Journal of Chromatography A, 659 (1994) 275–287
Elsevier Science B.V., Amsterdam

CHROM. 25 641

Novel affinity separations based on perfluorocarbon emulsions

Use of a perfluorocarbon affinity emulsion for the direct extraction of glucose-6-phosphate dehydrogenase from homogenised bakers' yeast

Graham E. McCreath* and Howard A. Chase

Department of Chemical Engineering, University of Cambridge, Pembroke Street, Cambridge CB2 3RA (UK)

Christopher R. Lowe

Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT (UK)

(First received July 22nd, 1993; revised manuscript received October 12th, 1993)

ABSTRACT

A perfluorocarbon affinity emulsion has been generated by homogenisation of perfluorodecalin with poly(vinylalcohol) and with subsequent cross-linking and derivatisation with Procion Red H-E7B. This affinity emulsion was tested for its applicability in direct extraction. The affinity emulsion exhibited limited fouling when contacted with a crude homogenate of bakers' yeast (*Saccharomyces cerevisiae*) and could be washed clear from cell debris using an aqueous buffer. Adsorption isotherm experiments showed that the capacity of the affinity emulsion for glucose-6-phosphate dehydrogenase (G6PDH) was not severely affected by the presence of whole yeast cell and cell debris. Two different contacting techniques were examined for G6PDH purification directly from a yeast homogenate. The first technique, expanded bed affinity chromatography (EBAC), which is an essentially batch operation, was carried out under conditions optimised through frontal analysis and was compared with a second technique, PERCAS (perfluorocarbon emulsion reactor for continuous affinity separations). G6PDH could be successfully purified using both techniques with an average purification factor of 18 directly from a crude homogenate. An analysis of system productivity showed that PERCAS had a productivity of some 2.25 times higher than expanded bed affinity chromatography under similar process conditions.

INTRODUCTION

While affinity chromatography is now well established as a popular unit operation for the purification of proteins, its position in the downstream processing flowsheet has frequently not been optimised. Classically, affinity chromatography has been reserved as one of the final stages of purification [1] even when it is obvious that the inclusion of a highly selective purification step early in the sequence of separation would generate more favourable process economics as greater step yields would be achieved with fewer steps necessary downstream [2–4]. Often, the implementation of affinity chromatography as one of the final steps used is due to the expense

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E1084-D

and lability of the affinity media [5] and the need for prior removal of particulates [3]. However, although the use of ultrastable matrices, linkage chemistries and affinity ligands may circumvent some of these problems [6,7]; even with ultrastable affinity media, it is not usually possible to use the technique early on in the process. The presence of particulate matter, be it whole cells or cell debris, totally prohibits the use of conventional packed bed techniques and hence early use of an affinity chromatography step requires practical innovations in the area of contactor design. For example, as an alternative to using a packed bed with prior clarification of the feed stock, affinity chromatography has recently been carried out using an expanded (liquid fluidised) bed of an adsorbent designed for conventional packed bed operation, Blue Sepharose Fast Flow [3]. Alternatively, affinity chromatography has been carried out in stirred tanks [8,9]. The physical nature of the adsorbent has also been changed and the use of affinity partitioning [10] and reversed micelles [11] is well documented.

We have recently described the development of a novel affinity support based on perfluorocarbon emulsions stabilised by an adsorbed layer of polymeric surfactant previously derivatised with affinity ligands and subsequently cross-linked *in situ* [12]. These emulsions were used successfully for the purification of human serum albumin from plasma in an expanded bed [13]. We later showed that continuous affinity chromatography is possible using perfluorocarbon emulsions and a four stage mixer-settler, a system we have named PERCAS (perfluorocarbon emulsion reactor for continuous affinity separations) [14]. In this paper we compare both techniques, expanded bed affinity chromatography (EBAC) and PERCAS in the direct extraction of glucose-6-phosphate dehydrogenase (G6PDH) from unclarified homogenised bakers' yeast using a perfluorocarbon affinity emulsion incorporating the reactive dye Procion Red H-E7B. Liquid perfluorocarbon emulsions have certain advantages over some of the techniques mentioned above for direct extraction in that they are inherently transportable by pumping due to their liquid nature and they have a high density resulting in rapid phase separation under gravity. They are also, chemically, very stable and hence are able to withstand cleaning and sterilisation. The purification of an enzyme from yeast cell homogenates was chosen as a model system showing the typical features of direct extraction. G6PDH was chosen as the target enzyme as it is used widely in clinical analysis for both glucose and hexokinase determinations [15] and purification protocols based on triazine dyes have been published previously [16–19].

EXPERIMENTAL

Materials

Poly(vinylalcohol) (PVA) (M_r 115 000, 100% hydrolysed) was purchased from Aldrich (Gillingham, UK) as was triethanolamine–HCl and magnesium chloride. Perfluorodecalin (Flutec PP6) was obtained from ISC Chemicals (Avonmouth, Bristol, UK). Non-ionic fluorosurfactant (Zonyl FSN) was obtained from E.I. du Pont de Nemours & Co. (DE, USA). Bakers' yeast was purchased in the form of pressed blocks from a local supplier. All other chemicals and reagents were purchased from Sigma (Poole, UK). Masterflex peristaltic pumps (Cole-Palmer, UK) were used for the fluidisation experiments and were also used, partly, as delivery pumps to PERCAS, the remaining delivery and extraction pumps were a Pharmacia P-1 (Pharmacia Biotechnology, Milton Keynes, UK) and a Minipuls II (Gilson, France).

