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Immobilisation of the D-2-haloacid dehalogenase from *Pseudomonas putida* strain AJ1/23

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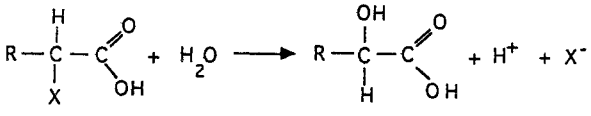
Abstract

A variety of procedures were used to immobilise D-2-haloacid dehalogenase. Natural polymer supports were insufficiently robust to withstand degradation by high concentrations of 2-chloropropionate. The best results were obtained with enzyme covalently attached to controlled-pore glass via a diazo linkage. The immobilisation procedure was optimised with respect to enzyme loading, pH, temperature and the presence of substrate during attachment. Immobilisation significantly modified the kinetics of the enzyme, in particular improving its temperature stability and ability to withstand mildly alkaline conditions where it is most active. The performance of the immobilised preparation in batch and plug-flow bioreactors was assessed. Biocatalyst half-life in plug-flow reactors was better than in batch bioreactors whereas effectiveness factors, although concentration dependent in the batch reactor, were similar at least with 200 mM D,L-2-CPA as substrate.

Introduction

Previously we have described the purification and characterisation of the chiral-specific D-2-haloacid dehalogenase from *Pseudomonas putida* strain AJ1/23 (Smith et al. 1989, 1990). This enzyme carries out the following hydrolysis with inversion of configuration:



where R is a short chain alkyl group or hydrogen and X is a halogen other than fluorine. Thus the enzyme converts D-2-chloropropionic acid (D-2-CPA) to L-lactic acid, and transforms racemic 2-chloropropionate into L-2-chloropropionate and L-lactic acid. The substrate range of the enzyme is fairly limited with a requirement for a free primary carboxyl group, the reactive halogen to be in the C-2 position and an overall chain length not exceeding four carbons (Smith et al. 1990).

D-2-haloacid dehalogenase has found commercial application in the production of L-2-chloropropionic acid as a chiral feedstock chemical for the production of herbicides (ICI Patent no. 179603). The experiments described in this paper were undertaken with a view to developing a model continuous bioreactor system for the conversion of racemic 2-chloropropionic acid to the L-isomer. This required that the dehalogenase be immobilised to an insoluble support and it was hoped that this might confer additional advantages over the soluble enzyme such as increased enzyme stability.

Materials and methods

Materials

Lactate dehydrogenase, glutamate pyruvate aminotransferase, bovine serum albumin, glutaraldehyde (25%), controlled pore glass (mesh size 120–200 μm and mean pore diameter 504Å), dithiothreitol, DEAE-Sephadex, CNBr-Sepharose 4B, acrylamide

and nicotinamide adenine dinucleotide (NAD) were obtained from Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset. BDH, Broom Road, Poole, Dorset suppled sodium alginate, ammonium sulphate (especially low in heavy metals), silicon antifoam reagent and Fractogel TSK DEAE-650 (Merck). D,L-2-chloropropionic acid was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset and DEAE-Sephacel from Pharmacia Fine Chemicals, Milton Keynes, Bucks.

Growth of Pseudomonas putida AJ1/23

Pseudomonas putida AJ1/23 was grown in a mineral medium supplemented with D,L-2-chloropropionate pH 7.0 (D,L-2-CPA) as sole carbon and energy source. A 15 litre working volume fermenter was used but otherwise the methods were as described by Smith et al. (1990). Stock cultures were maintained on mineral medium plus 20 mM D,L-2-CPA solidified with 1.5% (w/v) agar. For long-term storage, aliquots of culture in 50 mM Tris-H_3PO_4 buffer, pH 7.5 containing 10% (v/v) glycerol as a cryoprotectant were sealed in glass ampoules and stored in liquid nitrogen.

Preparation of partially purified dehalogenase

Partially purified D-2-haloacid dehalogenase was prepared from sonicated washed cell suspensions by DEAE-cellulose chromatography and ammonium sulphate fractionation as described by Smith et al. (1990). Enzyme was dialysed overnight at 2 °C against 1000 volumes of 50 mM K_2HPO_4/NaH_2PO_4 buffer, pH 7.5 and then drop frozen in liquid nitrogen for storage at −70 °C.

Determination of soluble enzyme activity

Standard ion chromatograph (IC) assay. The assay described by Smith et al. (1989) was used for the quantitative determination of dehalogenase activity in partially purified enzyme preparations for use in immobilisation procedures.

Protein estimation. Enzyme preparations were analysed for protein content by the modified Lowry method (Larson et al. 1986).

Definition of enzyme unit. In this study one unit of D-2-CPA dehalogenase activity is defined as the amount of enzyme that produced 1 μmol Cl^- min^{-1} from D,L-2-CPA at 30 °C and pH 7.5.

Immobilised enzyme preparations

Protein determinations and enzyme activity measurements were made before and after all the immobilisation procedures. Immobilised enzyme preparations were stored in Pharmacia AC 10/20 columns containing 50 mM Tris-H_3PO_4 buffer pH 7.5 (buffer A) at 4 °C prior to the assessment of their activity using the column reactor assay described below. All buffers contained 1 mM DTT.

