Journal of Chromatography, 449 (1988) 413–422
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 716

PURIFICATION OF HUMAN 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM HUMAN HAEMOLYSATE WITH CHROMATOGRAPHY ON AN IMMOBILIZED DYE AS THE ESSENTIAL STEP AND USE OF AUTOMATION

SIMULTANEOUS PURIFICATION OF LACTATE DEHYDROGENASE

Y. KROVIARSKI, S. COCHET, C. VADON, A. TRUSKOLASKI*, P. BOIVIN and O. BERTRAND*
INSERM U160, Hôpital Beaujon, F-92118 Clichy Cedex (France)
(First received October 21st, 1987; revised manuscript received June 6th, 1988)

SUMMARY

The screening procedure described in the preceding paper allowed a practical purification procedure to be devised that was automated for human 6-phosphogluconate dehydrogenase. The purification needed only two chromatographic steps, first on immobilized Procion Blue HE-GN and then on Phenyl-Sepharose. This technique also gave purified lactate dehydrogenase. Both enzymes showed single bands in SDS polyacrylamide gel electrophoresis.

INTRODUCTION

The results of the screening procedure described in the preceding paper prompted us to devise a complete purification procedure for human 6-phosphogluconate dehydrogenase (6PGD) from human haemolysate using an immobilized dye, Procion Blue HE-GN. The first trial on a large scale showed that 6PGD could be obtained in a fairly purified state by NADP elution. Only one major contaminant could be seen by gel electrophoresis, and this 36 kDa contaminant was identified as lactate dehydrogenase (LDH). Separation of the two enzymes was easily achieved by differential elution procedures from the immobilized dye column. Diverse procedures for the specific elution of the enzymes from the dye columns were tried and the results are presented in this paper.

EXPERIMENTAL

The dye, chemicals and most of the methods employed were the same to those described in the preceeding paper. Nucleotides were obtained from Boehringer (Mannheim, F.R.G.) or Sigma (Saint Louis, MO, U.S.A.).

* INSERM U24.

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The procedure used for dye immobilization was also essentially the same. However, the results described in this paper were obtained with dye immobilized on Sepharose CL 4B from Pharmacia (Uppsala, Sweden) and not on Ultrogel A4 (IBF, Villeneuve la Garenne, France). The dye incorporation was 6.2 mg/ml of wet Sepharose [the dye incorporated in Ultrogel A4–B16 III (see the preceding paper) was 6.0 mg/ml]. Bleed volume measurements (made with 1.6-ml columns, see the preceeding paper) gave values of 90 ml for Procion Blue HE-GN–Ultrogel and of 155 ml for Procion Blue HE-GN–Sepharose. The better performance of the latter gel is reminiscent of similar results obtained by Dean *et al.*[1], who studied the effect of the matrix used for immobilization on the performance of dye columns.

The buffers used for the purification procedure were as follows:

(A) 10 m*M* potassium hydroxide adjusted to pH 6.5 with solid morpholinoethane-sulphonate containing 30 m*M* sodium chloride and 2 m*M* magnesium chloride[2]; (B) the same as buffer A but adjusted to pH 7.5; (C) the same as buffer A but the sodium chloride concentration was 2 *M* and magnesium chloride was absent; (D) 6 *M* urea; (E) the same as buffer A but containing ammonium sulphate at 20% saturation; (F) the same as buffer A but containing ammonium sulphate at 80% saturation.

The nucleotide solution for the elution of LDH from the Procion Blue HE-GN column was 5 m*M* AMP dissolved in buffer A, and that for the elution of 6PGD from the Procion Blue HE-GN column was NADP dissolved in digestion buffer hydrolysed completely to NMN and 2′5′-ADP by snake venom phosphodiesterase (see below for details).

Lactate dehydrogenase (LDH) was assayed according to Beutler[3].

The isozymic pattern of LDH was determined by electrophoresis (Helena Labs., Beaumont, TX, U.S.A.).

The protein (haemoglobin) content in the starting material was determined according to Drabkin[4]. Other protein assays were performed according to the Bradford procedure[5] using reagents from Bio-Rad Labs. (Richmond, CA, U.S.A.). However, the protein contents of totally purified 6PGD pools were evaluated from optical density measurement at 280 nm using an $A_{1\,\mathrm{cm}}^{1\%}$ value of 10 (ref. 6).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was conducted according to Laemmli[7]. Molecular weight markers were obtained from Pharmacia.

