Application of Enzymes

Chapter 22,p.349

I-Enzymatic Analyses:Analyses based on use of enzymes as reagents(analytical aids) for determination of food components

-as substrates
-as inhibitors and activators
-for structural studies

II-Analyses based on determination of enzyme activity

a-For assessment of food quality

b-For determining efficiency of enzyme preparations (Enzyme assays).

Enzymes are protein-biocatalysts which govern, initiate and control biological reactions involved in life processes.

Enzymes are produced only by living cells and are not synthetic chemicals but can be isolated from the living organism.
Their content of amino acids and nitrogen ratios are very similar to proteins, they are denatured by heat, are amphoteric, exhibit an isoelectric point. They need an optimal temperature and optimal pH for showing optimal activity. Each enzyme is characteristically specific in that each attacks a specific type of compound, called "substrate". **During enzymatic reactions, the substrates are either converted to other new compounds or are broken into smaller pieces,** producing typical "endproducts".
Any physical or chemical property that is related to concentration of substrate or endproduct can therefore be used to follow an enzyme reaction.

• Enzyme and Substrate Concentrations:

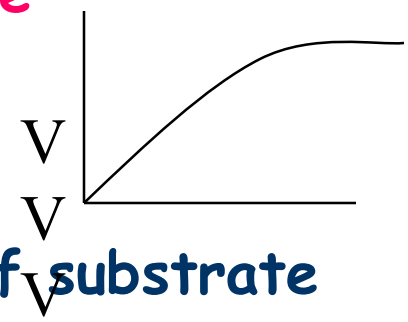
In the reaction:

S+E ⇒ES ⇒P+E

Enzyme+Substrate⇒Enzyme Substrate complex

⇒ NewProduct+Enzyme

Enzyme activity is affected by:



•Temperature

•pH, ionic strength

•Concentration of both enzyme and of substrate

•Presence of activators-inhibitors.

Therefore when working with enzymes, you have to make sure that you are at the optimum range of affecting parameters for the first-order reaction. Enzymes should be preserved at refrigeration temperatures not to loose their enzymatic activity.

- When working with samples containing other interfering substances, enzymes are more preferrable than classical chemical reagents because of their substrate specificity since they can interact only with their specific substrates (i.e. Lactase acts only on Lactose, amylases will break only only amylose chains). Enzymes are also being preferred as analytical aids because they make reactions possible at much milder conditions (Activation Energy Ea is much lower) therefore protecting more labile food components.

- Any physical or chemical property of the system that relates to substrate or product concentration (absorbance, fluorescsence, manometrics, titration, viscosity, isotope measurements) can be used for quantitative enzyme analyses.

I. Enzymes as Analytical Reagents:

I.1. SUBSTRATE Assays: For determination of substrates or end products of enzyme-catalyzed reactions

1.a. Determination of starch in foods: By using first amyloglucosidase enzyme that breaks down starch into glucose units,and then using glucosoxidase enzyme for oxidising glucose to gluconolacton and H_2O_2. Finally H_2O_2 +dianisidine(a dye)+peroxidase will yield a yellow-colored endproduct that can be quantified spectrophotometrically,because reaction is stoichiometric.

1.b. Glucose in blood: When blood is spotted on special filter paper strips soaked with glucose oxidase enzyme and [dianisidine-toluidine coloring reagent], a coupled reaction with blood glucose will produce colors correlated with concentration of glucose in blood. The reaction, however,has to go to completion so that the concentration of product will be related directly to substrate composition.

I.2. For determination of inhibitors or activators :

An inhibitor decreases (linearly with its increasing concntration) the rate of an enzyme reaction by reacting with either the enzyme or substrate.

Example:Even 10^{-9}g concentrations of Organophosphorous pesticides are inhibitors of the vital cholinesterase enzyme. A screening method was developed for detecting the presence or absence of pesticides,which also can yield semi-quantitative results in positive samples.

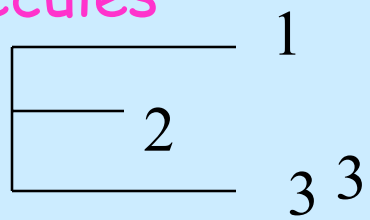
Principle of the method:

Acetylcholine is chosen as the "substrate" and DTNBA (Dithionitrobenzoic acid) is the color reagent with which acethylcholinesterase forms a yellow color(410nm) that turns pale if pesticides are present, because they inhibit the enzyme.

[Interpretation of Results:EA>%70 is (-);EA<50 is (+)].

