1. INTRODUCTION
Enzymes are the functional units of cell metabolism. They are proteins specialized to catalyze biological reactions. They have extraordinary specificity and catalytic power, which are far greater than that of synthetic catalysts. The unique catalytic properties of enzymes have led to effective exploitations in the food industry (Djordjevic et al., 1984; Reed, 1975) analytical chemistry (Guilbault, 1970), preparative organic chemistry (Jones et al., 1976) and medicine (Ohnishi, 1984; Ohnishi et al., 1976). Despite their great potential, there are not many enzymes that are being used in industry and medicine.

There are a number of factors responsible for the limited use of enzymes in industries. Cost of the enzymes, their short half-lives and their inability to act under conditions other than the physiological ones are some of the limitations. To overcome these limitations, biotechnologists employ different techniques to immobilize enzymes. There are three major reasons for immobilizing enzymes. Firstly, immobilized enzymes offer a considerable operational advantage over freely mobile enzymes. Secondly, they may exhibit selectively altered chemical or physical properties. Thirdly, immobilized enzymes may serve as model systems for natural in vivo membrane-bound enzymes. General operational advan-
tages of immobilized enzymes are reusability, possibility of batch or continuous operational modes, rapid termination of reactions, controlled product formation, greater variety of engineering designs for continuous process and possible greater efficiency in consecutive multistep reactions.

Immobilized enzymes are now being successfully used in diverse areas including chemical and food industries (Chibata and Tosa, 1976; Dogu et al. 1985; Nagashima et al., 1984), chemical analysis (Amarant and Bohak, 1981), synthesis of pharmaceuticals (Abbott, 1976), medicine (Duarte and Lilly, 1984; Mochida Pharmaceutical Co., 1984), preparative biochemistry (Schepet et al., 1984; Jakoby and Wilchek, 1974) and for commercial purposes (Iqbal and Saleemuddin, 1985). Nucleic acid modifying enzymes have also been immobilized with considerable success (Nilsson and Mosbach, 1981; Bulow and Mosbach, 1982).

The choice of method of immobilization and that of carrier is largely responsible for successful immobilization of enzymes. The procedures used for immobilizing different enzymes for various purposes are discussed in the following pages. A schematic representation of the many available methods of immobilization is shown in Figure 1.
1.1 Covalent Attachment

The covalent attachment of water soluble enzyme molecules through non-essential amino acid residues to water insoluble functionalized supports is a prevalent method for immobilizing enzymes. It can involve the activation of support material for reaction with a protein group or the activation of protein group for binding to support. In order to overcome diffusional restrictions and steric hindrance, a spacer arm together with a coupling reagent may also be used for attaching the protein with the support.

The water insoluble supports can be either organic or inorganic. A number of supports like agarose, dextran, cellulose, chitin, glass, etc, have been used. Fibrous supports are also used for immobilization (Ichijo et al., 1982). Some new polymers like crosslinked acrylic acid-styrene copolymers and poly(vinyl) alcohol activated with maleimide groups (Manecke and Vogt, 1980) and cellulose-O-ethylamine and cellulose dialdehyde (Kurtossy, 1982) are also used as supports for the purpose of enzyme immobilization. Some of the common coupling agents used are cyanogen bromide, glutaraldehyde, isocyanates, carboxyethyl cellulose azide, thiophosgene and carbodiimide (Zaborsky, 1973). The reactions involved in covalent attachment of enzymes to an insoluble carrier are shown in Figure 2.
Figure 2. **Schematic representation of covalent attachment of enzymes to water insoluble carriers.**

(a) Preparation of cyanogen bromide activated derivative and its attachment to an enzyme.

(b) Preparation of carboxymethyl cellulose and attachment of enzyme to active derivative.

(c) Preparation of diazonium chloride and attachment of enzyme through azolinkage.

(d) Preparation of isocyanate and isothiocyanate and their attachment to an enzyme.

