Enzyme Assays

Enzyme assays are performed to serve two different purposes:

- To identify a special enzyme, to prove its presence or absence in a distinct specimen, like an organism or a tissue
- To determine the amount of the enzyme in the sample under defined condition so that the enzyme activity can be compared between one sample and another (tissue homogenate, fluid or partially purified preparation)

While for the first, the qualitative approach, a clear positive or negative result is sufficient, the second, the quantitative approach must deliver data as exact as possible.

Enzyme activity measured in *In vitro* condition is not similar to *In vivo* conditions.

Factors affecting enzyme activity

- It is important to treat sample carefully prior to assay.
  Specimens for enzyme assay are usually stored at temperatures below 4°C – death or isolation of tissue changes the cellular organization of cell, leading to autolysis. Autolysis is accompanied by changes to enzymes and its cofactors. Autolysis is minimized tissue is kept in cold both before and after the assay.
  Autolysis - destruction of cells or tissues by their own enzymes, especially those released by lysosomes.
  Many enzymes are denatured at high or even moderate temperatures, hence storage at 4°C.
  Again the assay must be carried out as soon as possible after the isolation of sample.
  Autolysis and denaturation of enzyme increases with storage.

- Effects of substrate concentration – most enzymes follow Michaelis-Menten hyperbolic response to substrate concentration. Those enzymes not following the hyperbolic curve i.e the allosteric enzymes, follow a sigmoid curve with substrate concentration.

- Effects of pH – enzymes are active only within a narrow pH range. At optimum pH, enzymes show maximum activity. Therefore it is important to maintain the required pH while assaying the activity of the enzyme.

- Effect of temperature – rates of enzyme activity increases by 4 to 8% per degree C, although at high temperatures denaturation of enzyme decreases product formation. Optimum temperature shows maximum enzyme activity.

- Effect of ionic strength, salts – concentration of salt, but also the identity of the ions, and the ionic strength of the solution can affect the activity of an enzyme. It is best to try out various salts at a range of concentrations to find the optimal conditions for the required enzyme.

- Further influences of compounds not directly involved in the reaction may occur, e.g. interactions of ions, especially metal ions, hydrophobic substances or detergents with the protein surface, either stabilizing, e.g. as counter ions, or destabilizing. For example, enzyme reactions dependent on ATP need Mg^{2+} as essential counter ions. If only ATP without Mg^{2+} is added to the assay mixture even in sufficient concentration, it can become limiting, especially if complexing compounds, like inorganic phosphates or EDTA are present.

Enzyme units and measurement

- **Enzyme activity** = amount of substrate converted per unit time. Or Amount of product formed per unit time.

  Enzyme activity is a measure of the quantity of active enzyme present which is dependent on conditions, *which should be specified.*
  The SI unit is the katal, 1 katal = 1 mol s^{-1}, but this is an excessively large unit.
  A more practical and commonly used value is enzyme unit (U) = 1 µmol min^{-1}.
  1 U corresponds to 16.67 nanokatals.

- **Specific activity** – amount of product formed per mg of protein.
  Unit = µmol/ min mg

- **Percent purity of enzyme=** Enzymatic purity or activity purity refers to the fraction of activity observed in an assay that comes from a single enzyme.

- **Enzyme Identity-** Enzyme identity determination is the confirmation that the protein preparation in fact contains the enzyme of interest. Enzyme identity is confirmed by demonstrating that the experimentally determined primary amino acid sequence matches the predicted primary amino acid sequence

- **Mass Purity -** Mass purity refers to the percentage of the protein in a preparation that is the target enzyme or protein. For instance, 90 µg of enzyme in a solution containing a total of 100 µg of protein is considered to be 90% pure.

Direct assay and Coupled assay: Wilson

Direct assays – product

Methods for observing the enzyme reaction

Colorimetric: In the simplest case an enzyme reaction can be observed by the appearance (or disappearance) of a coloured compound, so that it can be even observed by eye. The advantage is not just to avoid the use of an instrument; rather the reaction can immediately and directly be controlled, excluding any operating error. Such a procedure, however, will yield no accurate and reproducible data and therefore an appropriate instrument, a colorimeter or a photometer, must be applied to determine the colour intensity.

In spectrophotometric assays, you follow the course of the reaction by measuring a change in how much light the assay solution absorbs. If this light is in the visible region you can actually see a change in the color of the assay, and these are called **colorimetric assays.**

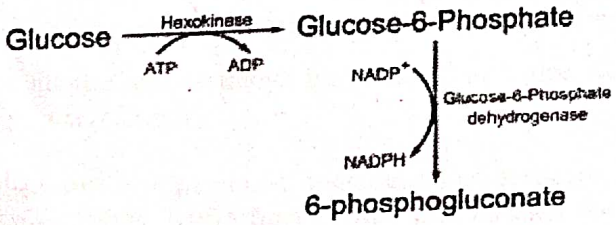
Example- **MTT** assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular **oxidoreductase enzymes** may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Other closely related tetrazolium dyes including XTT, MTS and the WSTs

Example - A specific colorimetric assay for the determination of glucose-6-phosphate (G6P) was developed. This assay is based on the oxidation of G6P in the presence of glucose-**6-phosphate dehydrogenase (G6PD)** and $NADP^+$; the NADPH thereby generated reduces the tetrazolium salt **WST-1** (2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl-2H tetrazolium, monosodium salt) to water-soluble yellow-colored formazan with 1-methoxy-5-methylphenazium methylsulfate (1-mPMS) as an electron carrier.