Ionic binding to DEAE anion exchange matrices. DEAE-Sephacel is a weakly alkaline soft-gel anion exchanger of bead diameter 40–60 μm (wet bead) and is supplied with chloride counterions. Fractogel TSK DEAE-650 is a weakly alkaline rigid anion exchanger of particle size 0.025–0.050 mm. It consists of hydrophilic vinyl polymers modified by the addition of numerous ion-exchanger groups on both internal and external surfaces. Before use with the enzyme, the ion-exchange matrices were washed several times with 50 mM Tris-H_3PO_4 buffer pH 7.5 to remove storage solvent and fines. A 3:1 ratio of enzyme (mg) to matrix (ml) was used. The enzyme in buffer was mixed with the matrix in a sterile screw-capped plastic bottle on a Denley Spiramix (Denley Instruments Ltd., Natts Lane, Billingshurst, Sussex.) for 2 hrs at room temperature. The gel was finally washed with buffer A to remove non-attached protein.

Entrapment in calcium alginate. Entrapment of enzyme in alginate was attempted using a 1:1 and a 1:2 ratio of protein (mg) to alginate (ml) (method amended from Kierstan & Bucke 1977). A 4% sodium alginate solution was prepared by steaming alginate in water until it dissolved. This was mixed with enzyme preparation to obtain the desired ratios of protein to alginate. The enzyme preparation consisted of 1 mg ml^{-1} protein in 50 mM Tris-H_2SO_4 buffer pH 7.5; the sulphate counter ion was used because phosphate ions disrupt the alginate structure. Enzyme and 4% alginate solutions were mixed to give a 2% and a 2.7% gel. These mixtures were dropped into a stirred 0.5 M $CaCl_2$ solution using a 21 g needle and left to harden for 30 min. The resultant beads were washed with glass distilled water, and then buffer A, before storage and assessment.

Covalent coupling to CNBR-sepharose 4B. 2.5 g of dried CNBr-Sepharose 4B matrix was washed and reswollen on a sintered glass filter with 1 mM HCl to produce about 9 ml of gel. The gel was mixed with 23 mg of protein in 0.1 M $NaHCO_3$ buffer pH 8.3 containing 0.5M NaCl in a screw capped bottle on a Denley spiramix for 2 hrs at room temperature. To block any remaining active groups the gel was mixed with 0.2 M glycine in buffer for 2 hrs at room temperature. Adsorbed protein was removed from the gel by washing alternately with 0.1 M $NaHCO_3$ buffer pH 8.3 and 0.1 M acetate buffer pH 4.0 both containing 0.5 M NaCl. Blocking agent was removed by washing the gel several times with 0.1 M $NaHCO_3$ buffer pH 8.3, and then buffer A, prior to storage.

Covalent coupling to nylon mesh with glutaraldehyde. Lengths of nylon mesh (9 cm), type 66 (Henry Simon, Special Products Division, P.O. Box 31, Stockport, Cheshire.), were sown into a series of concentric tubes. The resultant tube was placed in 3.65 M HCl at 45 °C for 40 min with occasional stirring to partially hydrolyse the nylon. The tube was then placed in a Pharmacia AC 10/20 column and washed three times with glass distilled water at room temperature. The column was filled with 12.5% w/v glutaraldehyde in 0.5 M borate buffer pH 8.5 and 10 ml of this solution pumped through the column at a flow rate of 0.5 ml min^{-1}. 20 ml of 0.1 M sodium phosphate buffer pH 7.5 was pumped through the column at 0.5 ml min^{-1} to wash the activated nylon. 5.5 ml of 0.5% w/v protein in 0.05 M sodium phosphate buffer pH 7.5 was placed in the column containing the activated nylon and left at 4 °C for 4 hrs. Non covalently bound protein was removed by pumping 0.1 M sodium phosphate buffer pH 7.5 containing 1 M NaCl through the column at 4 °C and 1 ml min^{-1} for 1 hr, followed by buffer A.

Covalent coupling to Sepharon TM-HEMA 1000 affinity chromatography cartridge. The cartridge (Anachem, Charles Street, Luton, Bedfordshire.) contained approximately 0.5 g of a copolymer of 2-hydroxyethylmethacrylate and ethylene diamethacrylate in a macroporous spherical form of controlled particle size 40–80 μm and a mean pore diameter of 350Å, giving an exclusion limit of about 1–3 $\times$ 10^6 daltons. The specific area of HEMA 1000 is approximately 50 m^2 g^{-1}. The cartridge was injected with 5 ml of glass distilled water and swelling of the matrix occurred over the following 2 hrs. The matrix was washed with 5 ml of 0.1 M sodium carbonate buffer pH 9.5, then loaded with 5 ml of enzyme preparation (8.25 mg) in 0.1 M sodium carbonate buffer pH 9.5 and 0.5 M ammonium sulphate and then capped and stored at 4 °C. The coupling mixture was washed out of the matrix with 5 ml of glass distilled water. To block any remaining active groups 1 ml of 1 M ethanolamine was injected into the cartridge, which was capped and stored at 4 °C overnight. The matrix was then washed with buffer A prior to storage.

Covalent coupling to gutaraldehyde activated Macrosorb. Macrosorb K (GA) (Sterling Organics Ltd., Dudley, Cramlington, Northumberland.), an inorganic support of 710 μm diameter and activated with glutaraldehyde, was mixed with protein in a 1:5 and 1:10 ratio (ml Macrosorb:mg protein). The Macrosorb and protein were mixed with 50 mM sodium phosphate buffer pH 8.0 in a screw capped bottle on a Denley spiramix overnight at 4 °C. The support was then washed with buffer A.

Covalent coupling to CNBR-controlled pore glass (CPG). 0.5 g of CPG mesh size 120–200 μm, mean pore diameter 504Å, was mixed with 12.5 ml of glass distilled water in a beaker which was kept in an ice-salt bath. To this mixture, 0.5 g of ground CNBr was added very slowly maintaining the pH between 11.0–11.5 using concentrated sodium hydroxide, and the temperature below 8 °C using ice. The reaction was continued until the pH remained constant. The CPG was washed on a sintered glass filter with cold glass distilled water and then mixed with enzyme preparation (6 mg) pH 9.0 and stored overnight at 4 °C. The beads were then washed with buffer A and stored.

