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Screening method for large numbers of dye-adsorbents for enzyme purification

DIRK H. A. HONDMANN and JAAP VISSER*

Department of Genetics, Section Molecular Genetics, Agricultural University, Dreyenlaan 2, 6703 HA Wageningen (The Netherlands)

ABSTRACT

A method is described by means of which 96 different dye-adsorbents can be tested simultaneously for their ability to bind enzymes and to test their biospecific elution. Small amounts of cell-free extract are applied to dye-adsorbents which are packed in a 96-well transplate cartridge. After biospecific elution, the amount of the eluted enzyme is tested in a microtitre plate assay. The method is illustrated by the purification of glycerol dehydrogenase (E.C. 1.1.1.72), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) from the hyphal fungus *Aspergillus nidulans*.

INTRODUCTION

Dye-ligand chromatography has been proved to be a very useful technique in small- and large-scale purification procedures for enzymes and proteins[1]. The reactive dye Cibacron Blue F3G-A has been used especially in the purification of dehydrogenases and kinases[2]. It was shown to bind to the nucleotide-binding domain of liver alcohol dehydrogenase, partly similar to the binding of NAD^+[3,4]. It is now accepted, however, that many other dyes can interact with proteins and other biomolecules, and are therefore of potential use in affinity chromatography[5]. Good purification factors and high yields can be achieved if a dye-adsorbent can be found that binds the desired protein selectively and in such a way that it can be eluted (bio)specifically.

Several strategies for the screening of dye-ligands have been proposed. Quadri and Dean[6] selected two dyes that inhibited free in solution the activity of the enzyme to be purified. These dyes were shown to be useful in the purification of 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus*. Hey and Dean[7] examined large numbers of dye-adsorbents to select useful dyes for the purification of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. They proposed a two-step purification using first a "negative" column that binds much protein but not the protein of interest, followed by a "positive" column that binds the wanted protein but not many other proteins. Scopes[8] divided reactive dyes in into five groups accord-

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ing to their protein-binding capacities. He also studied factors that can affect protein binding. Guidelines were derived by which systematic screening of dye-adsorbents became possible. Kroviarski *et al.*[9] constructed an automated set-up by means of which eight mini-columns, containing 1.6 ml of dye-adsorbent, can be screened for their ability to retain certain enzymes.

We present here a screening method by means of which 96 different dye-adsorbents can be tested simultaneously within several hours for their ability to bind enzymes in such a way that they can be eluted biospecifically. The method is illustrated by screening which dyes are suitable in the purfication of three enzymes from the hyphal fungus *Aspergillus nidulans:* glycerol dehydrogenase (GLYDH), 6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD).

EXPERIMENTAL

Materials

Remazol dyes were gifts from Hoechst (Frankfurt, F.R.G.), Cibacron dyes from Ciba-Geigy (Basle, Switzerland), Levafix dyes from Bayer (Leverkusen, F.R.G.), Basilin dyes from BASF (Ludwigshafen, F.R.G.) and Procion dyes from ICI (Manchester, U.K.). Sepharose 4B-Cl, fast protein liquid chromatographic (FPLC) equipment and a MonoQ column were supplied by Pharmacia (Uppsala, Sweden). Phenazine methosulphate (PMS) and nitro blue tetrazolium chloride (NBT) were obtained from Serva (Heidelberg, F.R.G.). The transplate cartridge was produced by Costar (Cambridge, MA, U.S.A.). Bovine serum albumin (BSA), glucose-6-phosphate, 6-phosphogluconate, $NADP^+$ and Tris were from Boehringer (Mannheim, F.R.G.). All other reagents were of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

Immobilization of dyes

Sepharose 4B-Cl agarose beads were washed extensively with deionized water on a sintered-glass funnel under suction. For every dye 2 g of moist agarose beads were resuspended in 6.5 ml of deionized water, to which were added 10–40 mg of a reactive dye and 1.3 ml of 3 *M* sodium chloride solution. These suspensions were stirred at room temperature for 1 h, then 1 *M* sodium carbonate solution was added to raise the pH to 10.5. After incubation for a further 1 h at 60°C, the gels were washed on a small glass funnel with deionized water, 4 *M* urea, deionized water, 2 *M* sodium chloride solution, deionized water and 0.05 *M* sodium phosphate solution (pH 7.0). The affinity adsorbents were stored in 0.05 *M* sodium phosphate solution (pH 7.0) containing 0.02% (w/v) of sodium azide.