I.3.For structural determination:configurational analysis of complex molecules

-Lipid Structure



Lipases from different sources differ in the site on the triglyceride molecule they attack. For example, pancreatic lipase (PL) shows preferential hydrolysis of fatty acids at (1) and (3) positions of triglycerides. Therefore, hydrolysis caused by PL will only break the fatty acids that are at triglyceride positions (1) and (3), leaving those at position (2).

Application:

The amount of total saturated (TSFA) fatty acids at β-position (2) is characteristic for natural virgin olive oils. By using pancreatic lipase, olive oil adulteration can be detected since the sum of TSFA (palmitic+stearic) at β-position will always be <1,5 % in virgin olive oils, <%2.2 in olive pomace cake oils, <%1.8 in refined olive oils and >%11 in esterified oils.

-Specific Protein Structural Studies:

a. Pepsin and tripsin are used for determining the amino acid sequence in peptides and proteins since they are quite selective in that they permit isolation of only uniform fragments characteristic for each enzyme.

b. Free or Bound Amino acids: Enzymes can be used for determining nutritional availability of essential amino acids (Ex: "bound" vs "free" lysine). Free lysine is determined calorimetrically by determining CO_2 formed during enzymatic decarboxylation of lysine by "lysine decarboxylase" enzyme, which can attack only free lysine specifically.

$$R-CH-COOH \xrightarrow{+DC} CO_2 + R-CH_2\_NH_2$$

-ENZYME IMMUNOASSAYS

Antibodies are able to make a significant contribution to characterization and quality assessment of agri-food materials. The unique properties of antibodies include a potential for extraordinary diversity of recognition, high affinity and specifity towards the target, and a stability and robustness well-suited to in vitro (and even 'in field') applications.

Immunoassays have been recognized for a number of properties:
-performance-specificity, sensitivity, precision, accuracy and reproducibility,
-high rates of sample through-put,
-dealing with analytes difficult to handle by alternative procedures,
-simplicity

"Enzyme immunoassays" employ "enzyme labels" (like Alkaline phosphatase, glucose oxidase) that catalyse reactions causing substrates to degrade and form coloured products which can be read spectrophotometrically or just observed by the eye. These are of two types:

a. Homogeneous EI- requiring no separation of unreacted reagents because immune reaction affects the enzyme activity

b. Heterogeneous EI-requiring washings between steps to remove unbound reagents(ELISA)

Modern, non-isotopic methods such as the enzyme-linked immunosorbent assay (ELISA) have basic, comparatively cheap equipment requirements.

Many of the applications of enzyme immunoassays in the agri-food area have been related to food safety:for rapid detection of trace food contaminants . Of the haptenic compouds, much interest has been devoted to the mycotoxins, the secondary metabolite mold products that can be found as contaminants in food and feed and which can have potent toxic bioactivities in animals and man. The group includes aflatoxins, the trichothecenes, and the fumosins. Many other applications exist for similar low molecular weight materials, including pesticides, phycotoxins and veterinary drugs. Also for specification of the origin of a sample of meat, immunoassays provides a rapid, simple and objective procedure that is difficult to reproduce with alternative methods of analysis.

II-Determination of Enzyme Activity

II.a. For assesment of food quality:

-Heated honeys have relatively lower diastase activity levels

-Lipase activity will increase in stored grains

-Phosphatase should have been inactivated in properly pasteurized milks.

-Efficiency of blanching operation done prior to freezing vegetables can be measured with their peroxidase activity, since the heat required for inactivating peroxidase in vegetables will also be sufficient to destroy other enzymes and microbes present.

-Bacteriologically contaminated milk will have very high catalase activity-

Example 1:

Phosphatase enzyme is always present in raw milk. The heat resistance of this enzyme is greater than the heat resistance of pathogenic microorganisms. It should not be present in duly pasteurised milks.

The principle is that the alkaline phosphatase enzyme if present in raw milk will liberate phenol from DSPP (disodium phenyl phosphate) substrate at certain T° and pH. The amount of phenol liberated will be proportional to the activity of the phosphatase enzyme.

Liberated phenol is quantified by measuring spectrophotometrically at 650 nm after its reaction with DCQC(2,6Dichloroquinonechloramide) to form the blue indophenol color.

