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Nature of Enzyme-Matrix Binding in Trypsin Immobilized on Molecular Sieve Type 4A

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Physicochemical studies on the nature of enzyme-matrix binding in trypsin immobilized on molecular sieve type 4A by physical adsorption method have been carried out. The effect of *p*H, temperature, ionic strength and presence of sorbitol and sucrose on the immobilization process indicates the formation of salt-like linkage, van der Waals interaction and hydrogen bonding between trypsin and the carrier. Experiment in the presence of *p*-chloromercuribenzoate suggests the complete absence of covalent binding between trypsin and the carrier. Studies on sorption isotherm indicate that a Langmuir type adsorption is involved, which is confirmed by X-ray diffraction data of enzyme-carrier conjugate and free carrier.

THE technique of immobilization of enzyme on a carrier by physical adsorption is simple and most economical. However, the nature of bonds between the enzyme and the carrier is not easily understood. Literature survey[1-3] shows that three types of bond formation have been reported so far during enzyme immobilization by adsorption method : (a) ionic, salt bridge, resulting from charge-charge interaction; (b) hydrogen bond; and (c) covalent bond. The enzyme matrix obtained by the adsorption procedure suffers from a serious drawback of leakage of enzymatic activity. Since stability of the conjugate increases with the strength of the bonds between the enzyme and matrix, it is, therefore, important to study the nature of enzyme-matrix binding and the contribution of the individual type of bond towards the whole process when the conjugate is prepared by the adsorption method.

In the present study, the nature of binding involved between the enzyme, trypsin, and the carrier, molecular sieve type 4A, has been investigated. Process engineering study of molecular sieve physically bound to trypsin has already been reported by the authors[4,5]. It is believed that the use of molecular sieve type 4A, a sodium aluminosilicate compound, as carrier would involve the formation of more than one of the different types of bonds mentioned above. From the results of various physicochemical studies a reasonably good idea may be obtained about the nature of matrix-enzyme binding.

Materials and Methods

The reagents and instruments used were: molecular sieve type 4A (Linde, USA); bovine trypsin (E. Merck); trishydroxymethylaminomethane (AR); disodium hydrogen phosphate (AR); potassium dihydrogen phosphate (AR); sodium chloride (AR); sorbitol (AR); sucrose (AR); *p*-chloromercuribenzoate (PCMB) (Sigma Chemicals, USA); and Debye-Scherrer camera of 114.6 cm diameter for X-ray studies.

Trypsin was immobilized on molecular sieve type 4A by the physical adsorption method. The detailed process has been described earlier[4,5].

The amount of bound trypsin was estimated spectrophotometrically at 280 nm[6] as well as by Kjeldahl's method. The average of two values obtained by each of the two methods (standard deviation $\pm$ 0.05) was taken.

Results and Discussion

Effect of pH on immobilization — The experimental results showing the relationship between amount of trypsin bound to that of *p*H of the immobilization reaction medium are plotted in Fig. 1. Experiments below *p*H 6.0 could not be carried out as the carrier was not stable at lower *p*H. It is evident from Fig. 1 that the amount of bound enzyme progressively increases with increase in *p*H from 6.5 to 7.5. With further increase in *p*H the amount of bound enzyme slowly falls off.

The change in the amount of bound enzyme with increasing *p*H indicates some sort of ion-ion interaction between the enzyme and the matrix surface, which is known to be charged. The contribution of this ion-ion interaction to enzyme-matrix bonding diminishes as the *p*H of the immobilization bath approaches the isoionic point of the enzyme (10.8) and the amount of bound trypsin decreases.

Effect of ionic strength on immobilization — The effect of ionic strength on enzyme loading was followed by adding requisite amounts of sodium chloride solution to the immobilisation bath (*p*H 8.0), the ionic strength being calculated employing Lewis and Randall relation. It is found that the enzyme loading

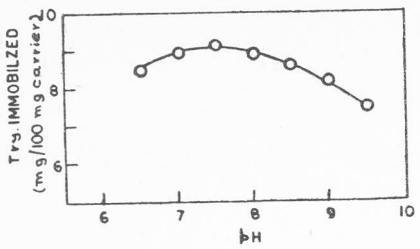

Fig. 1 - Effect of varying *p*H on the amount of trypsin (Try) immobilised.

