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ACRYLIC COPOLYMERS AS MATRICES FOR THE IMMOBILIZATION OF ENZYMES

I COVALENT BINDING OR ENTRAPPING OF VARIOUS ENZYMES TO BEAD-FORMED ACRYLIC COPOLYMERS

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SUMMARY

The preparation of various cross-linked acrylic copolymers in bead-form, suitable for the immobilization of enzymes, is described The enzymes bound were ribonuclease A (EC 3 1 4 22), trypsin (EC 3 4 4 4), β-glucosidase (EC 3 2 1 21), yeast alcohol dehydrogenase (EC 1 1 1 1) and urease (EC 3 5 1 5) The enzymes were either entrapped within polyacrylamide or covalently bound to (a) polyacrylamide using glutaraldehyde, (b) copolymers of acrylamide–2-hydroxyethylmethacrylate using cyanogen bromide, or (c) copolymers of acrylamide–acrylic acid using a water-soluble carbodiimide Attempts were made to optimize immobilization conditions

INTRODUCTION

The breakthrough observed in the last few years in the technique of biospecific affinity chromatography, as well as the advent of immobilized enzymes for practical application and as model systems for in vivo situations, has largely been made possible by the development of suitable matrices or supports for ligands and enzymes The various agarose polymers most widely used as matrices appear particularly ideal for affinity chromatography of enzymes due to their high degree of porosity [1] For the immobilization of enzymes, inorganic carriers such as substituted glass derivatives [2] and acrylic polymers are useful alternatives The latter have good chemical and mechanical stability and are not susceptible to microbial attack [3, 6] Moreover, due to the number of different acrylic monomers available, a variety of copolymers can be prepared "tailor-made" for specific purposes In Part II of this paper, use is made of the flexibility in the composition of the copolymers to conduct a study on the effect of hydrophobicity of the support on enzyme activity by variation of one parameter, i e the amount of hydrophobic component in an acrylic copolymer

Abbreviations Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester, BIS, *N,N'*-methylenebisacrylamide, TEMED, *N,N,N',N'*-tetramethylethylenediamine, EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl

In Part I we report on a simple method for the preparation of a number of acrylic copolymers by a bead polymerization process previously applied to polyacrylamide see Nilsson, Mosbach R H and Mosbach, K [4]) To copolymers of acrylamide–2-hydroxyethylmethacrylate thus prepared the enzymes, including some of clinical interest, were covalently bound using the cyanogen bromide method [5] This coupling method applied to such copolymers has hitherto only been tested for the proteolytic enzyme trypsin [6] and more recently for α-chymotrypsin [3] The enzymes were also bound to polyacrylamide using the bifunctional agent glutaraldehyde and to copolymers of acrylamide–acrylic acid using water-soluble carbodiimides

Attempts were made to optimize coupling conditions by variation of a number of parameters such as the cyanogen bromide or carbodiimide concentrations as well as the pH during activation and coupling A simplified entrapment technique was also applied to the enzymes tested, leading to bead preparations of defined diameter

MATERIALS

The enzymes ribonuclease A (bovine pancreas, Type IA, 60 Kunitz units/mg), trypsin (bovine pancreas, 10 000 α-*N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) units/mg), β-glucosidase (almonds, 5 1 units/mg), alcohol dehydrogenase (yeast, 344 units/mg) and urease (jack bean, 3 49 Sigma units/mg) and the substrates cytidine 2′ 3′-cyclic monophosphate (sodium salt), Bz-Arg-OEt, *p*-nitrophenyl-β-D-glucoside as well as NAD^+ (grade III) and the coupling agent 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma Chem Co (St Louis, Mo , U S A) Acrylamide, *N*,*N*′-methylenebisacrylamide (BIS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and the liquid monomers 2-hydroxyethylmethacrylate and acrylic acid, which were distilled before use, were obtained from Eastman, Org Chem (N Y , U S A) The acrylic acid was adjusted to pH 7 0 by addition of NaOH pellets Ammonium persulphate, urea and glutaraldehyde (distilled before use) were obtained from Merck (Darmstadt, G F R), Sorbitan sesquioleate from Pierce Chem Co (Rockford, Ill , U S A), and cyanogen bromide from Fluka AG, Buchs SG (Switzerland)

