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COMPARATIVE STUDY OF FREE ENZYME & IMMOBILIZED ENZYME ON BSA DIGESTION

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ABSTRACT

Enzyme engineering is a fast-growing application in the pharmaceutical market. Enzymes are key to new processes because they are environmentally friendly and reduce hazardous waste. The uses of immobilized enzyme eliminate the enzyme separation step from the main process thus simplifying and increasing the overall process yield. The use of immobilized enzyme has several advantages as compared with an application of free enzymes. Immobilized enzymes can be recovered from reaction mixture and can be made available for reuse again. This work was undertaken to study the rate and duration of digestion of Bovine Serum Albumin by pepsin in free enzyme and immobilized states. Immobilization of pepsin was done by entrapment in calcium chloride. After studying the observations, it is found that in the reaction catalyzed by free pepsin, after a certain period of time; there was no more increase in optical density even if the enzyme concentration was increased. This indicates that the total liberation of amino acids related with increased absorbance gets in limiting stage due to exhaustion of substrate. While in the case of immobilized pepsin, the activity was increasing depending upon enzyme concentration after 4 hour of incubation. This clearly indicates the retention of enzyme activity for longer periods so; they are preferred to free enzymes. At last, it can be concluded that enzyme immobilization is the best method for retention of enzymes activity for longer periods.

INTRODUCTION:

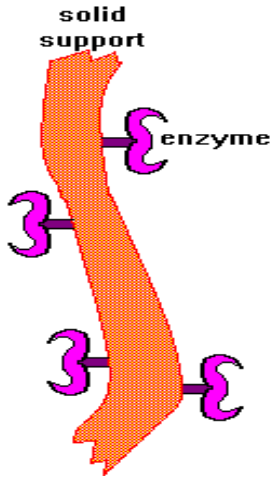
1. Enzymes are protein catalysts.
2. After synthesis within a cell, enzymes can function independently of the cell provided certain conditions are maintained.
3. Enzyme technology involves the production, isolation and purification of enzymes.
4. Commercial enzymes are obtained generally from;
    a. Plants
    b. Animals
    c. Microbes

Properties of enzyme:

1. The action of enzyme is specific, for example that the Enzyme that acts in protein will not act on starch. This Specific nature of enzyme is known to be generally as lock and key.
2. The enzyme acts like a catalytic agent i.e. it promotes the chemical reaction in the substances without changing itself.
3. Only a small amount of enzyme is sufficient for a Particular reaction.

Methods of immobilization:

1. **Carrier-Binding**: The binding of enzymes to water-insoluble carriers. The carrier-binding method is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. The following picture shows how the enzyme is bound to the carrier:



The selection of the carrier depends on the nature of the enzyme itself, as well as the: Particle size, Surface area, Molar ratio of hydrophilic to hydrophobic groups, Chemical composition In general, an increase in the ratio of hydrophilic groups and in the concentration of bound enzymes, results in a higher activity of the immobilized enzymes. Some of the most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel.

According to the binding mode of the enzyme, the carrier-binding method can be further sub-classified into:

A. **Physical Adsorption Mode:** This method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme or destruction of its active center. If a suitable carrier is found, this method can be both simple and cheap. However, it has the disadvantage that the adsorbed enzyme may leak from the carrier during use due to a weak binding force between the enzyme and the carrier. The earliest example of enzyme immobilization using this method is the adsorption of beta-D-fructo-furanosidase onto aluminum hydroxide. The processes available for physical adsorption of enzymes are:

   - **Static Procedure**
   - **Electro-deposition**
   - **Reactor Loading Process**
   - **Mixing or Shaking Bath Loading**

Of the four techniques, the most frequently used in the lab is Mixing-Bath Loading. For commercial purposes the preferred method is *Reactor* Loading. A major advantage of adsorption as a general method of immobilizing enzymes is that usually no reagents and only a minimum of activation steps are required. Adsorption tends to be less disruptive to the enzymatic protein than chemical means of attachment because the binding is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. In this respect, the method bears the greatest similarity to the situation found in natural biological membranes and has been used to model such systems. Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed.

