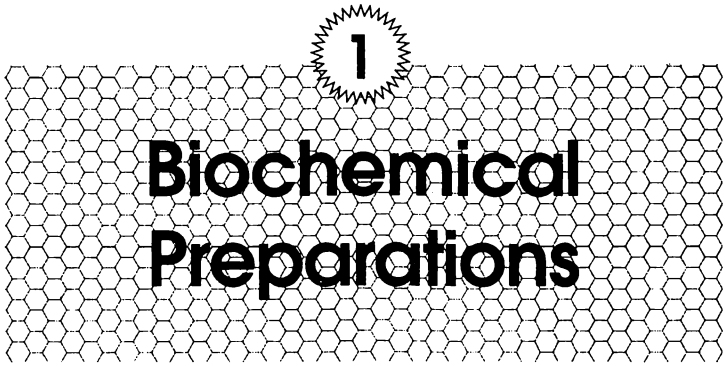


INTRODUCTION

The enzymes are critical in the functioning of cells. Life is a dynamic process that involves constant changes in chemical composition. These changes are regulated by catalytic reactions, which are regulated by enzymes. At one time, the cell was actually conceived of as a sac of enzymes. It was believed that on the basis of the reactions and their rates of action, cell and indeed, life itself could be defined. Few biologists continue to think of this as a simple task, but we know that life could not exist without the function of enzymes. Ideally, enzymes could be examined within an intact cell, but this is difficult to control. Consequently, enzymes are studied *in vitro* after extraction from cells. In a simpler way enzymes can be defined as biocatalysts. A catalyst is a substance that accelerates the reaction but does not get itself modified in the process so that it can be used again and again. Enzymes are the largest and most specialized class of protein molecules. More than a thousand different enzymes have been identified of which many of them have been obtained in pure and even in crystalline form.

An important feature of enzyme activity is its substrate specificity, i.e. a particular enzyme will act only on a certain

specific substrate. Some enzymes have nearly absolute specificity for a given substrate and will not act on even very closely related molecules, e.g. stereoisomers of the same molecule. Other enzymes have relative specificity, since they will act upon a variety of related compounds. Some enzymes require small non-protein components called cofactors for their activity, for example, some enzymes are conjugated proteins having tightly bound prosthetic groups, as in case of cytochrome, which have an iron porphyrin complex. Other enzymes cannot function without the addition of small molecules called coenzymes, which become bound during the reaction. When joined with a coenzyme, these inactive enzymes (also called apoenzymes) form active holozymes.

Holozymes ⟶ Apoenzymes + Coenzymes

Since all enzymes are proteins, which are differentially soluble in salt solutions, enzyme extraction procedures often begin with salt (typically, ammonium sulphate) precipitation. On the simplest level, proteins can be divided into albumins and globulins on the basis of their solubility in dilute salt solutions. Albumins are considered to be soluble while globulins are insoluble. Solubility is relative, however, and as the salt concentration is increased, even proteins tend to precipitate.

Thus, if a tissue is homogenized in a solution that retains the enzyme in its soluble state, the enzyme can be subsequently separated from all insoluble proteins by centrifugation or filtration. The enzyme will be impure, since it will be in solution with many other proteins. If aliquots of a concentrated ammonium sulphate solution are then added serially, individual proteins will begin to precipitate according to their solubility. By careful manipulation of the salt concentrations, thus fractions, which contain purer solutions of enzymes, or at least are enriched for a given enzyme can be produced. In order to determine the effectiveness of the purification, each step in the extraction procedure must be monitored for enzyme activity. The monitoring can be accomplished in many ways, which usually

involves a measurement of the decrease in substrate, or the increase in product specific to the enzyme.

It is important to remember that enzymes act as catalyst to a reaction and affect only the reaction rate. The general scheme for the action of an enzyme is given by the following equation:

$$E+S \underset{K_2}{\overset{K_1}{\rightleftarrows}} ES \underset{K_4}{\overset{K_3}{\rightleftarrows}} E+P$$

Where

E = concentration of the enzyme

S and P = concentrations of substrate and product respectively

ES and EP = concentration of enzyme-substrate complex and enzyme-product complex

$K_1 - K_4$ = rate constants for each step.

From above equation, the rate (velocities) of each reaction can be given by:

$V_1 = K_1[E][S]$ Formation of enzyme-substrate complex

$V_2 = K_2[ES]$ Reformation of free enzyme and substrate

$V_3 = K_3[ES]$ Formation of product and free enzyme

$V_4 = K_4[E][P]$ Reformation of enzyme-product complex.

At steady state, $(V_1 - V_2) = (V_3 - V_4)$ and, if all product is either removed or does not recombine with the enzyme, then $K_4 = 0$, then

$$K_1[E][S] - [K_2][ES] = K_3[ES]$$

This equation can then be rearranged to yield:

$$K_1[E][S] = (K_2+K_3)\ [ES]$$

The left side of this equation can be given as a single constant, known as K, the rate constant, or the Michaelis constant. Note that the units for this constant will be that of concentration.

One of the important concepts of metabolism is that enzymes from differing sources may have the same function

(i.e. the same substrate and product), but possess significantly different K values. Since biological function is as dependent on the rate of a reaction as it is on the direction of a reaction, it becomes necessary to measure the K value for any enzyme under study.

Enzymes act as catalysts because of their three dimensional protein structure. This structure is controlled by many factors, but is particularly sensitive to changes in pH, salts and temperature. Small changes in the temperature of a reaction can significantly alter the reaction rate, and extremely high temperatures can irreversibly alter both the three dimensional structure of the enzyme and its activity. It may even render the enzyme non-functional; that is, to denature the enzyme. Salts can also cause denaturation, but the effect of ammonium sulphate is usually reversible.

Active Sites

An enzyme works by binding to a substrate in a geometrical fashion so that the substrate is able to undergo the reaction (product formation) at a more rapid rate. This type of reaction is commonly referred as the lock and key model for enzyme action. It implies that there is a particular part of the enzyme structure, the active site, which specifically binds sterically to a substrate. The enzyme does not actually react with the substrate but merely brings the substrate into the proper alignment or configuration for it to react spontaneously or in conjunction with another substance. Since a reaction proceeds normally by a random kinetic action of molecules colliding with each other any time molecules come in contact and react in a faster manner. Thus, for any given enzyme there will be a best-fit configuration to the protein in order to align the substrate and to facilitate the reaction. When the enzyme is in its ideal configuration, the reaction proceeds at its maximum rate, and the overall rate of action will depend on substrate concentration.

Maximum reaction rate assumes that an optimal pH, salt environment and temperature have been established. Maximum rate further assumes the presence of any co-

enzymes and/or cofactors that the enzyme requires. Coenzymes are organic molecules, which must bind to the protein portion of the enzyme in order to form the correct configuration for a reaction whereas cofactors are inorganic molecules which do the same.

To measure the concentration of an enzyme via its action rate (i.e. the velocity of the catalyzed reaction), the effect of temperature, pH, salt concentration, coenzymes, cofactors, and substrate concentration on reaction rate must be controlled. Each of these parameters affects the rate of an enzyme reaction. Thus, each must be carefully controlled if we attempt to study the effects of changes in the enzyme itself.

The relationship between substrate concentration and enzyme concentration was mathematically established by two biochemists, L. Michaelis and M.L. Menten in 1913. In recognition of their work, the plots of enzyme activity vs. substrate concentration are known as Michaelis-Menten plots. These are relatively simple plots in which the substrate concentration is on the X-axis, and the velocity of reaction is on the Y-axis. The plot demonstrates that as the substrate increases, the velocity increases hyperbolically, and approaches a maximum rate known as V_{max}. This is dependent upon saturation of the enzyme. At V_{max}, all enzyme molecules are complexed with substrate, and thus any additional substrate added to the reaction has no effect on the rate of reaction.

However, this situation becomes more complex on changing the enzyme concentration, V_{max} will also change. Thus, V_{max} is not a constant value, but is constant only for a given enzyme concentration. Consequently, the value of V_{max} cannot be used directly to infer enzyme concentration. It is dependent upon at least two variables, enzyme concentration and substrate concentration (assuming temperature, pH and cofactors have all been controlled). Michaelis and Menten gave a simple means of solving the equations for two variables. If multiple plots of enzyme activity vs. substrate

concentration are made with increasing enzyme concentration, the value of V_{max} continues to increase, but the substrate concentration, which corresponds to $1/2V_{max}$, remains constant. This concentration is the Michaelis Constant for an enzyme. As mentioned, it is designated as K and is operationally the concentration of substrate, which will give exactly $1/2V_{max}$ when reacted with an enzyme with maximum pH, temperature and cofactors.

According to the Michaelis-Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

Above equation is derived from the formula for a hyperbola (c=xy)

Where $K_m = [S](V_{max}/V-1)$

When $V = V_{max}/2$,

$K_m = [S][V_{max}/(V_{max}/2)-1] = [S]$,

This confirms that the units of this constant is that of concentration.

A Michaelis-Menten plot thus provides an easy way to measure the rate constant for a given enzyme. An immediate difficulty is apparent, however, when Michaelis-Menten plots are used. V_{max} is an asymptote. Its value can only be certain if the reaction is run at an infinite concentration of substrate. In 1934, two individuals, Lineweaver and Burke made a simple mathematical alteration in the process by plotting a double inverse of substrate concentration and reaction rate (velocity).

