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# THE CHEMICAL ATTACHMENT OF CHYMOTRYPSIN TO WATER-INSOLUBLE POLYMERS USING 2-AMINO-4,6-DICHLORO-*s*-TRIAZINE

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SUMMARY

1. A method is described for the preparation of water-insoluble derivatives of α-chymotrypsin using 2-amino-4,6-dichloro-*s*-triazine. The insoluble support materials used were cellulose, DEAE-cellulose, CM-cellulose, Sephadex and Sepharose.

2. Results for the effect of enzyme concentration, pH, temperature and time on the attachment process are given.

3. The influence of ionic strength, pH and particle size on the measured activities of these insoluble derivatives of chymotrypsin have been measured.

4. The results show that the overall reaction rate may be restricted by the rate of diffusion of substrate into and product out of the insolubilised enzyme derivative.

## INTRODUCTION

A wide range of techniques have been used for the chemical attachment of enzymes to water-insoluble support materials[1,2]. Some methods require the presence of amino or carboxyl groups on the support material. We have favoured the use of chemical linking agents that react with hydroxyl groups on the support material because it is then possible to choose the net electrostatic charge on the insoluble support by using, for instance, DEAE- or CM-cellulose.

Several chemical agents have been used to link enzymes to support materials containing hydroxyl groups, including bromacetyl bromide[3], cyanogen bromide[4] and cyanuric chloride (2,4,6-trichloro-*s*-triazine) and its derivatives. In previous papers we have described the preparation of insoluble derivatives of chymotrypsin[5], lactate dehydrogenase[6], pyruvate kinase[7], creatine kinase[8], β-galactosidase[9], penicillin amidase[10] and amyloglucosidase[11] to cellulose and its derivatives using various *s*-triazinyl compounds, but the methods of preparation were not investigated in detail.

From these experiments we concluded that 2-amino-4,6-dichloro-*s*-triazine was

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the most convenient and useful of the various compounds we had tried. It is easy to prepare in large quantities, gives convenient reaction times, and has no charged groups, but is not very soluble in water. In this paper we describe a general method for reacting this compound with a variety of support materials and investigate some of the factors affecting the amount of enzyme attached and the proportion of the enzyme activity retained. Although the experiments were confined to chymotrypsin, our previous experience with other enzymes indicates that the results may be of more general application.

## MATERIALS AND METHODS

### *Materials*

Crystalline α-chymotrypsin and acetyl-L-tyrosine ethyl ester were obtained from Severac Laboratories (Pty) Ltd., Maidenhead, Berks. Cyanuric chloride was supplied by British Drug Houses Ltd., Poole, Dorset. DEAE-cellulose (grade DE 52) and CM-cellulose (grade CM 32) were supplied by W. R. Balston (Modified Cellulose) Ltd., and Sephadex G-200 and Sepharose 4B from Pharmacia Ltd., Uppsala. Analar grade reagents were used where possible.

### *Preparation of 2-amino-4,6-dichloro-s-triazine*

This compound was prepared by a modification of the method of THURSTON *et al.*[12]. A stream of gas, generated by blowing $N_2$ through a gently warmed solution of 0.88 ammonia (35%) and dried by passage through NaOH pellets, was introduced into a cold slurry (5–8°) of 184 g of cyanuric chloride dissolved in a mixture of 1 l of dioxane and 200 ml of toluene, until the products of the reaction formed a thick suspension. This solid was filtered off, washed with 500 ml of dioxane and discarded. The filtrate and washings were bulked and evaporated to dryness under reduced pressure on a rotary film evaporator. The product was recrystallized first by dissolving it in an equivolume mixture of acetone and water followed by removal of the acetone under reduced pressure, and secondly by dissolving the solid in boiling water and cooling to room temperature within 10 min to prevent any hydrolysis. The yield was about 90%.

### *Preparation of aminochloro-s-triazinyl derivatives of polymers*

Two solutions were required. Solution A was made by dissolving 10 g of 2-amino-4,6-dichloro-*s*-triazine in 250 ml of acetone at 50° and adding 250 ml of water of the same temperature. Solution B was 15% (w/v) aqueous solution of sodium carbonate to which 0.6 vol. of 1 M HCl had been added.