Another disadvantage is non-specific, further adsorption of other proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate. Adsorption of the enzyme may be necessary to facilitate the covalent reactions described later in this presentation. Stabilization of enzymes temporarily adsorbed onto a matrix has been achieved by cross-linking the protein in a chemical reaction subsequent to its physical adsorption.

B. **Ionic Binding Mode:** The ionic binding method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues. Polysaccharides and synthetic

polymers having ion-exchange centers are usually used as carriers. The binding of an enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme. Therefore, this method yields immobilized enzymes with high activity in most cases. Leakage of enzymes from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH. This is because the binding forces between enzyme proteins and carriers are weaker than those in covalent binding. The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages is much stronger for ionic binding although weaker than in covalent binding.

C. Covalent Binding Mode: The most intensely studied of the immobilization techniques is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics:

(1) The binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and;

(2) The active site of the enzyme must be unaffected by the reagents used. The covalent binding method is based on the binding of enzymes and water-insoluble carriers by covalent bonds.

The functional groups that may take part in this binding are listed below:

- Amino group
- Carboxyl group
- Sulfhydryl group,
- Hydroxyl group
- Imidazole group
- Phenolic group
- Thiol group
- Threonine group
- Indole group

This method can be further classified into diazo, peptide and alkylation methods according to the mode of linkage. The conditions for immobilization by covalent binding are much more complicated and less mild than in the cases of physical adsorption and ionic binding. Therefore, covalent binding may alter the conformational structure and active center of the enzyme, resulting in major loss of activity and/or changes of the substrate.

However, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength. Covalent attachment to a support matrix must involve only functional groups of the enzyme that are not essential for catalytic action. Higher activities result from prevention of inactivation reactions with amino acid residues of the active sites. A number of protective methods have been devised:

1. Covalent attachment of the enzyme in the presence of a competitive inhibitor or substrate.
2. A reversible, covalently linked enzyme-inhibitor complex.
3. A chemically modified soluble enzyme whose covalent linkage to the matrix is achieved by newly incorporated residues.
4. A zymogen precursor. Hence, covalent binding can be brought about by the following:

- Diazotization: SUPPORT--N=N--ENZYME.
- Amide bond formation: SUPPORT--CO-NH--ENZYME
- Alkylation and Arylation: SUPPORT--CH_2-NH-ENZYME SUPPORT--CH_2-S--ENZYME
- Schiff's base formation: SUPPORT--CH=N--ENZYME
- Amidation reaction: SUPPORT--CNH-NH--ENZYME
- Thiol-Disulfide interchange: SUPPORT--S-S--ENZYME
- UGI reaction

- Mercury-Enzyme interchange
- Gamma-Irradiation induced coupling
- Carrier binding with bifunctional reagents: SUPPORT-O $(CH_2)_2$ N=CH $(CH_2)_3$ CH=N-ENZYME

The active site of the enzyme must not be hindered. There must be ample space between the enzyme and the backbone.

It is possible in some cases to increase the number of reactive residues of an enzyme in order to increase the yield of the immobilized enzyme. This provides alternative reaction sites to those essential for enzymatic activity. As with cross-linking, covalent bonding should provide stable, immobilized enzyme derivatives that do not leach enzyme into the surrounding solution. The wide variety of binding reactions and insoluble carriers (with functional groups capable of covalent coupling or being activated to give such groups) makes this a generally applicable method of immobilization. This is true even if very little is known about the protein structure or active site of the enzyme to be coupled.

2. **Cross-linkage:** intermolecular cross-linking of enzymes by bi-functional or multi- functional reagents. The enzyme is covalently bonded to a matrix through a chemical reaction. This method is by far the most effective method among those listed here. As the chemical reaction ensures that the binding site does not cover the enzyme's active site, the activity of the enzyme is only affected by immobility. However the inflexibility of the covalent bonds precludes the self-healing properties exhibited by chemo adsorbed self-assembled monolayer.