METHODS

Preparation of bead-formed acrylic copolymers

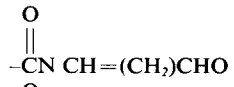
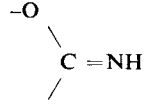
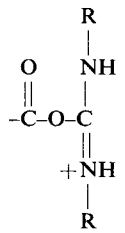
The various copolymers referred to in Methods are summarized in Table I

Bead polymerization was carried out in a 1-l round-bottom flask, equipped with a stirrer, a N_2 inlet and outlet, and a tap funnel for admission of the monomer solution The flask was cooled in ice water, and contained 400 ml of the hydrophobic phase, toluene–chloroform (290 110, v/v) This phase also contained the emulsion-stabilizing agent Sorbitan sesquioleate (3, 4 and 5 ml for copolymers 1, 2 and 3 respectively) Prior to the polymerization, this phase was stirred under N_2 at 260 rev /min, the stirring was maintained during the entire polymerization process For each copolymer preparation a solution of 5 7 g monomers, (Table I) and 0 3 g BIS (cross-linking agent) was prepared in 0 1 M Tris–HCl buffer, pH 7 3 the total volume was 58 5 ml To the cold monomer solution was added the catalyst system, which

TABLE I

VARIOUS COPOLYMERS PREPARED IN BEAD-FORM AND USED FOR COVALENT BINDING OF ENZYMES

The total concentration of monomer in the polymer was 10% and the relative concentration of cross-linking agent (BIS) was 5%

	Percent monomers (w/w) present			Functional group involved in coupling	Activating or coupling agent	Active intermediate
	Acrylamide	2-Hydroxyethyl-methacrylate	Acrylic acid			
Polymer 1	100	—	—	$-NH_2$ (amide)	glutaraldehyde	
Polymer 2	50	50	—	-OH	CNBr	
Polymer 3	50	—	50	-COOH	EDC	

consisted of 0 5 ml TEMED and 1 0 ml ammonium persulphate solution (0 4, 0 6 and 0 8 g/ml buffer for the copolymers 1, 2 and 3 respectively The resulting mixture was immediately added to the stirred hydrophobic phase, except in the case of polymer 3, when it was necessary to wait 5 min for the polymerization to start before adding the mixture to the organic phase Polymerization of the aqueous phase of the dispersion began within 5 min and was complete within 30 min The copolymer beads were washed on glass filter, first with toluene to remove the chloroform, and then with 4 l of distilled water prior to freeze-drying

Entrapment of enzymes in polyacrylamide beads (polymer 4)

10 mg of enzyme (ribonuclease A, trypsin, β-glucosidase, yeast alcohol dehydrogenase or urease (in presence of 5 mg bovine serum albumin and 1 mM mercaptoethanol) were dissolved in 14 7 ml ice-cold monomer solution, containing 2 85 g acrylamide and 0 15 g BIS After addition of 150 μl TEMED and 150 μl ammonium persulphate solution (0 4 g/ml) the solution was poured into a 250-ml beaker containing a hydrophobic phase (72 5 ml toluene, 27 5 ml chloroform and 1 2 ml Sorbitan sesquioleate) which had been equilibrated for 10 min under nitrogen at 4 °C stirred magnetically at 240 rev /min The polymerization started within 2 min and was complete after 20 min The beads were first washed with 50 ml toluene and then as described under washing procedures

Covalent binding of enzymes to glutaraldehyde-treated polyacrylamide beads (polymer 1)

Polyacrylamide beads (100 mg dry weight) were activated for 14 h at 40 °C using a 6% solution of glutaraldehyde, dissolved in 0 2 M phosphate buffer, pH 7 4 The glutaraldehyde-treated beads were thoroughly washed with distilled water, at 4–8 h intervals, for one day, at 4 °C The beads were filtered on a glass filter and transferred to a tube 2 mg of enzyme (ribonuclease A, trypsin, β-glucosidase, yeast alcohol dehydrogenase or urease) dissolved in 2 ml 0 1 M phosphate buffer, pH 7 4, were added to the beads and coupling was allowed to proceed for 14 h at 4 °C Due to the labile nature of yeast alcohol dehydrogenase this enzyme was coupled for only 3 h