Spectrophotometers- In spectrophotometric assays, you follow the course of the reaction by measuring a change in how much light the assay solution absorbs. When the absorbance maxima falls in the UV region, these are known as **spectrophotometric assay**.

UV light is often used, since the common coenzymes NADH and NADPH absorb UV light in their reduced forms, but do not in their oxidized forms.

Example- An **oxidoreductase** using **NADH** as a substrate could therefore be assayed by following the decrease in UV absorbance at a wavelength of 340 nm as it consumes the coenzyme



If an enzyme reaction cannot be observed photometrically, other optical methods may be used.

Fluorimetry - is more sensitive than absorbance measurements (about hundredfold), but only a few enzymatic substrates or products emit fluorescence, such as NADH and some artificial substrate analogues.

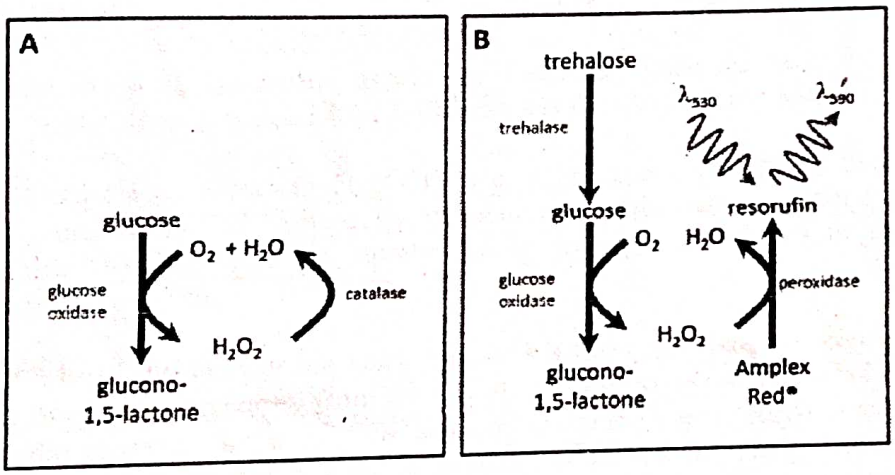
Fluorescence is an emission phenomenon where an energy transition from a higher to a lower state is accompanied by radiation. Only molecules in their excited forms are able to emit fluorescence; thus, they have to be brought into a state of higher energy prior to the emission phenomenon.

To be measured with a fluorescence-based assay the reaction needs to proceed with either the formation of a fluorescent product from a nonfluorescent substrate or *vice versa*.

Example of the first case - is the use of the non-fluorescent butyl ester of resorufin as a substrate in a **hydrolase assay**, where enzymatic cleavage of the ester bond yields highly fluorescent resorufin.

An example of the latter is any enzyme reaction involving the oxidation of NAD(P)H to the non-fluorescent $NAD(P)^+$

Example - Principle of the fluorometric assay of trehalose. (A) First, glucose is eliminated from the tissue extract by incubation with glucose oxidase and catalase. (B) After inactivation of the glucose oxidase and catalase, trehalose is then hydrolysed to glucose by trehalase. Glucose is determined by coupling to peroxidation of a fluorogenic substrate, Amplex Red®, using glucose oxidase and peroxidase and measuring the increase in fluorescence at 590 nm.



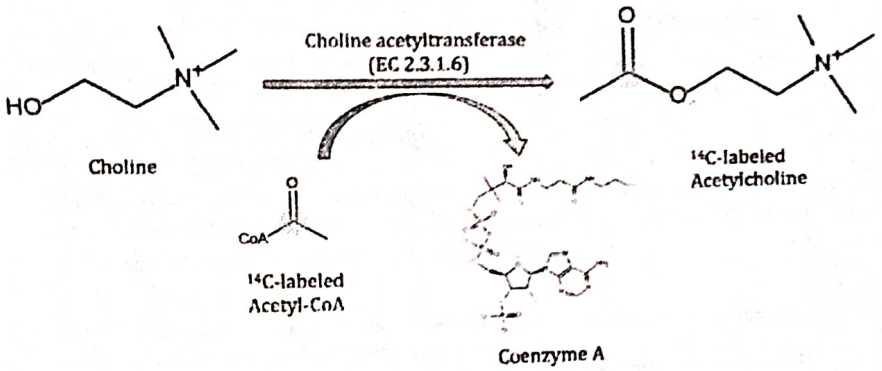
For all the above assays based on absorbance, substrate and product should not absorb at the same wavelength.