Chromatographic procedure using a transplate cartridge

From a transplate cartridge, which is normally used in hybridoma technologies for filling 96-well plates, the plastic membrane was removed. In each of the 96 wells glass-wool was placed, on which 150 μl (bed volume) of affinity adsorbent were loaded. A different dye-adsorbent was placed in each well. The position of each dye was well documented. In this way a holder containing 96 "mini-columns" was created.

Before use, the mini-colums were equilibrated with a 10-fold volume of extrac-

tion buffer, after first having been rinsed with deionized water, 4 *M* urea, deionized water, 2 *M* sodium chloride solution and deionized water. Using a multi-channel pipette, 100 μl of cell-free extract were deposited on each of the mini-columns. After 10 min, non-adsorbed protein was washed away with a 10-fold volume of extraction buffer. The transplate cartridge was then placed above a 96-well microtitre plate. Proteins were eluted by pipetting into each well 250 μl of elution buffer, *viz.*, extraction buffer containing 1 m*M* $NADP^+$, in portions of 50 μl at 2-min intervals. The eluted fractions (one per well) were collected in a microtitre plate, which had previously been incubated for several hours with 1% (w/v) BSA in extraction buffer to block protein-binding sites. After washing it extensively with deionized water and cooling it to 4°C, the plate was ready for use. The microtitre plate containing the fractions was kept on ice. All operations were performed at 4°C.

After use, the transplate cartridge was washed with deionized water, 4 *M* urea, deionized water, 2 *M* sodium chloride solution and deionized water and then stored in 0.05 *M* sodium phosphate solution (pH 7.0) containing 0.02% (w/v) sodium azide at 4°C.

Colorimetric microtitre plate assays

G6PD and 6PGD were assayed in a microtitre plate assay essentially as described by Cairns[10]. In the case of G6PD, samples of 25 μl of the eluted fractions were added to 225 μl of 50 m*M* Tris–HCl (pH 8.0), 5 m*M* magnesium chloride, 5 m*M* glucose-6-phosphate, 0.4 m*M* $NADP^+$, 0.25 m*M* PMS and 0.2 m*M* NBT. 6PGD was assayed by adding 25 μl of the eluted fractions to 225 μl of 50 m*M* glycylglycine (pH 8.0), 2 m*M* 6-phosphogluconate, 0.2 m*M* $NADP^+$, 0.25 m*M* PMS and 0.2 m*M* NBT. Assays were performed at 25°C.

Spectrophotometric microtitre plate assay

GLYDH was assayed by adding 25 μl of the eluted fractions to 225 μl of 50 m*M* glycine–sodium hydroxide (pH 9.6), 50 m*M* glycerol and 0.2 m*M* $NADP^+$. The change in the absorbance at 340 nm was measured in a UVmax microtitre plate reader (Molecular Devices, Palo Alto, CA, U.S.A.). The results of the assay computations were printed using a Hewlett-Packard printer. Assays were performed at 25°C.

Enzyme assays

The activities of enzymes were measured spectrophotometrically with an Aminco (Silver Spring, MD, U.S.A.) DW-2 UV–VIS spectrophometer using the double-beam mode at 340 nm *versus* 380 nm.

G6PD was assayed in a reaction mixture containing 50 m*M* Tris–HCl (pH 8.0), 5 m*M* glucose-6-phosphate, 5 m*M* magnesium chloride and 0.4 m*M* $NADP^+$. 6PGD was assayed in 50 m*M* glycylglycine (pH 8.0), 2 m*M* 6-phosphogluconate and 0.2 m*M* $NADP^+$. GLYDH was assayed in 50 m*M* glycine–sodium hydroxide (pH 9.6), 50 m*M* glycerol and 0.2 m*M* $NADP^+$. Reactions were performed at 25°C.

Protein determination

Protein in cell-free extract and in the eluted fractions after affinity chromatography was determined by the micro-biuret method[11]. Protein in the fractions after MonoQ chromatography was determined by the bicinchoninic acid method accord-

ing to the instructions of the supplier (Pierce, Rockford, IL, U.S.A.). For both methods BSA was used as a standard.