(f) Preparation of an active pseudourea from a carboxyl and carbodiimide and reaction with an enzyme forming amide-linkage.
1) \(\text{CH-OH} + \text{CNBr} \rightarrow \text{CH-O-C=NH}\)
   
iminocarbamic acid

2) \(\text{CH-O-C=NH} \cdot \text{NH} \cdot \text{NH}_2 - \text{Enzyme}\)

1) \(\text{OCH}_2\text{COOH} \xrightarrow{\text{CH}_3\text{OH}} \text{OCH}_2\text{COOCH}_3\)
   \(\xrightarrow{\text{HCl}} \text{OCH}_2\text{CONH}_2\)
   
   CM-cellulose

2) \(\text{OCH}_2\text{CONH}_2 \xrightarrow{\text{NaNO}_2} \text{OCH}_2\text{CON}_3\)

3) \(\text{OCH}_2\text{CON}_3 + \text{NH}_2 - \text{Enzyme} \rightarrow \text{OCH}_2\text{COONH} - \text{Enzyme}\)
   
   CMC-Protein conjugate

1) \(\text{R} - \text{NH} \xrightarrow{\text{NaNO}_2} \text{R} - \text{N}_2\text{Cl}^-\)

2) \(\text{R} - \text{N}_2\text{Cl}^- + \text{HO} - \text{Enzyme} \rightarrow \text{R} - \text{N} = \text{N} \cdot \text{Enzyme}\)

   O

\(\text{C} = \text{C} - \text{NCO}\)
   
isocyanate

2) \(\text{R} - \text{NCO} + \text{NH}_2 - \text{Enzyme} \rightarrow \text{R} - \text{N} = \text{NH} - \text{Enzyme}\)

3) \(\text{R} - \text{NCS} + \text{NH}_2 - \text{Enzyme} \rightarrow \text{R} - \text{N} = \text{NH} - \text{Enzyme}\)

1) \(\text{COOH} + \text{CN} - \text{N} + \text{H}^+ \rightarrow \text{COO} - \text{NH}^+\)
   
   Carbodiimide

2) \(\text{COO} - \text{NH}^+ - \text{Enzyme} \rightarrow \text{CONH} - \text{Protein} + \text{O=C} + \text{H}\)

   NHR'
A few recent examples of enzymes coupled covalently to solid supports are pepsinogen on Sepharose (Shamsuzzaman and Haard, 1984), epoxide hydrolase to dextran activated by imidazole carbonate (Ibrahim et al., 1985), urease on a polyacrylamide type support having carboxylic functional groups (Dala and Szajani, 1984), pepsin to gel-forming albumin in the presence of glutaraldehyde (Buerger and Hartmeier, 1983), cellobiase on a dialdehyde dextran (Lenders et al., 1985), carboxypeptidase on an acrylic polymer (Boross et al., 1985), α-amylase on partially imido-esterized granular polyacrylonitrile by an amidation reaction (Handa et al., 1984), glucose oxidase on Con A cellulose and Con A Sepharose (Iqbal and Saleemuddin, 1983a, 1983b) and invertase to silica gel and porous glass via glutaraldehyde (Mansfeld and Schellenberger, 1986).

The enzymes that are immobilized to solid support by covalent coupling are usually attached very strongly. However, chemical inactivation of the enzymes occurs often and thus the active center residues should be carefully avoided during immobilization.

1.2 Adsorption

This method of preparing water-insoluble enzyme conjugates is one of the simplest. It consists of contacting
an aqueous solution of an enzyme with a surface-active adsorbent and washing the resulting conjugates to remove any non-adsorbed enzymes. The idea of adsorption originates from the fact that these enzymes in immobilized forms will behave in vitro as models for in vivo membrane bound enzymes such as those participating in active transport, respiration, protein biosynthesis and photosynthesis (Melrose, 1971).

The adsorption of an enzyme onto a water-insoluble material is dependent on variables as pH, nature of the solvent, ionic strength, concentration of protein and adsorbent, and temperature. A good review of these variables has been given by McLaren and Packer (1970). The adsorbents are either organic (Kuraray, 1984) or inorganic (Samoshina et al., 1984) in nature, and often require special pretreatments in order to ensure good adsorption.