For the dry polymers, cellulose and CM-cellulose, 20 g of the material was added to Solution A (100 ml) and stirred for 5 min at 50°. Solution B (40 ml) was added and the slurry stirred for a further 5 min at 50°. The pH value of the suspension was reduced rapidly to below 7 by the addition of concentrated HCl. The product was recovered by filtration, washed with an equivolume mixture of acetone and water, followed by water and then stored at 2° in 0.1 M sodium phosphate buffer (pH 6.7).

For DEAE-cellulose (40 g wet weight) identical reaction conditions were used except that the mixture of DEAE-cellulose and Solution A was stirred for only 30 sec before addition of Solution B.

Before use, Sephadex G-200 was boiled in water for 30 min and Sepharose 4B was washed thoroughly to remove preservatives. After removal of excess water with gentle suction on a Buchner funnel, these materials (equivalent in each case to about 2.5 g dry weight) were reacted in the same way as for 20 g cellulose.

*Preparation of insoluble derivatives of chymotrypsin*

An aqueous solution of chymotrypsin (20 mg/ml) and a 0.5 M sodium borate buffer (pH 8.75) (unless otherwise stated) were added to the suspension of amino-chloro-*s*-triazinyl derivative, to give final concentrations of 7–15 mg/ml and 0.07–0.2 M, respectively, depending on the experiment. The reaction was allowed to proceed at 23°. The product was washed with an equivolume mixture of 5 M NaCl and 8 M urea unless otherwise stated.

The amount of enzyme attached was obtained from the difference in absorbance at 280 m$\mu$ between the chymotrypsin added and that present in the washings.

*Enzyme assay*

Both chymotrypsin and its insoluble derivatives were assayed using acetyl-tyrosine ethyl ester in a pH-stat (model TTlc, Radiometer Ltd., Copenhagen). The temperature was 23°. As the substrate is not very soluble in water, it was dissolved in 10% dioxane at 50° for some experiments, so that a final concentration of 25 mM could be reached in the assay mixture. The amount of enzyme solution or suspension added in the assay varied, giving final concentrations of dioxane between 5 and 6%. In most assays of the insolubilised enzyme derivatives, phosphate buffer and NaCl were added to give final concentrations of about 0.02 and 0.3 M, respectively.

## RESULTS

*Factors determining the amount of enzyme attached*

*(a) Support material.* Chymotrypsin was attached to various support materials as described in MATERIALS AND METHODS. The amounts of enzyme attached after 18 h in each case are shown in Table I. The quantity of enzyme used in each experiment was not identical but in all cases the enzyme concentration was sufficiently high (7–15 mg/ml) not to affect the amount attached. Each preparation was washed thoroughly with 5 M NaCl to remove adsorbed enzyme and the results at this stage

TABLE I

THE EFFECT OF SUPPORT MATERIAL ON THE AMOUNT OF ENZYME ATTACHED

| *Support material* | *Water regain (wet wt./dry wt.)* | *Proportion of available enzyme attached (as %)* | *Amount of enzyme attached (mg/g support material)* | |
|---|---|---|---|---|
| | | | *1* | *2* |
| CF 11-cellulose | 2.0 | 10.0 | 4.3 | 3.2 |
| CM 32-cellulose | 3.9 | 21.6 | 113 | 90 |
| DE 52-cellulose | 4.2 | 37.8 | 133 | 117 |
| Sephadex G-200 | 18 | 75.0 | 212 | 186 |
| Sepharose 4B | 25 | 74.0 | 392 | 340 |

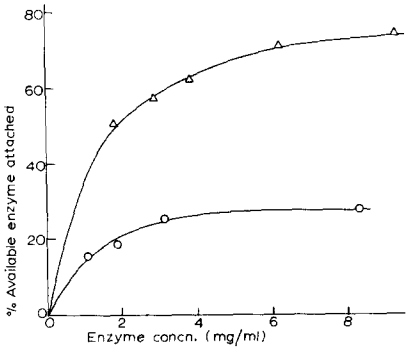



Fig. 1. Effect of enzyme concentration on the amount of available enzyme attached to CM-cellulose (○) and Sepharose (△) after reaction at 23° and pH 8.7 for 18 h.

are shown in Column 1 of Table I. Repeated washing with $NH_4OH$–$NH_4Cl$ (pH 8.6) for 4 days followed by washing with 5 M NaCl removed some more enzyme, as shown in Column 2 of Table I. Further washing with an equivolume mixture of 5 M NaCl and 8 M urea removed small amounts of enzyme from the Sepharose 4B preparation and trace quantities from the Sephadex G-200 and DEAE-cellulose preparations. After this exhaustive washing procedure no more enzyme was solubilised when the preparations were left to stand for several weeks at 2°. However when the slurry of Sepharose 4B–chymotrypsin was stirred overnight in 5 M NaCl some further enzyme was solubilised.