Strains and growth conditions

A. nidulans wild type strain WG 096 (*yA2, pabaA1*) was used. For the preparation of conidiospores. *A. nidulans* was grown on complete medium[12] solidified with 1.2% agar using 25 m*M* sucrose as carbon source. Mycelium was grown by inoculating 10^6 ml^{-1} in minimal medium[12], supplemented with 2 μg ml^{-1} *p*-aminobenzoate. For the purification of G6PD and 6PGD 100 m*M* glucose was used. The mycelium was grown for 18 h at 37°C in a New Brunswick Scientific (Edison, NJ, U.S.A.) orbital shaker (200 rpm) using 1-l flasks containing 300 ml of minimal medium. The mycelium was harvested by filtration, washed with cold saline and squeezed to remove excess of fluid. The mycelium was then frozen with liquid nitrogen and stored at −60°C.

Preparation of cell-free extract

Small volumes of cell-free extract were prepared by disrupting 1.0 g frozen mycelium using a Braun micro-dismembrator. The mycelial powder obtained was extracted with 2.0 ml of extraction buffer [20 m*M* Bis–Tris (pH 7.0)–1 m*M* $MgCl_2$–0.5 m*M* EDTA). Larger amounts of cell-free extract were prepared by grinding 20 g of frozen mycelium in a Waring blender with liquid nitrogen for 8 min. After evaporation of the liquid nitrogen, 50 ml of extraction buffer were added. Extraction was performed for 1 h at 4°C with gentle stirring of the suspension. This suspension was then centrifuged for 15 min at 20 000 *g*. The supernatant was used as the cell-free extract.

Electrophoresis

Electrophoresis in 10% polyacrylamide gel containing 0.1% SDS (SDS-PAGE) was performed according to Laemmli[13] in a minigel system (LKB, Bromma, Sweden). Carbonic anhydrase (mol.wt. 29 000), ovalbumin (45 000), BSA (68 000) and phosphorylase B (92 500) were used as protein standards.

Chromatographic procedures using 1- or 10-ml columns

Columns of 1 ml (5 cm × 0.50 cm I.D.) or 10 ml (6.5 cm × 1.4 cm I.D.) were packed with dye-absorbent and equilibrated with extraction buffer. One bed volume of cell-free extract was applied to each of the columns. After washing with three volumes of extraction buffer, extraction buffer containing 1 m*M* $NADP^+$ was used to elute. Fractions of 0.5 or 1 ml were collected.

Ion-exchange chromatography

Pooled fractions eluted from one of the dye-adsorbents were loaded onto a MonoQ ion-exchange column. After washing with extraction buffer until the absorbance at 280 nm had decreased to less than 0.01, a 20-ml linear sodium chloride gradient (0–0.4 *M*) was applied. The fraction collector was programmed to collect only peak fractions (1.0 ml).

RESULTS

Screening of dyes using a spectrophotometric microtitre plate assay

The screening procedure involves applying cell-free extract to the 96 mini-columns in the transplate cartridge, washing away unbound protein, followed by the elution of proteins, which are then collected in a microtitre plate. These fractions are used as samples in a spectrophotometric microtitre plate assay.

Cell-free extract (100 μl) of *A. nidulans* WG 096 was applied to each of the 96 mini-columns. After washing away unbound proteins, 250 μl of extraction buffer containing 1 m*M* $NADP^+$ were used for elution. In the eluted fractions GLYDH activity was measured, using a kinetic microtitre plate reader. Protein content was determined in the six most active fractions. Table I lists the data for these fractions, which show that there are several dye-adsorbents that are capable of binding GLYDH in such a way that it can be eluted biospecifically with $NADP^+$. Despite the fact that the yields and purification factors are low, the data suggest that Cibacron Brilliant Red 3B-A will give the best results in the purification of GLYDH.

TABLE I

BINDING AND ELUTION BEHAVIOUR OF GLYCEROL DEHYDROGENASE OF *A. nidulans* WITH RESPECT TO SIX DYE-ADSORBENTS

In the 96 eluted fractions GLYDH activity was measured using a kinetic microtitre plate reader (see Experimental for details). The yield is related to the amount of GLYDH applied to each of the mini-columns.

Well	*Dye*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
A5	Cibacron Brilliant Red 3B-A	0.408	44	8.5
D2	Basilin Red E-B	0.186	20	3.9
D12	Basilin Orange E-2R	0.209	23	4.4
E3	Procion Black 2-RPC	0.091	25	1.9
F3	Procion Red P-8B	0.096	28	2.0
H3	Levafix Blue E-3GLA	0.150	33	3.1

A 1-ml Cibacron Brilliant Red 3B-A column was used to test this dye-adsorbent. Cell-free extract (1 ml) of *A. nidulans* WG 096 was loaded on this column. After washing with 5 ml of extraction buffer, 1 m*M* $NADP^+$ was added. Fractions of 0.5 ml were collected. GLYDH appeared at the $NADP^+$ front as a sharp peak of activity. A 97-fold purification with 91% recovery was achieved (Table II). The active fractions were pooled and loaded on a MonoQ ion exchanger. A linear sodium chloride gradient (0–0.4 *M*) was used to elute GLYDH and the enzyme eluted at 80 m*M* sodium chloride. $NADP^+$ also binds to this column, but it is eluted at a higher sodium chloride concentration, so the GLYDH fraction will be free of $NADP^+$.