Hydrolysis of NADP by phosphodiesterase from *Crotalus durissus* (Boehringer) was carried out in a buffer of the same composition as buffer A but containing 20 m*M* Tris and adjusted to pH 8. Completion of digestion was checked by chromatography at 37°C on an octadecylsilica column (25 × 0.46 cm I.D.) (Merck, Darmstadt, F.R.G.). The mobile phase[8] was 75 m*M* dipotassium hydrogenphosphate buffer adjusted to pH 4.12 with phosphoric acid, containing 1.4 m*M* undecanoic acid (Aldrich, Milwaukee, WI, U.S.A.) and 12% (v/v) methanol. This procedure gives a clear separation of NADP, NMN and 2′5′-ADP.

Several experiments intended to study the conditions for elution by nucleotides were conducted on a small scale on columns of 1.5-cm I.D. filled to a height of 2.7 cm. The volume of deposited haemolysate (prepared as mentioned below) was 60 ml, the flow-rate was 12 ml/h and volume of collected fractions was 3 ml.

After deposition, the columns were rinsed for 2.5 h with buffer B, then for 2 h with buffer A. The columns were then developed with 15 ml of the various solutions

of nucleotides in buffer A (readjustment of pH was sometimes necessary) and further rinsed with buffer A.

RESULTS AND DISCUSSION

Elution of enzymes by nucleotides

A large number of enzymes were purified by taking advantage of the fact that inclusion of the enzyme cofactor elutes the protein from the immobilized dye column. Nevertheless, this fact obviously does not prove that the dye binding site is identical with the substrate binding site. In at least one instance (with Cibacron Blue and alcohol dehydrogenase[9]) it was definitely proven by careful crystallographic studies that the dye did overlap with the nucleotide binding site, but was linked to the protein by other residues not involved in nucleotide enzyme interactions. If the enzyme does bind to the dye by just one part of the nucleotide binding site, one can expect that a fragment of the nucleotide will be effective in promoting enzyme desorption. Moreover, one can speculate that a combination of fragments may be effective. The impetus to search for eluents other than the coenzyme itself can also be based on economic reasons: fragments of the nucleotide can be cheaper than the nucleotide itself.

TABLE I

RECOVERY OF 6PGD AND LDH FROM IMMOBILIZED PROCION BLUE HE-GN

Numerical values given are percentages of enzymatic activities deposited on the columns which were found in the eluate after switching to nucleotide containing developer.

Eluent	*Concentration (mM)*	*6PGD (%)*	*LDH (%)*
NADP	0.5	0	n.d.*
	1	23	5
	2	60	n.d.
	5	80	23
2'(3'),5'-ADP	0.5	0	0
	1	43	n.d.
	2	60	n.d.
	10	75	0
2',5'-ADP	1	65	n.d.
3',5'-ADP	1	0	n.d.
NAD	5	0	67
AMP	5	0	72
NMN	5	0	0
NMN + 2'(3'),5'-ADP	0.5 + 1	56.5	n.d.
	1 + 2	95	0
NMN + AMP	1 + 2	0	16
	2.5 + 2.5	n.d.	32
	5 + 5	n.d.	67
NADP + 2'(3'),5'-ADP	1 + 1	71	n.d.
	1 + 2	100	0.5
NAD + 2'(3'),5'-ADP	1 + 2	74	n.d.
NADP hydrolysed by phosphodiesterase I (snake venom)	2	96	0

* Not determined.

The aim of the studies described below was to find rapidly an efficient and cheap way of eluting the enzymes from the immobilized dye column. It is understood that results obtained by the empirical method used (see Experimental) are only qualitative and mostly interesting from a practical point of view. In no way do we claim that they could give a true quantitative description of the interactions of the protein with the dye in the presence of interacting substances.

The results of trials with several eluting agents for 6PGD and LDH are given in Table I.

NADP was observed to be able to elute 6PGD. The mixed isomer 2′(3′),5′-ADP was also able to elute the enzyme efficiently. The active species in the isomer mixture was shown to be the 2′,5′-ADP isomer. The phosphate grafted in the 2′-position of the ribose ring seems to be of the greatest importance for competition with the dye because neither NAD, AMP nor 3′,5′-ADP is able to elute 6PGD. Nicotinamide mononucleotide has no eluting power for 6PGD but a mixture of 1 m*M* NMN and 2 m*M* 2′(3′),5′-ADP is clearly more efficient than 1 m*M* NADP or 2 m*M* 2′(3′),5′-ADP used alone to elute the enzyme from the dye column. This suggests on the one hand that NMN at least interacts with the enzyme and on the other that it assists the other half of the cofactor molecule to elute the enzyme from the column. From these results one can guess that it would be interesting to check analogues of NADP possessing a longer or possibly more flexible link between 2′(3′),5′-ADP and NMN parts with regards to their ability to elute the enzyme from the dye column.