*(b) Enzyme concentration.* The effect of enzyme concentration on the amount of enzyme attached was examined for Sepharose 4B and CM-cellulose. The damp amino-chloro-*s*-triazinyl derivative (0.1 g dry weight) of Sepharose 4B was mixed with 2 ml chymotrypsin solution, 1 ml borate buffer and water to give the desired enzyme concentrations. For the CM-cellulose derivative only 1 ml of the chymotrypsin solution was used. The results for the amounts of enzyme attached after 18 h are shown in Fig. 1.

*(c) Time of reaction.* The amounts of enzyme attached to derivatives of DEAE-cellulose and CM-cellulose after different reaction times were measured and the results are shown in Tables II and III. In both experiments the amount of chymo-

TABLE II

THE EFFECT OF REACTION TIME ON THE AMOUNT OF ENZYME ATTACHED TO DE-52-CELLULOSE

| *Time (h)* | *Protein attached (mg/g support material)* | *Activity retained by attached enzyme (%)* |
|---|---|---|
| 0.17 | 42 | 42 |
| 0.34 | 58 | |
| 0.67 | 77 | |
| 1.0 | 84 | 40 |
| 2.0 | 96 | |
| 3.0 | 102 | |
| 19.5 | 110 | 42 |

TABLE III

THE EFFECT OF REACTION TIME ON THE AMOUNT OF ENZYME ATTACHED TO CM 32-CELLULOSE

| *Time (h)* | *Protein attached (mg/g support material)* | *Activity retained by attached enzyme (%)* |
|---|---|---|
| 1.7 | 11 | |
| 2.4 | 11 | |
| 4.25 | 19 | 70 |
| 21 | 38 | |
| 48 | 52 | 46 |

trypsin available was 110 mg/g support material and the final borate concentration was 0.08 M. The pH of the reaction mixture was 8.85 for DEAE-cellulose and 8.6 for the CM-cellulose experiment.

*(d) Effect of pH.* Since the isoelectric point of chymotrypsin is close to the pH at which the enzyme was attached, experiments were done with DEAE-cellulose and CM-cellulose derivatives at pH values above and below the isoelectric point. The reaction conditions were as described in MATERIALS AND METHODS except that for the experiments at pH 9 the borate buffer was brought to the correct pH with alkali and for the experiment at pH 8, 1 M phosphate buffer was used instead of borate buffer. The results are shown in Table IV.

*(e) Effect of temperature.* All the previous experiments were done at 23°. It is possible that some enzymes may be unstable during attachment at this temperature. Therefore an experiment at 2° was done. The amino-chloro-*s*-triazinyl derivative of Sephadex (0.14 g dry weight) was reacted for 18 h with 2 ml of chymotrypsin and 1 ml borate buffer (pH 8.75). During this time 82 mg enzyme/g Sephadex were attached. When treated with fresh solutions of chymotrypsin and borate buffer for a further 60 h, an additional 85 mg enzyme/g Sephadex were attached.

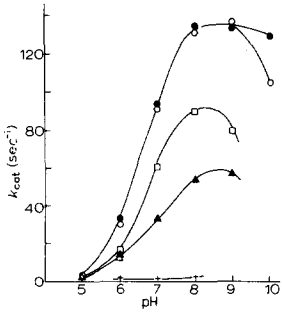
*Factors affecting the enzymic activity of the insolubilised enzyme*

It is not very meaningful to characterise an insolubilised enzyme simply by measuring its apparent $K_m$ and $v_{max}$ values under one set of conditions. Many factors,