Covalent coupling of enzymes to CNBr-treated copolymers of acrylamide–2-hydroxyethylmethacrylate (polymer 2)

Swollen beads (100 mg dry weight) were activated in 4 ml distilled water with varying amounts (16–550 mg) of CNBr The activation step was allowed to proceed for 6 min at 4 °C while pH was maintained at 10 8 by addition of 0 5–4 M NaOH The activated beads were thoroughly washed on a glass filter with ice-cold 0 1 M $NaHCO_3$ and were then transferred to the enzyme solution (2 mg enzyme in 2 ml 0 1 M $NaHCO_3$) The coupling reaction proceeded for 12 h at 4 °C Urease was coupled in the presence of 1 mM mercaptoethanol

Covalent binding of enzymes to copolymers of acrylamide–acrylic acid (polymer 3) using EDC

Swollen beads (50 mg dry weight) were washed in 0 2 M $NaHCO_2$ after which 1 ml EDC solution (2 mg EDC per ml water) and 1 ml enzyme solution (2 mg enzyme per ml 0 2 M $NaHCO_3$) were added The coupling proceeded for 2 5 h at 4 °C

Washing procedures

The washing procedures were all carried out at 4 °C but differed for some of the enzymes

The immobilized derivatives of ribonuclease A, trypsin and β-glucosidase were first washed on glass filters with 0 1 M $NaHCO_3$, and were then stirred for one hour each in 0 1 M $NaHCO_3$, 0 5 M HCl, distilled water and finally with the solution used for the assay

The alcohol dehydrogenase derivatives were washed throughout with solutions having a pH of 7 5–8 5 These were 0 1 M $NaHCO_3$, 0 1 $NaHCO_3$ made 0 1 M in NaCl and finally 0 05 M phosphate buffer, pH 7 8 No protecting agents were used

Urease-containing beads were washed with 0 1 M $NaHCO_3$, 0 5 M NaCl and finally with 0 1 M Tris–HCl buffer, pH 8 0 All washing solutions were 1 mM in mercaptoethanol

Enzyme assays

All enzyme assays were carried out at 25 °C

Ribonuclease A activity was measured titrimetrically (Automatic titrator, Radiometer) by following the rate of hydrolysis of 7 9 μmoles cytidine 2′ 3′-cyclic phosphate at pH 7 5, in 2 5 ml 0 1 M NaCl made 0 4 mM in EDTA

The esterase activity of the trypsin derivatives was determined titrimetrically at pH 8 5 with 2 5 μmoles Bz-Arg-OEt in 2 5 ml 0 1 M NaCl

β-Glucosidase activity was measured spectrophotometrically at 400 nm following the rate of hydrolysis of 30 μmoles *p*-nitrophenyl-β-D-glucoside in 8 ml 0 1 M phosphate buffer, pH 6 8 From the incubation mixture, contained in a 25-ml conical flask, the reaction solution was pumped through a flow-cuvette and back again to the enzyme suspension A net filter prevented the beads from entering the flow-system [7]

Alcohol dehydrogenase was assayed as describ edfor β-glucosidase by following the formation of NADH at 340 nm from 15 μmoles NAD^+ and 50 μmoles ethanol in 8 ml 0 05 M phosphate buffer, pH 7 8 in the presence of 750 μmoles semicarbazide Immobilized β-glucosidase and yeast alcohol dehydrogenase were alternatively assayed in a spectrophotometer (Zeiss, PMQ II) provided with a cuvette stirrer which kept the beads suspended in 2 ml of the incubation solution in a 3-ml cuvette No fragmentation of the beads was observed at the high rates of stirring (250 rev /min) used

Urease activity was determined by following the hydrolysis of 167 μmoles urea with an NH_4^+-sensitive electrode (Beckman, 39 137 Cationic Electrode) in 25 ml 0 1 M Tris–HCl buffer, pH 8 0