The Lineweaver/Burke equation is:

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]}$$

On simplification

$$\frac{1}{V} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}}$$

This equation fits the general form of a straight line, y=mx+b, where m is the slope of the line and b is the intercept. Thus, the Lineweaver/Burke plot for an enzyme is more useful than Michaelis-Menten, since as velocity reaches infinity, $1/V_{max}$ approaches 0. The slope of straight line is equal to K_m/V_{max}, the Y intercept equals $1/V_{max}$ (1/[S]=0). Projection of the line back through the X-axis yields the value $-1/K_m$ (when 1/V=0). These values can easily be determined by using a linear regression plot and calculating the corresponding values for x=0 and y=0. The inverse of the intercept values will then yield V_{max} and K_m. The aim of all of these calculations is to determine the true activity and thus the concentration of the enzyme. If the reaction conditions are adjusted so that the substrate concentration is at Km, then alterations in the rate of reaction are linear due to alterations in enzyme concentration. Kinetic analysis is the only means of accurately determining the concentration of active enzyme.

Specific Activity

Specific activity is defined in terms of enzyme units per mg enzyme protein. An enzyme unit is the amount of substrate converted to product per unit time under specific reaction conditions for pH and temperature. As generally accepted, an enzyme unit is defined as that which catalyzes the transformation of 1 μM of substrate per minute at 30°C with optimal chemical environment (pH and substrate concentration). Specific activity relates the enzyme units to the amount of protein in the sample. Though it is relatively easy to measure the protein content of a cell fraction, there may be a variable relationship between the protein content and a specific enzyme function. Remember that the initial extraction of an enzyme is accomplished by differential salt precipitation.

Many proteins may precipitate together due to their solubility, but have no other common characteristics. Determination of both protein content and enzyme activity requires two different procedures. One can measure the

amount of protein, or one can kinetically measure the enzyme activity and combining the two will give the specific activity.

TYROSINASE AS A MODEL ENZYME

Tyrosinase is the common name for an enzyme that is formally termed monophenol monooxygenase (Standard Enzyme Nomenclature 1.14.18.1). It is also known as phenolase, monophenol oxidase and as cresolase. Functionally, it is an oxygen oxidoreductase enzyme. Enzyme tyrosinase was discovered in animal systems and named for its action on the amino acid tyrosine specifically for its ability to form dopaquinone (an intermediate metabolite) in the production of melanin. The same enzyme isolated from plant materials had been examined for its ability to oxidize phenolic residues, and thus the name phenolase, monophenol oxidase and cresolase were given. Since it has been extensively studied in melanin production and known commonly as tyrosinase. The enzyme tyrosinase is fairly ubiquitous and present in nearly all cells. It is isolated and purified from the fungi, *N. crassa* by freezing fungal mycelia in liquid nitrogen, homogenizing the frozen tissue with a French Press, precipitating the proteins in ammonium sulphate, and purifying the enzyme chromatographically on Sephadex and Celite columns. Tyrosinase has also been extracted from hamster melanomas by modifying the technique and with the addition of acetone extractions as well as DEAE-cellulose (diethyl aminoethyl cellulose) chromatography and alumina treatments. Tyrosinase has also been separated from many plant tissues utilizing a far simpler technique based principally on ammonium sulphate precipitation of proteins.

The catalytic action of this enzyme is the conversion of tyrosine in presence of O_2 to yield dihydroxyphenylalanine (DOPA), which is then converted to dopaquinone and H_2O. Dopaquinone in turn can be readily converted to dopachrome, an orange to red pigment (found in human red hair), which can then be converted to the black/brown melanin pigments (found in virtually all human pigments).

$$\text{Tyrosine} + 1/2O_2 \longrightarrow \text{DOPA}$$

$$2\ \text{DOPA} + O_2 \longrightarrow 2\ \text{Dopaquinone} + 2H_2O$$

$$\text{Dopaquinone} \longrightarrow \text{Leukodopachrome}$$

$$\text{Leukodopachrome} + \text{Dopaquinone} \longrightarrow \text{Dopachrome} + \text{DOPA}$$

The enzyme takes part in catalysis of the first two of these reactions, which are the conversion of tyrosine and the conversion of DOPA. The formation of dopachrome from dopaquinone is spontaneous. Activity of the enzyme can be monitored by analyzing the disappearance of tyrosine and/or DOPA as substrates, the appearance of leukodopachrome or dopachrome as products, or by monitoring the use of oxygen. Natural pigment dopachrome could be measured at l_{max} 475 nm. This absorbance allows us to use standard spectrophotometric analysis by analyzing the formation rate of dopachrome from the substrate DOPA. The summary reaction for tyrosinase activity, which makes the basis of its estimation in the reaction is

$$\text{DOPA} + 1/2\,O_2 \longrightarrow \text{Dopachrome}$$

To study the various parameters for the evaluation of an enzyme, tyrosinase is selected as model enzyme. This exercise involves the isolation (extraction) of the enzyme tyrosinase from potatoes and subsequent measurement of its activity.

PROTOCOL 1.1

Extraction of Tyrosinase

The enzyme tyrosinase is insoluble in 50% ammonium sulphate, but is soluble in the citrate buffer.

Equipments

Blender, volumetric cylinders (50, 100, 250ml), cheesecloth, beakers (100 ml, 250 ml), chilled centrifuge tubes (30-50 ml), refrigerated centrifuge, glass stirring rod

Materials

Potatoes

0.1 M Sodium fluoride

0.1 M Citrate buffer (pH 4.8),

Saturated ammoniumsulphate (4.1 M at 25°C).

Procedure

- Peel a small potato and cut into pieces. Put 100 g of potato in a blender and add 100 ml of sodium fluoride (NaF). Homogenize for about one minute at high speed. Pour the homogenate (mixture) through several layers of cheesecloth.
- Measure the volume of the homogenate and add an equal volume of saturated ammonium sulphate and allow to stand till a flocculent white precipitate appears as many of the soluble potato proteins become insoluble. The enzyme tyrosinase is one of these proteins.
- Centrifuge at 1500g for 5 minutes at 4°C and collect the centrifuge tubes, and carefully discard the supernatant. Collect the pellets. Combine all of the pellets into a 100 ml beaker.
- Add 60 ml of citrate buffer (pH 4.8) to the beaker and break the pellet using glass rod. Again divide the solution into centrifuge tubes and recentrifuge at 300g for 5 minutes at 4°C. Collect the supernatant. Place it in an Erlenmeyer flask, label it as Enzyme Extract and keep it in an ice bucket in which tyrosinase is stable for about an hour. If enzyme is not to be used within this period then extract the enzyme from fresh potatoes at the time of its use.

PROTOCOL 1.2

Preparation of Standard Curve of Tyrosinase

Equipments

Test tubes, 5 ml pipette, spectrophotometer and cuvettes

Materials

8 mM DOPA

Enzyme extract

0.1 M Citrate buffer, pH 4.8

Procedure

- Prepare a standard solution of the orange colored dopachrome from L-DOPA. To 10 ml of 8 mM DOPA, add 0.5 ml of enzyme extract and keep the solution to stand for 15 minutes at room temperature. During this period, all the DOPA gets converted to dopachrome, and solution contains 8 mM dopachrome. Dopachrome is somewhat unstable in the presence of light and should be stored in an amber bottle in a dark place.
- Prepare dilutions of the 8 mM dopachrome to yield the concentrations 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125 mM of dopachrome. One tube containing no dopachrome is used as blank.
- Take 3.0 ml of each indicated concentration in tubes. As the units of concentration are millimolar (mM). A 1.0 mM solution contains 0.001 moles per liter or 0.000001 moles per ml. Thus, with a volume of 3.0 ml, there are 0.000003 moles of dopachrome, or 3 µM. Correspondingly, tubes 2 to 8 contain 1 to 24 µM of dopachrome.
- Measure the absorbance of each solution at 475 nm using content of the tube 1 as blank. Average the values of obtained absorbance that is the average extinction coefficient and can be used in subsequent determinations of dopachrome concentrations according to the Beer-Lambert law.
- Once more accurately determine the extinction coefficient by performing a linear regression analysis of the data, and computing the slope and Y-intercept. The slope of the linear regression will represent the extinction coefficient for a sample. Plot a scattergram of the absorbance value against the concentration of dopachrome. The known concentration of dopachrome is taken on the X-axis, while absorbance on the Y-axis.

Plot the computed slope and intercept of the linear regression as a straight line overlaying the scattergram. The equation for a straight line is y = mx + b, where 'm' is the slope and 'b' the intercept.

Note: Since tyrosinase catalyzes the conversion of L-DOPA to dopachrome, this exercise measures the conversion of colorless DOPA to the dark orange dopachrome. Substrate and product are in a 1:1 ratio for this reaction, thus the amount of product formed equals the amount of substrate used. The optical density of dopachrome at 475 nm is directly proportional to the intensity of orange colour formation in solution (Beer-Lambert Law).

PROTOCOL 1.3

Assessment of Optimum Enzyme Concentration for Reaction Kinetics

Equipments

10 ml pipette, ice bath, spectrophotometer and cuvettes

Materials

Enzyme extract

0.1 M Citrate buffer, pH 4.8

8 mM DOPA.

Procedure

- To determine the kinetic effects of the enzyme reaction, first determine an appropriate dilution of enzyme extract. Appropriate dilution can be assessed by measuring the rate of reaction given by the diluted enzyme. The rate of reaction of diluted enzyme should be in between 5–10 μm of DOPA converted per minute. For this prepare a serial dilution of enzyme extract.
- Place 9.0 ml of citrate buffer into each of three test tubes and label them 1/10, 1/100 and 1/1000 and pipette 1.0 ml of enzyme extract to make the 1/10 dilution in first test tube (labeled as 1/10) and mix by

inversion. Then pipette out 1 ml of the 1/10 dilution into the second tube (labeled as 1/100) and mix by inversion. Pipette 1.0 ml of the 1/100 dilution into the third tube (labeled as 1/1000) and mix by inversion.