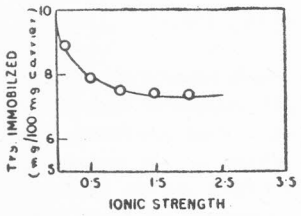

Fig. 2 - Effect of varying ionic strength on the amount of bound trypsin

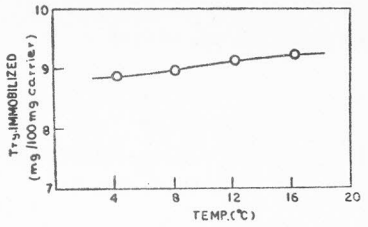

Fig. 3 - Effect of temperature on the amount of immobilised trypsin.

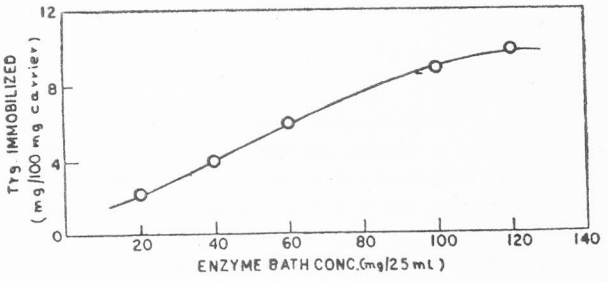

Fig. 4 - Sorption isotherm for trypsin.

decreases with increase in the ionic strength (Fig. 2). However, increase in ionic strength beyond 1.0 has no appreciable effect on enzyme loading.

This study indicates the formation of some salt-type linkages between the enzyme and the carrier. With increase in ionic strength of the reaction medium there is probably a competition between the micro-ions and the enzyme to attach to the molecular sieve. As the micro-ions are smaller in size compared to the enzyme molecule, it is easier for them to interact with sites of the carrier[1]. As a result, they saturate such binding sites depriving the enzyme the chance for attachment to the matrix. Consequently, enzyme loading decreases at higher ionic strength. Thus there is indications of some salt-type linkages between the enzyme and the carrier.

Effect of pretreatment of enzyme with p-chloromercuribenzoate (PCMB) — The effect of this well known reagent reacting on sulphydryl group in the process of immobilization was studied by pretreatment of trypsin with PCMB at *p*H 7.0 by the method described by Liener[7]. Another experiment was performed without this treatment, i.e. with unblocked trypsin. The results of the experiments indicate that there is no difference in bound protein content when trypsin is pretreated with PCMB before immobilization. Hence, any involvement of thiol group in the formation of enzyme-matrix conjugate is ruled out. In other words, experiment with PCMB indicates complete absence of any covalent binding between trypsin and the carrier. This is in contrast to the observation of Messing[8] who observed formation of partial covalent binding even in the samples immobilised by physical adsorption technique.

Effect of temperature on immobilization and sorption isotherm — The effect of temperature on immobilization was studied by adding equal amounts of trypsin and molecular sieve in the immobilization bath at 4°, 8°, 12° and 16°C maintaining other conditions the same as before. The results, plotted in Fig. 3, show that the enzyme loading increases with temperature. It is probable that Langmuir isotherm is obeyed[9].

The experiment on sorption isotherm was carried out by adding different amounts of enzyme to a fixed amount of carrier (100 mg) at 4°C under stirring for 18 hr. The sorption isotherm is shown in Fig. 4. The progressive saturation of the matrix as the enzyme loading increases is a further indication of a Langmuir type adsorption.

