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Entrapment of Enzymes and Microorganisms in Synthetic Cross-linked Polymers and their Application in Column Techniques

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A simple technique for the entrapment of enzymes and microorganisms in synthetic cross-linked polymers is described. The obtained gels containing enzyme or microorganism were granulated and used in continuous column processes. The activity of gel granules containing orsellinic acid decarboxylase was measured using Warburg technique and found to be as high as 30 % in relation to free enzyme. Leakage of enzyme from the gel matrix was negligible over a period of 14 days.

The study of fixed enzymes has attained considerable attention in recent years. Primarily, three different methods of preparation have been employed: 1) The use of ion-exchangers as supports for enzymes such as in the preparation of insoluble ribonuclease on Dowex-50 cation exchange resin,[1] 2) Commonly, preparation of insoluble enzymes by covalent binding to derivatives of cellulose [2] or dextran,[3] amino acid polymers [4] and synthetic resins,[5] 3) The entrapment of enzymes in a gel matrix as has been reported for cholinesterase in starch gel [6] and for several enzymes in a polymer preparation from N,N′-methylene bis(acrylamide) and $AlNH_4(SO_4)_2 \cdot 12H_2O$.[7]

In the present investigation an entrapment technique using cross-linked polyacrylamide has been chosen since it promised to fulfil the following requirements: high yield, no chemical modification of the enzyme, easy preparation and granulation and good flexibility by varying the size of the lattice formed. The entrapped enzymes thus obtained have been studied as to their properties with particular reference to their activity, stability and their applicibility to continuous column processes. Entrapped microbial cells on columns have also been studied.

EXPERIMENTAL

Materials. The enzymes used were orsellinic acid decarboxylase which was prepared according to the literature,[8] and cristalline trypsin (Worthington Biochemical Corp.). In the experiments with intact microbial cells, the lichen species *Umbilicaria pustulata* was used.

Preparation of entrapped orsellinic acid decarboxylase in gel granules. Preparation of 5 % (w/w) gel granules. To 9.5 ml of an orsellinic acid decarboxylase preparation (4.5 mg protein/ml, specific activity as μmoles CO_2 evolved per min and mg protein = 0.82) in 0.02 M phosphate buffer (pH 6.2) were added 0.475 g of acrylamide and 0.025 g of N,N′-methylene bis(acrylamide). After addition of the catalyst system consisting of 20 mg of ammonium persulfate and 50 μl of β-dimethylaminopropionitrile, the solution was carefully deaerated in a small test tube by applying vacuum with a water pump. After polymerisation the stiff gel thus obtained was passed through a 30-mesh sieve. The granules formed were then washed several times in the above buffer after which they were ready for use.

Preparation of 20 % (w/w) gel granules. The above procedure was followed. To reduce heat evolving during polymerisation the solution was kept at 4°. The amounts used were: 8 ml of enzyme solution, 1.9 g of acrylamide, 0.1 g of N,N′-methylene bis(acrylamide), 20 mg of ammonium persulfate and 20 μl of β-dimethylaminopropionitrile. Gels were then sieved as described above.

In the studies on the effect of storage on the decarboxylase granules so prepared, the different samples (0.4 g) used were taken from one polymerisation batch. They were kept in 5 ml of 0.02 M phosphate buffer (pH 6.2) at 20°. Before assaying, the buffer was filtered off and the granules washed twice.

Preparation of entrapped trypsin in gel granules. 5 % (w/w) and 20 % (w/w) trypsin granules were prepared from an enzyme solution containing 0.1 mg/ml of crystalline trypsin in 0.15 M borate-HCl buffer and 0.02 M $CaCl_2$ at pH 7.5. The same polymerisation procedure was used as described above.

Assay of orsellinic acid decarboxylase in gel granules. The decarboxylase was assayed manometrically by measurement of CO_2 evolved at 32°. 36 μmoles of orsellinic acid (2-methyl-4,6-dihydroxybenzoic acid) dissolved in 3 ml of 0.02 M phosphate buffer (pH 6.2) were placed in the main-chamber of the Warburg vessels. In the side-arm 0.4 g of the granules were placed together with 0.1 ml of the same buffer. Blanks were run with a heat-denaturated enzyme preparation entrapped in gel granules. The enzymic activity of free enzyme (100 μl) was determined as described above. Readings were made for a total of 10 min and enzyme activity calculated as μmoles CO_2 evolved per minute.