   Use of a spacer molecule like poly (ethylene glycol) helps reduce the stearic hindrance by the substrate in this case. Intermolecular cross linking of enzyme by bi-functional or multi-functional reagents. Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein either to other protein molecules or to functional groups on an insoluble support matrix. Cross-linking of an enzyme to itself is both expensive and insufficient.

   As some of the protein material will inevitably be acting mainly as a support. This will result in relatively low enzymatic activity. Generally, cross-linking is best used in conjugation with one of the other methods. It is used mostly as a means of stabilizing adsorbed enzymes and also for preventing leakage from polyacryamide gels. Since the enzyme is covalently linked to the support matrix. Very little desorption is likely using this method.

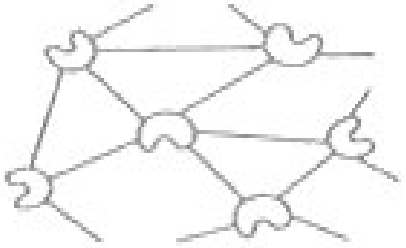


FIG: CROSS LINKAGE OF ENZYME

The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively sever conditions. These harsh conditions can change the conformation of active center of enzyme; and so may lead to significant loss of activity.

3. **Entrapping:** Enzyme Immobilization by Adsorption: : incorporating enzymes into the lattices of a semi-permeable gel or enclosing the enzymes in a semi-permeable polymer membrane. Immobilization of enzymes by adsorption is probably the mildest method available, being mediated by ionic, hydrophobic or hydrogen bonds. Adsorption of enzymes onto insoluble supports is a very simple method of wide applicability and capable of high enzyme loading (about one gram per gram of matrix).

   Simple mixing of the enzyme with a suitable adsorbent, under appropriated conditions of pH and ionic strength, followed after a sufficient incubation period, by washing off loosely bound and unbound enzyme will produce the immobilized enzyme in a directly usable form. The driving force causing this binding is usually due to the combination of hydrophobic effects and the formation of several salt-links per enzyme molecule.

The particular choice of adsorbent depends principally upon minimizing leakage of the enzyme during use. Although the physical links between the enzyme molecules and the support are often very strong, they may be reduced by many factors including the introduction of the substrate. Examples of suitable adsorbents are ion-exchange matrices, porous carbon, clay, hydrous metal oxides, glasses and polymeric aromatic resins. Manufacture of vinegar by naturally immobilized A. aceti cells on Birchwood twigs is an established method. Adsorption is also easy to perform simply by stirring the biocatalysts with an ion-exchange resin.

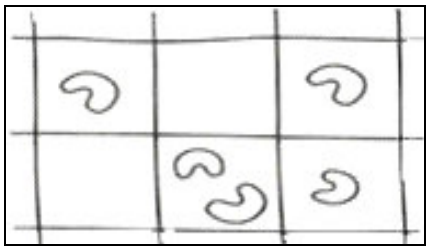


FIG: ENTRAPMENT OF ENZYME

In entrapment, the enzymes or cells are not directly attached to the support surface, but simply trapped inside the polymer matrix. Thus, loss of enzyme activity upon immobilization is minimized. Entrapment is carried out by mixing the biocatalyst into a monomer solution, followed by polymerization initiated by a change in temperature or by a chemical reaction. The polymer is formed either in particulate form, or as a block which can be disrupted to form discrete particles. The most common methods of entrapment use polyacrylamide, collagen, cellulose acetate, calcium alginate or carrageenan as the matrices.

MATERIAL AND METHODS:

Materials : Pepsin (Hi media), Bovine serum albumin (Hi media) 2%, Citrate phosphate buffer (0.1M), TCA solution (5%), Sodium alginate, Calcium chloride.