Synthesis of Procion Red H-E7B perfluorocarbon affinity emulsion

A solution of PVA (M_r 115 000, 100% hydrolysed, 40 mg/ml, 200 ml) was added to perfluorodecalin (90 g) and homogenised (Ultra-Turrax T-25, half speed, 1 min) in a conical flask. The emulsion suspension was then made to a total volume of 900 ml by the addition of distilled water and heated to 40°C while stirring; HCl (5 *M*, 64 ml) was added and stirring continued for 20 min followed by the dropwise addition of glutaraldehyde [25% (w/v), 0.5 ml], the suspension was stirred for a further 2 h to allow cross-linking. Following cross-linking, the emulsion phase was allowed to settle and the supernatant decanted; the emulsion was washed

(5×) with distilled water (300 ml) and then transferred to a 500-ml conical flask containing a solution of PVA (M_r 115 000, 100% hydrolysed, 20 mg/ml, 40 ml) and HCl (5 *M*, 20 ml). Glutaraldehyde [25% (w/v), 0.2 ml] was then added and the emulsion suspension stirred for 20 min after which dilute HCl (0.17 *M*, 380 ml) was added and the suspension was stirred rapidly for a further 2 h. The emulsion was allowed to settle and the top phase decanted and replaced with distilled water to a final volume fraction of 50%. The emulsion suspension was transfered to a Buchner flask where it was vigorously degassed for 1 h after which the settled emulsion was washed (10×) with distilled water (200 ml) and finally suspended as a 50% emulsion phase volume fraction in distilled water.

Washed emulsion suspension (120 ml, 50% emulsion phase volume fraction) was added to a solution of Procion Red H-E7B (3.5 g, 80 ml) and heated to 65°C for 20 min with mixing after which NaOH (5 *M*, 10 ml) was added and mixing continued for 3 h. The emulsion was allowed to settle and washed with distilled water until washings were clear of free dye as determined spectrophotometrically. The emulsion was additionally washed (2 ×) with a solution of Zonyl FSN (100 ml, 0.01% in distilled water) to displace any remaining unattached dye and finally it was washed with distilled water (5 × 200 ml) before being suspended as a 50% emulsion phase volume fraction in distilled water.

Homogenisation of bakers' yeast

Glass ballotini (0.50–0.59 mm, 30 ml) were de-ashed by adding to a solution of concentrated nitric acid (100 ml) followed by stirring overnight. The beads were then vacuum washed with concentrated nitric acid (100 ml) followed by distilled water (1000 ml) and 0.1 *M* triethanolamine–HCl, pH 8.0 (200 ml). Bakers' yeast (4.5 g, net mass) was added to ice-cold 0.1 *M* triethanolamine–HCl, pH 8.0 (4.5 ml) and mixed; to this suspension was added phenylmethylsulphonyl fluoride (PMSF) to 1 m*M* and β-mercaptoethanol to 5 m*M*. Ice-cold de-ashed glass ballotini (9 ml) were then added and the whole suspension further cooled on melting ice (4°C) for 30 min. The suspension was vortex mixed (Vortex Genie 2, full speed) for a total vortexing time of 10 min carried out intermittently (1 min vortexing, 1 min cooling on melting ice). After this time the supernatant (8.0–8.5 ml) was removed and the ballotini washed with ice-cold extraction buffer (0.1 *M* triethanolamine–HCl, pH 8.0, 1 m*M* PMSF, 5 m*M* β-mercaptoethanol, 2 ml). The wash was then added to the previously collected supernatant to produce what will now be referred to as stock homogenate. In experiments involving the use of whole homogenates, the stock homogenate was used directly or after dilution with extraction buffer. In experiments involving cell free extracts, the stock homogenate was centrifuged (Eppendorf, 8800 *g*, 10 min) and filtered (0.22 μm) before use.

Protein determination and G6PDH assay

Protein determination was carried out with the Pierce Coomassie protein assay reagent using BSA as a standard. Calibrations were performed on stock solutions whose initial concentrations were determined at 280 nm assuming an extinction coefficient of 0.66 ml mg^{-1} cm^{-1} for BSA [20]. Spectrophotometry was carried out using a Shimadzu UV-160A spectrophotometer (VA Howe, Oxon, UK). Serial dilutions of stock solutions (20 μl) were incubated with assay reagent (1 ml) for 10 min at room temperature. The absorbance was then read at 595 nm against a buffer blank in order to prepare a standard curve. In order to determine the total protein concentration in homogenates, dilutions (1 in 50–1 in 100) of stock homogenate were taken and centrifuged (Eppendorf, 8800 *g*; 10 min) and the supernatant assayed. By carrying out dilutions on stock homogenates, the variation in apparent concentration between unclarified and clarified solutions caused by the presence of cells/cell debris could be determined.

G6PDH was assayed by adding enzyme solution (33 μl) to a 1-ml disposable polystyrene cuvette containing the following solutions: 0.1 *M* triethanolamine–HCl (pH 8.0) (870 μl), 0.2 *M* $MgCl_2$ (33 μl), 38.5 m*M* glucose-6-phosphate in ice-cold 5 m*M* potassium phosphate buffer (pH 7.4) (33 μl) and 13 m*M* $NADP^+$ in ice-cold 5 m*M* potassium phosphate buffer (pH 7.4) (33

μl). Enzyme activity was measured spectrophotometrically (20°C) at 340 nm by monitoring the reduction of $NADP^+$ to NADPH. Results are expressed in units of enzyme activity where 1 unit is defined as that amount of enzyme needed to convert 1 μmol of substrate to product in 1 min at 20°C.

Determination of the degree of fouling of the affinity emulsions

Aliquots (1 ml) of settled Procion Red H-E7B perfluorocarbon affinity emulsion and stock yeast cell homogenate were added to each of 3 test tubes. The tubes were mixed by rotary tumbling for 1 h, after which the emulsions were allowed to settle under gravity (1 min) before the supernatant was carefully removed. A sample of wash buffer (100 m*M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0) was then added to each tube to generate emulsion volume fractions of 50, 33 and 20%, respectively. The tubes were mixed for 10 min, the emulsion was then allowed to settle and the supernatant removed. Wash buffer was added to the settled emulsion to generate the original emulsion volume fraction and the emulsions were then re-washed. This procedure was carried out until the emulsions had been washed 6 times. After the last wash, 1 ml of a solution containing ethanol (60%), NaOH (0.5 *M*) and urea (4 *M*) was added to each tube. The tubes were again mixed for 30 min at room temperature, after which the emulsions were allowed to settle and the supernatant removed. The absorbance of samples of each of the collected supernatants was measured at 600 nm, and this was taken as an indication of the turbidity of the solution. Where necessary, dilutions were taken so that the absorbance was less than 1 AU.

