multienzyme complex and hydroxylases. Examples of enzyme based applications include the use of bacterial luciferase in bioluminescent biosensors and as a reporter for gene expression studies. The Old Yellow Enzyme family of flavoproteins was
covered in the lectures, as was the applications of these flavoenzymes in biocatalysis and bioremediation. Illustrations should be used where possible.

Module-level learning outcome being addressed:

- Students will be able to describe, through the use of examples the role of coenzymes in enzyme catalysis.
- An appreciation from studying specific examples of the use of enzymes for biotechnological applications.

B) Using the bacterial protease subtilisin as an example, discuss the extent to which structure-informed, site-directed mutagenesis is an appropriate tool for engineering enzymes for altered or improved performance in industrial reactions.

Subtilisin is an excellent example of a readily available, robust and commercially significant biocatalyst that has been the subject of a great many mutation studies using both site directed mutagenesis techniques and SDM combined with chemical modification. The essay should describe a general method for SDM. It should then establish the natural reaction catalysed by subtilisin, the reasons for the intense research effort devoted to engineering improved performance, and also detail some examples of how both point mutations and point mutations in combination with chemical modification, have led to subtilisin variants of, for example; increased resistance to oxidation; altered substrate specificity, by point mutations in specificity pockets; increased activity toward different peptide bonds by the mutation of active site residues to cysteine, followed by highly selective chemical modification.

Module-level learning outcome being addressed:

- To be able to explain how ‘random mutagenesis’ or ‘in vitro’ evolution experiments can be used to improve or alter the catalytic properties of enzymes.
Discuss the role of primary, secondary and tertiary structure in natural ribozymes, and compare and contrast with protein-based enzymes.

The essay should highlight the similarities and essential differences between ribozymes (catalytic RNA) and enzymes (catalytic protein) with regard primary structure (high intrinsic negative charge of RNA and only four nucleotides versus diverse chemical properties of 20 amino acids), secondary structure (hairpins/junctions/pseudoknots versus alpha-helix/beta-sheet), and tertiary structure (docking of RNA duplexes versus folding of alpha-helices/beta-sheets to generate 3D structure). It is important to note here the important stabilisation role of Mg\(^{2+}\) in most RNA structures. With regard this structural hierarchy, the answer should note that RNA structures utilise Watson-Crick, non-Watson-Crick and Hoogsteen base pairing to create stable secondary and tertiary structures. Both ribozymes and enzymes utilise their tertiary structures to generate a 3D scaffold in which the active site is located, and there is evolutionary pressure to conserve the structural features that generate this 3D scaffold (e.g. Groups I and II ribozymes). For catalysis, RNA has a more limited range of chemical groups (4 nucleobases) to utilise. However, like amino acids in enzymes, ribozymes utilise the different tautomeric/ionised forms of the nucleobases in the active site (e.g. pK\(_a\) shifts for key chemical groups) and use Mg\(^{2+}\) as a cofactor (e.g. metal-dependent catalytic mechanism of Group I introns and T7 DNA polymerase). Like enzymes, ribozymes have strategies for accelerating the phosphoryl-transfer reaction which they catalyse: general acid-base catalysis (HDV ribozyme compared to RNase A), charge stabilisation for transition state, and conformational effects (stabilise transition state). Unlike enzymes, many ribozymes undergo large-scale conformational dynamics (e.g. hairpin ribozyme) as an integral part of the catalytic cycle. A complete answer would incorporate details for all of the natural nucleolytic (hammerhead, hairpin, hepatitis delta virus) and self-splicing (Groups I and II intronic) ribozymes covered in the lecture material. An excellent essay would incorporate the experimental evidence covered in the lectures and extra reading which demonstrates the above key points.

Module-level learning outcome being addressed:

- To be able to explain using specific examples how RNA can act as a catalyst and how ‘in vitro’ evolution can be used to generate novel RNA catalysts.
- To be able to select and describe the appropriate experimental technique required to identify novel catalytic RNAs, to study RNA structure and dynamics, or to measure the mechanical stability of RNA.