Reagent preparations:

1. **Preparation of pepsin solution:** 20 mg of pepsin was dissolved in 50 ml of acetate buffer.
2. **Preparation of 2% BSA solution:** 2 mg of BSA was dissolved in 100ml of distilled water to produce 2% BSA solution.
3. **Preparation of 0.2 M Acetate buffer (pH 4), 100 L:**
   a. 1.15 ml of acetic acid (CH_3COOH) was dissolved in 100 ml of distilled water
   b. 1.64 of sodium acetate ($C_2H_3O_2Na$) was dissolved in 100 ml of distilled water. Then, 41 ml of acetic acid was added to 9 ml sodium acetate and diluted to a total of 100 ml to make Acetate buffer of pH 4.0.
4. **Preparation of citrate- phosphate buffer, 100 ml:**
   a. 2.10 g of citric acid ($C_6H_8O_7$) was dissolved in 100 ml of distilled water.
   b. 3.5 g of dibasic sodium phosphate ($Na_2HPO_4.2H_2O$) and diluted to a total of 100 ml make citrate- phosphate buffer of pH 2.6.
5. **Preparation of Trichloroacetic acid solution (5%):** 5 gm of TCA was dissolved in 100 ml of distilled water to make 5% TCA solution.
6. **Preparation of 3% sodium alginate solution:** 3 gm of sodium alginate was dissolved in 100ml of Acetate buffer and heated to dissolve it in order to make the 3% solution. The solution was stored in 4^oC.
7. **Preparation of 0.2 M $CaCl_2$ solution:**

Molecular weight of $CaCl_2$ = 110.99g.

110.99 g dissolved in 1000 ml=1 ml

Therefore, 0.1 M solution = 110.99/10(in 1000ml) = 11.099 g (1000 ml).

Therefore, 0.2 M solution = (11.099)*2 = (22.1 g in 1000 ml)

Therefore, 2.21 g of $CaCl_2$ was dissolved in 100ml distilled water.

Methods:

1. **Free enzyme:** it involves the following steps:
   a. A set of test tubes was taken and in each tube: 0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml and 1.6 ml of enzyme solution was taken. Then, 2 ml of BSA solution followed by 1 ml of 0.1 M buffer was

added and incubated at 37°C for 15 min, 30 min, 1 hour, 2 hour and 4 hour respectively.

b. After desired period of incubation, 6 ml of 5% TCA solution was added to all the test tubes to stop the reaction.

c. The solution was filtered. Then the O.D of filtrate substrate was recorded at 280 nm in UV spectrophotometer.

2. **Immobilized enzyme:** Immobilization of pepsin was done by entrapment in calcium chloride.

Alginate Enzyme Mixture: About 2 ml of alginate solution was taken in each beaker and 0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml and 1.6 ml of enzyme solution were added to each tube mixed thoroughly.

Immobilization: The alginate enzyme mixture was transferred in drop wise manner from a approx. 20cm in stirred solution of 0.2 M calcium chloride to form beads. Pipette (1ml) was used for preparation of large beads and a syringe (2ml) with 24 no. needle was used for small bead preparation. Both beads were prepared separately. Beads were kept for 20 min for hardening in $CaCl_2$ solution. $CaCl_2$ was discarded thereafter and beads were washed with acetate buffer.

Test: Beads along with respective concentration of enzyme were put in beakers, and then 2 ml of 0.2 % BSA solution was added to each. Then 1ml of 0.1M buffer was added and kept in incubation for 15min, 30 min, 1 hour, 2 hour, 4 hour and 6 hour at 37°C. After desired period of incubation, 6 ml of 5% TCA solution was added to stop the reaction. Then solution was filtered and The O.D. of filtrate substrate was taken at 280nm in UV spectrophotometer.

