IMMOBILIZATION OF ENZYMES ON NATURAL AND SYNTHETIC POLYMERIC MATERIALS

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Enzymes are the catalysts specially designed by nature to bring about chemical reactions essential for life. They are globular proteins with enormous flexibility that permits enzymes to be characterized by very high degree of catalytic efficiency and selectivity. Industrially useful catalysts should be stable, so that they can remain in use for an economically acceptable period, and solid so that they are not washed away with the chemical components of the reaction catalyzed. Enzymes as provided by nature are not usually either stable or solid, and a great deal of research has gone into finding ways of immobilizing them to make them more useful for technology. At its crudest, immobilization can simply means attachment of the enzyme to a solid supports such as natural or synthetic, either by physical or chemical means.

Immobilized enzymes exhibit better stability over broad pH range, temperature and organic solvents. Due to possibility of repeated use, they can be successfully used in the development of continuos processes.

In the present study covalent binding and entrapment of alcohol dehydrogenase from yeast, α-chymotrypsin from bovine pancreas and peroxidase from horse radish has been investigated. A copolymer of acrylamide and 2-hydroxyethyl methacrylate has been synthesized at low temperature and used for the entrapment of the enzymes, whereas natural polymer chitosan has been used for the covalent coupling of the enzymes.

Immobilization of these enzymes was carried out through amino group activation of chitosan by glutaraldehyde. Various coupling conditions such as concentration of cross-linking reagent, cross-linking time, pH of the coupling medium, enzyme/support ratio and coupling time were optimized for all the enzymes to get maximum loading and retention of enzyme activity. It was

observed that the coupling reaction is influenced by enzyme/support and activator/support ratios, pH and coupling time. Protein coupled was determined by Lowrys' method and the activity of the coupled protein was estimated by standard assay procedures using ethanol, casein (or L-BTEE) and pyrogallol substrates respectively for alcohol dehydrogenase, α-chymotrypsin and peroxidase.

Insitu entrapment of these enzymes was carried out by polymerizing acrylamide and 2-hydroxyethyl methacrylate using N-N' methylene bis-acrylamide as a cross-linking agent, ascorbic acid and hydrogen peroxide as initiator at 293 °K. Maximum entrapment was obtained by optimizing the enzyme addition time and concentration of cross-linking agent. Leakage of enzyme from the polymer network and its effect on entrapped enzyme activity due to swelling of polymer were also studied.

The activity of these immobilized enzymes was compared with the free enzymes at different pH and temperatures. The stability of free and immobilized enzyme was also compared at different temperatures, storage conditions and organic solvents. Kinetic behaviour of enzymes was studied through Linweaver-Burk plots. The selected immobilized systems were used in fixed bed reactor for the continuous process.

Optimized conditions for covalently immobilized enzymes onto porous chitosan beads are summarized in Table 1.

Table - 1

Optimized conditions for covalently immobilized enzymes

Conditions	Alcohol dehydrogenase	Chymotrypsin	Peroxidase
Substrate	Ethanol	L-BTEE / Casein	Pyrogallol
Conc. of cross-linking reagent (mg g^{-1})	20	16	30
Cross-linking time (minutes)	90	45	60
pH of coupling medium	7 0	8 0	6.0
Coupling time (h)	10	6	14
Enzyme loaded (U g^{-1})	9 9	59	45
Retention of activity (%)	24	60	32

Immobilization by entrapment resulted in 90% retention of activity at 5 minutes enzyme addition time with 2% crosslinking reagent concentration.

All immobilized enzymes were thoroughly characterized and their performance was compared with free enzymes. The comparative account is given in Table 2, 3 and 4.

Table - 2

Comparison of properties of free and immobilized alcohol dehydrogenase

Properties	Free enzyme	Covalent binding	*Insitu* entrapment
Optimum pH	9	8	8 - 9
Optimum temperature (°C)	30	30	30
Storage stability at 30 °C (days)	2	30	60
Durability (cycles)	-	11	6
Thermodeactivation constant (K_d) at 70 °C	0 077	0 037	0 045
Michaelis constant, K_m (mM)	8 33 x 10^{-2}	6.66 x 10^{-2}	1 57 x 10^{-2}
Maximum velocity, V_{max} (mM min^{-1})	9 09	5.26	3 58

Table - 3

Comparison of properties of free and immobilized α-chymotrypsin

Properties	Free enzyme	Covalent binding	*Insitu* entrapment
Optimum pH	8	7-8	8-9
Optimum temperature (°C)	35	35	40
Storage stability at 30 °C (days)	9	23	70
Durability (cycles)	-	9	9
Thermodeactivation constant (K_d) at 60 °C	0.056	0.035	0 041
Michaelis constant, K_m (mM)	2.71 x 10^{-3}	2.50 x 10^{-3}	4.23 x 10^{-3}
Maximum velocity, V_{max} (mM min^{-1})	5.95 x 10^{-2}	9.60 x 10^{-2}	4 84 x 10^{-2}
Stability in methanol (%)	58	88	95
Effect of Cu^{+2} inhibitor (%)	21	17	9

Table - 4

Comparison of properties of free and immobilized peroxidase

Properties	Free enzyme	Covalent binding	*Insitu* entrapment
Optimum pH	6 0	7.0	7 0
Optimum temperature (°C)	20	30	30
Storage stability at 30 °C (days)	2	11	29
Durability (cycles)	-	15	6
Thermodeactivation constant (K_d) at 60 °C	5×10^{-3}	2×10^{-3}	$0\ 92 \times 10^{-3}$
Michaelis constant, K_m (mM)	6 67	4 17	4 65
Maximum velocity, V_{max} (mM min^{-1})	1.11	0 8	1 10
Stability in methanol (%)	39	61	73

From the study the following observations are made :

- A mild and reproducible method has been developed for the entrapment of enzymes into cross-linked copolymer poly(AAm-co-HEMA) synthesized by free radical copolymerization at low temperature, using bis-AAm as a cross-linking agent. Natural polysaccharide chitosan in bead form was effective and useful supports for the immobilization of enzymes through covalent binding.

- Activity of the entrapped enzyme was observed to be critically dependent on the time of addition of enzyme during polymerization and percentage cross-linker used. While extent of covalent coupling of protein and retention of enzyme activity depend on the enzyme/support ratio,

concentration of cross-linking reagent, cross-linking time and pH of the coupling medium.

- Immobilized enzymes achieved using both the techniques showed improved storage, thermal and solvent stability than free enzymes. The immobilized enzymes also showed excellent durability for their repeated use. Kinetic study reveals that no conformational changes in the enzymes were observed upon immobilization. However, covalently coupled enzymes showed more stability than entrapped enzymes in major cases.

- Effect of metal ions on free and immobilized enzymes was also studied. It was observed that enzyme activity was enhanced by addition of metal ions such as calcium and magnesium while, heavy metal ions such as copper and nickel had less inhibitory effect on immobilized enzymes than free enzymes.

- Further, the immobilized enzymes were used in continuous packed bed reactor. It was observed that immobilized chymotrypsin was useful for the hydrolysis of casein, immobilized alcohol dehydrogenase for the oxidation of C_2 to C_5 primary alcohols while immobilized peroxidase was useful for the removal of phenol.