Example 2:

Peroxidase activity of blanched vegetables is an indication of the adequacy of the blanching operation for freezing. Therefore it is also an indication of the future storage stability of frozen vegetables. Peroxidase activity can be determined by adding known amount of H_2O_2 to sample, which when reacted in presence of peroxidase enzyme will oxidize guaiacol or PPDA(ParaphenyleneDiamine) to a colored compound , the absorbance of which when measured at 450 or 490nm is correlated to activity of peroxidase enzyme in sample.

$$H_2O_2 \xrightarrow{peroxidase} orange-brown$$

Example 3:

Soybeans contain urease enzyme which is an anti-nutritional factor and which specifically will convert urea into ammonia.

Desolventizer-Toaster "DT" units at modern oil factories apply heat treatment for drying the oilseed cakes, this drying resulting in inactivating the urease enzyme.
Efficieny of the DT unit can be measured with amount of urease activity remaining in cake.

II b. For determining efficiency of enzyme preparation

-Enzyme Activity Assays:

Enzyme assays are analyses for determining the potency of enzyme preparations, and their results are expressed in enzyme units specific for the enzyme investigated. Enzyme concentrations on commercial enzyme products should be expressed in either EU/cm^3 or EU/gr.

General Definition of "Enzyme unit": The amount of enzyme required to modify (under standardized conditions of T°, pH, time) 1 m mole of substrate per minute.

Standardized methods for determining activities of specific enzymes are given in FCC (Food Chemicals Codex), which differ in the specific definitions of respective enzyme units.

Examples:

1.α-Amylase Activity:

Also called "diastatic power",and/or "dextrinizing activity", it is important for food technologists.When it acts on starch paste, it reduces its viscosity."Falling number" test for characterisng wheat flour is a measure of this acitivity level.

1) Fungal a-amylase activity is expressed in DU (dextrinizing units)

•1 DU: The quantity of α-amylase that will dextrinize soluble starch at a rate of 1 gram starch per hour at 30°C.

2) Bacterial a-amylase: is expressed in BAU (Bacterial a-amylase activity)

•1 BAU: The quantity of bacterial a-amylase enzyme that will dextrinize 1 mg of starch per minute under test conditions.

2) Cellulase Activity:

•1 EU: The quantity of enzyme required to reduce the viscosity of 200 gr of 5% solution of carboxymethyl cellulose(CMC) from 400 to 300 cps at 35°C, pH= 5 in one hour. The viscosity is expected to decrease as cellulase enzyme breaks down the cellulose chains,the decrease rate being correlated to enzyme activity level.

3) Catalase Activity : Arbitrarily selected unit is Baker unit

•1 Baker Unit: The amount of catalase that will decompose 266 mg of H_2O_2 under standardized conditions of assay.

4) Rennet activity

- Rennet, an extract of bovine stomach, is used as a coagulating agent in cheese production.
- Rennet activity can be determined by either of the following two methods:

  12%non-fat dry milk is dispersed in 10mM $CaCl_2$ solution and warmed to 35°C. An aliquot of rennet preparation is added and the time of milk clotting is observed visually and compared in relation to a rennet with standardized potency.

Or,

- A dye is covalently attached to casein(azocasein).The rennet preparation is incubated with 1% azocasein for a set period after which the enzymatic reaction is sytopped by adding trichloroacetic acid that will precipitate proteins not hydrolysed by the rennet. The small fragments of colored azocasein produced by rennet hydrolysis are left in solution and the absorbance reading at 345nm will indicate enzyme activity after comparison with a rennet of standardised potency.

•IMMOBILIZED ENZYMES and BIOSENSORS

Commercial enzyme preparations are now available and thus making enzymatic analyses much cheaper than before, due to the development of physical and chemical immobilization techniques based on following techniques:

a-adsorption

b-entrapment within a gel matrix

c-covalent cross-linking it to itself or to a second protein.

d-Covalent attachment to an insoluble carrier, like Sephadex or polyacrylamide.

Even though enzyme stability increases after immobilization, its optimal ranges may change. Therefore, determination of enzyme activity is always necessary before beginning an enzymatic analysis.

An immobilized enzyme, working in connection with a sensor is called a "BIOSENSOR": A device made up of a biological sensing element coupled to a suitable transducer(optical or electrochemical).

Enzyme Electrodes: These biosensors make use of electrochemical sensors.Here, the enzyme is located at the intermediate layer of a semipermeable membrane and the substrate diffuses through this membrane; or the immobilized enzyme is supported on an inorganic matrix (Glass electrode) where the enzyme is stored in dry , immobilized state. These electrodes may be used for measuring the concentration of specific substrates or end products (glucose ,CO_2, PO_4).

Example: "Glucose electrode" contains glucosoxidase enzyme combined with an oxygen electrode and is used to determine glucose concentration.