Covalent coupling to CPG activated with stable diazonium salt (SDZS) and hydrophobic attachment to CPG derivatised with long chain alkylamine (LCA). The CPG used (Pierce and Warringer (UK) Ltd., 44, Upper Northgate Street, Chester.) was of 125–177 μm particle size and pore diameter 500Å. Two derivatives were used; stable diazonium salt (SDZS) (No. 23749) for covalent linkage and long chain alkylamine (LCA) (No. 24875) commonly the starting material for further derivatization but here used for hydrophobic bonding. Derivatized CPG (1 g) and protein (11.5 mg) were mixed with 0.1 M sodium phosphate buffer pH 7.5 in a screw capped bottle on a Denley spiramix overnight at 4 °C and then the support washed with buffer A.

Determination of immobilised enzyme activity

Column reactor assay. This assay was the method by which all the immobilised enzyme-conjugates were assessed. Firstly dehalogenase activity was quantitatively determined and secondly conjugate tolerance to 0.5 M D,L-2-CPA pH 7.0 was evaluated. The reactor was a Pharmacia AC 20 cm × 1.0 cm (I.D.) glass column with an outer water-jacket connected to a Haake E15 thermostatic circulator (Berlin, W. Germany) to maintain 30 °C constant temperature. Immobilised enzyme-support conjugates were packed into the column and washed with 0.5 M sodium phosphate buffer pH 7.5 at 0.2 ml min^{-1}. Immobilised enzyme activity was determined by pumping 0.5 M D,L-2-CPA pH 7.0, in 0.5 M sodium phosphate buffer pH 7.5 through the column at 0.2 ml min^{-1} and measuring Cl^- concentration in the effluent using a Marius chlorocounter (Marius Instrumenten, J.C.Th. Marius N.V., Hollantlaan 18, 3526 AM Utrecht, Holland). The counter was calibrated in mmol $litre^{-1}$ using 100 mM NaCl solution and the minimum reliable titration was equivalent to 700 ppm (700 $\mu g\ ml^{-1}$) Cl^-.

Modified IC assay. This assay was used to determine the immobilised enzyme activity produced when dehalogenase enzyme was loaded onto SDZS/CPG under differing conditions. The loading experiments all produced 0.1 g of washed enzyme-SDZS/CPG conjugate in a universal bottle. The assay involved adding 5 ml of reaction mixture (1 mmol D,L-2-CPA and 0.9 mmol Tris-H_3PO_4 buffer pH 7.5) equilibrated at 30 °C, to the conjugate in the universal bottle. The bottle was shaken and placed on a Denley spiramix in a 30 °C incubator. 50 μl samples were taken at t = 0, 2.5, 5, 7.5 and 10 min, diluted 100-fold in ice-cold glass distilled water, filtered to 0.2 μm and the Cl^- content determined by ion chromatography. The resultant immobilised enzyme activity was determined for all conjugates produced after loading under a variety of conditions.

Experimental and results

Partial purification of D-2-CPA dehalogenase.

D-2-CPA dehalogenase was partially purified from crude soluble extract of *P. putida* by anion-exchange chromatography. The pooled and ammonium sulphate-concentrated DEAE-eluate had a specific activity about 15-fold greater than the crude soluble extract and contained 75% of the starting enzyme activity.

Comparison of different immobilisation methods

All carbohydrate supports collapsed within 2–4 hrs contact with 0.5 M D,L-2-CPA (Table 1). For this reason, although DEAE-Sephacel, DEAE-Sephadex A50 (adsorption) and CNBr-Sepharose 4B (covalent linkage) conjugates showed good immobilised enzyme activity with low D,L-2-CPA concentrations, they were omitted from further studies. Support collapse was also evident with alginate-entrapped enzyme. Glutaraldehyde, one of the most commonly used immobilisation agents, was unsuccessful when used to covalently attach the enzyme to Macrosorb and nylon 66. Coupling occurred but the resulting conjugate had little activity. Fractogel TSK DEAE-650 (adsorption), SDZ/CPG (covalent linkage), LCA/CPG (hydrophobic binding), and Sepharon TM-HEMA 1000 (covalent linkage) immobilised enzyme preparations all showed D-2-CPA dehalogenase activity and were resistant to 0.5 M D,L-2-CPA (4 hr contact). Of these supports SDZS/CPG produced the most active immobilised enzyme-support conjugate and was used for all further studies.

Optimisation of the immobilisation procedure

In these experiments, all immobilised enzyme preparations were washed, assayed for protein and then their activity determined using the modified IC assay.

Effect of protein loading. When considering enzyme loading onto a support there are two important factors:- (i) the amount of protein it is possible to load on (i.e. the saturation level), and (ii) the protein loading which produces the most active immobilised enzyme preparation.

Five samples of 0.1 g SDZS/CPG were mixed with 5–25 mg partially purified enzyme in coupling buffer, (K_2HPO_4/NaH_2PO_4 pH 7.5) for 40 min. The results are shown in Figs 1 & 2. The total protein present was not removed in any immobilisation mixture. With loadings of 50–150 mg protein/g SDZS/CPG about 80% of protein was bound. Saturation of the SDZS/CPG occurred at about 200 mg/g but the greatest immobilised enzyme activity (37 U g^{-1} loaded SDZS/CPG) occurred at 150 mg/g protein loading (Fig. 2). Loading was completed after 30–40 min incubation.

Table 1. Results of the survey of immobilisation methods. Immobilisation of D-2-CPA dehalogenase was attempted by a variety of protocols as described in the Materials and Methods section. The effect of these methods on enzyme activity (% conversion of substrate), and on the integrity of the supports, are recorded in the table.