Effect of sorbitol and sucrose on the degree of immobilization — This study was undertaken with a view to finding out the contribution of hydrogen bonds in the trypsin and molecular sieve conjugate. It is well known that sucrose and sorbitol have a great tendency to form hydrogen bonds. Hence, in the presence of these reagents during immobilization, due to the preference of their binding to the carrier through hydrogen bonds, the formation of hydrogen bonds in the carrier-enzyme conjugate is expected to be inhibited.

Immobilization was carried out at 4°C for 18 hr with enzyme to carrier ratio of 1:1 in the presence of 20% sorbitol and 20% sucrose, respectively. Control experiments were also caried out under identical conditions without any sorbitol and sucrose. The amount of trypsin immobilised in the control experiment and in the presence of 20% each of sorbitol and sucrose comes out to be 8.86, 7.76 and 7.98 mg per 100 mg carrier, respectively.

It is found that with 20% each of sorbitol and sucrose the amount of bound enzyme decreased by 13.4% and 10%, respectively, indicating that sorbitol and sucrose are also attached to the carrier through hydrogen bonds thereby reducing enzyme loading

to the carrier. This also indicates that trypsin is perhaps also attached to the carrier by hydrogen bond.

X-ray analysis — A powder photograph of molecular sieve—trypsin conjugate was obtained using Cu-K radiation. A comparison of *d* and *I* values calculated from the photograph with those of the molecular sieve type 4A (from ASTM index) shows that the values are almost unchanged, indicating that the enzyme has not penetrated into the pores of the zeolite structure. This is what is to be expected, because of the large size of the enzyme molecule as compared to the pore size. Thus the crystal lattice structure of molecular sieve remains virtually unchanged. This confirms that there is only Langmuir type adsorption of enzyme on the matrix surface.

The use of an additional binding agent is essential in enzyme immobilization by covalent binding or covalent crosslinking. Sometimes chemical modification of the protein structure is also required. This may disturb the native conformation of the enzyme leading to either reduced activity or complete loss of activity of the enzyme. On the contrary, the adsorption procedure followed in the present investigation does not require the use of any additional reagent. Furthermore, the chance of conformational change of the protein is meagre.

The effects of *p*H, ionic strength, temperature and sorbitol and sucrose on the immobilization process confirm the role of van der Waal interaction, salt linkage and hydrogen bond formation in enzyme-molecular sieve binding. Due to this strong bond formation, the trypsin-molecular sieve conjugate is fairly stable with respect to leakage even for adverse changes in *p*H towards the alkaline region. This confirms the observation of Weldes[10] who found that the strong stability of protein-glass bond was due to the combined effect of formation of ionic amine silicate bond and hydrogen bond between enzyme and alkali metal silicate.

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References

1. VENKATASUBRAMANIUM, K., SANI, R. & VEITH, W. R., *J. Ferment. Technol.*, **52** (1974), 268.
2. BERNATH, F. R. & VEITH, W. R., *Immobilized enzymes in food and microbial process*, edited by A. C. Olson & C. L. Cooney (Plenum, New York), 1974, 157.
3. MESSING, R. A., *Immobilized enzymes for industrial reactors* edited by R. A. Messing (Academic Press, New York), 1975, 93.
4. MUKHERJEA, R. N., BHATTACHARYA, P., GHOSH, B. K. & TARAPHDAR, D. K., *Biotechnol. Bioengng*, **19** (1977), 1259.
5. MUKHERJEA, R. N., BHATTACHARYA, P., GHOSH, B. K. & GANGOPADHYAY, T., *Biotechnol. Bioengng*, **22** (1980), 543.
6. WORTHINGTON MANUALS *Enzymes and enzyme reagents* (E. C. 3.4.4.4) (1967).
7. LIENER, I. E., *Biochim. Biophys. Acta*, **53** (1961), 332.
8. MESSING, R. A., *Biotechnol. Bioengng*, **16** (1974), 1419.
9. GLASSTONE, S., *Text book of physical chemistry* (MacMillan, London), 1966, 1204.
10. WELDES, R., *Adhes. Age*, **10** (1967), 32.