TABLE IV

THE EFFECT OF pH ON THE AMOUNT OF ENZYME ATTACHED

| *Support material* | *pH of reaction* | *Amount of enzyme attached (mg/g support material)* |
|---|---|---|
| CM-cellulose | 9 | 84 |
| CM-cellulose | 8 | 22 |
| DEAE-cellulose | 9 | 220 |
| DEAE-cellulose | 8 | 125 |



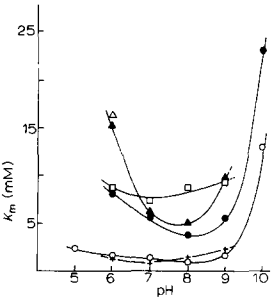




Fig. 2. The variation with pH of $k_{cat}$ for chymotrypsin and its derivatives in presence of dioxane, buffer and NaCl. Chymotrypsin (●), DEAE-cellulose–chymotrypsin (110 mg/g support) before grinding (△) and after grinding (▲), DEAE-cellulose–chymotrypsin (11 mg/g support) (□). Also shown are values for chymotrypsin in the absence of dioxane (○) and for DEAE-cellulose–chymotrypsin (110 mg/g support) in the absence of dioxane, buffer and NaCl (+).

Fig. 3. The variation with pH of the $K_m$ of chymotrypsin and apparent $K_m$'s of its derivatives. Symbols as for Fig. 2.

including electrostatic interaction and diffusional limitation, may affect the kinetics of the system. As we show in this paper, even when the reaction rate is partly diffusion-limited it is possible to obtain data which result in an almost straight Lineweaver–Burk plot but give misleading values for $K_m$ and $v_{max}$. Therefore we have examined the activity of chymotrypsin attached to DEAE-cellulose over a wide range of conditions.

The values of $k_{cat}$ and $K_m$ for the soluble enzyme at various pH values and in the presence and absence of dioxane are given in Figs. 2 and 3. Values for $k_{cat}$ were calculated assuming a molecular weight for chymotrypsin of 24 000. The results agree well with those obtained previously by KAPLAN AND LAIDLER[13] but the value of $k_{cat}$ at pH 10 with dioxane present is subject to some error as the $K_m$ was very high.

Similar measurements were made for a preparation of DEAE-cellulose–chymotrypsin containing 110 mg of enzyme/g support material. With no buffer and no dioxane present in the assay mixture the values for $k_{cat}$ were very low but increased with pH indicating that the pH optimum was much higher than for the free enzyme (Fig. 2). Also the apparent $K_m$ values, although close to those for the free enzyme, were shifted by almost two pH units judging by the position of minimum values in each case (Fig. 3). This was thought to be a reflection of a difference between the pH of the micro-environment around the attached enzyme molecules and the bulk solution.

When buffer but no NaCl was added, $k_{cat}$ at pH 6 was 36 $sec^{-1}$, *i.e.* several times that when buffer and NaCl were added. Although this result was rather inaccurate because of the high apparent $K_m$ (25 mM), it indicated that a shift to high values of the pH of the micro-environment of the enzyme was occurring as would be expected with a positively-charged support material[10].

To overcome this all other experiments were done with dilute buffer and NaCl in the assay mixture. When the DEAE-cellulose–chymotrypsin was assayed under these conditions its pH–activity profile was now similar to that for the free enzyme. The much higher activities that were now obtained, particularly at pH's 8 and 9, gave rise to another effect, giving a distorted Lineweaver–Burk plot. This is very

clearly shown in Fig. 4 when the insolubilised enzyme was assayed in the presence of dioxane so that high substrate concentrations could be reached. Extrapolation of the line through points obtained at low substrate concentrations gave a value for $v_{max}$ greater than for the free enzyme although all of the measured reaction rates were considerably less than those for the free enzyme at corresponding substrate concentrations. The apparent $K_m$ measured in this way was extremely high (greater than 0.1 M). At pH where the insolubilised enzyme was less active the Lineweaver–Burk plot was linear and $k_{cat}$ and $K_m$ could be measured. These are shown in Figs. 2 and 3.