Immobilisation method	% conversion at various D,L-2-CPA CONCNS.	observations
DEAE-Sephacel (adsorption)	10mM: 76% 100mM: 32% 500mM: 2%	support collapse with 500mM substrate
Fractogel TSK DEAE-650 (adsorption)	100mM: 20%	support integrity maintained
Alginate (entrapment)	10mM: 0%	immediate support collapse with 10mM
CNBr-Sepharose-4B (covalent linkage)	10mM: 90% 100mM: 42% 500mM: 2%	support collapse with 500mM
Glutaraldehyde-Nylon 66 (covalent linkage)	10mM: 88%	activity on day 1 only
Sepharon TM-HEMA 1000 affinity chromatography cartridge (covalent linkage)	100mM: 5%	no access available
Glutaraldehyde-Macrosorb (covalent linkage)	10mM: 0%	support integrity maintained
CNBr-controlled pore glass (covalent linkage)	100mM: 2%	support integrity maintained
Long chain alkylamine-controlled pore glass (hydrophobic binding)	10mM: 80% 100mM: 56% 500mM: 8%	support integrity maintained
Stable diazonium salt-controlled pore glass (covalent linkage)	10mM: 100% 100mM: 100% 500mM: 33%	support integrity maintained

Effect of temperature. This experiment was designed to investigate the rate of protein binding over a range of temperatures. The objective was to determine whether the loading time might be decreased by increasing the loading temperature, perhaps resulting in a more active immobilised enzyme preparation (Wang et al. 1979).

Five samples of 0.1 g SDZS/CPG were loaded with partially purified enzyme in coupling buffer at a 150 mg/g ratio and one at 4 °C, one at 15 °C, 25 °C, 37 °C and 50 °C over 90 min. Between 100 and 118 mg protein/g SDZS/CPG was bound in the tests. Protein was bound most quickly at 50 °C and most slowly at 4 °C. Increasing the temperature improved the rate of protein binding to the SDZS/CPG but had little effect on the total amount bound. Binding at 50 °C resulted in very little immobilised activity, presumably due to thermal inactivation of the soluble enzyme prior to coupling, and/or to the inactivation of the coupled enzyme post-immobilisation (Fig. 3). Immobilised enzyme activities of conjugates where the enzyme was bound at 4 °C, 15 °C, 25 °C or 37 °C were very similar at about 20 U ml^{-1}.

Effect of pH. The pH environment affects the charge of the amino acids on the surface of D-2-CPA dehalogenase, so that the immobilised enzyme conjugates produced at different pH may show varying activities.

Five samples of 0.1 g SDZS/CPG were loaded with partially purified enzyme at a 150 mg/g ratio for 40 min; one at pH 6.2, one at pH 7.1, and the others at pH

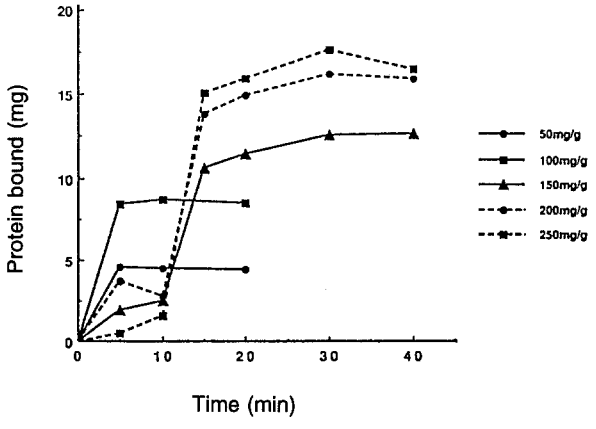


Fig. 1. Protein loading v time for a range of protein concentration. Five samples of 0.1 g SDZS-CPG were each mixed with 5 ml of partially purified D-2-CPA dehalogenase in 50 mM K_2HPO_4/NaH_2PO_4 buffer pH 7.5. Protein concentrations ranged from 50–250 mg per g of support. These test immobilisations were incubated at room temperature on a Denley spiramix and the supernatant sampled over the 40 min incubation. Supernatant samples were snap-frozen in microfuge tubes in liquid nitrogen and stored at −70 °C until assayed for protein.

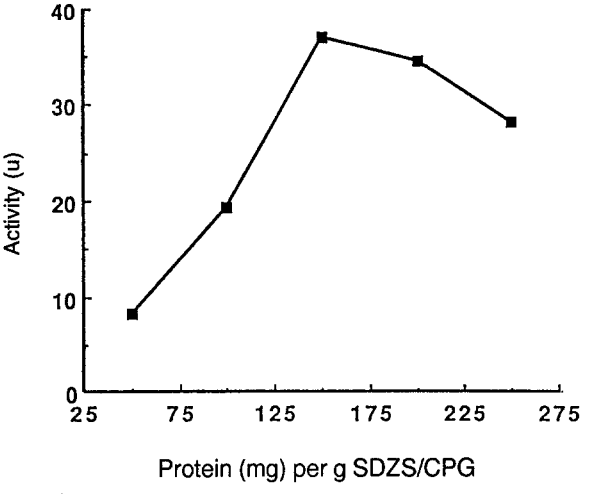


Fig. 2. Immobilised enzyme activity against protein loading ratio. Conjugates were prepared as described in the legend to Fig. 1. Immobilised enzyme activities were determined using the modified IC assay.