- Place all of the dilutions in the ice bath until ready to use. Turn on a spectrophotometer, adjust to 475 nm and with a blank in a tube containing 2.5 ml of citrate buffer and 0.5 ml of enzyme extract. Add 2.5 ml of 8 mM DOPA to each of 4 cuvettes or test tubes. Note that each tube contains 0.0025 × 0.008 moles or 20 µM of DOPA.
- Pipette 0.5 ml of undiluted enzyme extract to one of the tubes containing 8 mM DOPA. Mix by inversion, place into the spectrophotometer and immediately begin timing the reaction. Carefully measure the time required for the conversion of 8 µM of DOPA into dopachrome. Note that since the cuvette will contain a volume of 3.0 ml, the concentration when 8 µM are converted will be 8/3.0 or 2.67 mM dopachrome. Use the data from the standard curve to determine the absorbance equal to 2.67 mM dopachrome. This absorbance value will be the end point for the reaction.
- As the reaction takes place within the cuvettes in the spectrophotometer the absorbance will increase with dopachrome formation. When the absorbance reaches the value above, note the required time from the mixing of the enzyme extract to the 10 µM DOPA.
- Express the time as a decimal rather than minutes, seconds. The time should be between three and five minutes. If the end point is reached before three minutes, repeat this step using the next dilution of enzyme (i.e. the 1/10 after the undiluted, the 1/100 after the 1/10 and the 1/1000 after the 1/100). For the enzyme dilution, which reaches the end point between 3 and 5 minutes, calculate the velocity of reaction.
- Divide the amount of product formed (10 µM) by the time required to reach the end point.

PROTOCOL 1.4

Effect of pH on Enzyme Activity

Equipment

10 ml pipette, ice bath, spectrophotometer and cuvettes, stopwatch

Materials

8 mM DOPA in citrate buffer adjusted to pH values of 3.6, 4.2, 4.8, 5.4, 6.0, 6.6, 7.2 and 7.8.

Enzyme extract.

Procedure

- Set up a series of test tubes each containing 2.5 ml of 8 mM DOPA, adjusted to the pH values, 3.6, 4.2, 4.8, 5.4, 6.0, 6.6, 7.2, and 7.8.
- Begin with the tube containing DOPA at pH 3.6, add 0.5 ml of the diluted enzyme extract which will convert 10 µM of Dopachrome in 3 to 5 minutes.
- Start timing the reaction, mix by inversion and insert into the spectrophotometer. Note the time for conversion of 10 µM of DOPA to dopachrome.
- Repeat last step for each of the indicated pH values. Calculate the velocity of enzyme reaction (µM/minute) at each pH. Plot pH (X-axis) versus reaction velocity (Y-axis).

PROTOCOL 1.5

Effect of Temperature on Enzyme Catalyzed Reaction

Equipment

10 ml pipette, ice bath, spectrophotometer and cuvettes, stopwatch, incubators or water baths adjusted to 10, 15, 20, 25, 30, 35 and 40°C.

Materials

Enzyme extract

8 mM DOPA pH 6.6

Procedure

- Set up a series of test tubes each containing 2.5 ml of 8 mM DOPA buffered to a pH 6.6. Place one tube in an ice bath or incubator adjusted to the following temperature; 10, 15, 20, 25, 30, 35 and 40°C.
- Add 0.5 ml of an appropriately diluted enzyme extract (that gives a yield of 10 µM dopachrome in 3–5 minutes) to each of a second series of tubes. Place one each in the corresponding temperature baths. Allow all of the tubes to equilibrate for 5 minutes at respective temperature. Do not mix the tubes.
- Begin with the tube equilibrated and kept at 10^0C, and adjust the spectrophotometer to 475 nm properly with the blank solution, pour the enzyme (0.5 ml stored at 10°C) into the tube containing the DOPA and start timing the reaction. Mix thoroughly. Note the time to reach the end point equivalent to the conversion of 10 µM of substrate.
- Repeat last step for each of the listed temperatures, complete the following and plot the data.
- Calculate the velocity of enzyme reaction (µM/minute) at each temperature.
- Plot the graph between temperatures (X-axis) versus reaction velocity (Y-axis).

PROTOCOL 1.6

Kinetic Analysis

Equipment

10 ml pipette, spectrophotometer and cuvettes, stopwatch

Materials

8 mM DOPA pH 6.6

Enzyme extract diluted to yield 10 µM of dopachrome in 3 to 5 minutes.

Procedure

- Prepare a reaction blank in a clean cuvette that contains 2.5 ml of citrate buffer and 0.5 ml of enzyme extract. Use this blank to adjust spectrophotometer for 100% transmittance.
- Add 2.5 ml of 8 mM DOPA, (pH 6.6) to a clean cuvette. Add 0.5 ml of appropriately diluted enzyme extract.
- Shake well and immediately insert the tube into the spectrophotometer. Record the absorbance or transmittance as quickly as possible. Designate this reading as time 0. At 30 second intervals read and record the transmittance until a transmittance value of 10% (or absorbance equal to 1.0) is reached.
- Calculate the amount of dopachrome formed as µM by using standard curve.
- Plot time in minutes (X-axis) versus the amount of dopachrome formed (Y-axis).

PROTOCOL 1.7

Determination of K_m and V_{max}

Equipments

10 ml pipette, spectrophotometer and cuvettes, stopwatch.

Materials

Enzyme extract, 8 mM L-DOPA adjusted to pH 6.6

Procedure

- Dilute the DOPA standard (8 mM) to obtain the concentrations of L-DOPA 0.5 mM, 1 mM, 2 mM, 4 mM and 8 mM respectively.
- Repeat kinetic analysis for each of the substrate concentrations listed, substituting the change in concentration.

- Plot each set of data and from the data calculate the time required to convert 10 µM of DOPA to dopachrome.
- Calculate the velocity of enzyme reaction (µM/minute) for each substrate concentration and convert that in 1/V. Calculate the 1/[S] with the help of various substrate concentration.
- Plot the rate of DOPA conversion (V) against substrate concentration. That will give a Michaelis-Menten plot. Plot a double reciprocal of the values that is, in between 1/[S] versus 1/V. This plot is Line weaver-Burke plot. Perform a linear regression analysis on the second plot and compute the slope and both Y- and X-intercepts.

Notes: In Michaelis-Menten plot the X-intercept is −1/Km and the negative inverse of it, is the Michaelis-Menten Constant. The Y-intercept is $1/V_{max}$ and the slope equals to Km/Vmax. To repeat the various experiments mentioned above with other enzymes some of the methods to extract and purify the enzymes are discussed below.

PROTOCOL 1.8

Kinetic Analysis of α- Amylase Activity

Principle

Starchy substances constitute the major part of the human diet for most of the people in the world, as well as many other animals. They are synthesized naturally in a variety of plants. Some plants for examples with high starch content are corn, potato, rice, sorghum, wheat, and cassava. Similar to cellulose, starch molecules are glucose polymers linked together by the α-1,4 and α-1,6 glucosidic bonds, as opposed to the β-1,4 glucosidic bonds for cellulose. In order to make use of the carbon and energy stored in starch, the human digestive system, with the help of the enzyme amylases, first breaks down the polymer to smaller assimilable sugars, which are eventually converted to the individual basic glucose units.

Since a wide variety of organisms, including humans, can digest starch, α-amylase is obviously widely synthesized in nature, as opposed to cellulase. For example, human saliva and pancreatic secretion contain a large amount of α-amylase for starch digestion. The specificity of the bond attacked by α-amylases depends on the sources of the enzymes. Currently, two major classes of α-amylases are commercially produced through microbial fermentation. Based on the points of attack in the glucose polymer chain, they can be classified into two categories— liquefying and saccharifying. Because the bacterial α-amylase to be used in this experiment randomly attacks only the α-1,4 bonds, it belongs to the liquefying category. The hydrolysis reaction catalyzed by this class of enzymes is usually carried out only to the extent that, for example, the starch is rendered soluble enough to allow easy removal from starch-sized fabrics in the textile industry. The paper industry also uses liquefying amylases on the starch used in paper coating where breakage into the smallest glucose subunits is actually undesirable.

On the other hand, the fungal α-amylase belongs to the saccharifying category and attacks the second linkage from the nonreducing terminals (i.e. C-4 end) of the straight segment, resulting in the splitting of two glucose units at a time. Of course, the product is a disaccharide called maltose. The bond breakage is thus more extensive in saccharifying enzymes than in liquifying enzymes. The starch chains are literally chopped into small bits and pieces. Finally, the amyloglucosidase (also called glucoamylase) component of an amylase preparation selectively attacks the last bond on the non-reducing terminals. The type to be used in this experiment can act on both the α-1,4 and the α-1,6 glucosidic linkages at a relative rate of 1:20, resulting in the splitting of simple glucose units into the solution. Fungal amylase and amyloglucosidase may be used together to convert starch to simple sugars.

The practical applications of this type of enzyme mixture include the production of corn syrup and the conversion of cereal mashes to sugars in brewing. Thus, it is important to specify the source of enzymes when the actions and kinetics

of the enzymes are compared. Four types of α-amylases from different sources will be employed in this experiment: three of microbial origin and one of human origin. The effects of temperature, pH, substrate concentration, and inhibitor concentration on the kinetics of amylase catalyzed reactions will be studied. Finally, the action of the amylase preparations isolated from microbial sources will be compared to that from human saliva.

Equipment

Erlenmeyer flasks, beakers, graduated cylinder, pipettes (1 ml, 10 ml), test tubes, temperature bath, thermometer, balance, syringe, filter holder and filter paper, spectrophotometer, brookfield viscometer.

Reagents

Enzymes

Bacterial amylase solution, 3000 SKB units/ml, fungal amylase powder, 40,000 SKB units/g (concentration of the fungal amylase solution to be used 7.5%), amyloglucosidase solution, 75 AG units/ml, human salivary amylase.

Corn starch

HCl stopping solution (0.1N HCl)

Iodine reagent stock solution (in aqueous solution, iodine: 5 g/l and KI 50g/l)

Potassium phosphate buffer (KH_2PO_4, $K_2HPO_4.3H_2O$),

$CaCl_2.2H_2O$ (0.1M solution)

Reagents for the analysis of reducing sugars.

