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Porous glass as a solid support for immobilisation or affinity chromatography of enzymes

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SUMMARY

Chymotrypsin (EC 3.4.4.5) and β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) were immobilised by attachment with glutaraldehyde to aminoalkylsilyl glass. The preparations contained 16 and 12 mg protein/g glass respectively. The retention of activity on immobilisation for chymotrypsin was 50% and for β-galactosidase was 36%. Glycyl-D-phenylalanine was immobilised in the same way and used for purification of carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.1.2) by affinity chromatography.

Support materials such as cellulose, polyacrylamide, and dextran derivatives used for enzyme immobilisation and affinity chromatography are not ideal for large-scale operation in columns. The attachment of biologically active molecules to porous glass beads[1] is therefore of interest. They are resistant to microbial attack and can be regenerated by heating to remove all organic material. Here we describe a cheap and rapid method of attachment of enzymes and enzyme inhibitors to porous glass beads using glutaraldehyde.

Porous glass beads (Corning CPG 10, 200 mesh, 2000A pore diameter) were dried by heating at 500°C for 6 h. The method used by Weetall[1] for the preparation of aminoalkylsilane glass was optimised to give higher yields of amino groups/g of support. These were measured with 0.1% trinitrobenzene sulphonic acid in saturated sodium tetraborate[2], and found to be 30–35 μmoles NH_2/g support. The glass was refluxed in 0.1% 3-aminopropyltriethoxysilane in toluene for 36 h. Another method however gave better results. The beads were immersed in a 2% solution of 3-aminopropyltriethoxysilane in acetone. Excess liquid was decanted off and the beads allowed to stand at 45°C for 24 h. This direct polymerisation produced beads containing 80–90 μmoles NH_2/g support, and was used in preparing the derivatives described in this paper.

Aminoalkylsilane glass (2 g) was stirred in a cold 1% aqueous solution of

glutaraldehyde for 30 min. The derivative was rinsed with water and suspended in 10 ml of 0.05 M phosphate buffer, pH 7.5, containing 40 mg of α-chymotrypsin (EC 3.4.4.5) (Miles–Seravac (PTY) Ltd., U.K.). After 2 h at 4°C the beads were washed thoroughly with 1 M NaCl until no further activity was detectable in the washings. The immobilised chymotrypsin was assayed using *N*-acetyl-L-tyrosine ethyl ester as described by Kay and Lilly[3]. The immobilised preparation contained 16 mg of chymotrypsin/g support. When assayed in the absence of buffer the immobilised chymotrypsin had an optimum at pH 9.5 of 2.0 μmoles/min per mg support, corresponding to a retention of enzyme activity of about 50%. Addition of 0.01 M phosphate buffer to the assay mixture shifted the pH–activity profile giving a broad optimum between pH 8 and 9, similar to that for the free enzyme. This effect was similar to that previously observed with chymotrypsin attached to DEAE-cellulose[3]. The glass–chymotrypsin derivative was more heat stable than the free enzyme. When incubated at pH 3 for 2 h at 50°C the derivative lost 8% of its activity. In an identical experiment at pH 8, 19% of the activity was lost.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was attached to glass beads by a similar procedure to that used for chymotrypsin. An ammonium sulphate fraction from an extract of *Escherichia coli* was dialysed against 0.01 M Tris buffer containing 0.01 M $MgCl_2$ and 0.01 M 2-mercaptoethanol at pH 7.5. Aminoalkylsilylglass beads were activated with glutaraldehyde, washed and then reacted with β-galactosidase solution for 2 h at 4°C. The product was washed with the above buffer solution which is also suitable for storing the immobilised enzyme. The glass–β-galactosidase contained 12 mg protein/g glass. When assayed with *o*-nitrophenyl galactoside at 25°C and pH 7.5 the preparation had an activity of 0.13 μmoles/min per mg support, corresponding to a retention of activity of 36%. The immobilised β-galactosidase lost no activity at room temperature for 3 days when 0.01 M mercaptoethanol was present.