Other assays -Enzymatic degradation of particles, like starch, can be observed by **turbidimetry** (Bock, 1980), while **luminometry** is applied for ATP dependent reactions (Campbell, 1989, DeLuca and McElroy, 1978).

Radiometric assay: Radiometric assays measure the incorporation of radioactivity into substrates or its release from substrates. The radioactive isotopes most frequently used in these assays are ^{14}C, ^{32}P, ^{35}S and ^{125}I. Since radioactive isotopes can allow the specific labeling of a single atom of a substrate, these assays are both extremely sensitive and specific. They are frequently used in biochemistry and are often the only way of measuring a specific reaction in crude extracts (the complex mixtures of enzymes produced when you lyse cells).

Radioactivity is usually measured in these procedures using a scintillation counter.

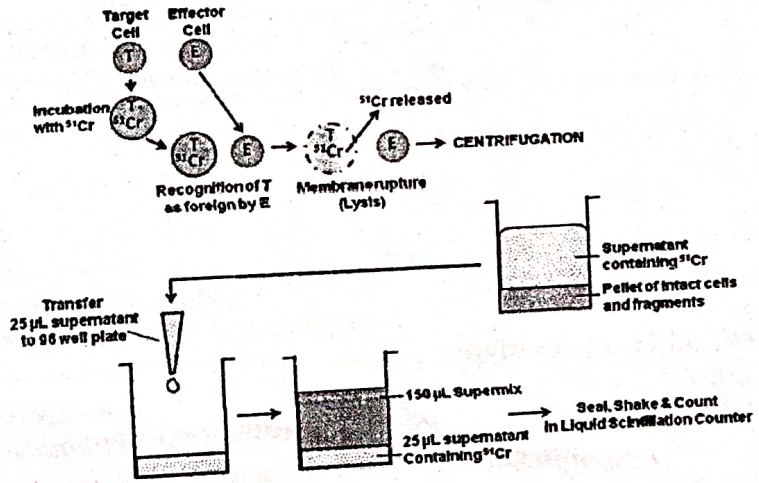
Example - Radiometric assays are often used in ligases activity testing. For example, the aminoacyl-tRNA synthases, which catalyze the ATP-dependent formation of aminoacyl-tRNA from amino acids and specific acceptor transfer ribonucleic acids. These enzymes are highly specific, each enzyme being specific for only one natural amino acid. They have been assayed almost exclusively by radiometric methods.



Example - Radiometric assays are frequently used for decarboxylases assays. The release of $^{14}CO_2$ from carboxyl-labeled substrates is the obvious and most often used technique.

Example - Chromium-51 (^{51}Cr) release assays are commonly used for the precise and accurate quantification of cytotoxicity, particularly in the study of tumor and viral cytolysis. The assay is used to determine the number of lymphocytes produced in response to infection or drug treatment.

A brief overview of the assay principle is illustrated below. Target cells are labeled with ^{51}Cr, the label is then released from the target cells by cytolysis. The label can be isolated by centrifuging the samples and collecting the supernatants. Supernatants from centrifugation can either be counted directly in a gamma counter, or mixed with scintillation cocktail in a microplate (or dried on a LumaPlate™) and counted in a liquid scintillation counter.



Name	Definition	Notation	Dimension	Conversion
Enzyme units (measure of enzyme activity)	Enzyme amount converting 1 mol substrate/s	katal (kat)	mol/s	1 kat=60,000,000 IU
				1 nkat=0.06 IU
	Enzyme amount converting 1 µmol substrate/min	International unit (IU)	µmol/min	1 IU=0.0000000167 kat
				1 IU=0.0167 nkat
Volume activity	Enzyme units per volume unit	katal/volume	kat/L	
		IU/volume	IU/mL	
Specific enzyme activity	Enzyme units per protein; volume activity/protein concentration	katal/protein	kat/kg	
		IU/protein	IU/mg	
Enzyme velocity	Turnover per time unit	v	mol/s	
			µmol/min	
Maximum velocity	Turnover per time unit at saturating conditions of substrates and cofactors under standard conditions	V_{max}	mol/s	
			µmol/min	
Turnover number (catalytic constant)	Maximum velocity divided by the enzyme concentration	$k_{cat}=V_{max}/[E]_0$	s^{-1}	
Michaelis constant	Substrate concentration for half-maximal velocity	$K_m=(k_{-1}+k_{cat})/k_1$	M	

References :

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6) A fluorometric assay for trehalose in the picomole range, Petronia Carillo
7) https://www.creative-enzymes.com/resource/radiometric-enzyme-assays_13.html
8) http://www.perkinelmer.com/ radiometric/125I-labeling.html