Procedures

Preparation of 20% Starch Solution.

- Mix 20 g of soluble potato starch in approximately 50 ml of cold water. While stirring, add the slurry to approximately 900 ml of gently boiling water in a large beaker. Mix well and cool the gelatinized starch solution to room temperature. Add more water to bring the total volume to 1 liter.

- Put a few drops of the starch solution on a glass plate. Add 1 drop of the iodine reagent and see that a deep blue colour is developed. The blue colour indicates the presence of starch in the solution.

Effect of pH

- Prepare 0.1 M pH buffer solutions ranging from pH 4.5 to pH 9 in increments of one pH unit. (Note that phosphate buffer is only good for pH 4.5–9 due to the dissociation constant). Add an equal volume of one of the above buffer solutions to 5.0 ml of the 2% starch solution. The resulting solution should contain 1% of starch in a buffered environment.
- Start the enzymatic digestion process by adding 0.5 ml of the bacterial amylase solution; shake and mix. Let the hydrolysis reaction proceed for exactly 10 minutes at 25°C. Add 0.5 ml of the reacted starch solution to 5 ml of the HCl stopping solution (0.1 N).
- Add 0.5 ml of the above mixture to 5 ml iodine solution to develop color. Shake and mix. The solution should turn deep blue if there is any residual, unconverted starch present in the solution. The solution is brown-red colored for partially degraded starch, while it is clear for totally degraded starch.
- Measure the absorbance with a spectrophotometer at 620 nm.

Effect of Temperature

- Take hot water from a hot temperature bath and adjust the temperatures of the temporary water baths in 500 ml beakers so that they range from 30°C to 90°C in increments of 10°C.
- Prepare the starch substrate by diluting 2% starch solution with an equal volume of pH 7 phosphate buffer solution. This results in a working starch concentration of 1%. Add 5 ml of the starch solution to each of the test tubes.

- Allow the temperature of each of the starch solutions to come to equilibrium with that of the water bath.
- Add 0.5 ml of the bacterial amylase solution to each of the thermostated test tubes to start the reaction.
- Stop the reaction after exactly 10 minutes and analyze the starch content by measuring the absorbance at 620 nm spectrophotometrically.

Effect of Heat

- Place 0.5 ml of the bacterial amylase solution in each of eleven test tubes. Heat-treat the enzyme solution by placing all the test tubes, except one, in a hot (90°C) water bath. The untreated enzyme is used as the control.
- Take out the first test tube after one min and quickly bring it to room temperature by immersing it in a cold water bath. Remove the second test tube after 2 minutes the third after 3 minutes, and so on.
- Add 5 ml of the 1% buffered (pH 7) starch solution to each of the test tubes containing the enzyme. Carry out the hydrolysis reaction at room temperature and analyze the sample after exactly 10 min. Mix an equal volume of the $CaCl_2$ solution to the enzymes and repeat the same procedure to investigate the heat stabilization of the enzymes in the presence of Ca^{2+} ions.
- This set of studies can be done quickly if the procedures are synchronized. If time permits, try 0.5 ml samples of the amyloglucosidase and 0.5 ml samples of the fungal amylase solution. Compare the sensitivity to heat for these related enzymes.

Effect of Substrate Concentration

- Add 0.5 ml of the bacterial amylase solution to 50 ml of a 1% starch solution buffered at pH 7.0. Note that less enzyme per ml of substrate is used in this part of the experiment than the previous parts. The objective here is to slow down the reaction so that multiple sampling is possible with reasonable accuracy before all the starch is consumed.

- Take samples periodically to monitor both the decrease in the starch concentration and the increase in the reducing sugars until most of the starch is hydrolyzed. The starch concentration is measured with the same steps outlined above and the sugar concentration with the dinitrosalicylic colorimetric method.
- Continuously monitor the viscosity of the substrate-enzyme mixture with a viscometer. Generate a calibration curve for the viscosity as a function of the starch concentration. Note that this part of the study is fruitful only when the starch solution is extremely thick.

PROTOCOL 1.9

Production of Corn Syrup Using Bacterial Amylase

In making industrial sugars, e.g. corn syrup, large gelatinized starch molecules are first chopped into smaller dextrins with the help of bacterial amylase. The liquefaction step is followed by saccharification with either fungal amylase or amyloglucosidase, depending on the end use of the sugar. These sequential enzymatic treatment steps will be simulated in this part of the experiment.

Procedure

- Add 0.5 ml of the bacterial amylase solution to 50 ml of the 2% non-buffered starch solution.
- Periodically place a few drops of the reaction mixture on a glass plate.
- Add one drop of the iodine reagent. The colour should finally turn red, indicating the total conversion of starch to dextrin. This liquefaction step should last for approximately 10 minutes.
- When the process of liquefaction is complete, adjust the pH of the starch solution to 4.7 with 1 N HCl.
- Filter the starch solution if it is turbid.
- Separate the solution into two equal parts.

- To the first solution, add 0.5 ml of amyloglucosidase.
- To the second solution add 0.5 ml of fungal amylase solution.
- Measure the sugar concentrations periodically.
- Note that appropriate calibration curves are needed because one is maltose and the other is glucose. Also the initial absorbance at the start of the saccharification process should be measured so that the increase in the sugar concentration can be correctly measured. This saccharification step should last for about 30–60 minutes.
- Taste the two sugar solutions and compare the sweetness.

PROTOCOL 1.10

Evaluation of Amino Transferases Activity

The major site of amino acid degradation in mammals is the liver. The α-amino group of many amino acids is transferred to α-keto glutarate to form glutamate, which is then oxidatively deaminated to yield NH^{4+}.

Aspartate amino transferase (Glutamate: Oxaloacetate Amino Transferase).

Principle

Aspartate, a four-carbon amino acid, is directly transaminated into oxaloacetate, a citric acid cycle intermediate. This reversible interconversion is catalyzed by the enzyme, glutamate oxaloacetate amino transferase (GOT).

$$\text{Aspartate} + 2\text{-Oxoglutarate} \rightleftharpoons \text{Glutamate} + \text{Oxaloacetate}$$

The oxaloacetic acid gives a brown coloured hydrazone with 2,4-dinitrophenyl hydrazine in alkaline medium, which is measured colorimetrically at 510nm. This constitutes the basis for quantitative estimation of catalytic activity of the enzyme.

Materials

Phosphate buffer, pH 7.4

Anhydrous disodium hydrogen phosphate 11.3 g,

Anhydrous potassium dihydrogen phosphate 2.7 g

Water q.s to Bring the pH to 7.4, store at 4°C. 1000 ml

Pyruvate Standard

Weigh accurately 22.0 mg sodium pyruvate and dissolve in 100 ml distilled water.

Substrate Solution

Dissolve 13.3 g D, L-aspartic acid in minimum amount of 1 N sodium hydroxide and adjust the pH 7.4. Add 0.146 g 2-oxoglutarate and dissolve it by adding little more sodium hydroxide solution. Adjust to pH 7.4 and make up to 500 ml with phosphate buffer. Store the substrate solution at –15°C.

2,4-Dinitrophenyl Hydrazine Solution

Weigh accurately 19.8 mg dinitrophenyl hydrazine and dissolve in 10 ml hydrochloric acid and make up the volume to 100 ml with distilled water. Keep the solution in an amber coloured bottle.

Enzyme Extract

Grind the plant tissue in 0.2 M potassium phosphate (pH 7.5) in a homogenizer for 2 minutes. Pass it through eight layers of cheesecloth and then centrifuge at 25000g for 15 minutes).

Sodium hydroxide (0.4 N)

Procedure

- Warm 0.5 ml substrate solution in a water bath at 37°C for 3 minutes and add 0.2 ml enzyme extract and mix.
- Incubate at 37°C for 1 hour. Then add 0.5 ml dinitrophenyl hydrazine solution and mix.
- Prepare a control by mixing 0.5 ml substrate, 0.5 ml

dinitrophenyl hydrazine solution and 0.1 ml enzyme extract. Keep the tubes at room temperature for 20 minutes. Add 5 ml 0.4 N sodium hydroxide, mix and keep for 10 minutes. Measure the absorbance colorimetrically at 510 nm.

- Pipette pyruvate standard 0.05 to 0.20 ml, make up to 0.2 ml then add 0.5 ml substrate and 0.5 ml dinitrophenyl hydrazine solution.
- Similarly, prepare a blank solution by mixing 0.5 ml substrate, 0.2 ml water and 0.5 ml dinitrophenyl hydrazine solution. Then keep the tubes at room temperature for 20 minutes, add 5 ml 0.4 N NaOH, mix and keep for 10 minutes. Measure the absorbance colorimetrically at 510 nm.

Calculation

The difference in absorbance between test and control is due to the pyruvate formed by the enzyme. The pyruvate in standard produces the difference between standard and blank.

Alanine Amino Transferase (Glutamate : Pyruvate Amino Transferase)

Principle

Alanine amino transferase, which is also prevalent in mammalian tissue, catalyzes the transfer of amino group of alanine to α-ketoglutarate (2-oxoglutarate).

$$\text{Alanine} + \alpha\text{-ketoglutarate} \longrightarrow \text{Pyruvate} + \text{glutamate}$$

The pyruvate formed after incubation for half an hour is made to react with 2,4-dinitrophenyl hydrazine and measured colorimetrically at 510 nm.

Materials

Substrate Solution

Dissolve 9.0 g alanine in 90 ml water with addition of about 2.5 ml sodium hydroxide (1 N) and adjust to pH 7.4. Then add 0.146 g 2-oxoglutarate and dissolve it by adding a little more sodium hydroxide solution and adjust to pH 7.4, make

up the volume to 500 ml with phosphate buffer, store at –15°C.