Equilibrium adsorption isotherms

Aliquots (0.5 ml) of settled Procion Red H-E7B perfluorocarbon affinity emulsion were added to a series of 1.5-ml Eppendorf microtest tubes. The emulsions were washed (3×) with 50 m*M* triethanolamine–HCl, pH 8.0 after which the supernatant was removed. To each set of test tubes was added serial dilutions of unclarified or clarified bakers' yeast homogenate (0–6.5 U/ml, 1 ml). The test tubes were mixed in a rotary mixer for 1 h after which the emulsions were allowed to settle (1 min) and the supernatant assayed for both total protein and specifically for G6PDH activity.

Frontal analysis of G6PDH in an expanded emulsion bed

The experimental apparatus for expanded bed affinity chromatography is shown in Fig. 1. The system was arranged around a 2 cm I.D. column (BDH, UK) fitted with a hemi-spherical inlet and P160 sintered disc (average pore size 160 μm) which acted as a distributor. Two delivery pumps were used connected through a 2-way valve which allowed the pumps to be used simultaneously, one for delivery and one for priming with feed solution or eluent. When expanded bed experiments were carried out, the head space between the top of the bed and the outlet was kept to a minimum (2–3 cm) to minimise the dead volume of liquid within the column. A mesh at the top of the column before the outlet ensured no elutriation of smaller emulsion droplets took place if the bed were to over expand.

Procion Red H-E7B perfluorocarbon affinity emulsion was poured into the column to give a settled bed height of 3.4 cm (10.7 ml). The emulsion was fluidised with buffer (50 m*M* triethanolamine–HCl, pH 8.0) at a flow-rate of

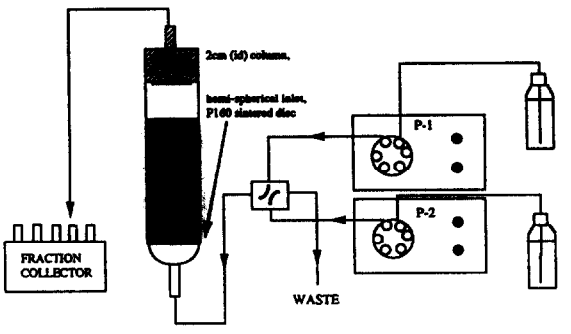

Fig. 1. Experimental apparatus used for expanded bed affinity chromatography of G6PDH using Procion Red H-E7B perfluorocarbon affinity emulsion. P-1 and P-2 are pumps.

5.75 ml/min (108 cm/h) to give a stable expanded bed height of 10.2 cm (31 ml). Stock yeast homogenate was diluted with buffer (50 m*M* triethanolamine–HCl, pH 8.0) to give 70 ml of a solution containing 9.4 mg/ml protein and 2.98 U/ml G6PDH. During the experiment, the flow-rate of solution to the column was progressively decreased to maintain a constant degree of bed expansion; the increased viscosity and density of the homogenate solutions decreases the terminal velocity of the emulsion droplets and hence to avoid elutriation of the emulsion, the superficial velocity must be decreased. The initial superficial velocity (108 cm/h) was decreased to 26 cm/h towards the end of the experiment. Fractions (2.2 ml), collected throughout the experiment, were immediately stored on ice and were subsequently assayed for turbidity, protein concentration and G6PDH concentration.

G6PDH purification in an expanded emulsion bed

Procion Red H-E7B perfluorocarbon affinity emulsion was poured into the column described above to give a settled bed height of 3.25 cm (10.2 ml). The emulsion was fluidised with buffer (50 m*M* triethanolamine–HCl, pH 8.0) at a flow-rate of 5.75 ml/min (108 cm/h) to give a stable expanded bed height of 9.8 cm (30.1 ml). Stock yeast homogenate (diluted with buffer, 50 m*M* triethanolamine–HCl, pH 8.0) (11.8 mg/ml protein, 2.71 U/ml G6PDH, 5 ml) was then pumped onto the expanded bed at the same flow-rate. Non-bound components were washed from the bed using 50 m*M* triethanolamine–HCl, pH 8.0. Elution of bound components was carried out firstly with 0.27 *M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0 and secondly with 0.88 *M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0. The eluent was washed from the bed using 50 m*M* triethanolamine–HCl, pH 8.0. Fractions (5 ml) collected throughout the experiment were assayed for turbidity, protein concentration and specific G6PDH activity. Fractions from the eluent washing stage were also measured for conductivity (Schott, Konduktometer CG 855).

Continuous G6PDH purification directly from a bakers' yeast homogenate using PERCAS

A description of PERCAS, and its use in the continuous purification of serum albumin from human plasma, has been reported in detail previously [14]. The basic operation is shown in Fig. 2, the system consists of four mixer-settlers arranged in series and in a loop. Perfluorocarbon affinity emulsion travels continuously through the system where it is contacted at different stages with different solutions, *i.e.* adsorption solution, wash buffer, eluent and re-equilibration buffer. In each stage, separation of emulsion from the bulk liquid phase is carried out by settling under gravity in the settler tanks. These tanks are separated from the mixing tanks by a weir, and the settled emulsion is transported by peristaltic pumps to the next stage.

PERCAS, containing Procion Red H-E7B perfluorocarbon affinity emulsion (35 ml, settled volume) was equilibrated at the flow-rates and stream compositions shown in Table I. After 1 h equilibration, the input to stage 1 (adsorption) was step changed from buffer to stock bakers' yeast homogenate (12.19 mg/ml protein, 2.29 U/ml G6PDH) at the same flow-rate. The experiment was carried out for 4 h with aliquots (1 ml) being removed from the top of each settler every 15 min. The collected fractions were stored on ice until analysis. Three sets of analysis were carried out; firstly, biomass profiles were determined by assaying the fractions spectrophotometrically (600 nm) diluting where necessary to obtain readings of less than 1 AU. Fractions collected from stage 1 (flow through) and stage 3

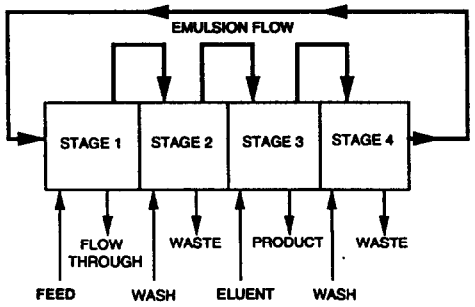

Fig. 2. Diagramatic representation of PERCAS operation as described in text.