The distorted Lineweaver–Burk plots for DEAE-cellulose-chymotrypsin assayed at pH's 8 and 9 were believed to be due to diffusional limitation of the reaction rate. The preparation was therefore ground to give a very fine powder and the experiments repeated. Values for $k_{cat}$ and apparent $K_m$ of this finely ground material are given in Figs. 2 and 3. Even at pH 9 a linear Lineweaver–Burk plot was obtained (Fig. 4). The

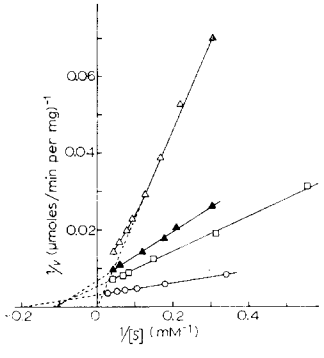



Fig. 4. Lineweaver–Burk plot for chymotrypsin (○), DEAE-cellulose-chymotrypsin (110 mg/g support) before grinding (△) and after grinding (▲), and DEAE-cellulose-chymotrypsin (11 mg/g support) (□) assayed at pH 9 in the presence of dioxane, buffer and NaCl.

large reduction in the diffusional limitation of the reaction rate resulting from the reduction in particle size was reflected in the apparent $K_m$ values which were much lower and close to the values for the free enzyme assayed under the same conditions. It is very interesting that grinding increased the activity of the preparation in the region of its optimum pH but there was no significant increase at pH 5 or 6 where the potential maximum activity of the enzyme is lower. Thus diffusional limitation of the reaction rate of the unground preparation occurs only under those conditions where the potential maximum reaction rate of the preparation is high.

A second preparation of DEAE-cellulose–chymotrypsin containing 11 mg of enzyme per g support material was also assayed in the presence of buffer and NaCl. Linear Lineweaver–Burk plots were obtained at all pH values examined (*e.g.* Fig. 4). The results are given in Figs. 2 and 3. The values for apparent $K_m$ are similar to those for the preparation containing 110 mg of enzyme per g support material after it had been ground except that at pH 8 where it is higher. Since this corresponds to the highest $k_{cat}$ value slight diffusional limitation may be occurring at this pH.

## DISCUSSION

Cyanuric chloride and its derivatives provide a wide range of compounds with differing reactivities. They are of considerable interest therefore as agents for linking enzymes to solid support materials. Cyanuric chloride itself is very reactive and it is very difficult to control the various reactions. In particular the support material often becomes cross-linked giving a material to which very little enzyme can be attached. 2-Carboxymethoxy-4,6-dichloro-*s*-triazine, linked to a hydroxyl group on the support material and to an amino group on the enzyme, has a structure similar to ammelide and the covalent links are not very stable. Dyes based on *s*-triazines, while commercially available and cheap, are not very reactive and contain large hydrophobic groups, which may denature the enzyme, and strongly electronegative sulphonic acid groups. Nevertheless, dyes can be used successfully with DEAE-cellulose[6]. 2-Carboxymethylamino-4,6-dichloro-*s*-triazine is a satisfactory reagent[5,10] but is difficult to prepare and carries a negative charge making it unsuitable for use with some support materials.

The reagent that we have used in this paper, 2-amino-4,6-dichloro-*s*-triazine has proved to be the most suitable so far. It is easily prepared, carries no net charge and has chlorines that react at convenient rates. Unfortunately it is not very soluble in water and it is necessary to use mixed solvents which may affect the support material. When dissolved in acetone–water to make Solution A, 2-amino-4,6-dichloro-*s*-triazine is stable at 2° for several days but at 50° for only about 3 h. On mixing with Solution B, a final pH of 10.5 is obtained at which the coupling reaction occurs rapidly at 50°. Under these conditions cross-linking of the support material is minimised and with the support materials used in this paper, their water regain was unchanged after reaction with the *s*-triazine derivative.

The amounts of enzyme that we have attached to various support materials using 2-amino-4,6-dichloro-*s*-triazine were considered adequate but in most cases at least they were probably not the maximum values attainable. The result for Sepharose was similar to that obtained by PORATH *et al.*[14] for agarose-linked chymotrypsin. We considered that preparations containing about 100 mg enzyme/g support material, assuming a reasonable recovery of enzyme activity (30–40%), were sufficiently active for most purposes. This amount of enzyme could not be attached to cellulose powder although 100 mg/g has been coupled to reprecipitated cellulose. Unfortunately we have found that this material is slightly water-soluble and because of its amorphous gelatinous nature is very difficult to filter.