Phosphate buffer pH 7.4

Pyruvate standard

2,4-Dinitrophenylhydrazine solution

Sodium hydroxide 0.4 N

Procedure

Use alanine as substrate and follow the steps described for aspartate amino transferase except incubate for 30 min instead of 1hour.

Calculation

Express the enzyme activity as micromoles of pyruvate formed per minute per mg protein.

PROTOCOL 1.11

Evaluation of Phospholipase Activity

These are the enzymes, which hydrolyze phospholipids. Four types of phospholipases having different sites of attack are known.

Phospholipase A

Principle

Phospholipase A acts upon lecithin with the release of lysolecithin which lyses the erythrocytes.

Materials

4% Suspension of saline washed rabbit erythrocytes

Lecithin

Enzyme source (several seeds, yeast, snake venom, etc.)

0.1M Veronal buffer of (pH 7.6) is used in which dissolve 1.25 g NaCl (0.22 M), 22 g $CaCl_2$ (20 mM) and 0.037g EDTA

Procedure

- Mix 1 ml of saline washed rabbit erythrocytes suspension (4%) with 1 ml of water and freeze quickly.

- Repeatedly thaw the frozen solid and freeze few times to lyse the erythrocytes.
- Determine the amount of hemoglobin released in the supernatant by centrifuging for 5 minutes at 100g and measure the absorbance/OD of the supernatant at 560 nm.
- Emulsify 100 mg of lecithin in saline (100 µg/ml) under vigorous shaking.
- Add some enzyme source to 1 ml of erythrocyte suspension and make up the final volume to 2.0 ml with veronal buffer.
- Incubate the mixture at 37°C for 30 minutes.
- Keep the mixture at 4°C for 20 minutes.
- Centrifuge and determine the amount of hemoglobin released in the supernatant by measuring the absorbance/OD at 560 nm and compare to that of completely lysed sample.

Phospholipase D

Principle

The enzyme releases choline by splitting lecithin (phosphatidyl choline), which forms a complex with iodine. The formed complex is measured spectrophotometrically at 365 nm.

Materials

Iodine reagent (as described in official Pharmacopoeias)

Enzyme Source

Following protocol is followed for the processing of the enzyme.

- Grind and homogenize 100 g fresh cabbage leaves with 75 ml distilled water in a blender.

- Keep it to stand for 1 to 2 hours at 5°C, filter through coarse cloth and centrifuge. The supernatant is a rich source of enzyme.
- Harvest yeast cells by centrifugation and mix the pellet with approximately equal volume of a mixture of carborundum/celite (1:1) mixture. Transfer the resulting dry powder to a mortar kept in ice and grind well till a viscous paste is formed. Extract the paste with citrate-phosphate buffer (0.1 M, pH 5.6, 10 ml/g cells). Decant the carborundum sediment and centrifuge at 5000 rpm for 10 minutes. The supernatant is a rich source of enzyme.

Substrate egg lecithin (commercially available) 10 mg/ml in ethyl ether.

Procedure

- Pipette 1 ml of ether solution of substrate into a tube and place in warm water bath to remove the solvent. To the residue, add 0.2 ml enzyme source and mix well to form an emulsion. Incubate at 25°C for 90 minutes. Add ether to make up the volume to 5 ml, mix well and keep stoppered for 45 minutes. Centrifuge, discard the ether layer and estimate choline in the aqueous layer spectrophotometrically by the procedure given below.
- To 0.5 ml of a standard solution of choline, add 0.2 ml of iodine reagent, mix well and keep in ice bath for 15 minutes to precipitate out the choline-iodine complex.
- Centrifuge and discard the supernatant and dissolve the precipitate in chloroform (10 ml) and measure the optical density/absorbance of the solution spectrophotometrically at 365 nm. The molar extinction coefficient of choline-iodine complex is 2.7×10^{-7}.
- Prepare a standard curve between absorbance/optical density and concentration. Treat the aqueous phase from the test solution similarly and measure the liberated choline.

PROTOCOL 1.12

Determination of Acetylcholinesterase Activity

Principle

Acetylcholine is made to react with hydroxylamine to form the corresponding acylhydroxamic acid, which forms a strongly coloured ferric hydroxamate with ferric salts, and the colour is measured spectrophotometrically at 490 nm. The hydrolyzed acetylcholine per unit time is measured by comparison of the initial concentration in a tube with the final concentration in the experimental tube.

Materials

Blood Source

Collect 0.2 ml blood from the test animal in a tube containing 5 ml water. Use the hemolysate for the assay.

Acetylcholine stock solution

3.64% solution of acetylcholine (200 mM) in distilled water.

Veronal Buffer (0.1M pH 8.6)

Dissolve 4.92 g sodium veronal and 3.24 g sodium acetate in 300 ml distilled water, add 3 ml 1N HCl and dilute to 500 ml with distilled water, check the pH.

Sodium hydroxide (10% w/v in distilled water)

Substrate (Acetylcholine solution, 1.33 mM)

Mix 1 ml of acetylcholine stock solution thoroughly in 150 ml veronal buffer.

Hydroxylamine (1N)

7% w/v Hydroxylammonium chloride in distilled water.

Iron Solution (0.7M)

Dissolve 2.5 g potassium nitrate in about 10 ml distilled water. Dissolve 33.75 g Fe $(NH_4)(SO_4)_2.12H_2O$ in about 70 ml distilled water with gentle warming. Mix both the solutions and make up the volume to 100 ml with distilled water.

Alkaline hydroxylamine solution

Mix equal volumes of 2.5 N sodium hydroxide and 1 N hydroxylamine solutions.

Citrate Buffer (1 M, pH 1.4)

Dissolve 2.10 g citric acid and 0.8 g NaOH in minimum quantity of distilled water, add 89 ml 1 N HCl and make up the volume to 100 ml with distilled water. Dilute 10 ml of this solution to 100 ml with distilled water. Adjust the pH between 1.2 and 1.4.

Enzyme Source

Grind the fresh sample material (root, leaf or any other part) from plant and extract in 10 mM veronal buffer (pH 8.6) followed by centrifugation at 20,000g for 10 minutes. Grind the pellet containing the enzyme and extract with the above buffer containing 5% ammonium sulphate. Centrifuge the extract at 20,000g for 10 minutes and use the supernatant as the enzyme source.

Procedure

- Pipette out the following into 50 ml volumetric flasks as given below (Table 1.1).

Table 1.1. *Volumes of samples and substrates for determination of cholinesterase activity*

	Reference (ml)	*Test (ml)*	*Blank (ml)*
Sample (enzyme Source)	-	2	-
Substrate solution	25	25	-
Mix well and incubate at 37°C for 30 minutes			
Alkaline hydroxylamine solution	5	5	5
Sample	2	-	-
Citrate buffer	5	5	5
Ferric solution	10	10	10

- Allow the ferric solution to run slowly down the wall of the flask. Dilute with distilled water to the mark

and shake thoroughly. Allow to stand for 20 minutes at room temperature. Filter the solutions and discard the first portion of filtrate.

- Measure the absorbance of the solution spectrophotometrically against blank at 490 nm.

Calculation

The absorbance difference (DE) between initial concentration of substrate (reference and final concentration of substrate (test) is used for calculation. The extinction of the dye is 0.961 at 490 nm. Hence the amount of dye formed from the non-hydrolyzed acetyl choline in 50 ml.
C = E × 50/ 0.961 × 1.0 (mM/50 ml).

The acetylcholinesterase activity in whole blood

= E × 50/ 0.961× 1.0 × 1/ 0.08 × 30 × 1000

= E × 21667 (U/liter)

PROTOCOL 1.13

Cellulases (CXb (1-4) Gluconase) Assay

Principle

Glucose, a reducing sugar is produced due to cellulolytic activity, which is colorimetrically, estimated by dinitrosalicylic acid reagent at 540 nm.

Materials

Sodium citrate buffer 0.1 M, pH 5.0

Dinitro Salicylic Acid Reagent

Dissolve 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 ml 1% NaOH and store at 4°C.

Potassium Sodium Tartrate

40 g in 100 ml of distilled water.

Carboxymethyl cellulose

1% in sodium citrate buffer, 0.1 M, pH 5.0.

Procedure

- Incubate the mixture of 0.45 ml 1% carboxymethyl cellulose solution and 0.05 ml enzyme extract at 55°C for 15 minutes.
- Remove the mixture from the bath.
- Add 0.5 ml dinitrosalicylic acid reagent.
- Heat the mixture in a boiling water bath for 5 minutes.
- Add 1.0 ml potassium sodium tartrate solution.
- Cool to room temperature.
- Make up the volume to 5 ml with distilled water.
- Measure the absorbance at 540 nm.
- Prepare a standard graph with glucose in the concentration range 50–1000 mg/ml.
- Report the enzyme activity as the mg glucose released per min per mg protein.

PROTOCOL 1.14

Evaluation of Papain Activity

Principle

Papain hydrolyzes benzoyl L-arginine p-nitroanilide (BAPNA) and releases p-nitroaniline which is measured colorimetrically at 410 nm.

Materials

Acetic acid 30% v/v

Enzyme Source

0.1 mg/ml papain in distilled water.

Tris-HCl buffer (50 mM pH 7.5)

Dissolve 0.60 g Tris in 50 ml distilled water. Adjust to pH 7.5 with 0.05 N hydrochloric acid and make up the volume to 100 ml. To the above buffer (100 ml) add 87.80 mg of cysteine hydrochloride (0.005 M) and 74.40 mg of EDTA (0.002 M) and dissolve completely.

Buffered Substrate Solution

Dissolve 43.5 mg BAPNA in 1 ml of dimethyl sulphoxide and make up the volume to 100 ml with Tris-HCl buffer containing 5 mM cysteine and 2 mM EDTA.