TABLE I

PERCAS STREAM COMPOSITIONS

Operational set-up of PERCAS using Procion Red H-E7B perfluorocarbon affinity emulsion for the continuous direct extraction of G6PDH from homogenised bakers' yeast.

Stage	Buffer concentration (*M*)	NaCl concentration (*M*)	Flow-rate (ml/min)	Emulsion volume fraction (%)
Adsorption	0.1	0.1	1.21	46
Washing	0.1	0.35	3.41	23
Elution	0.1	2.0	0.84	55
Washing	0.1	0	3.86	21

(elution) were assayed for total protein and G6PDH activity, and appropriate dilutions were carried out where necessary.

RESULTS AND DISCUSSION

Generation of flocculated Procion Red H-E7B perfluorocarbon affinity emulsions

In our previous reports of the preparation of perfluorocarbon affinity emulsions incorporating C.I. Reactive Blue 4 [13] or C.I. Reactive Blue 2 as affinity ligands [14], the emulsions were prepared by first derivatising the polymeric surfactant with the ligand followed by homogenisation with perfluorodecalin. However, the presence of two equally reactive triazine rings on Procion Red H-E7B precluded the use of this technique as PVA was precipitated from solution during derivatisation. To overcome this problem, the emulsion floccule was prepared first and then derivatised with dye–ligand in much the same way as a solid support. After homogenising the PVA with perfluorodecalin the emulsion was cross-linked firstly at low emulsion volume fraction (5%) to ensure emulsion stability and secondly at high emulsion volume fraction (50%) to generate floccules. After this, the emulsion was stirred in dilute HCl at a low emulsion phase volume fraction (14%) to allow any more stabilising cross-links to form on the inside of the floccule. The ability to generate affinity emulsions in this way opens up possibilities for immobilising a range of ligands. For example, Protein A could be immobilised for the purification of IgG and enzymes could be immobilised in a similar fashion to generate new supports for use in biotransformations, which would be resistant to attrition.

Washing of the affinity emulsion was carried out first using water to remove bulk unreacted dye and secondly with a solution of non-ionic fluorosurfactant (Zonyl FSN, 0.01%) to displace any dye adsorbed to the surface of the perfluorocarbon oil though gaps in the PVA layer. The Zonyl FSN wash was successful in removing unreacted dye as subsequent storage of emulsions in distilled water, Zonyl FSN (0.2%) and NaOH (0.5 *M*) have revealed no further evidence of dye leakage. Under microscopic examination, the affinity emulsions appeared similar to those described previously [14] with the same range of floccule diameters (100–150 μm).

Homogenisation of bakers' yeast

Vortexing bakers' yeast with ballotini routinely produced soluble protein concentrations in stock homogenates of approximately 30 mg/ml (before washing or dilution) for a 50% (w/v) yeast suspension.

Emulsion fouling

In order that a selective adsorbent can be used for direct extraction, it must be shown that non-specific adsorption of contaminating substances is minimal. Non-specific adsorption may occur on both the ligand and the support matrix. Therefore, an assessment of the degree of fouling of adsorbents is a prerequisite to their

possible application in direct extraction. In this experiment perfluorocarbon affinity emulsion was contacted with a stock homogenate solution for 1 h, after which the samples of the emulsion were washed with a solution of aqueous buffer. Increasing volumes of buffer were added to generate progressively lower emulsion phase volume fractions. In the operation of the PERCAS unit, phase volume changes can be made by increasing or decreasing the appropriate input flow-rates to generate higher or lower emulsion phase volume fractions. The results from this experiment are shown in Fig. 3 where it can be seen that increasing the wash buffer volume fraction from 50 to 80% decreases the number of washes required to obtain a baseline absorbance from 6 to 2. It is evident therefore, that the most efficient washing in PERCAS will be carried out using a low emulsion phase volume fraction as this leads to maximum dilution of contaminants in the system.

When the emulsions were washed with a solution containing ethanol (60%), NaOH (0.5 *M*) and urea (4 *M*) there was little evidence of release of adsorbed components as assessed by measuring the absorbance of the supernatant at 600 nm, assuming that the release of adsorbed components would increase the absorbance of the solution at that wavelength. In each of the three test tubes the absorbance (at 600 nm) was 0.06–0.08 which does indicate that very little material had adsorbed and subsequently been released by this cocktail. More importantly, there was no evidence to suggest that dye leakage had occurred even after incubation in the cocktail for 72 h at room temperature.

Equilibrium adsorption isotherms

Adsorption isotherms were carried out to ascertain if the presence of suspended cellular material influenced the adsorption of G6PDH from a crude preparation. Hence, both unclarified and clarified homogenates were used. Fig. 4 presents the results from this experiment where it can be seen that the presence of cell debris has little effect at low concentration but has a slight effect at higher concentrations. The parameter q_m (maximum adsorption capacity) was determined for both systems by using a least-square fit analysis of a linearised form of the Langmuir equation [21]. In the case of the clarified homogenate q_m for G6PDH was equal

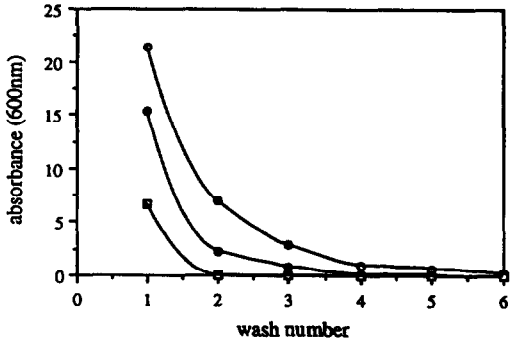

Fig. 3. Cell debris washout profiles for Procion Red H-E7B perfluorocarbon affinity emulsions. Samples of settled emulsion (1 ml) were incubated with yeast homogenate suspension (1 ml) by rotary tumbling for 1 h. After settling under gravity, the supernatant was removed and replaced with a solution of buffer (50 m*M* triethanolamine–HCl, pH 8.0) to generate final emulsion volume fractions of 50, 33 and 20%. The emulsions were washed with these solutions for a total of 6 washes. After each wash the supernatant was carefully removed and replaced with fresh solution. After each wash, the collected supernatant was assayed for turbidity by measuring the absorbance at 600 nm. Wash buffer volume fractions: ○ = 50%; ● = 67%; □ = 80%.