Adsorption of enzymes to support can be attributed to ion-exchange mechanism, to simple physical adsorption or to physicochemical bonds created by hydrophobic interactions, vander Waals attractive forces, etc. (Messing, 1970). Some recent examples of adsorption are β-galactosidase on silochrome modified with silylundecanoic acid (Samoshina et al., 1984), alkaline phosphatase and trypsin on activated alumina particles (Koga et al., 1984a; 1984b), invertase on polyacrylamide gel and on cheese cloth (Dogu et al., 1985;
Yamazaki et al., 1984), glutamate dehydrogenase and pepsin on Triton X-100-substituted Sepharose 4B (Nemat-Gorgani and Karimian, 1984), lysozyme, glutamate dehydrogenase and β-galactosidase on palmityl-substituted Sepharose 4B and on dimethylaminated poly (vinyl) alcohol (Nemat-Gorgani and Karimian, 1982; Ichijo et al., 1986) and cellulase on CM cellulose (Kumakura, 1986).

Although this method has a number of advantages like operational simplicity, mildness causing little or no enzymic inactivation, and reversibility permitting the reuse of both the support and the enzyme for other purposes, it is not without its disadvantages. The optimal conditions for immobilization are achieved by trial and error. Leakage of the enzymes also occurs when operating at high substrate concentrations.

1.3 Chemical Aggregation

The preparation of water-insoluble enzyme derivatives using low molecular weight bi- or multi-functional reagents is an easy method of enzyme immobilization. It involves the covalent bond formation between molecules of the enzymes and the reagent to give intermolecularly cross-linked species. The commonly used bi-functional reagents include dimethyl adipimate, 2,4-diisocyanotoluene, diazobenzedene, 1,5-difluoro-
2,4-dinitrobenzene and glutaraldehyde. Excellent reviews on the utility and scope of the bi- and multifunctional reagents are available (Fasold et al., 1971; Wold, 1972; Zaborsky, 1973; Stanley and Olsen, 1973; Peters and Richards, 1977). Among the cross-linking agents studied, glutaraldehyde is considered the best, with the added advantage of being highly soluble in aqueous media. It readily reacts and binds covalently with the ε-amino groups of lysine residues (Habeeb and Hiramoto, 1968). This procedure has been proved as one of the good coupling methods (Figure 3).

Glutaraldehyde was first applied for cross-linking of ribonuclease crystals (Quicho and Richards, 1966). Later, Habeeb (1967) and Habeeb and Hiramoto (1968) immobilized some other proteins and enzymes using glutaraldehyde. A chemically aggregated preparation of trypsin was made by Bano et al. (1980). Some water insoluble derivatives were also prepared by adsorption of enzymes on surface active support followed by crosslinking (Brown et al., 1970; Yamazaki et al., 1984). Recently, a major glucoamylase from Rhizopus species was polymerized by crosslinking with glutaraldehyde (Takahashi et al. 1986).

Advantages of this procedure of immobilization of enzymes include no increase in the mass of the preparation due
Figure 3. Schematic representation of the preparation of an active aldehyde derivative and its attachment to an enzyme.
Figure 23. **Immunodiffusion of rabbit antisera raised against commercial F(ab')₂.**

The antiserum was taken in the central well. The commercial and experimental F(ab')₂ as antigen were taken in the peripheral wells.

- C = commercial F(ab')₂
- E = experimental F(ab')₂
to absence of carrier and very high stabilization due to inter- as well as intramolecular crosslinking. However, chemical aggregation of enzymes has also its disadvantages. There may be unavoidable inactivation of the enzyme caused by chemical modification, and the gelatinous nature of these enzyme derivatives makes it difficult to use them in some column operations.

1.4 **Entrapment within Crosslinked Polymers**

In order to entrap an enzyme, either a crosslinked polymeric network is formed around the enzyme molecule or the enzyme is mixed with the polymeric material and the polymeric chains are then crosslinked. Enzyme molecules are physically entrapped within the polymer lattice and cannot permeate out of the gel matrix, but appropriately sized substrate and product molecules can transfer across this network to ensure a continuous transformation. Polyacrylamide gel is the most commonly used polymeric system, though starch, silica gel and silicon rubber have also been used (Kennedy et al., 1985; Sharma et al., 1982). Recently, alginic acid, fibrin network and carrageenan have also been used for the entrapment of enzymes and living cells (Bucke and Wiseman, 1981; Inada et al., 1980; Matsushita Electric Works Ltd., 1984; De Taxis du Poet et al., 1984).
A great number of enzymes and living cells have been entrapped and brought in different uses. These include papain, trypsin and \( \alpha \)-chymotrypsin in polyacrylamide gel (Kawabe and Kawabe, 1981; Martinek et al., 1982), glucoamylase, invertase and cellobiase in crosslinked gelatin beads (Kennedy et al., 1984), tyrosinase in Enzacryl AA (Vilanova et al., 1984), glucoamylase in starch (Kennedy et al., 1985), \( \alpha \)-amylase in dried spheres made from an alginate solution containing magnetic particles (Burns et al., 1985), E. coli B218 in carrageenan beads (De Taxis du Poet, 1984), Concanavalin A-glucose oxidase and yeast cells in calcium alginate beads (Hussain et al., 1985; Nagashima et al., 1984) and cellulase and cellobiase in hydrophilic glass forming monomers with application of radiation technology (Higa et al., 1986). The stability of most of the entrapped enzymes was greater than that of the soluble enzymes and the immobilized living cells could be stored for much longer periods. The production of ethanol and cholesterol degradation by entrapped cells has also been studied (Fang et al., 1984; Duarte and Lilly, 1984).