RESULTS AND DISCUSSION

The various acrylic copolymers, prepared in bead form, to which the enzymes have subsequently been covalently coupled are given in Table I In addition to covalent binding the entrapment technique has been applied The results of the various immobilization procedures used for the different acrylic copolymers are summarized in Table II (Polymer-bound enzymic activity is given as a percentage of added enzymic activity) Amino acid analyses, according to Spackman et al [8], for the determination of the amount of bound protein have not been carried out except for ribonuclease A because of the large number of analyses necessary (Ribonuclease A

TABLE II

VARIOUS ENZYMES IMMOBILIZED TO DIFFERENT ACRYLIC COPOLYMERS BY FOUR IMMOBILIZATION TECHNIQUES

To determine enzymic recovery the optimal activities of bound enzyme in each series were averaged To polymers 1 and 2, 20 mg enzyme were added per g dry polymer, to polymer 3, 40 mg Polymer 4 represents polyacrylamide entrapped enzyme preparations in which only 3 35 mg of enzyme per g dry polymer were used The units given refer to international units (one unit of enzyme converts 1 0 μmole of substrate per min at 25 °C)

Enzyme	Polymer-bound enzyme activity expressed as percent of added activity to polymer				Enzyme units/g dry weight polymer			
	1 Polyacrylamide (glutaraldehyde)	2 Acrylamide–2-hydroxyethyl-methacrylate (CNBr)	3 Acrylamide–acrylic acid (EDC)	4 Polyacryl-amide (entrapped)	1	2	3	4
Ribonuclease A	10	18	9	11	60	110	110	11
Trypsin	8	19	38	23	70	160	640	33
β-Glucosidase	65	35	52	74	65	35	105	13
Alcohol dehydrogenase, yeast	2	0	2	0	130	0	260	0
Urease	56	0	3	90	140	0	13	38

polymer 1, binding yield of enzyme 98% = 20 mg/g dry polymer, polymer 2, binding yield 50% = 10 mg/g dry polymer and polymer 3, binding yield 100% = 40 mg/g dry polymer) However, Table II lists the number of units found per g of dry polymer, which will provide information of practical use and give a general idea of the potential of the method All the polymers studied were prepared following a bead-polymerization procedure previously published [4], except when enzymes were entrapped in polyacrylamide beads, where a simplified procedure (Nilsson, H and Mosbach, K , unpublished) was used The average diameter of the beads was 50–250 μm, except for the entrapped preparation which were 40–110 μm The total concentration of monomer in the polymer used for enzyme entrapment was 20% and the relative concentration of cross-linking agent (BIS) was 5% The corresponding figures for the other polymers were 10 and 5% It should be pointed out however, that there is no guarantee of absolutely identical degree of cross-linking in the various copolymers described, since different monomers are involved In the acrylamide–2-hydroxyethylmethacrylate and acrylamide–acrylic acid copolymers the monomers were present in a ratio of 1 1 The covalent coupling of enzyme was done in the same total volume of 3 ml with a ratio of enzyme/dry polymer of 2 mg/100 mg, except in the case of the acrylic acid-containing polymer where only half of the amount of polymer was used (50 mg of the latter occupies the same gel volume as 100 mg of polymer 1 and 2, i e 1 ml in the swollen state)

For the entrapment preparations however, a different ratio was chosen, i e 0 3 mg enzyme/100 mg dry polymer, in order to reduce the amount of enzyme used Entrapment in polyacrylamide has been applied to all five enzymes tested This technique has been widely used for both the immobilization of enzymes [9–11] and intact cells [12, 13] Recently, entrapment of enzymes within another hydrophilic cross-linked acrylic polymer, 2-hydroxyethylmethacrylate has been reported [14] In the present study a simplified version of our previously published bead-polymerization technique was applied Bead-polymerization has the advantage of yielding preparations of mechanically stable beads of uniform and defined diameter and represents an improvement over previously applied bulk polymerization followed by physical fragmentation Of the enzymes tested, yeast alcohol dehydrogenase gave inactive preparations, probably as a result of denaturation of the enzyme during the polymerization process On the other hand, as much as 90% of the total enzymic activity of the added urease was found polymer-entrapped This high recovery was obtained when bovine serum albumin and mercaptoethanol were present during the polymerization Upon omission of the stabilizing agents the recovery in the entrapped preparations was considerably reduced There appears to be a direct correlation between activity recovered and the molecular weight of the entrapped enzyme (Table II)