Procedure

- To 0.5 ml papain solution in a test tube add sufficient Tris-HCl buffer to make up the volume to 1.0 ml.
- Add 5 ml of substrate solution and incubate for 25 minutes at 25°C.
- To terminate the enzyme action, add 1 ml acetic acid (30%).
- Measure the absorbance of the released p-nitroaniline at 410 nm against a control spectrophotometrically.
- Prepare a standard graph using p-nitroaniline.
- Calculate and report the activity per g sample.

Molar Extinction Coefficient of p-nitroaniline

(E^{1M}_{1cm}) = 8,800/mole/cm

1 mM/l = 8.800/1000 = 8.8 Absorbance (A)

1 µM/l = 8.8/1000 = 0.0088 A

1 µM/ml = 8.8.

If the absorbance is 8.8, amount of p-nitroaniline = 1 µM/ml

If X is the absorbance of the sample $= \frac{1\mu M/ml}{8.8} \times X$

$$= \frac{X \times \mu M/ml}{8.8}$$

This is for one ml of the sample for 25 minutes.

For 7 ml (volume of the assay mixture) of the sample solution

$$= \frac{X\mu M \times 7.0}{8.8 \times 7.0 \times 25}$$

The assay mixture contains 0.5 ml of the enzyme.

Therefore, activity of the enzyme in 1 ml

$$= \frac{X\mu M x}{8.8 \times 0.5 \times 25} (\mu M \text{ of p-nitroaniline released per minute})$$

- Report the activity per g sample.

PROTOCOL 1.15

Evaluation of Glucose Oxidase Activity

Glucose oxidase catalyses the oxidation of α-D-glucose to D-glucono-1,5-lactone (gluconic acid) with the formation of hydrogen peroxide. The oxygen liberated from hydrogen peroxide by peroxidase reacts with the O-dianisidine and oxidizes it to a red choromophore product. The enzyme can be assayed either colorimetrically or polarographically.

Colorimetric Estimation

In colorimetric assay, a coupled peroxidase-o-dianisidine is used. The oxygen liberated from hydrogen peroxide is directly coupled to the dye o-dianisidine, which turns to a brownish red colour which is measured colorimetrically at 460 nm.

Enzyme Source

The moulds like *A. niger, A. oryzae, P. notatum, P. vitale* and *P. chrysogenum* and red algae like *Iridophycus flaccidum* are the good sources of glucose oxidase. Glucose oxidase has not been found in animal tissues, with the exception of the pharyngeal glands of the honeybee. The enzyme from *Aspergillus* is intracellular whereas that from Penicillium is extracellular.

Materials

Phosphate buffer (0.1M, pH 6.0).

Coupling Enzyme

Aqueous solution of purified horseradish peroxidase containing 60 units/ml.

Dye

1% Aqueous solution of o-dianisidine. Add 0.1 ml of this to 12 ml buffer.

Substrate

18% Solution of glucose.

Enzyme

Aqueous solution of the crude enzyme in 0.1 M phosphate buffer pH 6.0

Procedure

- Prepare a control by adding 2.6 ml dye buffer solution, 0.3 ml glucose and 0.1 ml peroxidase.
- Prepare a test solution by adding 2.5 ml dye buffer solution, 0.3 ml glucose and 0.1 ml peroxidase.
- Adjust the OD of the test solution to zero against the control at 460 nm. Add 0.1 ml of enzyme to the test solution, mix well. Measure the increase in OD at 460 nm at 30 seconds intervals for 3 to 5 minutes. Plot a graph and calculate the rate.

Polarographic Method

Glucose oxidase causes the conversion of glucose to gluconic acid and the oxygen consumed in this conversion is measured using an oxygen electrode.

Materials

Phosphate buffer 0.1M, pH 6.0

Substrate

18% Aqueous solution of glucose.

Enzyme

Aqueous solution of crude enzyme in 0.1M phosphate buffer pH 6.0

Azide Solution

0.002% Sodium azide in the buffer.

Procedure

- Add 3 ml of well aerated buffer and 0.1 ml of suitably diluted crude extract containing the enzyme in the

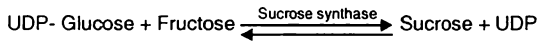
oxygen electrode cuvette. Add 0.1 ml of glucose solution and record the rates for at least 2 minutes.

- Calculate the enzyme activity from the amount of oxygen consumed per minute, which is equal to the micromoles of oxygen consumed per minute.

PROTOCOL 1.16

Sucrose Synthase (UDP Glucose: D-fructose 2-α-d-glucosyl Transferase)

It catalyzes the following reversible reaction in plants.



Principle

The activity of sucrose synthase is estimated by coupling the formation of UDP-glucose to the reduction of NAD+ in the presence of excess UDP-glucose dehydrogenase and the change in absorbance at 340 nm followed.

Materials

Sucrose 0.5 M (17.11 g/100 ml)

UDP 0.01 M (4 mg/ml)

NAD+ 0.015 M (9.95 mg/ml)

HEPES-KOH buffer 0.1M, pH 7.5

UDP-glucose dehydrogenase (0.25 mg/ml).

Enzyme Extract

Homogenize 10 g tissue with 20 ml potassium phosphate buffer (10 mM, pH 7.2) containing 1 mM EDTA and 5 mM 2-mercaptoethanol. Filter the homogenate through eight layers of cheesecloth and centrifuge the filtrate at 30,000 rpm for 15 min, preferably at 4°C. The supernatant is a rich source of enzyme.

Procedure

Pipette out all reagents as given in Table 5.2. Set the spectrophotometer to get zero absorbance at 340 nm without adding NAD+ in the test against blank in a cuvette. Add

NAD+, quickly to the test, mix and record the initial absorbance and set a timer. Record the decrease in absorbance every min until no further change is observed.

Table 1.2. Volumes of reagents for determination of sucrose synthase activity

Reagent	Assay (ml)	Blank (ml)
HEPES-KOH	0.2	0.3
Sucrose	0.2	0.2
UDP	0.2	0.2
Enzyme extract	0.2	0.2
UDP-glucose dehydrogenase	0.1	0.1
NAD+	0.1	-

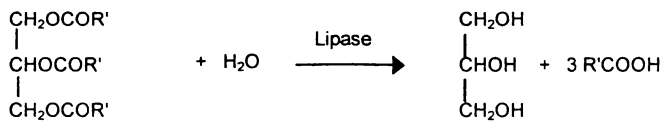
Calculation

The change in absorbance at 340 nm is 12.0 for each µM of UDPG per ml. Report the enzyme activity as µM UDPG formed per mg of protein.

PROTOCOL 1.17

Lipase Enzyme Activity Determination

Lipase hydrolyzes triglycerides, releasing free fatty acids and glycerol.



The germinating seeds of groundnut, castor bean and sunflower are good source of lipase.

Principle

The amount of fatty acid released in unit time is measured by the volume of NaOH required to maintain constant pH. The milliequivalent of alkali consumed is taken as a measure of the activity of the enzyme.

Materials

50 mM phosphate buffer (pH 7.0)
0.1N NaOH
Substrate

Neutralize 2 ml of any clear vegetable oil to pH 7.0 if necessary and stir well with 25 ml water in presence of any emulsifying agent (sodium taurochloate, 100 mg) till an emulsion is formed.

Enzyme Source

- Germinating oil seeds like castor seeds is a good source. They are pounded in a mortar and pestle. The minced tissue is homogenized with twice the volume of ice-cold acetone. The suspension is filtered and quickly washed with successive portions of acetone, acetone-ether (1:1), ether and then air-dried. The dry powder, called the acetone dry powder can be stored for a long time in a refrigerator. Just before use 1 g of the powder is stirred with 20 ml of ice-cold water (or buffer) for 15 minutes and the residue is removed by centrifugation (15,000 rpm for 10 minutes). The supernatant is used as the enzyme source.
- The pancreatic tissue obtained from a slaughterhouse is also a good source. The tissue is kept in ice during transportation, then cut into very small pieces.

Procedure

- To 20 ml of substrate, add 5 ml of phosphate buffer (pH 7.0).
- Stir the contents slowly at 35°C on a magnetic stirrer cum hot plate. Note the pH of the reaction mixture and adjust it to 7.0.
- Add enzyme extract (0.5 ml), immediately note the pH and set the timer on.
- At frequent intervals (10 min) or as the pH drops by about 0.2 unit add 0.1 N NaOH to bring pH to initial value. Continue the titration for 30–60 minutes and note the volume of alkali consumed.

Calculation

The enzyme activity is expressed as the amount of enzyme which releases one milliequivalent of free fatty acid per minute per gram sample.

Note: Cellulose nitrate tube should be filled full otherwise it will collapse during centrifugation.

PROTOCOL 1.18

Isolation of Crystalline Animal Cytochrome C

Principle

Cytochrome c is extracted from muscle at neutral pH after decomposition of cells and particles by a weak acid treatment. The protein is adsorbed on a column loaded with a carboxylic acid cation exchange resin at neutral pH and eluted with neutral buffer of high concentration after washing with a lower concentration. Cytochrome c is oxidized and then further purified by employing a careful fractional elution procedure on a resin column. Some impurity may still present, that is removed by making the solution nearly saturated with ammonium sulphate at weak alkalinity. After dialysis, the preparation is separated into several cytochrome c fractions by chromatography on a resin column and the largest fraction, which contains unmodified cytochrome c, is used for the crystallization. The highly concentrated protein following resin treatment is crystallized in reduced form from nearly saturated ammonium sulphate solution containing ascorbic acid at a alkaline pH.

