## BBA Report

BBA 61244

# The use of bead polymerization of acrylic monomers for immobilization of enzymes*

H. NILSSON[a], R. MOSBACH[b] and K. MOSBACH[a]

[a]*Chemical Centre, Biochemical Division, University of Lund, P.O. Box 740, S-220 07, Lund 7 (Sweden), and* [b]*Robert Koch Institut, Abtlg. Virologie, 1 Berlin 65, Nordufer 20 (West-Berlin)*

(Received February 18th, 1972)

SUMMARY

A new procedure for immobilizing enzymes is described, based on the technique used in conventional bead-polymerization processes. An aqueous solution of the enzyme trypsin and acrylic monomers is dispersed in a hydrophobic phase, and polymerization is initiated.

The obtained spherical polymer beads contain the entrapped active enzyme and show excellent mechanical stability and high flow rates when used in column processes. The average bead diameter can be reduced from 100-250 μm to 10 μm by changing polymerization conditions. Such small preparations have potential use in enzyme replacement therapy. The versatility of the above procedure is underlined by the fact that similar entrapment of a labile enzyme, a fungal lactase, also yields preparations with high residual enzymic activity.

During the last few years an ever-increasing amount of work has been reported on different aspects of immobilized enzymes[1,2]. The three major techniques widely used in immobilizing enzymes are covalent binding, adsorption and entrapment of enzymes within a gel lattice. The latter technique offers the advantages of leaving the biocatalyst unchanged yet eliminating the risk of desorption. Thus, entrapment, particularly within acrylic polymers, has been used in a number of cases for the immobilization of enzymes[3-5], antigen antibodies[6,7], DNA[8] and cells[3,9-11]. All these reports describe the preparation of a gel block containing the entrapped biological material followed by mechanical fragmentation. This procedure results in a wide range of size and shape of particles which

* Correspondence may be addressed to either institute.

also show decreased mechanical stability. A method of entrapment akin to that used in bead-polymerization processes, however, should lead to well-defined spherical beads of specific size, which should allow high flow rates when used in column processes and having good mechanical stability. Such preparations may, if small enough, be of potential use in future enzyme replacement therapy.

In a typical experiment 60 mg of trypsin (11 000 *N*-benzoyl-L-arginine ethyl ester units/mg) were dissolved together with 8.55 g of acrylamide and 0.45 g of *N,N'*-methylenebis(acrylamide) in 0.05 M triethanolamine-HCl buffer (pH 7.0) (total volume 60 ml). A total concentration of acrylamides of T=15% (w/v) was chosen since it has been shown to give maximal activity of *e.g.* cholinesterase immobilized by entrapment in polyacrylamide blocks[12]. After addition of the catalyst system (0.25 g of ammonium persulfate dissolved in 0.5 ml of the above buffer and 0.5 ml of *N,N,N',N'*-tetramethylethylenediamine) the solution was poured into a 1-l wide neck reaction vessel (Quickfit FR 1 LF, MAF 2/32) equipped with a paddle stirrer (the blade of which just fits the flask) and containing 400 ml of the organic phase (toluene-chloroform, 290:110) and 1 ml of the stabilizer Sorbitan sesquioleate (Pierce Chem. Co., Rockford, Ill.). The bead-polymerization procedure[13] here applied is similar to that reported for the preparation of cross-linked polyacrylamide used in gel chromatography[14]. Stirring at about 240 rev./min was initiated prior to the addition of the monomer enzyme solution. The polymerization reaction was carried out at 4 °C under nitrogen atmosphere. After 30 min the formed bead suspension was filtered on a sintered glass funnel, the beads were then washed twice with toluene to remove traces of chloroform and were stirred for 30 min each at 4 °C in 0.1 M $NaHCO_3$, 1 mM HCl, 0.5 M NaCl and water to ensure removal of enzymes not properly entrapped. The 'enzyme beads' are then ready for use but can be freeze-dried until required.

Protein analysis of the 'enzyme beads' was carried out according to Spackman, Moore and Stein after acid hydrolysis. The yields of protein were calculated on the basis of alanine, aspartic acid, glutamic acid and leucine; no interference of the matrix material was observed. Enzymic activity of entrapped trypsin was determined as the rate of hydrolysis at pH 9.0 of *N*-benzoyl-L-arginine ethyl ester by the pH-stat method with an automatic titrator. The final concentration in the assay mixture was 0.01 M *N*-benzoyl-L-arginine ethyl ester, 0.1 M NaCl, 0.02 M $CaCl_2$, 2–10 mg of freeze-dried preparation (previously swollen in the buffer) in a total volume of 3.0 ml.