In this two-step purification GLYDH is purified to homogeneity as judged by SDS-PAGE (Fig. 1). The specific activity of 25.1 U mg^{-1} is in good agreement with another purification method for *A. nidulans* GLYDH[14].

TABLE II

PURIFICATION OF GLYCEROL DEHYDROGENASE, 6-PHOSPHOGLUCONATE DEHYDROGENASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE OF *A. nidulans* BY USING THREE TWO-STEP PURIFICATION METHODS

1 ml of cell-free extract (GLYDH) or 10 ml of cell-free extract (6PGD and G6PD) was loaded onto columns of 1 ml (GLYDH) or 10 ml (6PGD and G6PD) packed with immobilized Cibacron Red 3B-A (GLYDH), Procion Black 2 RPC (6PGD) or Levafix Navy P-RRL (G6PD). After washing, the columns were eluted with 1 m*M* $NADP^+$ in the extraction buffer. Pooled active fractions were applied to a MonoQ column and eluted with a linear sodium chloride gradient (see Experimental for details).

Step	*Volume (ml)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
GLYDH extract	1.0	0.23	0.048	100	1
Red 3B-A	1.5	0.21	4.67	91	97
Mono Q	1.4	0.20	25.1	87	623
6PGD extract	10.0	18.4	0.74	100	1
Black 2 RPC	4.0	15.6	48.9	85	66
Mono Q	1.0	14.4	115.0	78	156
G6PD extract	10.0	75.0	3.0	100	1
Navy P-RRL	5.0	40.5	270.0	54	90
Mono Q	2.8	37.5	357.8	50	119

Selection of dyes using a colorimetric microtitre plate assay

As kinetic microtitre plate readers are not widely available, we investigated the possibility of using less expensive means, *e.g.*, colorimetric assays. 6PGD and G6PD activity can be coupled to the reduction of the chomophore NBT[15]. As an intermediate electron donor PMS is used. Reduced NBT changes colour from yellow to blue, wich can be assessed by eye if the dehydrogenase activities are not too low.

A. nidulans cell-free extract (100 μl per well) was applied to the mini-columns. After washing with extraction buffer, 250 μl of extraction buffer containing 1 m*M* $NADP^+$ were used as the elution buffer. G6PD and 6PGD activity were determined in a microtitre plate assay by coupling the formation of NADPH to the reduction of NBT. In Fig. 2 a picture of the microtitre plate is shown in which the G6PD activity was determined. The G6PD microtitre plate assay gave a similar result (data not shown). We also measured the 6PGD activity and G6PD activity in a "normal" spectrophotometric assay of the ten most active fractions, ten non-active fractions and ten fractions with intermediate activities. The measured activities showed a good correlation with the colour development in the corresponding wells. The only clear exception was well D2, which showed as prominent a colour development in the 6PGD assay as in the G6PD assay, whereas the measured enzyme activities were about half as high as the activities in the most active fractions. The protein content in fraction D2 was 5–10 times higher than that content in other fractions. Cairns[10] has shown that colour development in a coupled NADP reduction to produce a formazan dye is enhanced by increased protein concentrations. This might explain why fraction