Starting Material

Beef heart is processed as soon as possible after removal from the animal. The heart should be frozen if the time between removal and processing exceeds 24 hours. Though cytochrome c has been crystallized adopting the present method, or a slightly modified one, from any of the tested animal tissues having a high concentration of cytochrome c, such as beef, pig, horse, human, bonito (*Katsuwonus vagans*) and tuna (*Thunnus alalunga*) hearts, horse thigh and pigeon breast

muscle, and beef kidney. Beef heart seems to be the most suitable source. The fishes' heart and pigeon breast muscle are also preferred sources in places where they are easily obtainable.

Procedure

Extraction

Beef heart (or other animal tissue) is made free of fat and ligaments and twice passed through a meat grinder. Two kilograms of the minced muscles are suspended in 1.6 liter of 0.5 N cold acetic acid and kept at 5°C for 1 hour, with stirring (pH 4.3). The suspension is adjusted to pH 6.2–6.5 by addition of about 0.4 liter of 2 N ammonium hydroxide under stirring and placed in a cold room (5–10°C) overnight. The suspension is mixed with 300 g of Celite 545 in the case of pigeon breast muscle or beef kidney (use double of this amount) and squeezed out through a thick cloth in a press. The residue is again suspended in 1 liter of water and squeezed again after 1hour. The combined extracts are adjusted to pH 6.2–6.5 (indicator paper), filtered through a Buchner funnel with 300 ml of water. The combined extract is a clear brown solution (except with pigeon muscle and beef kidney), volume 4 liters, NH_4^+ concentration 0.2 N, pH 6.2–6.5.

First Resin Treatment

The combined extracts are passed through a resin bed, which has been prepared by introducing a suspension of 100–200 mesh X E-64 equilibrated with a buffer of pH 7.0 containing 0.1 N ammonium ion (abbreviated 0.1 N NH_4^+ buffer, pH 7.0) into a sintered glass funnel of 7 cm diameter until the bed height becomes 5 cm. This rate of flow is achieved by applying suction. The brown solution passing through is discarded, and the resin bed (upper part is reddish brown from the adsorption of cytochrome C and possibly denatured myoglobin) is washed with about 500 ml of 0.1 N NH_4^+ buffer, pH 7.0. The dark colored part of the resin is separated after strongly sucking out the washing with a spatula from the colorless part and

suspended in 500 ml of 0.05 N NH_4^+ buffer, pH 7.0. The pH of the suspension is adjusted to 7.3–7.6 by the dropwise addition of concentrated ammonium hydroxide and transferred to a 7 cm glass funnel containing about a 1 cm layer of the fresh resin, and the resulting resin layer (upper part, red; lower part, white) is washed with about 2 liter of 0.1 N buffer, pH 7.0 and all the reddish elute is collected (100 ml, NH_4^+ 0.4 N, pH 6.5, purity 60%).

Second Resin Treatment

The solution is diluted with 3 volumes of water and adjusted to pH 7.0 with ammonia. Then the cytochrome c is completely oxidized with 0.01 M $K_4Fe(CN)_6$ (1 ml). The solution is then made to pass through a resin column (4 × 10 cm, 100-200 mesh, equilibrated with 0.1 N NH_4^+ buffer at pH 7.0) at a rate of 10 ml per minute, and the column is washed with 0.15 N NH_4^+ buffer, pH 7.0, until the red band moves down and cytochrome c just begins to be eluted. This step is carried out at room temperature with pressure applied as needed. Cytochrome c is eluted from the washed column with 0.5 N NH_4^+ buffer, pH 7.0 as before to yield about 30ml of solution approximately 1% with respect to cytochrome c purity 90%.

Ammonium Sulphate Treatment

To the elute collected in a small beaker, 0.7 g of solid ammonium sulphate and 0.02 ml of 30% ammonia are added per ml of the elute (80% saturation). The solution is kept at about 10°C for 2 hours, and passed through a small sintered glass funnel to remove the precipitate with the aid of 0.2 g Hyflow Super-Cel. The filter is washed with 5 ml of 95% saturated ammonium sulphate, pH 8.0, to yield about 35 ml of a solution of cytochrome c (about 35 ml), NH_4^+ concentration 7 M, pH 8.5, purity nearly 100%.

Chromatography

The combined filtrate and washings are dialyzed in a cellophane bag against 100 volumes of distilled water at 0–5°C overnight. The column is loaded with the resin (200–300 mesh, equilibrated with 0.25 N NH_4^+ buffer, pH

7.0) suspended in buffer into a tube of diameter 2.5 cm and length about 70 cm until the settled resin bed becomes 50 cm high. When the supernatant buffer over the resin bed has just passed down, the dialyzed cytochrome C solution is introduced along the side of tube. Then 0.25 N NH_4^+ buffer is introduced and allowed to pass at the rate of 20–40 ml per hour on a fraction collector at room temperature. The colored solution is collected (belonging to the second fraction) which contains more than 90% of the total cytochrome C and used for crystallization. It is possible to collect the fractions without using the collector by merely observing the band and elute color. This procedure yields about 200 ml of solution, purity about 100%.

Crystallization

The solution (about 200 ml) is dialyzed against 10 volumes of distilled water in the cold overnight (not more than 24 hours) in order to reduce its cationic concentration to 0.03–0.05 N. The solution is then passed through a column (2 × 2 cm) of the resin (100–200 mesh), held in a sintered glass funnel equilibrated with 0.1 N NH_4^+ buffer, pH 7.0. After the column is washed with 20 ml of 0.1% ammonium sulphate solution pH 8.0 the dark red resin in the upper part of the column is transferred with 0.1% ammonia solution to another column (0.7 cm in diameter). Then 5% ammonium sulphate solution, is slowly passed through the column to elute cytochrome c. A very dark band moves down the column and more than 98% of the pigment adsorbed on the resin is collected in a volume of less than 4 ml (4–8% cytochrome c) in a small centrifugation tube of known weight. After the addition of a drop of octanol, 0.43 g of ammonium sulphate is added per g of the solution. When the salt gets dissolved completely, about 5 g of ascorbic acid and a few drops (1 drop per ml) of 30% ammonia are added and the solution is kept at 10°C for 10 minutes. To the solution, which has been grown light in color by the reduction of cytochrome c, small quantities of fine powdered ammonium sulphate are added, each portion being dis-

solved completely by a glass rod until, the solution becomes turbid. The solution, tightly stoppered, is allowed to stand at 15–25°C for 1 to 2 days while the cytochrome c crystallizes out as fine needles or in grouped form of needles such as leaflets or rosettes. About 0.02g of ammonium sulphate is added per milliliter of the suspension and allowed to stand for a few more days. The crystals are collected by centrifugation at 5000g, suspended with a minimum volume of water and recrystallized as above, after the addition of 1 drop of ammonia and 1mg of ascorbic acid. The yield is about 200 mg from 2 kg of the minced muscle. The crystals may be stored as a suspension in saturated ammonium sulphate solution at 0°C or lyophilized after dialysis against 0.08 M NaCl or 0.1 M sodium buffer.

Properties and Purity of Product

The purity of cytochrome c preparations has been checked by iron content or the ratio of extinction at 280 nm for maximum extinction (this should be altered to 278 nm for maximum extinction) in oxidized form to that at 550 nm in reduced form. The iron content of the present preparation is 0.45% and the extinction ratio (550 nm/ 278 nm) is 1.28 (1.20 and 1.05 with pigeon and fish preparations respectively). Although a few preparations of extinction ratio greater than 1.4 are obtainable from another fraction in the resin chromatography of trichloroacetic acid-treated cytochrome c, it is unlikely that the present crystalline preparation is not a pure one, since the former preparations are thought to be modified. The present preparation is an essentially homogeneous substance that results from ultracentrifugation under several conditions, electrophoresis at weak alkalinity, ion exchange resin chromatography and constancy of the extinction ratio on further recrystallization. Biological activity of the present preparation in the succinic oxidase and the cytochrome oxidase systems is the same as that of the Kieilin and Hartree preparation if based on the extinction at 550 nm in reduced form. However, the cytochrome c described herein is not autoxidizable and is resistant to protease action, in contrast

to preparations of cytochrome c, which have been subjected to trichloroacetic acid precipitation.

Method of Preparation

In order to get a high yield of crystalline cytochrome c from sources other than beef heart, some steps should be modified slightly. More extensive modifications are necessary for the preparation of the crystals from yeast and wheat germ. The yield of crystalline preparations per unit weight are as follows: pigeon breast muscle >bonito or tuna heart >beef and horse heart > pig or human heart > baker's yeast > wheat germ. There are other reports about the preparation of crystalline cytochrome c from penguin muscle and beef heart.

PROTOCOL 1.19

Digestion of Proteins into Amino Acids

Principle

Various species of organisms cannot synthesize or are not efficient in generating all of the twenty amino acids needed to construct the proteins and enzymes essential for their survival. To sustain growth and to maintain metabolic functions, these amino acids must be provided from outside sources. This can be accomplished by the intake of proteins. Humans are a good example of living organisms that ingest proteins as part of their nutritional requirements. Some organisms secrete proteolytic enzymes extracellularly to break down the protein to its component monomeric amino acid units by hydrolyzing peptide bonds at the end of the polymer chain. A series of shorter polypeptides of different lengths are also formed if the broken peptide bonds are not at the end of the polymer chain. Thus, depending on the location of the attack, proteases can be further classified into exopeptidases (attack on the terminal group) and endopeptidases (attack on internal linkages).

In this experiment, an accurate and generally accepted color method is introduced. In this method, an organic

compound called ninhydrin is reacted with the amino acids released during the hydrolysis of the protein. The original unreacted ninhydrin is yellowish in color, but the reacted product of ninhydrin has a deep purple-blue color. For example, the procedure given at the end of this section yields an absorbance of 0.27 for 1×10^{-4} M of glutamic acid. Since ninhydrin does not react with the undegraded protein, one can measure the amino acid concentration by following the development of the purple color by measuring the absorbance of the solution with a spectrophotometer. Because the color intensity is a measure of the amino acid present, the color should intensify as more protein is degraded to amino acid over time. The upper limit in color intensity is reached when all the ninhydrin originally present in the solution has been consumed. Thus, the amount of ninhydrin originally present in the reaction mixture determines the maximum amino acid concentration that can be detected.