An inhibitor of carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.2.1), the dipeptide, glycyl-D-phenylalanine, was coupled to porous glass by suspending 2 g of the glutaraldehyde-activated glass in 5 ml of phosphate buffer, pH 7.5, containing 25 mg of the dipeptide. The suspension was stirred for 24 h at room temperature and subsequently washed with 2 M NaCl. After being packed in a column the glass derivative was equilibrated at 25°C with 0.02 M Tris buffer, pH 7.5, containing 0.2 M NaCl. The column was charged with 1 ml of a solution containing 2 mg of carboxypeptidase A (Sigma London Chemical Co.) and 8 mg of a mixture of lysozyme, ribonuclease and bovine serum albumin. The column was washed through with the starting buffer. The bound carboxypeptidase A was eluted from the column with 0.01 M acetic acid, pH 3.0. Carboxypeptidase A activity in the column effluent was determined by the colorimetric procedure of Ravin and Seligman[4].

The results for a small column (0.5 cm × 4 cm) are shown in Fig. 1. The flow rate was 7.5 ml/h. The recovery of protein was 96% and of carboxypeptidase A activity was 80%, 10% of the protein and 85% of the recovered activity was in the fraction retained by the column. The best preparations of dipeptide glass removed 2 mg of enzyme from solution for each g of glass. α-Chymotrypsin, which is inhibited by D-amino acids[6], exhibited some affinity for the column.

The attachment of enzymes to porous glass by diazotisation of an arylamine derivative and by sulphonamide linkage using an isothiocyanate derivative has been reported by Weetall[1]. Glutaraldehyde has been used to cross-link enzymes to form insoluble

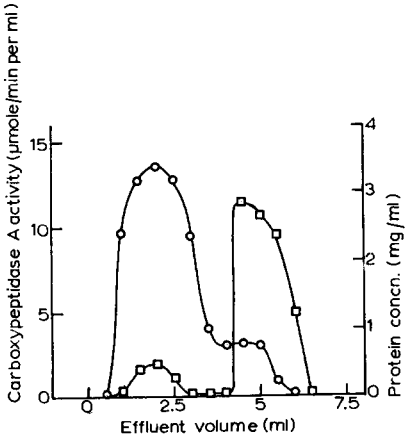

Fig. 1. The separation of carboxypeptidase A from a mixture of proteins by affinity chromatography. ○—○, protein; □—□, carboxypeptidase activity. 0.01 M acetic acid was added after 4 ml of effluent had been collected.

aggregates[5] and to immobilise enzymes to solid supports such as AE-cellulose[6]. Here we have shown that it may be used to link enzymes and an enzyme inhibitor to porous glass in three simple steps. It should be pointed out that glutaraldehyde solutions normally contain condensation products[7], and it is not known which of these is the active species. The two enzyme preparations retained a high proportion of their activity on immobilisation. The amount of protein bound to the glass was higher than in most of the preparations previously described[1]. However, as might be expected with the highly active chymotrypsin derivative, there was some sign of diffusional limitation of the reaction rate including a shift in the pH optimum. Diffusional limitation may account for part of the decrease in activity on immobilisation.

REFERENCES

1 H.H. Weetall, *Science*, 166 (1969) 616.
2 W.E. Hornby and Filippusson, *Biochem. J.*, 120 (1970) 215.
3 G. Kay and M.D. Lilly, *Biochim. Biophys. Acta*, 198 (1970) 276.
4 H.A. Ravin and A.M. Seligman, *J. Biol. Chem.*, 190 (1951) 391.
5 C.K. Glassmeyer and J.D. Ogle, *Biochemistry*, 10 (1971) 786.
6 L. Cunningham, in M. Florkin and E.H. Stotz, *Comprehensive Biochemistry*, Vol. 16, Elsevier, Amsterdam, 1965, p. 85.
7 F.M. Knowles and J.R. Richards, *J. Mol. Biol.*, 37 (1968) 231.