Derivatization of polyacrylamide with glutaraldehyde [15] gave preparations well suited for enzyme binding All enzymes tested in this study gave active preparations The widely used cyanogen bromide method [5] usually applied to agarose gels, has been applied to copolymers of acrylamide–2-hydroxyethylmethacrylate A preliminary report on the subject [6] is followed up here by a more systematic study As is seen from Table II, the enzymes ribonuclease A, trypsin and β-glucosidase give good recoveries, whereas urease and yeast alcohol dehydrogenase are not active, which may in part be due to inactivation by traces of cyanogen bromide present It appears that the concentration of cyanogen bromide used for activation of the

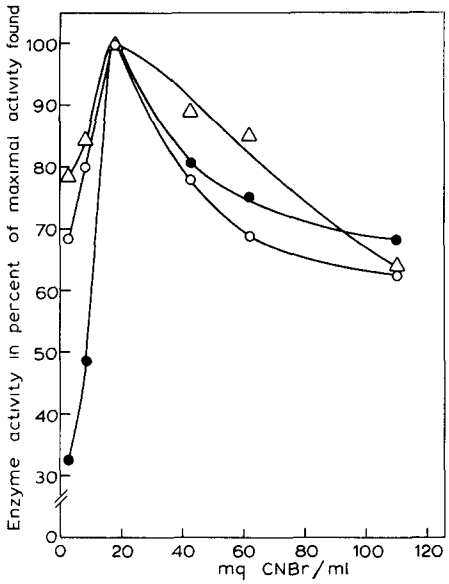


Fig 1 Enzyme activity bound to 100 mg acrylamide–2-hydroxyethylmethacrylate polymer, pre-activated with different amounts of CNBr in distilled water (total volume of 5 ml) for 6 min at pH 10 8 and 4 °C The maximal activity found, as total units for each enzyme, has been set as 100% Trypsin, △—△, β-glucosidase, ●—●, ribonuclease A, ○—○

copolymer has a critical bearing on the amount of enzymic activity recovered (Fig 1) A concentration of 18 mg cyanogen bromide per ml of total activation volume appears to be optimal for all three enzymes If the concentration of the activating agent is too high there is a drastic decrease in the swelling capacity of the polymer It seems that vicinal hydroxyl groups on the same polymer chain are not required for the production of active intermediates on the polymer As was the case with glutaraldehyde, all the enzymes tested gave active preparations when bound by a water-soluble carbodiimide to copolymers of acrylamide–acrylic acid The carbodiimide used, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide was found to be more reactive than 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide which was previously used in the coupling of the enzymes trypsin [6] and pullulanase [16] to similar, commercially available copolymers (Bio-Gel CM 100) It was found that for the enzymes tested, best recoveries were obtained from activation and coupling at an alkaline pH 8 4, using a concentration of 2 mg carbodiimide in 3 ml of total coupling solution The advantage of the procedure described lies in its rapidity, since it requires only about three hours for the coupling procedure This is of particular value when immobilizing labile enzymes Active enzyme preparations, as tested with ribonuclease A, were also obtained when the matrix was first treated with the carbodiimide at pH 3 0, permitting formation of the *O*-acylisourea derivatives [17], followed by washing with 1 mM HCl to remove excess coupling agent Coupling of the enzyme was then allowed to take place in 0 1 M $NaHCO_3$, at pH 8 4 This same modification has also been recently applied by other authors in the coupling of lactate dehydrogenase to succinylpropyl glass [18]

Besides the three different acrylic copolymers described here an additional derivative to which enzymes can be bound has been described [19] It is an acid azide which is prepared through modification of the carboxamide group in polyacrylamide to hydrazide, followed by treatment with HNO_2

In conclusion, the examples given for the immobilization of enzymes to various copolymers based on acrylic monomers indicate the potential of these preparations From the data obtained, no general statement can be made as to what coupling procedure and what acrylic matrix is ideal for the immobilization of any one enzyme However the availability of various acrylic polymer matrices with different properties together with the different immobilization techniques known will provide enough flexibility to obtain a "tailor-made" immobilized enzyme preparation

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