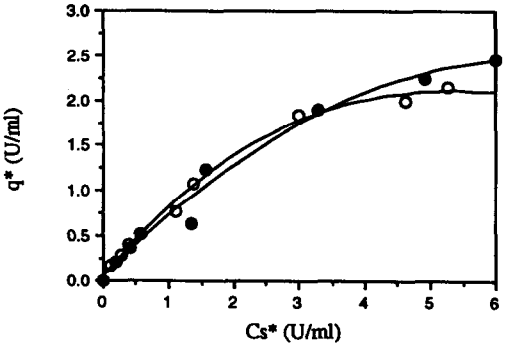

Fig. 4. Equilibrium adsorption isotherms for crude G6PDH on Procion Red H-E7B perfluorocarbon affinity emulsion. Aliquots (0.5 ml) of settled emulsion were washed (3×) with 50 m*M* triethanolamine–HCl, pH 8.0. Following this, serial dilutions of either clarified or unclarified bakers' yeast homogenate (0–6.5 U/ml, 1 ml) were added and the tubes rotary mixed for 1 h after which the supernatant was assayed for both total protein and specifically for G6PDH activity as described in Experimental. ○ = Homogenate, ● = clarified homogenate. q^* is the equilibrium capacity of the affinity emulsion of G6PDH (units of enzyme activity bound per ml of settled emulsion). Cs* is the equilibrium concentration of G6PDH in the non-emulsion phase.

to 2.6 U/ml which decreased to 2.1 U/ml in the presence of cell debris. This small drop in capacity is encouraging as it demonstrates that the capacity of the emulsion for G6PDH does not decrease significantly even in the presence of the high concentrations of cell debris.

Frontal analysis of G6PDH

An examination of the breakthrough curves presented in Fig. 5 shows that cell debris appeared first in the outlet followed by non-G6PDH protein and finally by G6PDH. This is a desirable situation as it demonstrates that G6PDH was being selectively removed from the feedstock by the emulsion. The capacity of the affinity emulsion for G6PDH was estimated by calculating the amount of enzyme contained in the difference of the volumes resulting between the breakthrough of non-G6PDH protein and the breakthrough of G6PDH. The capacity was found to be 1.8 U/ml and is similar to the equilibrium capacity obtained from the adsorption isotherm experiments in unclarified homogenates (2.1 U/ml) and shows that capacity was not compromised by the dynamic mode of operation.

Expanded bed affinity chromatography of G6PDH

The results from this experiment are shown graphically in Fig. 6. The volume of homogenate

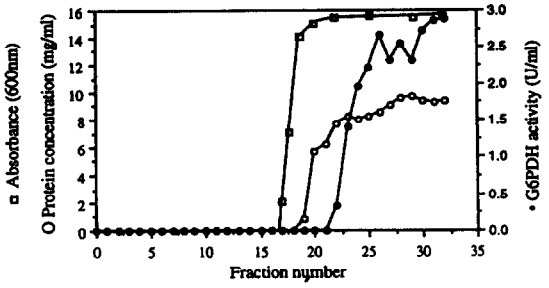

Fig. 5. Determination of dynamic binding capacity for G6PDH by frontal analysis on Procion Red H-E7B perfluorocarbon affinity emulsion. Settled bed height 3.4 cm (10.7 ml) expanded to 10.2 cm (31 ml) when fluidised with 50 m*M* triethanolamine–HCl, pH 8.0 at 5.75 ml/min (108 cm/h). Bakers' yeast homogenate (9.4 mg/ml protein, 2.98 U/ml G6PDH) was injected onto the expanded bed. Fractions (2.2 ml) collected throughout the course of the experiment were assayed for turbidity (absorbance at 600 nm), protein concentration and G6PDH activity as described under Experimental.

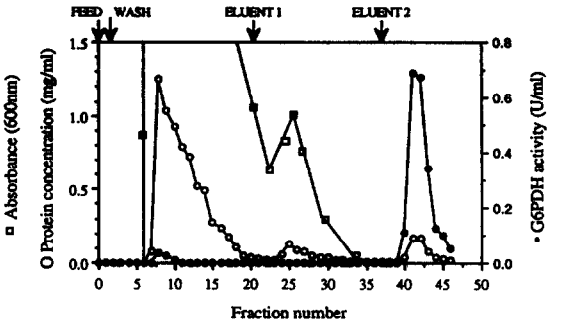

Fig. 6. Expanded bed affinity chromatography of G6PDH on Procion Red H-E7B perfluorocarbon affinity emulsion. Settled bed height 3.25 cm (10.2 ml) expanded to 9.8 cm (30.1 ml) when fluidised with 50 m*M* triethanolamine–HCl, pH 8.0 at 5.75 ml/min (108 cm/h). Bakers' yeast homogenate (5 ml, 11.8 mg/ml protein, 2.71 U/ml G6PDH) was injected onto the expanded bed. Non-bound fractions were washed from the bed using 50 m*M* triethanolamine–HCl, pH 8.0, followed by elution with 0.27 *M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0, followed by elution with 0.88 *M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0. The column was then re-equilibrated with 50 m*M* triethanolamine–HCl, pH 8.0, where the conductivity was monitored to establish when re-equilibration was complete. Fractions (5 ml) collected throughout the experiment were assayed for turbidity (absorbance at 600 nm), protein concentration and specific G6PDH activity as described under Experimental.