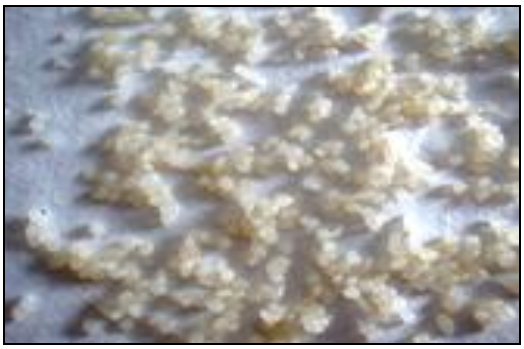


FIGURE 1: SMALL BEADS OF IMMOBILIZED

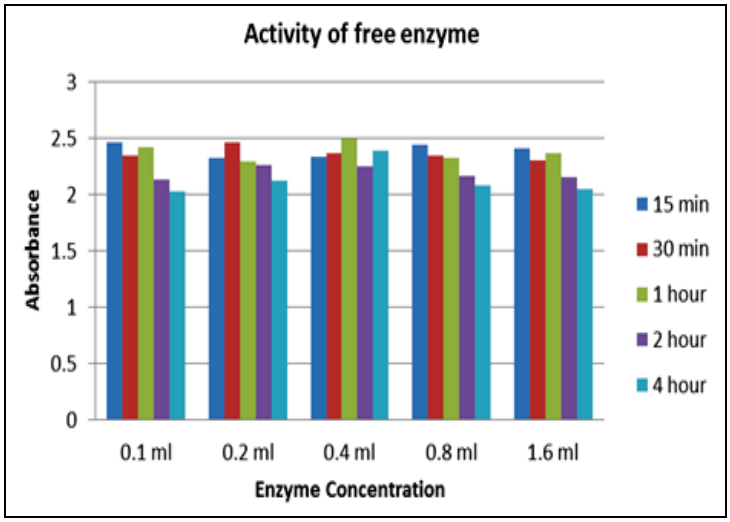
FIGURE 2: LARGE BEADS OF ENZYME IMMOBILIZED ENZYME

RESULT:

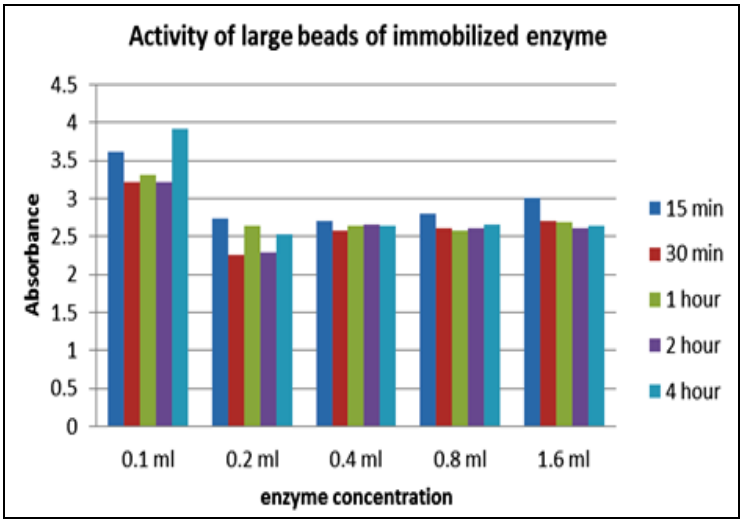
1. Absorbance of Free Enzyme:

TABLE 1: ABSORBANCE OF FREE ENZYME

Conc. of enzyme	Amt of Substrate	Digestion period	Absorbance
0.1 ml	2 ml	15 min	2.466
0.2 ml	2 ml	15 min	2.346
0.4 ml	2 ml	15 min	2.422
0.8 ml	2 ml	15 min	2.128
1.6 ml	2 ml	15 min	2.027
0.1 ml	2 ml	30 min	2.323
0.2 ml	2 ml	30 min	2.466
0.4 ml	2 ml	30 min	2.290
0.8 ml	2 ml	30 min	2.260
1.6 ml	2 ml	30 min	2.121
0.1 ml	2 ml	1 hour	2.334
0.2 ml	2 ml	1 hour	2.370
0.4 ml	2 ml	1 hour	2.498
0.8 ml	2 ml	1 hour	2.251
1.6 ml	2 ml	1 hour	2.382
0.1 ml	2 ml	2 hour	2.436
0.2 ml	2 ml	2 hour	2.346
0.4 ml	2 ml	2 hour	2.323
0.8 ml	2 ml	2 hour	2.165
1.6 ml	2 ml	2 hour	2.081
0.1 ml	2 ml	4 hour	2.408
0.2 ml	2 ml	4 hour	2.301
0.4 ml	2 ml	4 hour	2.370
0.8 ml	2 ml	4 hour	2.157
1.6 ml	2 ml	4 hour	2.045



GRAPH 1: ABSORBANCE OF FREE ENZYME V/S CONCENTRATION OF ENZYME



GRAPH 2: ABSORBANCE OF LARGE BEADS OF IMMOBILIZED ENZYME V/S CONCENTRATION OF ENZYME

2) Absorbance of large beads of Immobilized Enzyme:

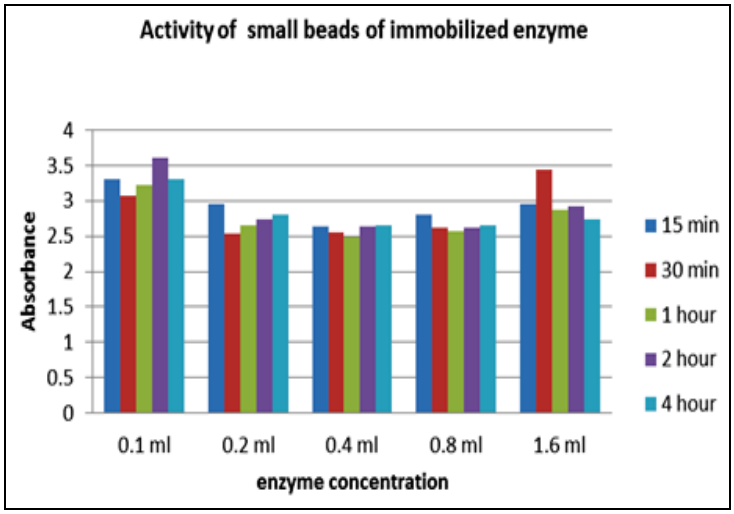
TABLE 2: ABSORBANCE OF LARGE BEADS OF IMMOBILIZED ENZYME

Conc. of enzyme	Amount of substrate	Digestion time	Absorbance
0.1 ml	2 ml	15 min	3.612
0.2 ml	2 ml	15 min	3.215
0.4 ml	2 ml	15 min	3.311
0.8 ml	2 ml	15 min	3.215
1.6 ml	2 ml	15 min	3.913
0.1 ml	2 ml	30 min	2.737
0.2 ml	2 ml	30 min	2.251
0.4 ml	2 ml	30 min	2.635
0.8 ml	2 ml	30 min	2.290
1.6 ml	2 ml	30 min	2.533
0.1 ml	2 ml	1 hour	2.709
0.2 ml	2 ml	1 hour	2.571
0.4 ml	2 ml	1 hour	2.635
0.8 ml	2 ml	1 hour	2.659
1.6 ml	2 ml	1 hour	2.635
0.1 ml	2 ml	2 hour	2.799
0.2 ml	2 ml	2 hour	2.613
0.4 ml	2 ml	2 hour	2.571
0.8 ml	2 ml	2 hour	2.613
1.6 ml	2 ml	2 hour	2.659
0.1 ml	2 ml	4 hour	3.010
0.2 ml	2 ml	4 hour	2.709
0.4 ml	2 ml	4 hour	2.683
0.8 ml	2 ml	4 hour	2.613
1.6 ml	2 ml	4 hour	2.635