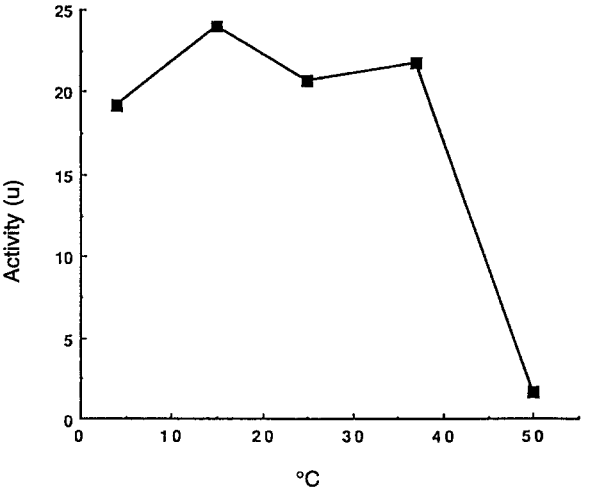


Fig. 3. Immobilised enzyme activity against loading temperature. Partially purified dehalogenase was immobilized to five samples of 0.1 g SDZS-CPG as described in the Materials & Methods section but at different temperatures covering the range 4–50 °C. Activities were determined using the modified IC assay.

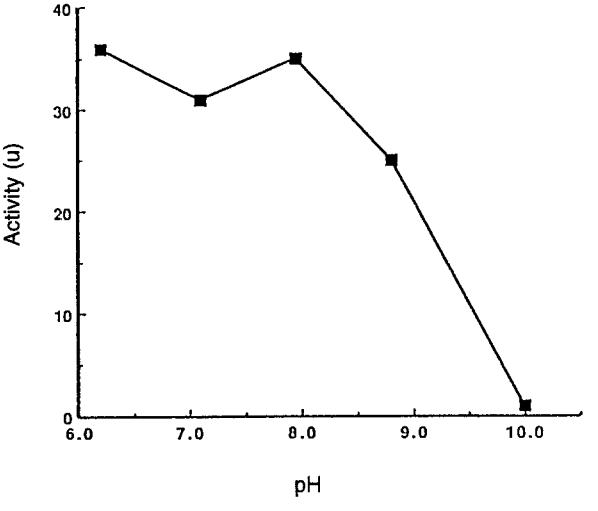


Fig. 4. Immobilised enzyme activity against loading pH. Partially purified dehalogenase was immobilized to five samples of 0.1 g SDZS-CPG as described in the Materials & Methods except that the coupling buffer was varied. The buffers used were pH 6.2 and pH 7.1 $KH_2PO_4/NaOH$, pH 7.1 NaH_2PO_4/Na_2HPO_4, pH 8.8 borate/H_3PO_4 and pH 10.0 Na_2HCO_3/NaH_2CO_3. Activities were determined using the modified IC assay.

8.0, pH 8.8 and pH 10.0. Approximately 80% of the available protein was bound in all cases except at pH 10.0 where 66% was bound. Protein was bound most slowly at pH 8.8 and pH 10.0. Immobilised enzyme activity determinations of the resultant conjugates (Fig. 4) showed that those produced at pH 6.2, pH 7.1 and pH 8.0 were more active (at about 35 U ml^{-1}) than those produced at pH 8.8 and pH 10.0 (at 25 U ml^{-1} and 2 U ml^{-1}, respectively).

From the above experiments a standard loading method was adopted for the experiments described

below. SDZS/CPG (0.1 g) was mixed with 5 ml of coupling buffer (K_2HPO_4/NaH_2PO_4 pH 7.5) containing 15 mg of partially purified enzyme, for 1 hr at room temperature.

Addition of DTT. When added to solutions to produce a 1 mM concentration, DTT provides reducing conditions which will maintain sulphydryl groups on proteins. D-2-CPA dehalogenase is thought to possess sulphydryl groups necessary for activity. A sample of 0.1 g SDZS/CPG was loaded using the standard conditions except that the coupling solution contained 1 mM DTT. The resultant conjugate had 102% of the activity of enzyme immobilised without DTT.

Effect of 20 mM D,L-2-CPA. It was thought that the presence of substrate might protect the active site during binding. A sample of 0.1 g SDZS/CPG was loaded with the usual coupling solution but additionally containing 20 mM D,L-2-CPA. The resultant conjugate had slightly less bound protein but 166% of the control activity (Table 2). The treatment thus resulted in a significant improvement in the immobilisation procedure and was adopted as part of the standard protocol in subsequent work.

Table 2. Comparison of enzyme immobilised in the presence or absence of D,L-2-chloropropanoic acid. The immobolisation procedure was as described in the Materials and Methods section.

Coupling conditions:-	no additions	20mM D,L-2-CPA added
Protein bound (mg per 0.1 g of SDZS-CPG)	10.2	9.8
Activity of conjugate (U per 0.1 g of SDZS-CPG)	18	29
% Activity expressed (U expressed per 100 U bound)	35	58
% Activity expressed (U expressed per 100 U present in coupling mixture)	23	38

Properties of soluble and immobilised enzyme

Immobilisation of an enzyme may alter its kinetic and other characteristics. The following experiments were done to investigate differences between the soluble and immobilised forms of the enzyme in terms of activity and stability over a range of conditions. Protein determinations were made using the modified Lowry method. Immobilised enzyme activity was determined using the modified IC assay using a standard amount (25 μl suspension) of conjugate in a 1 ml assay in a Universal bottle.

Effect of temperature on activity. Maximum soluble D-2-CPA dehalogenase activity was found at 45 °C with activity falling off sharply either side of the maximum. Activity was reduced to about 45% of the maximum at 30 °C and to 55% at 50 °C. Immobilised D-2-CPA dehalogenase had a broader, flattened temperature profile with maximum activity again measured at 45 °C. About 65% of the maximum activity was observed at 30 °C and 65% at 50 °C.