It is necessary to wash insoluble derivative very thoroughly with NaCl–urea mixtures preferably in a packed bed over a long period. In our experience we were satisfied when no enzyme was detectable in the washings when the material was stirred very gently in NaCl overnight. This means that the amount of enzyme solubilised in this time was less than about 1 ppm of bound enzyme. In the case of Sepharose this could not be attained; which may be due either to small particles being produced by grinding—these particles would then pass through the filter and be detected as soluble enzyme–or because the support material gradually dissolves. In this respect it is interesting that Sepharose is the only support material which is not chemically cross-linked, except for cellulose itself, which is very strongly cross-linked by hydrogen bonding.

It is evident from the time-course of the reaction (Tables II and III) that the reaction is much more rapid with DEAE-cellulose than with CM-cellulose. This is due probably to the catalytic effect of the tertiary amine groups although the precise nature of the catalysis is not understood.

Although DEAE-cellulose may be reacted effectively at pH 8, the reaction with CM-cellulose at this pH is too slow (Table IV). In general a pH greater than 8.75 is required.

The effect of the charge on the support material on the amount of enzyme attached as shown in Table IV is not typical. In general we have found that to get large amounts of enzyme attached it is necessary for the charge on the support to be opposite to that on the enzyme[8]. Chymotrypsin is an exception because its isoelectric point is 8.6, *i.e.* it is almost electrically neutral at the pH of attachment.

In nearly all the experiments on enzyme attachment we have not measured the activities of the preparations since, as we have shown for DEAE-cellulose derivatives, it is necessary in each case to ensure that the results obtained, especially by extrapolation of Lineweaver–Burk plots, are meaningful. For the activity measurements in Tables II and III the preparations were assayed at pH 6 with buffer and NaCl, and we have shown in other experiments in this paper that this was justified as no diffusional limitation occurred under these conditions.

It has not been our object to undertake a detailed kinetic analysis of the insolubilised chymotrypsin but merely to find a basis on which a sound comparison of the enzymic activities of the various preparations could be made. It is evident from the results that it is necessary to assay the material in the presence of buffer and moderately high ionic strength. In the absence of buffer the proportion of enzymic activity remaining after insolubilisation is very low, about 2–5% of the activity in free solution. This is almost certainly caused by acid that is evolved in the reaction. The rate of diffusion of acetyl-L-tyrosine ion out of the DEAE-cellulose particles is probably about the same as the rate of diffusion in of the ester, which is considerably less than the rate at which the enzymic reaction can occur (as measured on the finely ground material). Consequently the pH in the particles falls until the rate of the enzymic reaction is equal to the rate of diffusion of product out of the particle. This would appear to occur at about pH 5. Similar changes in the pH optimum due to acid production were observed by GOLDMAN *et al.*[15] for papain–collodion membranes. It should be noted that both phosphate buffer and NaCl were needed in our assays to shift the pH optimum of the insolubilised enzyme to correspond with that for the free enzyme.

The reaction rate of an insolubilised enzyme can be restricted by the rate of mass transfer of the substrate either from the bulk of the solution to the surface of the support material[16,17] or into the pores of the support[9]. The present experiments on DEAE-cellulose-chymotrypsin prove that pore diffusion can very seriously restrict the reaction rate. This leads to a large increase in the apparent $K_m$ giving Lineweaver–Burk plots that, though linear, give nonsensical values of the maximal velocity when extrapolated. It must be remembered, however, that the preparations described in this paper were made with a very active enzyme and therefore had maximal activities of about 12 $\mu$moles/min per mg of preparation. Thus it is possible with less active enzymes to make preparations with the same amount of enzyme attached but which do not suffer from severe diffusion limitation of the reaction rate.

In the absence of diffusion limitation the $k_{cat}$ values for the DEAE-cellulose-chymotrypsin derivatives containing 11 mg enzyme per g support material and the other containing 110 mg of enzyme per g of support material after grinding were a constant proportion of those for the free enzyme over the pH range examined. The attached enzyme in the two preparations had 62 and 41%, respectively, of the free enzyme activity. In both of our preparations almost all of the available enzyme in the attachment reaction mixture became attached. These results for amount of enzyme attached and recovery of enzymic activity compare favourably with those reported for insolubilised forms of chymotrypsin and other enzymes prepared by chemical attachment to a support material.

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