Using this technique, enzyme-containing polyacrylamide beads were obtained with an average diameter of 100–250 $\mu$m. The measured activity of the entrapped enzyme is about 67% of free trypsin, which is remarkably high (Table I). This indicates that using the entrapment technique trypsin is at most only slightly denatured, and that diffusion hindrance of substrate and product within the gel matrix under the conditions described is either non-existent or small. In a comparative study, in which trypsin was immobilized by the conventional block polymerization followed by mechanical fragmentation and an identical washing procedure, the enzyme continued to

TABLE I

Binding yields and enzymic activities of trypsin (mean of two determinations) and lactase F immobilized through bead polymerization of acrylic monomers

| *Polymer* | *Amount of bound enzyme (mg enzyme/g dried polymer)* | *Binding yield based on added amount of enzyme (%)* | *Enzymic activity* | | |
|---|---|---|---|---|---|
| | | | *μmoles/min per mg enzyme* | *μmoles/min per g dried polymer* | *Ratio bound to free enzyme (%)* |
| Poly(AAm)-entrapped trypsin | 1.9 | 31 | 19 | 36 | 67 |
| Copoly(AAm–AA)+ CMDI | 5 | 83 | 18 | 90 | 63 |
| Copoly(AAm–AA)– CMDI | 1.5 | 25 | 25 | 38 | 90 |
| Poly(AAm)-entrapped fungal lactase[16] | 5.6 | 51 | 0.31 | 1.75 | 36 |

Abbreviations: AAm, acrylamide; AA, acrylic acid; CMDI, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide.

leak out from the obtained particles during enzyme assay contrary to what is observed with the above 'enzyme beads'.

Entrapping biological material of low molecular weight, such as trypsin, may not entirely prevent escape through the pores of the gel. This can be avoided by combining the general encagement technique with that of covalent binding previously used for block polymerisates[15]. The enzyme should then be kept firmly bound besides being protected from *e.g.* interfering interactions with proteins present in the outer medium by being embedded in the gel network. To this end the above procedure was modified by substituting potassium acrylate (6.52 g) for half of the amount of acrylamide, subsequent adjustment of the pH to 7.0 using HCl which was followed by addition of 250 mg of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (Aldrich Chem. Co.). In addition the ratio of solvents of the organic phase had to be changed (toluene–chloroform, 265:135). After polymerization, the beads were stirred for 2 h at 20 °C prior to the washing procedure to ensure chemical coupling. The total enzymic activity per amount of polymer was higher than that observed under comparable conditions either for enzyme entrapped in polyacrylamide alone or when diimide was omitted (Table I).

The general applicability of the bead-polymerization technique is demonstrated by the fact that even a more labile enzyme such as lactase F, a fungal lactase, also shows high residual enzymic activity after entrapment (Table I). Details of the latter preparation and use in preparing lactose-free milk is given elsewhere[16].

Smaller beads are desirable for possible future applications such as in enzyme replacement therapy and can be prepared by a modification of the above procedure in which up to 50 ml of either stabilizer Sorbitan monooleate (Span 80, Pierce Chem. Co.) or Sorbitan sesquiolate is added to 350 ml of organic phase of

toluene–chloroform (255:95). Between each washing step, the beads had to be centrifuged (5000 × *g*, 20 min) rather than filtered. Following this procedure, active trypsin beads entrapped in cross-linked polyacrylamide down to 10 μm in diameter were prepared.

The 'enzyme beads' obtained following the described procedure resemble preparations obtained using the established micro-encapsulation technique[17] by which a free-solution enzyme is enclosed within a very thin semipermeable membrane. As was recently demonstrated for carbonic anhydrase[18], the enzyme, once entrapped, retained its activity, but the trapping yield of protein at present is still poor. The method we have developed appears to result in a situation we describe as 'molecular entrapment' of the enzyme within the three-dimensional network. These preparations show the valuable properties of permitting immobilization of even autodigestable enzymes, and resistance to rupturing by mechanical and other means. They also might prove valuable kinetics model systems.

REFERENCES

1 E. Katchalski, I. Silman and R. Goldman, *Adv. Enzymol.*, 34 (1971) 445.
2 K. Mosbach, *Sci. Am.*, 224 (1971) 26.
3 K. Mosbach and R. Mosbach, *Acta Chem. Scand.*, 20 (1966) 2807.
4 G.P. Hicks and S.J. Updike, *Anal. Chem.*, 38 (1966) 726.
5 T. Wieland, H. Determann and K. Bünn:g, *Z. Naturforsch.*, 21b (1966) 1003.
6 T. Goodfriend, D. Ball and S. Updike, *Immunochemistry*, 6 (1969) 181.
7 S. Carrel and S. Barandun, *Immunochemistry*, 8 (1971) 39.
8 L.F. Cavalieri and E. Carroll, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 807.
9 K. Mosbach and P.O. Larsson, *Biotechnol. Bioeng.*, 12 (1970) 19.
10 S.J. Updike, D.R. Harris and E. Shrago, *Nature*, 224 (1969) 1122.
11 N.E. Franks, *Biochim. Biophys. Acta*, 252 (1971) 246.
12 Y. Degani and T. Miron, *Biochim. Biophys. Acta*, 212 (1970) 362.
13 S. Hjertén, *Methods in Immunology and Immunochemistry*, II (1968) 142.
14 S. Hjertén and R. Mosbach, *Anal. Biochem.*, 3 (1962) 109.
15 K. Mosbach, *Acta Chem. Scand.*, 24 (1970) 2084.
16 A. Dahlquist, B. Mattiasson and K. Mosbach, *Biotechnol. Bioeng.*, to be published.
17 T.M.S. Chang and M.J. Poznansky, *Biomed. Method Res.*, 2 (1968) 187.
18 R.C. Boguslaski and A.M. Janik, *Biochim. Biophys. Acta*, 250 (1971) 266.