loaded onto the expanded bed column was such that the column would not be saturated with G6PDH and any loss of yield should not be due to breakthrough of unadsorbed G6PDH. Therefore, 13.55 U was loaded which corresponded to 74% of the maximum dynamic capacity of the column. As can be seen from Fig. 6, almost all the G6PDH was removed from the homogenate solution. Washing of the expanded bed was carried out with aqueous buffer and viscosity enhancers were not added to the wash solution [3] as the wash superficial velocity was sufficiently high to remove cell debris effectively in approximately 3–4 (expanded) bed volumes. The first elution step was carried out with 0.27 *M* NaCl in wash buffer (50 m*M* triethanolamine–HCl) as the results from a previous investigation [22] showed that irrigation with this concentration of NaCl does not elute G6PDH but does elute some other adsorbed protein. As can be seen from the trace of absorbance at 600 nm, there was some evidence of turbid material also being eluted and we attribute this to result from the desorption of some cell debris or cellular

material. Further investigations are being carried out to identify this material, but it was present in very low concentration and was completely removed by this first elution step. G6PDH was eluted by irrigation of the column with 0.88 *M* NaCl. This step elution is not optimal for the achievement of high purification factors but as our investigations were more concerned with the contacting rather than the elution stage, at this time, we did not deem it necessary to use elaborate gradients or specific elution strategies. However, we are currently investigating the optimisation of the elution protocol to obtain G6PDH of the highest specific activity. In order to prepare the column for a possible further purification cycle, the eluent was washed from the bed using wash buffer (50 m*M* triethanolamine–HCl, pH 8.0). Washing was followed by monitoring the conductivity of effluent. Eluent was washed out, and the column re-equilibrated, after approximately 2 (expanded) bed volumes had been passed through. The purification details from this experiment are shown in Table II, and an overall purification factor of 16.8 for G6PDH was achieved in 77% yield with the eluted fraction being clear from particulate material as assessed by nephelometric analysis.

One possible disadvantage of the use of perfluorocarbon emulsions in expanded bed affinity chromatography is that, as a result of their compressible liquid nature they are deformable under pressure. This necessitates that elution is carried out in an expanded rather than packed bed mode which does lead to some dilution of the product. Another problem is encountered in the batch mode of operation, in that purified product is only being obtained in 1 out of 4 stages (adsorption, washing elution, re-equilibration). This being the case, direct extraction using perfluorocarbon affinity emulsions may be better carried out using some other contacting technique, and we therefore decided to investigate direct extraction using a continuous affinity separation system, PERCAS.

Continuous direct extraction of G6PDH from homogenised bakers' yeast using PERCAS

The stock yeast homogenate was diluted with buffer (100 m*M* triethanolamine–HCl, pH 8.0) to a total protein concentration of 12.2 mg/ml (2.3 U/ml G6PDH, 0.19 U/mg) before being pumped into PERCAS at a flow-rate of 1.21 ml/min (2.77 U/min). Fig. 7 shows both protein and enzyme profiles from stage 1 (adsorption) and stage 3 (elution). As can be seen from the graphs, steady state is achieved in stage 1 in about 2 h. By multiplying the average G6PDH concentration (U/ml), over the steady state region, by the flow-rate, the rate of non-adsorbed enzyme loss from this stage can be calculated at 0.53 U/min which corresponds to 19% of the amount of G6PDH in the input to the system.

The same characteristic profile is observed in the bottom graph showing the profile in the elution stage (stage 3). Steady state is achieved

TABLE II

EXPANDED BED AFFINITY CHROMATOGRAPHY OF G6PDH USING PROCION RED H-E7B PERFLUOROCARBON AFFINITY EMULSION

G6PDH was purified from bakers' yeast homogenate (5 ml, 11.8 mg/ml protein, 2.71 U/ml G6PDH) using an expanded bed of Procion Red H-E7B perfluorocarbon affinity emulsion. Column volume, 10.5 ml settled, 30.1 ml when fluidised at 5.75 ml/min (108 cm/h) with 50 m*M* triethanolamine–HCl, pH 8.0. Elution carried out firstly with 0.27 *M* NaCl, followed by elution with 0.88 *M* NaCl, both in running buffer (50 m*M* triethanolamine–HCl, pH 8.0). Fractions (5 ml) were collected and assayed for turbidity (A^{600nm}), protein concentration (Pierce Coomasie assay) and specific G6PDH activity.

Stage	Volume (ml)	Total protein (mg)	G6PDH (U)	G6PDH activity (U/mg)	Yield (%)	Purification (fold)
Homogenate	5	59	13.55	0.23	(100)	(1)
Elution 1	65	3.23	0	0	–	–
Elution 2	35	2.71	10.47	3.86	77	16.8

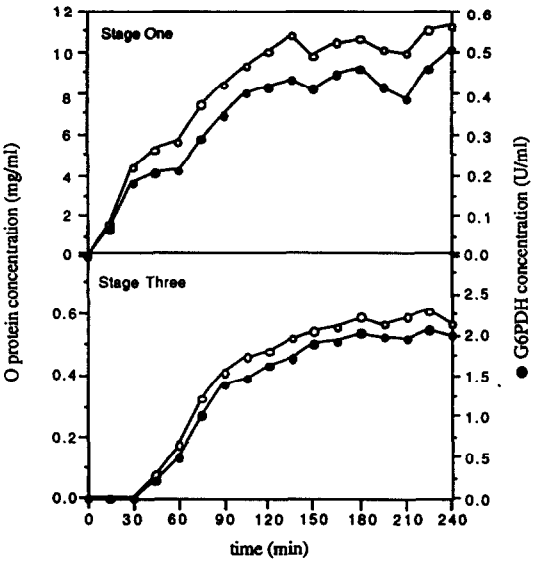

Fig. 7. Total protein and enzyme profiles appearing in PERCAS during the continuous purification of G6PDH from a bakers' yeast homogenate in the liquid leaving stage 1 (flow through) and the liquid leaving stage 3 (the eluted product). Bakers' yeast homogenate (12.19 mg/ml protein, 2.29 U/ml G6PDH) was pumped into PERCAS (stage 1) at a flow-rate of 1.21 ml/min. Purified G6PDH was collected from stage 3 after elution with 2.0 *M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0 at a flow-rate of 0.84 ml/min. Other experimental conditions described in text. Assays performed as described under Experimental.

in this stage after about 3 h. G6PDH is eluted from this stage at a concentration of 2.0 U/ml which corresponds to a rate of 1.68 U/min. By dividing the output rate of G6PDH from stage 3 by the input rate to stage 1, the yield of G6PDH in stage 3 can be calculated at 61%. At steady state, the specific activity of G6PDH leaving stage 3 is 3.42 U/mg which corresponds to a purification factor of 18.4. These results have been summarised in Table III, which compares steady state results from all 4 stages. The total recovery of G6PDH from PERCAS was calculated by addition of all individual stage yields at 93%. In terms of individual stages, 19% of G6PDH was lost in the flow through from stage 1, 8.3% at stage 2 and 4.7% at stage 4. The majority of the biomass flows over in stage 1 with each subsequent stage showing a progressively lower biomass concentration. Based on an initial biomass input to PERCAS of 37.5 (absorbance at 600 nm), 82% of the biomass was washed out in stage 1, 89% by stage 2, 93% by stage 3 and 98% by stage 4. This implies that the eluted stream (stage 3) contained 7% of the original biomass which demonstrates that clarification was reasonably effective although not complete.