Effect of pH on activity. Soluble enzyme showed a sharp pH profile with a definite optimum of pH 9.0. pH values of 8.0 and 10.0 resulted in less than 50% of the maximum activity. There was a less dramatic fall in activity at pH values below the optimum than for increased pH values; at pH 11.0 no activity was observed. Immobilised enzyme had a flattened pH profile with an indistinct optimum. Maximum activity was observed at pH 9.0 but 90% of this activity was observed at pH 8.0. As with the soluble enzyme, no activity was observed at pH 11.0. However the range was extended as immobilised enzyme showed some activity at pH 5.0 which soluble enzyme did not.

K_m (apparent). Standard 1 ml asssays were started by the addition of varying amounts of soluble or immobilised enzyme to allow an accurate rate of reaction to be determined at several substrate concentrations. K_m (apparent) was calculated using the Lineweaver-Burk method. The K_m(apparent) of soluble enzyme for D-2-CPA was 4 mM and for immobilised enzyme 45 mM. The increase in K_m(apparent) as a result of immobilisation could be due to some combination of the following factors:- (i) steric hinderance by the CPG preventing the substrate coming into contact with the enzyme, (ii) diffusional limitation of the reaction, or (iii) the enzyme may have bound in a configuration making the active site inaccessible.

Substrate specificity. Stereospecificity was shown by both soluble and immobilised D-2-CPA dehalogenase (Table 3). Both forms of the enzyme were more active with bromo-substrates than chloro-substrates, which

Table 3. Substrate range of soluble and immobilised D-2-CPA dehalogenase. Standard 1 ml IC assays containing 0.1 mmol of substrate were started by the addition of soluble or immobilised enzyme. Samples (50μl) were removed at t=0, 2, 4, 6 and 8 min, dilluted 100-fold and the rate of reaction calculated from rate of release of CL^- or Br^-. Rates are expressed as % of the rate with D,L-2-CPA (5.3 U/mg protein for soluble enzyme and 3.1 U/mg protein for immobilised enzyme.

Substrate	soluble enzyme	immobilised enzyme
D,L-2-chloropropionate	100	100
L-2-chloropropionate	0	0
D,L-2-bromopropionate	363	218
bromoacetate	405	247
chloroacetate	140	67
2,2-dichloropropionate	7	0
2-chlorobutyrate	3	0
2-bromobutyrate	13	28
2,3-dichloropropionate	0	0
3-chloropropionate	0	0
2-chloropropionamide	0	1
chloroacetaldehyde	0	0
bromoethanol	0	0

was expected due to the decreased electronegativity of Br^- compared with Cl^- making Br^- more easily removed than Cl^- (Smith et al. 1990). The decrease in activity with increasing number of carbon atoms in the molecule is a noticeable trend with soluble enzyme and to a lesser extent with immobilised enzyme i.e. bromoacetate, bromopropionate and bromobutyrate are listed in order of increasing number of carbon atoms and decreasing enzyme activity. The halogen must be in the 2-carbon position in order to be removed and the presence of an extra halogen severely reduced activity; there was no soluble enzyme activity and only 7% of immobilised enzyme activity with 2,2-dichloropropionate. The second halogen probably causes steric problems to the substrate inserting into the active site cleft. An absence of activity with bromoethanol indicated the necessity of a terminal carboxyl group for dehalogenation of molecules by this enzyme.

Temperature stability. Soluble and immobilised enzyme were incubated in 200 mM Tris-H_3PO_4 buffer pH 7.5 over a range of temperatures between 30 °C and 50 °C. Samples were taken over 600 min and 1440 min for soluble and immobilised enzyme respectively and assayed at 30 °C.

Both forms of the enzyme were most stable at 30 °C with soluble enzyme having a half-life 6-fold greater at 30 °C than at 35 °C and immobilised enzyme half-life 2.5-fold greater than at 35 °C. The half-life of immobilised enzyme at 30 °C was about 2500 min compared with a soluble enzyme half-life of about 1200 min indicating that immobilisation had a significant stabilising effect on D-2-CPA dehalogenase to temperature inactivation.

pH stability. Soluble and immobilised forms of the enzyme were incubated at 30 °C over a range of pH values from pH 4.0 to pH 9.0 (succinic acid-NaOH or phosphate buffers) for 1500 min. Samples were taken and assayed at pH 7.5.

Soluble enzyme incubated at pH 6 and pH 7, and immobilised enzyme incubated at pH 8 were so stable the half-lives could not be determined over the time-course of this experiment. The pH requirements for retention of activity were very different for soluble and immobilised enzyme. Soluble enzyme was most stable between ph 6 and pH 7, immobilised enzyme between pH 7 and pH 9. At pH 9 the half-life for immobilised enzyme was 3500 min and for soluble enzyme was 20 min whereas at pH 6 immobilised half-life was about 150 min and soluble half-life was too long to be determined. This change in pH stability of D-2-CPA dehalogenase due to immobilisation is a possible advantage for use of the conjugate in a bioreactor as it could be operated at pH 8–9 which confers maximum stability and also maximum activity.

Stability to 1 M D,L-2-CPA. Soluble enzyme was incubated at 30 °C in 1 M buffer pH 7.5 and 1 M D,L-2-CPA pH 7.0 for 420 min. Samples were taken at intervals and assayed in the usual asssay but with 100 μmol D,L-2-bromopropionate as the substrate. After 2–3 hrs in the presence of 1 M substrate half of the original activity of the soluble enzyme remained. This value was important as an indicator of the performance of soluble enzyme with 1 M D,L-2-CPA pH 7.0. Immobilised enzyme performance with D,L-2-CPA was investigated in the bioreactor experiments described below and appropriate comparisons made.