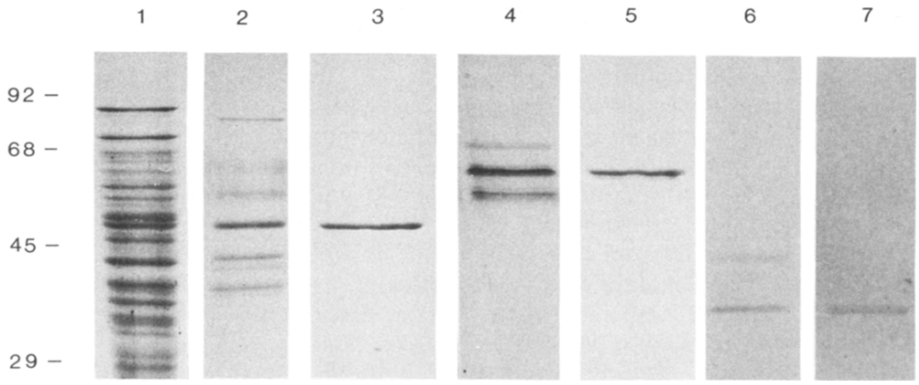

Fig. 1. SDS-PAGE of 6PGD, G6PD and GLYDH at different stages of purification. 1 = Cell free extract of *A. nidulans* 2 = 6PGD after chromatography on Procion Black 2 RPC; 3 = 6PGD after MonoQ chromatography using sample of lane 2; 4 = G6PD after chromatography on Levafix Navy p-RRL; 5 = G6PD after MonoQ chromatography using sample of lane 4; 6 = GLYDH after chromatography on Cibacron Red 3B-A; 7 = GLYDH after MonoQ chromatography using sample of lane 6. Positions and mass (in kilodalton) of molecular weight markers are indicated on the left.

D2 produced the strongest signal although it was not the fraction with the highest enzyme activity

Procion Black 2 RPC (well E3), Procion Rubine MX-B (well E9) and Procion Yellow P5-GN (well G6) were selected as potential dye-adsorbents for the purifica-

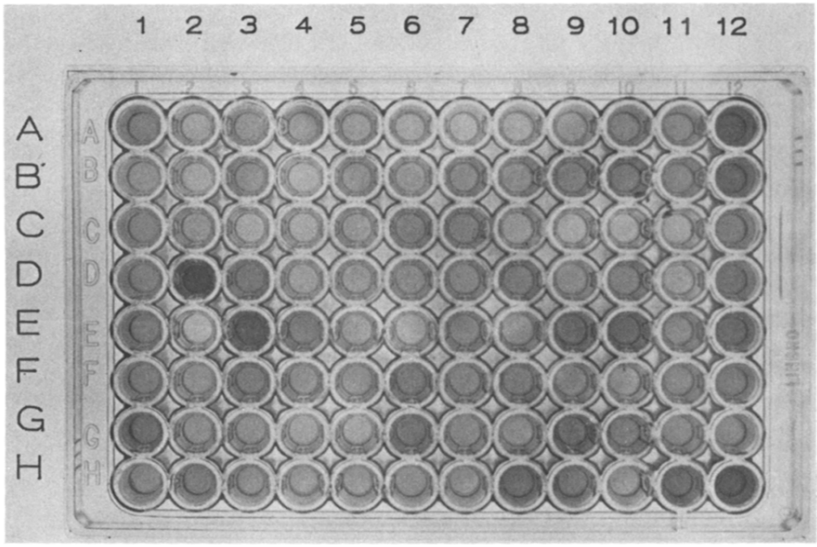

Fig. 2. Colorimetric microtitre plate assay of 6PGD. Collected fractions, eluted from the mini-columns in a transplate cartridge, were screened for 6PGD activity by coupling the reaction to the reduction of NBT, leading to darkening of the wells. The positions of the wells correspond to the positions of the dye-adsorbents in the transplate cartridge.

tion of 6PGD. These dyes were selected because in the corresponding wells the 6PGD activity was high (E3) or intermediate (E9 and G6), whereas the G6PD activity was low. The dye-adsorbents corresponding to wells A12, B12, E10, G9, H11 and H12 were rejected because they showed a clear colour development in the G6PD assay. These findings were confirmed by the measured enzyme activities.

The elution profiles of 6PGD from 10-ml columns of the three selected dye-adsorbents differ. From Procion Black 2 RPC 6PGD is eluted with 1 m*M* $NADP^+$ in a sharp peak (4 ml) with a yield of 85% (Table II). The use of Procion Rubine MX-B or Procion Yellow P5-GN resulted in peak broadening (6 and 10 ml) and lower yields (58% and 69%). These findings on a 10-ml scale confirm the indications obtained in the "mini-column scale" screening where Procion Black 2 RPC gave the strongest signal (apart from well D2) in the 6PGD microtitre plate assay.

The pooled fractions eluted from Procion Black 2 RPC were applied to a MonoQ column. Using a linear sodium chloride gradient, 6PGD was eluted at 230 m*M* sodium chloride. This fraction contained homogeneous 6PGD as judged by SDS-PAGE (Fig. 1). An overall purification factor of 156 and a yield of 78% were achieved in this two-step purification (Table II).