TABLE III

CONTINUOUS PURIFICATION OF G6PDH FROM HOMOGENISED BAKERS' YEAST USING PROCION RED H-E7B PERFLUOROCARBON AFFINITY EMULSION AND PERCAS

G6PDH was purified on a continuous manner from homogenised bakers' yeast using Procion Red H-E7B perfluorocarbon affinity emulsion. Settled emulsion volume in PERCAS, 35 ml; running buffer (in stage 1), 100 m*M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0. Washing (primary elution) buffer (stage 2), 0.35 *M* NaCl in 50 m*M* triethanolamine–HCl, pH 8.0; elution buffer (stage 3), 2 *M* NaCl in 50 m*M* triethanolamine buffer–HCl, pH 8.0. Washing (re-equilibration) buffer (stage 4), 50 m*M* triethanolamine–HCl, pH 8.0. Flow-rate of applied homogenate stream, 1.21 ml/min; flow-rate of eluted stream, 0.84 ml/min. Affinity emulsion recycle flow-rate, 1.06 ml/min. Fractions were collected and assayed for turbidity (A^{600nm}), protein concentration (Pierce Coomasie assay) and specific G6PDH activity.

Stage	Flow-rate (ml/min)	Protein concentration (mg/ml)	G6PDH specific activity (U/mg)	G6PDH concentration (U/ml)	G6PDH flow-rate (U/min)	Yield (%)	Purification (fold)	Absorbance (600 nm)
5 —in broth	1.21	12.19	0.186	2.29	2.77	(100)	(1)	37.5
1 —out	1.21	10.47	0.033	0.44	0.53	19.1	–	30.75
2 —out	3.41	1.46	0.046	0.67	0.23	8.3	–	4.12
3 —out	0.84	0.586	3.42	2.00	1.68	61	18.4	2.62
4 —out	3.82	0.014	2.49	0.034	0.13	4.7	–	0.75

Therefore, using the PERCAS system, G6PDH has been purified 18.4-fold in a single step with 61% yield and with a 93% reduction in particulate content. Optimisation of this procedure could be carried out at all stages in the operation. In stage 1, the capture of G6PDH could be improved by increasing the volume fraction of emulsion, alternatively more than one adsorption stage could be used. This is also true for stage 2 where a reduction in the emulsion volume fraction or the inclusion of multiple washing stages would increase contaminant dilution and therefore decrease contamination of the eluted product. Similarly, elution could be optimised by operating a series of elution stages either of increasing ionic strength or containing different eluents.

G6PDH has been purified previously from bakers' yeast using triazine dyes as selective ligands in a variety of techniques. For example, a 7-fold purification (to 41% pure) from a partially purified extract [16] has been reported using a packed bed of Procion Red H-8BN Sepharose using $NADP^+$ as a selective eluent. These authors also pointed out that the success of the dye–ligand affinity chromatography step depends on the degree of substitution of the ligand. Separations using aqueous two-phase affinity partitioning have also been reported. Using Procion Yellow HE-3G liganded PEG an elaborate 4-step partitioning procedure resulted in a 43.6-fold purification of the enzyme (to 2.4% pure) in 74% yield after an initial PEG precipitation [17]. The same authors have successfully applied their techniques to counter-current distribution with similar results [18]. More recently, techniques have been described where G6PDH has been purified using Cibacron Blue F3GA immobilised onto readily available microporous membranes. A 26-fold purification of the enzyme (to 56% pure) was achieved using specific elution with $NADP^+$ and ethylene glycol from a partially purified extract [19].

Comparison of techniques

As both PERCAS and the expanded bed adsorption studies were carried out under similar conditions of adsorption, washing and elution, they can be compared directly. Firstly, in terms of product quality, both techniques delivered partially purified G6PDH with approximately the same specific activity and final yield. The somewhat lower yield associated with PERCAS was mainly attributed to un-adsorbed G6PDH appearing in the flow through. This un-adsorbed fraction could possibly be reduced by increasing the emulsion phase volume fraction in stage 1 or, alternatively, by including a second adsorption stage. The modular design of PERCAS readily allows for the incorporation of additional stages. Both techniques could be readily automated, for example we have previously described the semi-continuous purification of serum albumin from human plasma using automated fast protein liquid chromatography [13]. PERCAS is a continuous process and as such has certain advantages over conventional affinity chromatography which, although can operate repetitively, only produces product during one out of the four batch stages of operation (load, wash, elute, wash). In the experiment described here, the time taken for the expanded bed column to go through one cycle of operation (including washing) was 48 min. During this cycle, 10.5 U of G6PDH was recovered from 10.2 ml of emulsion; this gives rise to a productivity of 1.28 U/h per ml of emulsion. In the case of PERCAS, even with flow-rates approximately 5 times lower, the productivity at 2.88 U/h per ml of emulsion was some 2.25 times higher than the expanded bed adsorption experiment under similar conditions.

Although, the productivity of both techniques appears rather low, it should be remembered that the separations were not optimised. Our present work has shown that G6PDH purification factors of at least an order of magnitude higher are possible from a crude homogenate using more selective elution strategies. Translating this into productivity, the PERCAS unit described in this paper, if run continuously, would be able to produce partially purified G6PDH at a rate of 2400 U/day.