A comparison of plug-flow and stirred batch bioreactor configurations

Previous experiments had shown pH 8.0 and 30 °C to be suitable operating conditions for the immobilised enzyme. At pH 8.0 and 30 °C the preparation showed 90% of maximum activity with no detectable loss of activity on storage for 27 h. Although enzyme activity was two-fold higher at 40 °C, longevity was considered of greater importance as lower activity could be overcome with increased enzyme or longer residence time of the substrate in the reactor. 30 °C was therefore chosen as the operating temperature.

Protein loss. Suitability of the SDZS/CPG-immobilised enzyme for use in a batch reactor was tested on a small scale by placing 25 μl conjugate and 975 μl of coupling buffer, pH 7.5 in a screw-capped Universal bottle and shaking overnight in a water bath. The supernatant was assayed for protein content which proved negative showing that shearing had not occured.

Diffusional effects. When an enzyme has been immobilised the substrate has to diffuse from the bulk solution through the film of stationary liquid on the surface of the support, and if the support is porous, on into the pores. Internal pore diffusional limitation has also been found with several enzymes immobilised to porous glass (Rovito & Kittrell 1973 and Marsh et al. 1973). The following experiments were designed to investigate the effects of external mass transfer limitation and internal pore diffusion limitation. The flow rate was varied for plug flow reactors and stirrer speed for batch reactors to observe the effect of external mass transfer limitation on enzyme activity reaction rate.

Plug flow reactor flow rate was varied from 0.16–1.96 ml min^{-1} and a graph of specific activity against flow rate was plotted for 200 mM, 400 mM, 600 mM and 800 mM D,L-2-CPA. Specific activity increased with increased flow rate up to a maximum of about 1.2 U mg^{-1} suggesting that external mass transfer limitation was occuring at low flow rates. Higher flow rates were required with 600 mM and 800 mM D,L-2-CPA than with 200 mM and 400 mM D,L-2-CPA to achieve the same specific activity. This need for increased flow rates with the higher D,L-2-CPA concentrations was probably an indication of product inhibition of enzyme activity.

The batch reactor was stirred by a two-bladed impeller and motor speeds were calibrated from 390 to 1290 rpm using a digital tachometer and reflective tape on the impeller shaft. A graph of μmol Cl^- against time was plotted for a range of stirrer speeds with immobilised enzyme and 200 mM D,L-2-CPA and the slopes calculated by linear regression. There was no obvious increase in rate of reaction with increased stirrer speed indicating that the minimum stirring speed possible with this experimental equipment was sufficient to remove the Nernst external layer responsible for diffusional limitation. A stirrer speed of 495 rpm was used for the following batch bioreactor experiments.

Table 4. Operational stability of immobilised enzyme. Half-livess ($t_{0.5}$) for SDZS-CPG immobilised enzyme in batch and plug-flow reactors were calculated from specific activity v. time curves as described in the text. The values in the table are in minutes.

Reactor Type:- D,L-2-CPA (mM)	Plug-flow	batch
200	360	200
400	420	175
600	300	220
800	260	120

Half-life. The plug flow bioreactor was operated at maximum flow rate and the % conversion of D-2-CPA to L-lactate, H^+ and Cl^- monitored by Cl^- production using the Chlorocounter. For each D,L-2-CPA concentration the flow rate was decreased stepwise until maximum conversion was obtained. To obtain half-life values ($t_{0.5}$) for the immobilised enzyme in the plug flow reactor (with 200 mM, 400 mM, 600 mm and 800 mM D,L-2-CPA), reaction mixture was pumped into the reactor at the rate which allowed maximum conversion of substrate to product and the % conversion noted over 24 hr. The specific activity against time curve was used to read off the $t_{0.5}$ (Table 4).

The batch reactor was operated with 50 ml of reaction mixture and at a stirrer speed of 495 rpm. The reaction mixture was added to the immobilised enzyme to start the reaction. The % conversion of substrate to product was followed by monitoring Cl^- production with time. After 1 hr the immobilised enzyme was allowed to settle and the supernatant was decanted off. Immobilised enzyme was used for 4 × 1 hr cycles, one enzyme batch for each D,L-2-CPA concentration. % conversion was plotted against time and specific activity determined by linear regression. The specific

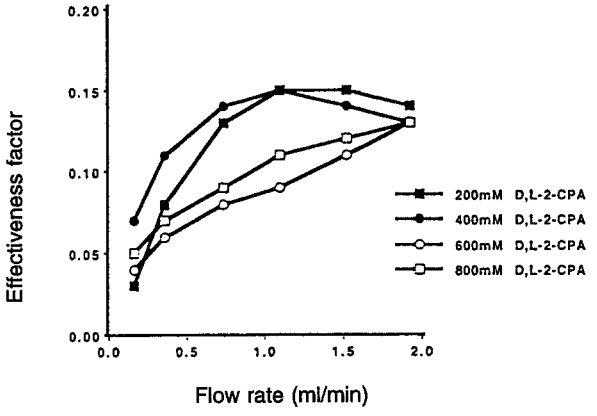


Fig. 5. Plug flow reactor: effectiveness factor v. flow rate for 200–800 mM D,L-2-CPA. Effectiveness factors were calculated for the first hour of the reaction from specific activity v. flow rate data using the formula given in the text.