3) Absorbance of small beads of Immobilized Enzyme:

TABLE 3: ABSORBANCE OF SMALL BEADS OF IMMOBILIZED ENZYME

Conc. of enzyme	Amount of substrate	Digestion time	absorbance
0.1 ml	2 ml	15 min	3.135
0.2 ml	2 ml	15 min	3.068
0.4 ml	2 ml	15 min	3.215
0.8 ml	2 ml	15 min	3.612
1.6 ml	2 ml	15 min	3.311
0.1 ml	2 ml	30 min	2.960
0.2 ml	2 ml	30 min	2.533
0.4 ml	2 ml	30 min	2.659
0.8 ml	2 ml	30 min	2.737
1.6 ml	2 ml	30 min	2.799
0.1 ml	2 ml	1 hour	2.635
0.2 ml	2 ml	1 hour	2.552
0.4 ml	2 ml	1 hour	2.482
0.8 ml	2 ml	1 hour	2.635
1.6 ml	2 ml	1 hour	2.659
0.1 ml	2 ml	2 hour	2.683
0.2 ml	2 ml	2 hour	2.613
0.4 ml	2 ml	2 hour	2.571
0.8 ml	2 ml	2 hour	2.613
1.6 ml	2 ml	2 hour	2.659
0.1 ml	2 ml	4 hour	2.960
0.2 ml	2 ml	4 hour	3.436
0.4 ml	2 ml	4 hour	2.872
0.8 ml	2 ml	4 hour	2.914
1.6 ml	2 ml	4 hour	2.737



GRAPH 3: ABSORBANCE OF SMALL BEADS OF IMMOBILIZED ENZYME V/S CONCENTRATION OF ENZYME

DISCUSSION: The project work was undertaken to study the rate and duration of digestion of Bovine Serum Albumin by pepsin in free enzyme and immobilized states. The results are summarized in **tables 1, 2, and 3 and figure A, B, and C** which indicates that the amount of BSA increases both in increasing enzyme concentration and digestion period.

It has been observed that. Increase of free enzyme (table 1) the activity of enzyme was increased as the concentration of the enzyme was increased along with the incubation period. But after four hours of incubation no raise of enzyme activity could be detected even when the concentration of enzyme was increased which indicates the total liberation of amino acids related with increased absorbance get in almost fixed maximum level due to exhaustion of substrate and so no more amino acid could be liberated from the given substrate (BSA) concentration.

In table 2 and 3, the concentrations indicate that the immobilized enzyme (both large and small beads) showed its increasing activity as the enzyme concentration and incubation periods were increased simultaneously. The immobilized enzyme showed to retain its activity even after 4 hours of digestion period and optical density was in enzyme concentration dependant increasing order. The differences in enzyme activity between large and small beads were marginal. There was no difference in enzymes activity for all the concentration in 15 min digestion but in all other duration of digestion, there was marginal activity of large beads in all the concentration level.

Therefore, studies on immobilized enzyme (larger and smaller beads) revealed that the immobilized enzymes had retained its activity for longer periods than the free enzyme because after 4 hours of incubation period, amino acids were liberated which showed increase absorbance thus revealing the retention of activity of immobilized enzymes.

CONCLUSION: After studying the observations, it is found that in the reaction catalyzed by free pepsin, after a certain period of time; these was no more increase in O.D. even if even if the enzyme concentration was increased. This indicates that the total liberation of amino acids related with increased absorbance gets in limiting stage due to exhaustion of substrate. While in the case of immobilized pepsin, the activity was increasing depending upon enzyme concentration after 4 hour of incubation. This clearly indicates the retention of enzyme activity for longer periods.

Since immobilized enzymes can be recovered from reaction mixture and can be made available for reuse again, so, they are preferred to free enzymes. At last, it can be concluded that enzyme immobilization is the best method for retention of enzymes activity for longer periods.

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