CONCLUSIONS

In this paper perfluorocarbon affinity emulsions have been assessed for their application in

direct extraction. Contacting experiments have showed that the affinity emulsions are very resistant to fouling and present a passive support. They have been shown to be stable to solvents commonly used in *in situ* cleaning and are readily autoclaved. Furthermore, they are reasonably inexpensive to manufacture as all the chemicals used in their synthesis are cheap. Their one disadvantage is their limited capacity which is due to their non-porous nature; for example, the capacity of the emulsion is some 10 times lower than an agarose support employing Procion Red H-8BN as a selective ligand [16]. Capacity comparisons are, however, difficult to make as in most other work capacity has been determined from partially pure G6PDH extracts in which possible adsorbing proteins may have been removed. In continuous operations, a smaller capacity may not be such a limiting problem as continuous recycle is employed. The lack of porosity may also be advantageous as it presents a smaller surface area for possible fouling agents and results in a support that is more easily washed clean. The purification of G6PDH from a crude homogenate was chosen as a model in which to assess the applicability of these novel supports in direct extraction and to compare modes of operation on the overall productivity. Both EBAC and PERCAS were successful in producing a partially purified and clarified preparation. PERCAS was perhaps the better system as it easily allows for changes in operating conditions, has a higher productivity per unit volume of adsorbent and could be easily integrated into a production system that is operated continuously. Although such continuous production processes are not in operation at present, chronic illnesses such as emphysema which require replacement therapy by α-1-antitrypsin at doses of up to 4 g/week per patient [23] may necessitate the use of such production strategies. Alternatively, the system could be thought of as providing trouble free continuous affinity chromatography from a batch fermentation.

However, although the purification factors obtained seem quite low in comparison to some of the techniques discussed above, our separations were not optimised and employed only step elution. One common aspect of all the above mentioned work on G6PDH purification from bakers' yeast is that the affinity chromatographic step has been reserved to the penultimate or final step following such techniques as centrifugation, precipitation and ion exchange which result in feed solutions with partial contaminant removal. To the best of our knowledge, this paper presents the first results for the direct extraction of G6PDH from a crude homogenate where no prior clarification/purification has taken place. The systems were successful in achieving partial purification and clarification in a single step with a high yield of eluted product. The affinity emulsion has proved to be re-usable showing no signs of deterioration over many months. An important aspect of continuous affinity separations is the maintenance of high yield over many cycles of purification and this is an aspect we are investigating. We believe that direct extraction procedures have great potential in industrial biotechnology where the rapid extraction of proteins from, sometimes, inhospitable environments, such as cell homogenates, is desirable. Our research into direct extraction is on-going, at present we are evaluating both liquid and solid perfluorocarbons for potential in direct extraction using both expanded bed techniques and novel continuous reactor formats.

ACKNOWLEDGEMENTS

G.E.McC. gratefully acknowledges the Biotechnology Directorate of the Science and Engineering Research Council (UK) for the provision of a research studentship.

H.A.C. and C.R.L. acknowledge support from the Separations Initiative of the Science and Engineering Research Council (UK).

REFERENCES

1 J. Bonnerjea, S. Oh, M. Hoare and P. Dunnill, *Bio/Technology*, 4 (1986) 954.
2 B.D. Kelley and T.A. Hatton, *Bioseparation*, 1 (1991) 333.
3 H.A. Chase and N.M. Draeger, *J. Chromatogr.*, 597 (1992) 129.
4 D. Sii and A. Sadona, *J. Biotechnol.*, 19 (1991) 83.

5 D.J. Stewart, D.R. Purvis, J.M. Pitts and C.R. Lowe, *J. Chromatogr.*, 623 (1992) 1.
6 C.R. Lowe, S.J. Burton, N. Burton, D.J. Stewart, D.R. Purvis, I. Pitfield and S. Eapen, *J. Mol. Recognit.*, 3 (1990) 117.
7 D.J. Stewart, D.R. Purvis and C.R. Lowe, *J. Chromatogr.*, 510 (1990) 177.
8 N.F. Gordon and C.L. Cooney, in M.R. Ladisch, R.C. Wilson, C.-D.C. Painton and S.E. Builder (Editors), *Protein Purification, From Molecular Mechanisms to Large-Scale Processes (ACS Symposium Series*, No. 427), American Chemical Society, Washington, DC, 1990, p. 118.
9 J.H.T. Luong and K.B. Male, *Bio/Technology*, 5 (1987) 514.
10 S.P. Foaden, *Ph.D. Thesis*, University of Cambridge, 1990.
11 J.M. Woll, T.A. Hatton and M.L. Yarmush, *Biotechnol. Prog.*, 5 (1989) 57.
12 G.E. McCreath, H.A. Chase, D.R. Purvis and C.R. Lowe, *Proceedings from I. Chem. E. Res. Event, Queens College, Cambridge, January 9 and 10, 1991*, p. 67.
13 G.E. McCreath, H.A. Chase, D.R. Purvis and C.R. Lowe, *J. Chromatogr.*, 597 (1992) 189.
14 G.E. McCreath, H.A. Chase, D.R. Purvis and C.R. Lowe, *J. Chromatogr.*, 629 (1993) 201.
15 H.U. Bergmeyer, *Methods of Enzymatic Analysis*, Vol. 2, Verlag Chemie, Weinheim, 1983.
16 E.E. Farmer and J.S. Easterday, *Anal. Biochem.*, 141 (1984) 79.
17 G. Johansson and M. Joelsson, *Enzyme Microb. Technol.*, 7 (1985) 629.
18 G. Johansson, M. Andersson and H.-A. Akerlund, *J. Chromatogr.*, 298 (1984) 483.
19 B. Champluvier and M.-R. Kula, *Biotechnol. Bioeng.*, 40 (1992) 33.
20 D.M. Kirschenbaum, *Handbook of Biochemistry and Molecular Biology*, Vol. 2, CRC Press, Cleveland, OH, 3rd ed., 1976, p. 54.
21 H.A. Chase, *J. Chromatogr.*, 159 (1984) 179.
22 G.E. McCreath, *Ph.D. Thesis*, University of Cambridge, Cambridge, 1993.
23 G. Wright, presented at the *Developments in the Isolation of Proteins, Society of Chemical Industry Meeting, London, May 24, 1993.*