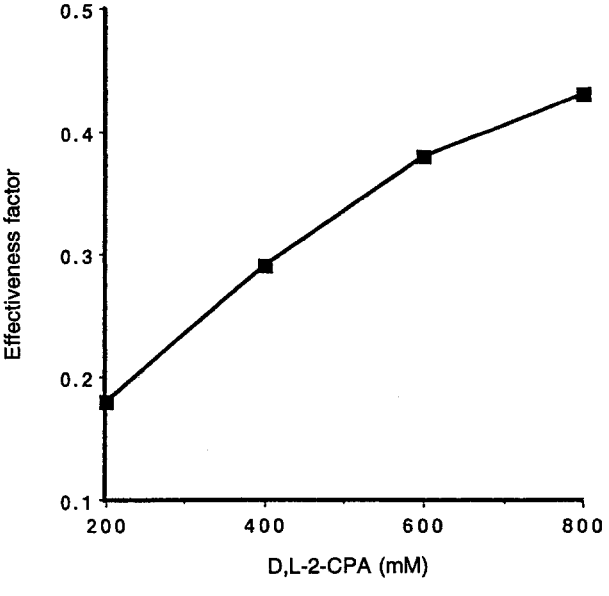


Fig. 6. Batch bioreactor: effectiveness factor v. D,L-2-CPA concentration. Effectiveness factors were calculated for the first hour of the reaction from specific activity v. D,L-2-CPA data using the formula given in the text.

activity values were then plotted against run number (hr) and $t_{0.5}$ read off the curve (Table 4).

Effectiveness factor. The effectiveness factor, which is the ratio of the reaction rate with and without pore diffusional effects, was calculated for immobilised enzyme under various conditions, in the plug flow and batch bioreactors using the equation:-

$$V = \pi \cdot V_{max} \cdot \frac{S}{K_m + S} \qquad (1)$$

where; V = specific activity (U mg^{-1})
V_{max} = maximum specific activity (U mg^{-1})
S = initial substrate concentration (mM)
π = effectiveness factor (0–1)
K_m = Michaelis constant (mM)

The values are given in Figs 5 & 6.

Discussion

Loading experiments were directed at gaining knowledge of the effects on immobilised enzyme activity of protein spacing, temperature/time relationships in loading, pH, and substrate presence (cf. Mosbach 1976; Garcia III et al. 1989). Maximum immobilised enzyme activity was observed at a loading of 150 mg protein/g SDZS-CPG although this was not the saturation point for the SDZS-CPG. The optimisation pattern obtained for protein loading involves attaching enzyme up to the point where the extra protein begins to sterically hinder the interaction between the enzyme and the substrate, observed as a reduction in enzyme activity. Fadda et al. (1989) observed a similar effect with β-glucosidase bound onto alumina where activity increased with increasing protein loading up to a maximum of 8 mg protein/g alumina and then decreased. Diffusional limitation may also be important. Carleysmith et al. (1980) found the optimisation of protein loading to be important because the penetration depth of the protein, and thus the diffusional limitation of the reaction rate, could be controlled by the conditions of preparation of the immobilised enzyme.

D-2-CPA dehalogenase was loaded onto SDZS-CPG most successfully at pH 6–8. Diazo coupling is one of the most stable linkages (Weetall 1976). Diazo linkage occurs most favourably at mildly alkaline pH values usually through a phenolic residue such as tyrosine (Adamich et al. 1978). However, other factors must be involved in linkage as Pifferi et al. (1989) found most binding via diazo linkages at lower pH values. At pH 8.5 and above imidazole groups as well as phenolic groups can be modified with the diazo linkage (Srere & Uyeda 1976).

Immobilised enzyme activity was substantially increased when D-2-CPA dehalogenase was bound to the SDZS-CPG in the presence of 20 mM D,L-2-CPA. A similar effect was observed by Naoi et al. (1978)

when D-aminoacid oxidase was complexed with benzoate before immobilisation. A preparation of enzyme-substrate or enzyme-benzoate complex protected the substrate binding site and the enzyme conformation during binding of that enzyme to an immobilisation support resulting in higher immobilised enzyme activity.

Stability to temperature and to mildly alkaline conditions (pH 8–9) were significantly improved by conjugation to controlled-pore glass allowing the immobilised preparation to be tested in batch and plug-flow reactors. Others have reported that immobilisation improves the stability of enzymes such as alcohol dehydrogenase (Johansson & Mosbach 1974) and phospholipase A2 (Adamich et al. 1978). In the case of phospholipase A2, the enzyme had only 1% of its original activity when bound to porous glass beads. Immobilised enzyme which has a relatively low percentage expressed activity will probably have its active site obscured in some way and this may prove protective, providing enhanced storage and operational stability. In the present study, improved stability to temperature and alkaline pH was achieved in immobilised preparations expressing a high % of activity.

Plug-flow $t_{0.5}$ values were consistently higher than batch values (Table 4). However, in the plug flow reactor the immobilised enzyme is not uniformly exposed to the substrate and often some of the enzyme is not fully active. Initially immobilised enzyme at the near end of the column will convert the substrate to product and the immobilised enzyme at the far end of the column will experience only a low concentration of substrate. As the near end enzyme becomes inactive the far end enzyme will convert the substrate to product; the half-life of the column will reflect this complicated situation. Moreover, in the plug flow reactor the enzyme was performing under conditions of external mass transfer limitation whilst in the batch reactor this was probably not so. In the batch reactor all the immobilised enzyme will be exposed to the full concentration of D,L-2-CPA.

The plug flow bioreactor effectiveness factor values increased with increasing flow rate as the external mass transfer diffusional limitation decreased (Fig. 5). At flow rates above 1 ml min^{-1} diffusional effects were insignificant. The batch reactor effectiveness factor for 200 mM D,L-2-CPA was about the same as that obtained in the plug flow reactor at flow rates of 1ml min^{-1} and above. However, in the batch reactor higher substrate concentrations produced greater effectiveness suggesting that internal pore limitation might be overcome with increased substrate concentration (Fig. 6).

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