The lattice entrapment method for immobilizing enzymes has certain advantages, like experimental simplicity, the need for only small amounts of enzyme and no chemical modifications in the enzyme. Leakage of the enzyme from the lattice
network and the limitation to only small sized substrates are the disadvantages of this procedure.

1.5 Microencapsulation

Enzymes can be immobilized within microcapsules that have either a permanent or non-permanent semipermeable membrane. Microcapsules can be formed either by coacervation (a physical phenomenon) or by interfacial polymerization (a chemical process). Non permanent or "liquid-surfactant" membranes are formed by the combination of appropriate surfactants, "additives" and hydrocarbons (Zaborsky, 1973). Like in entrapment of enzymes, this method also applies to substrate and products of low molecular weight.

Permanent microcapsules used for encapsulation of enzymes are made from different materials like polystyrene (Chang, 1964) cellulose nitrite collodion (Chang, 1964; Chang and Mac Intosh, 1964; Chang et al., 1971) and ethyl cellulose (Gardner et al., 1971). Membrane formation has also been achieved with silicone type polymers (sesquiphenyldimethylsiloxane and silastic) through a secondary emulsion technique (Kitajima and Kondo, 1971; Chang, 1966). In this procedure, an initially produced water-oil emulsion is added to another aqueous phase in order to form permanent microcapsules.
Immobilization of enzymes by entrapping the molecules within permanent semipermeable microcapsules was first reported in the mid 60's by Chang (1964, 1965). Since then, various enzymes have been immobilized in microcapsules of different chemical composition, like catalase in collodion microcapsules (Chang, 1971), L-asparaginase and urease in nylon microcapsules (Chang, 1969; Sparks et al., 1969) urease in microcapsules formed by a complex membrane from cellulose sulfate and polydimethyl diallyl ammonium chloride (Ristan et al., 1985) and invertase in polyamide microcapsules for possible hydrolysis of the accumulated sucrose in the intestinal lumen (Rambourge et al., 1982).

The immobilization of an enzyme in non-permanent microcapsules involves encapsulating the protein solution within a semi-permeable liquid surfactant membrane (May and Li, 1972). Using this technique, urease and phenolase have been immobilized (May and Li, 1972) and they showed substantial catalytic activity and presented no leakage difficulties. An excellent review has recently been given by Kotenko and Trimus (1985) upon the microencapsulation of physiologically active substances for sustained release of drugs.

Immobilization of enzymes by microencapsulation results in no change in the inherent properties of the enzymes. The enzymes remain chemically unmodified and free in solution.
However, apparent changes in activity, Michaelis constant, pH-activity profile etc., could be observed due to diffusional effects of the membrane.

1.6 Immobilized Proteolytic Enzymes

Water-insoluble derivatives of proteolytic enzymes are useful for controlling digestion of proteins and determination of protein sequence. They are used frequently in industry, and in medicine in digestive disorders. In addition, many immobilized proteases have been used as activators of their respective zymogens.

Numerous attempts have been made to immobilize different proteolytic enzymes. A number of methods have been employed to prepare water-insoluble trypsin. Covalent linking of trypsin to cross-linked dextran (Tanizawa and Kanaoka, 1984), immobilization of trypsin on activated alumina particles (Koga et al., 1984b), entrapment of the enzyme in polyacrylamide gels (Martinek et al., 1982), binding it to cellulose and to polysilicic acid (Nozawa et al., 1981; Yankevich et al., 1983) and aggregating it chemically (Bano et al., 1980) are some of the recent examples of immobilized trypsin. All these preparation have been found to be highly stable with considerable activity and are used for various purposes like in the treatment of inflammation and hemorrhage (Nozawa
et al., 1981), in the transesterification of L-lysine esters (Tanizawa and Kanoaka, 1984) and for hydrolysis of casein (Bano et al., 1980).