Equipments

Beakers (100 ml and 400 ml), graduated cylinder, pipettes (1 ml and 10 ml), temperature bath or bunsen burner or hot plate), thermometer, stirring rod, funnel and filter paper, centrifuge, test tubes, balance, blender, spectrophotometer

Reagents

Amino acid standard (for calibrating spectrophotometer)

Protein source

Cottage cheese or curds (10 g/l)

Protease

Ninhydrin solution

Procedure

- Make a 1% protein solution by dissolving casein in water. Make a saturated protease solution by adding 0.5 g of powder protease to 1 liter of water.

- Spin the solution in a centrifuge to separate the undissolved powder. Keep the supernatant.
- Mix equal volumes of protein solution and protease solution. Note the time at the start of the hydrolysis reaction.
- Withdraw 5 ml of the solution and measure the amino acid concentration of the solution as a function of time by using the ninhydrin colorimetric method. Suggested sampling time interval: 10 minutes for at least 1 hour.

Notes: Gelatin and albumin may be used in lieu of casein. The hydrolysis rate for different substrates may be studied. Alternatively, add 0.05 g protease directly to 50 ml of the protein mixture. However, one must perform the ninhydrin test on the supernatant obtained either through centrifugation or filtration for each sample.

If reagent grade casein is not available, a 1% mixture can be made by the following steps:

- Add 1 g of protein (cheese) using a blender, liquify the mixture; pour into a 100 ml graduated cylinder or a 100 ml volumetric flask.
- Rinse the blender with about 20 ml of water from a squeezable plastic bottle; pour the rinse into the measurement device and add water to 100 ml.

PROTOCOL 1.20

Determination of Serum Uric Acid

Uric acid in blood arises as a result of purine catabolism. The normal level of uric acid in serum varies from 2.0 to 6.0 mg per 100 mL. The uric acid content of the body in normal subjects has been found to be about 1–13 g. In mild cases of gout the level of serum uric acid increases (approx. 6.9 mg/ 100mL).

Principle

The blood serum is deproteinized by tungstic acid and filtered. The filtrate containing phosphotungstic acid in the presence of sodium carbonate silicate gives a blue colored compound (reduced phosphotungstate) and the color is measured in a photoelectric colorimeter at 700 nm (red filter).

Materials

Serum

Sodium tungstate 10 % w/v in distilled water

Sodium carbonate 10% w/v of anhydrous salt in distilled water

Sulphuric acid 2/3N

Tungstic Acid

Mix 25 ml sodium tungstate (10% w/v), 25 ml of 2/3 N sulphuric acid, a drop of phosphoric acid and 400 ml of distilled water. Keep in an amber colored bottle.

Phosphotungstic Acid (stock solution)

Dissolve 25 g sodium tungstate in about 200 ml distilled water. Add 20 ml phosphoric acid (85%) and reflux gently for 2 hours. Cool the content. Transfer to a 250 mL volumetric flask and make up the volume to 250 mL with distilled water. Store in a brown bottle.

Working Phosphotungstic Acid Solution

Dilute 10 ml of the stock solution to 100 ml with distilled water and keep in a brown bottle.

Standard Solution of Uric Acid

Weigh 100 mg of uric acid in a small beaker. Dissolve 60 mg of lithium carbonate in 15 to 20 ml of water in a test tube and heat it to 60°C. Pour this solution onto the uric acid, stir to dissolve the uric acid. Transfer quantitatively to a 100 ml volumetric flask and add 2 ml of formalin (40%) and 1 ml of 50 %v/v acetic acid (slowly with shaking). Make up the volume to 100 ml with distilled water. Protect this solution from light.

Standard Solution of Uric Acid (5 mg/ml)

Dilute 1 ml of the stock solution to 200 ml with distilled water and store in a brown bottle.

Procedure

- Mix slowly with stirring 1.0 ml serum and 9.0 ml of dilute tungstic acid reagent and centrifuge.
- Label three test tubes, 'T' for test, 'S' for standard and 'B' for blank.
- Add 1.0 ml sodium carbonate to each of the tubes, mix well and add 1.0 ml phosphotungstic acid color reagent. Mix the contents.
- In tube 'B' pipette 5.0 ml distilled water in 'S' tube pour 5.0 ml standard uric acid solution and in 'T' tube, pour 5.0 ml supernatant of serum as prepared above.
- Place all the tubes at 25°C in a water bath for 30 minutes.
- Read the color in a photoelectric colorimeter at 700 nm (red filter) using a red filter and calculate the amount of uric acid per 100 ml serum.

Calculation

If the colorimetric readings for blank, standard and test samples are B_1, S_1 and T_1 respectively the amount of uric acid may be calculated as follows:

$$\text{Uric acid (mg/100 mL serum)} = \frac{T_1 - B_1}{S_1 - B_1} \times 5$$

PROTOCOL 1.21

Estimation of Serum Bilirubin

Principle

This method is based on the methods of Malloy and Evolyn. Estimation of serum bilirubin is based on the formation of

purple colored azobilirubin when bilirubin reacts with diazotized sulphanilic acid. This color is proportioned to the amount of bilirubin present and is measured at 540 nm using a green filter.

Reagent

Serum

Absolute methanol

Hydrochloric acid (1.5% v/v in distilled water)

Diazo Reagent

Mix 0.3 ml of Van derr Bergh solution B and 10 ml of Van der Bergh solution A. Always use freshly prepared diazo reagent.

Van der Bergh Solution A

Dissolve 1 g of sulphanilic acid in 15 ml of 0.2 N HCl and make up to 100 ml with distilled water.

Van der Bergh Solution B

0.5% Sodium nitrite in distilled water.

Standard solution of bilirubin

Weigh 10 mg bilirubin and dissolve in 100 ml chloroform.

Procedure

- Label five tubes A, A1, A2, A3 and A4 and proceed as follows:

	A Standard Bilirubin	*A1 Serum total bilirubin*	*A2 Serum direct bilirubin*	*A3 Serum blank*	*A4 Standard blank*
Standard Bilirubin, ml	0.2	-	-	-	0.2

Contd...

Contd...

	A Standard Bilirubin	*A1 Serum total bilirubin*	*A2 Serum direct bilirubin*	*A3 Serum blank*	*A4 Standard blank*
Serum, ml	-	0.2	0.2	0.2	-
Water, ml	2.3	2.3	5.3	2.3	2.3
Diazo reagent, ml	0.5	0.5	0.5	-	-
Absolute methanol, ml	3.0	3.0	-	3.0	3.0
1.5% HCl, ml	-	-	-	0.5	0.5

- Mix the contents of each of the tubes and keep them in dark for 30 minutes.
- Read the % Transmittance / absorbance of standard and serum total bilirubin and direct bilirubin against blank at 540 nm using a green filter.
- Calculate the total bilirubin in 100 ml serum and direct bilirubin in 100 ml serum.

Calculation

$$\text{Total bilirubin in 0.2 ml serum} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.02 \text{ mg}$$

where 0.02 mg is the concentration of bilirubin

$$\text{Total bilirubin in 100 ml serum} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.02 \times \frac{100}{0.2} \text{ mg}$$

Similarly direct bilirubin in 100 ml serum

$$\text{Total bilirubin in 100 ml serum} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.02 \times \frac{100}{0.2} \text{ mg}$$

PROTOCOL 1.22

Estimation of Amylase in Saliva

Principle

The production of blue colour by starch on addition of iodine is not quantitative. Therefore, maltose is estimated using dinitrosalicylic acid.

Procedure

- Prepare a standard graph for maltose as given in Table 1.3. Pipette out different sets of solutions into different tubes following the schedule given below

$$\text{Aldehyde group} \xrightarrow{\text{Oxidation}} \text{Carboxylic acid}$$

$$\text{3,5-dinitrosalicylic acid} \xrightarrow{\text{Reduction}} \text{3-amino- 5-nitrosalicylic acid}$$

Table 1.3: *Volumes of reagents for estimation of amylase in saliva*

Ingredients	*Tube No.**				
	1	*2*	*3*	*4*	*5*
Phosphate buffer (pH 6.7, 0.1 M)	2.5	2.5	2.5	2.5	2.5
Starch solution	2.5	–	–	2.5	2.5
NaCl, 1%	1.0	1.0	1.0	1.0	1.0
Mix well and keep the tubes for 10 minutes at 37 °C					
Water	1.0	1.0	0.5	0.5	0.5
Appropriate diluted saliva	–	–	0.5	0.5	0.5

****Prepare tubes in duplicate***

- After adding the saliva, immediately add 0.5 ml 2 N NaOH to tube 5 to stop the reaction. This is called the 'zero time control'.
- Incubate the rest of the tubes at 37 °C for 15 minutes.

- Add 0.5 ml 2 N NaOH to stop the reaction. Add 0.5 ml of dinitrosalicylic acid reagent and mix well. Heat the tubes in a boiling water bath for 5 minutes.
- Cool the tubes to room temperature and measure the OD at 520 nm using tube 1 as blank. This does not contain any enzyme but it is a mixture of enzymes that are being used. Tube 2 is also a control. Any reading in this tube is to be subtracted from the value of reading from tube 4 and 5. Comparing tubes 4 and 5, it is seen that they contain complete mixtures, except that the reaction in tube 5 has been stopped immediately on addition of enzyme. In other words it is the zero time control. Thus the amount of maltose formed in 15 minutes by the amount of saliva added is:

  Maltose formed = (OD of tube 4-OD of tube 3)-
  (OD of tube 5-OD of tube 3)

- From the standard graph of maltose, calculate the corresponding amount of maltose formed per ml of saliva.

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