Levafix Navy P-RRL (well H8) was selected for study on a 10-ml scale. It was selected because an intense colour development was observed in well H8 (and also in wells H12 and D2) in the G6PD microtitre plate assay. In the 6PGD assay only a faint colour development was observed in well H8, whereas wells H12 (Cibacron Blue F3G-A) and D2 (Basilin Red E-B) showed an intense colour development. This indicated that Levafix Navy P-RRL would be more selective than Cibacron Blue F3G-A or Basilin Red E-B in the binding of G6PD.

A 10-ml Levafix Navy P-RRL column was loaded with 10 ml of cell-free extract. Almost all the 6PGD activity (83%) was found in the flow-through. The elution profile when using 1 m*M* $NADP^+$ in the extraction buffer showed a peak of G6PD activity at the $NADP^+$ front. This peak contained about half of the G6PD activity applied. Also, a long tailing effect was observed. Even after 10 ml a low G6PD activity was still found. This might be explained by the fact that two (or more) isoenzymes exist, which could behave differently on this dye-adsorbent or could differ in their affinity for $NADP^+$. Isoenzymes of G6PD differing only at the N-terminus are found in several organisms[16].

The fractions containing more than 3 U ml^{-1} were pooled and applied to a MonoQ column. G6PD activity was eluted at 200 m*M* sodium chloride using a linear 0–400 m*M* sodium chloride gradient. As judged by SDS-PAGE, this sample contained almost pure G6PD (Fig. 1). A purification factor of 119 and a yield of 54% were obtained in this two-step purification.

DISCUSSION

The development of a rapid and easy to set up purification procedure for a certain protein can be an important step in recombinant DNA work. Apart from being able to study the purified protein, it can be used as a means of obtaining a probe for the corresponding gene. Gas-phase microsequencing of small amounts of pure protein can be used to obtain data for the generation of oligonucleotide probes[17]. Purified protein can also be used to raise antibodies, which can be used to screen a

cDNA expression library[18]. For the characterization of mutant proteins or proteins modified by protein engineering, the availability of a rapid small-scale purification procedure is also of great importance, as these proteins may be less stable[19].

A particular enzyme or protein is generally found to have affinity for different textile dyes. Screening of a large number of different dyes to find the most suitable one is usually time consuming and therefore kept to a minimum in most instances. Here a method is presented by means of which 96 dye affinity adsorbents can be screened in several hours. This method uses components that have the same structure and therefore make it possible to handle 96 mini-columns at the same time. A transplate cartridge is modified to create 96 mini-columns. Fractions eluted from these columns can easily be collected in a microtitre plate. The amount of enzyme present in each of the fractions can be determined in a microtitre plate assay.

An important feature of the method presented is its versatility. Adsorption and (biospecific) elution conditions can be varied and tested in an integrated fashion in one cycle. In the eluted fractions the activities of more than one enzyme can be determined, in addition to the protein content. From these data a purification strategy may already be derived. A great diversity of enzyme detection methods can be used. In this paper a spectrophotometric assay (for GLYDH) and two assays based on colour development (for 6PGD and G6PD) are used. We have also succesfully used various discontinuous assays and some coupled enzyme assays in the screening of some other enzymes (data not presented).

As an illustration of the method we screened for dye-adsorbents that can be used in the purification of GLYDH, 6PGD and G6PD from *A. nidulans*. In the screening for GLYDH a spectrophotometric microtitre plate assay was used. Information about yields and purification factors on a microlitre purification scale were used to select Cibacron Red 3B-A as the most suitable dye. A yield of 91% and a purification factor of 97 were achieved using this dye-adsorbent in a millilitre scale purification. Colorimetric microtitre plate assays were used in the screening for dye-adsorbents for the purification of 6PGD and G6PD. Dye-adsorbents were selected that showed a strong (or intermediate) signal in one assay and a weak signal in the other assay. Using Procion Black 2 RPC a purification factor of 66 and a yield of 85% were achieved in one purification step. For G6PD a purification factor of 90 and a yield of 54% were obtained using Levafix Navy P-RRL. These three examples show that data obtained on a microlitre purification scale can be used to select from 96 dye-adsorbents the most suitable ones to be used on a millitre scale.

We currently use this screening method as a first routine step each time a new enzyme has to be purified. The transplate cartridge has so far been used for the purification of about ten different enzymes in several months. It always resulted in the identification of a suitable dye that proved was useful.

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