Chymotrypsin has also been immobilized by various procedures. It has been conjugated to cyanogen-bromide activated Sephadex and Sepharose (Axen and Ernback, 1971; Clark and Bailey, 1984). It has also been coupled to CM-cellulose, polyglutamic acid and polyacrylic acid (Patel et al., 1967), in polyacrylamide gel (Martinek et al., 1982) and to ethylene maleic anhydride (1:1) copolymer (Goldstein et al., 1970). Chymotrypsin entrapped in polyacrylamide gel showed high catalytic activity at elevated temperature (Martinek et al., 1982).

Papain is another proteolytic enzyme that has been immobilized by covalent linkage on Amberlite IRA 400 resin using glutaraldehyde as the coupling agent (Vaidya et al., 1979), by entrapment in crosslinked polyacrylamide gel (Kawabe and Kawabe, 1981) and by radiation polymerization of various monomers at low temperatures (Kumakura and Kaetsu, 1984).

The structure and amino acid composition of pepsin is shown in Figure 4 and Table I, respectively. It undergoes rapid inactivation above pH 6 and has been fixed to albumin in the presence of a crosslinking agent (Buerger and Hartmeier, 1983), to yeast cell surface (Hartmeier, 1981) on carboxyl
Structure of Pepsin
**TABLE - I**

**Amino Acid Composition of Pepsin** *

(Moles of amino acid per mole of protein)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>mol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>2</td>
</tr>
<tr>
<td>Cystine</td>
<td>6</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>40</td>
</tr>
<tr>
<td>Threonine</td>
<td>25</td>
</tr>
<tr>
<td>Serine</td>
<td>43</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>26</td>
</tr>
<tr>
<td>Proline</td>
<td>16</td>
</tr>
<tr>
<td>Glycine</td>
<td>34</td>
</tr>
<tr>
<td>Alanine</td>
<td>16</td>
</tr>
<tr>
<td>Valine</td>
<td>20</td>
</tr>
<tr>
<td>Methionine</td>
<td>4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>23</td>
</tr>
<tr>
<td>Leucine</td>
<td>28</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>16</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>44</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6</td>
</tr>
<tr>
<td>NH$_3$</td>
<td>27</td>
</tr>
</tbody>
</table>

containing PVC fibres (Gorakhova et al., 1982) and on porous glass (Lee et al., 1977). It has also been immobilized on amino hexyl Sepharose (Tomono et al., 1981), chitosan (Nithianandan et al., 1979) and on sand (Puvanakrishnan and Bose, 1984).

Immobilized pepsin preparations have been used in medicine for the removal of circulating immune complexes from blood (Mochida Pharmaceutical Co. Ltd., 1984), in industry for clearer wine and its faster fermentation (Hartmeier, 1981) and for estimation of amino acid residues in a protein (Mikuni-Takagaki and Hotta, 1977) and in the preparation of \( F(ab')_2 \) fragments by limited cleavage of IgG (Tomono et al., 1981). The cleavage of IgG by pepsin is shown in Figure 5.

1.7 Scope of The Thesis

The present studies describe the preparation of insoluble pepsin by crosslinking it with glutaraldehyde, by its covalent attachment to cyanogen bromide activated Sephadex G-200 and by its adsorption to polyethyleneimine coated cotton cloth, and their properties. The immobilized preparations show considerable enhancement in stability. They are quite stable under adverse conditions of temperature and pH, and chemical denaturants. They can be stored for considerable periods and also show good reusability.
Figure 5. Schematic representation of cleavage of immunoglobulin G by pepsin.
The diagram illustrates the process of cleavage of an antibody (Ag) by pepsin into fragments. Initially, the antibody is cleaved into two large fragments (F(ab')₂) by pepsin. These fragments are then treated with RSH (reducing agent) to form disulfide bonds, resulting in two smaller fragments (2 F(ab')₂) and the fragments of Fc.