Assay of trypsin in gel granules. To 15 ml of the borate buffer 1 g of trypsin granules was added. After addition of 3 mg of α-N-benzoyl-L-arginine methyl ester·HCl dissolved in 0.5 ml of borate buffer, the reaction was permitted to proceed in a test tube at 20°. Samples were taken over a period of 30 min from the supernatant solution. After filtration to remove any solid particles the increase of absorption at 253 mμ was measured.

Assay of orsellinic acid decarboxylase column. A solution of orsellinic acid (12 μmoles/ml) in the above phosphate buffer was passed through a column (dimensions 1.5 × 10 cm) packed with enzyme granules (20 % w/w) at 20°. A flow-rate of 0.25 ml/min was chosen and the orcinol present in the effluent analysed by paper-chromatography in different solvent systems.[9] The column was kept for 2 months during which time it was run repeatedly for 12 h periods.

Assay of lichen column. The enzymic activity of lichen cells entrapped in granules was determined by passing a solution of the depside evernic acid (0.4 μmoles/ml) in 0.02 M phosphate buffer (pH 6.2) containing 15 % (v/v) acetone through a column (dimensions 1.5 × 10 cm). The polymerisation of the column material followed the procedure described for 20 % gel granules except for the addition of 0.5 g of air-dried, powdered lichen thallus which prior to polymerisation had been washed extensively to remove soluble enzymes present. The products obtained by the esterase and the decarboxylase activity of the cells, orcinol and orcinol monomethyl ether, were analysed by paper-chromatography.[9] The column was run at intervals for 3 months at 20°.

Table 1. Activity of orsellinic acid decarboxylase in gel granules on storage.

Time	Enzyme activity in 5 % gel		Enzyme activity in 20 % gel	
	$\frac{\mu\text{moles } CO_2}{\text{min}}$	$\frac{\text{entrapped (\%)}}{\text{free}}$	$\frac{\mu\text{moles } CO_2}{\text{min}}$	$\frac{\text{entrapped (\%)}}{\text{free}}$
1 hour	0.42	30	0.31	26
3 days	0.43	31	0.31	26
7 days	0.39	28	0.31	26
14 days	0.38	27	0.30	26

100 μl of free enzyme solution yielded 0.37 μmole CO_2/min. The 5 % and 20 % samples assayed contained 380 μl and 320 μl of entrapped enzyme solution, respectively.

RESULTS AND DISCUSSION

The entrapment of enzymes in a hydrophilic gel matrix as described above for cross-linked polyacrylamide constitutes an economic way of fixing enzymes. In the preparation of 20 % granules containing orsellinic acid decarboxylase, only about 3 % of the original enzyme activity could be found in the buffer solution one hour after granulation. This low figure probably represents both leakage from the surface of the granules and traces of none-polymerized enzyme solution. Table 1 shows the following: a) Both the 5 % and 20 % preparations show remarkably high activity as well as high stability on storage; b) On comparing the two preparations no decrease in activity of the 20 % granules can be found whereas the 5 % granules show a slight decrease which might be due to some leakage from the granules and/or denaturation of the enzyme. The Warburg technique used in the course of the present investigation proved most valuable in the kinetic studies of enzyme granules described.

Trypsin-containing granules have been studied as well. The activities obtained from 5 % and 20 % preparations showed only about 2 % of activity retained in relation to free enzyme. The reason for the relatively low activity obtained as compared to 30 % found with the decarboxylase granules is possibly a function of the larger size of the trypsin substrate.

The granules have also been studied on columns. The decarboxylase column which was run repeatedly with substrate during longer intervals was still active after two months at 20°. The lichen column with cells entrapped in the granules had also retained part of its esterase and decarboxylase activity after a period of three months at 20°.

Providing the substrate and product are able to penetrate the cell wall of an organism, the entrapping of whole cells should save the often laborious and difficult steps involved in enzyme preparation. The advantages offered by entrapping cells or homogenates, as well as enzymes are manyfold and have been discussed elsewhere. We would like to draw the attention in particular

to two aspects. By the above method it should be possible to trap enzymes which appear transiently in a biosynthetic sequence thus being able to isolate intermediate metabolites in greater quantities. Another possible advantage of this technique is in situations in which only a small amount of enzyme is available for the study of substrate specificity and it is desirable to use the same preparation repeatedly. When polymerizing labile enzymes the described catalyst system may be omitted and photopolymerisation can be used instead.

Note added in proof. In the meantime a similar approach towards entrapment of enzymes has appeared in *Anal. Chem.* **38** (1966) 726 by G. P. Hicks and S. J. Updike.

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