A mixture with low concentrations of 2′(3′),5′-ADP and NADP is extremely powerful for elution of the enzyme from the column. Yet more striking is the fact that a mixture of 2′(3′),5′-ADP and NAD is able to elute efficiently 6PGD from the column. Therefore, one can guess that in this instance NAD is an analogue of NMN and that the other part of the molecule (*i.e.*, the "AMP part") does not play any role in the elution of the enzyme from the immobilized dye.

The fact that a mixture of NMN and 2′(3′),5′-ADP was very efficient in eluting 6PGD from the column gave the opportunity to prepare easily a (relatively) inexpensive eluent for the enzyme from the immobilized dye column: NADP was dissolved to a final concentration of 4 m*M* in digestion buffer and submitted to the action of phosphodiesterase from snake venom (final concentration 0.26 μg/ml). Digestion was complete after incubation for 18 h at 37°C. The mixture was then diluted once with buffer A and adjusted to pH 6.5 and used directly to elute the enzyme. The results in Table I demonstrate that, as expected, hydrolysed NADP is more effective than unhydrolysed NADP at the same concentration.

The results obtained with LDH will be discussed in the same way as those described previously for 6PGD. However, first, one should note that a significant part of the LDH activity was not retained by the dye, thus explaining that yields evaluated as mentioned in Table I are never quantitative, even with a powerful eluent such as NAD or AMP. The isozymic patterns of LDH both retained and not retained by the column were checked by cellulose acetate electrophoresis. Haemolysate showed three bands corresponding to the A2B2, A1B3 and B4 isozymes; only the last two were seen in the breakthrough volume. LDH eluted from the column consisted of a small proportion of B4 but mostly A1B3 and A2B2, with the latter isozyme eluted last in the activity peak. This finding suggests that Procion Blue HE-GN does not have the same affinity for the two different constitutive subunits of LDH. A similar observation was

made with Blue dextran agarose[10] and also with Cibacron Blue linked to silica, which was used to separate rapidly B4 and A4 isoenzymes[11].

NAD and AMP can elute LDH retained by the dye; NMN is ineffective. NADP can elute a significant portion of retained LDH activity, showing that grafting a phosphate group on the 2′-position of the ribose ring does not impair totally the power of the other part of the molecule to compete with the dye [however, 2′(3′),5′-ADP is ineffective]. It is interesting that at "equivalent" concentrations, mixtures of AMP and NMN are less powerful than NAD in eluting the enzyme, which is contrary to the result obtained with 6PGD; 2 m*M* hydrolysed NADP could not elute any LDH activity.

AMP is preferred to NAD for elution of LDH because of cost. Hydrolysed NADP is the cheapest eluent for 6PGD.

Large-scale elution of enzymes by a salt gradient from the immobilized dye column

Chromatography was performed on a column of 5 cm I.D. filled with immobilized dye to a bed height of 5 cm; the flow-rate was 150 ml/h. The deposited haemolysate volume was 1400 ml. After sample deposition the column was rinsed with 1000 ml of buffer B and then developed with a linear gradient obtained by automated mixing (with a Mixograd apparatus; Gilson, Villiers Le Bel, France) of 450 ml of initial buffer (buffer A) and 450 ml of final buffer (buffer C).

This linear gradient could not separate LDH and 6PGD significantly. SDS-PAGE of aliquots of the collected fractions showed the presence of several protein bands. This result is in sharp contrast to the satisfactory results obtained with nucleotide elution in a separate pilot experiment on the same scale. We therefore used the latter procedure for the elution of enzymes from the immobilized dye column in the routine automated purification procedure described below.

Automated complete purification of human 6PGD and purification of LDH from the same starting material

The rationale for using automation for the relatively large-scale production of enzymes in the research laboratory has been described[6]. Briefly, with the equipment available in a standard research laboratory, it is easier to repeat purifications starting from a small amount of starting material (*i.e.*, around 1 l) than to purchase specially dedicated columns or centrifuges necessary for the processing of large volumes of starting material. The drawback of such an approach is obviously the time needed to repeat the purifications. The solution for us was, as before, to automate the purification procedure.

The instrument, which is operated in a cold room, is under the control of a simple programmer made by associating electromechanical timers, liquid and voltage level sensors and electronics incorporating a diode matrix. The working principle of the machine has already been described[12] and a detailed description is beyond the scope of this paper. However, Fig. 1, showing a flow chart of the machine, and Fig. 2, showing the time schedule of activation of its elements and a description of the purification procedure itself given below, will allow the reader to understand how the machine works. Full details of the construction of the automatic machine and its simple programmers can be obtained from the authors on request.

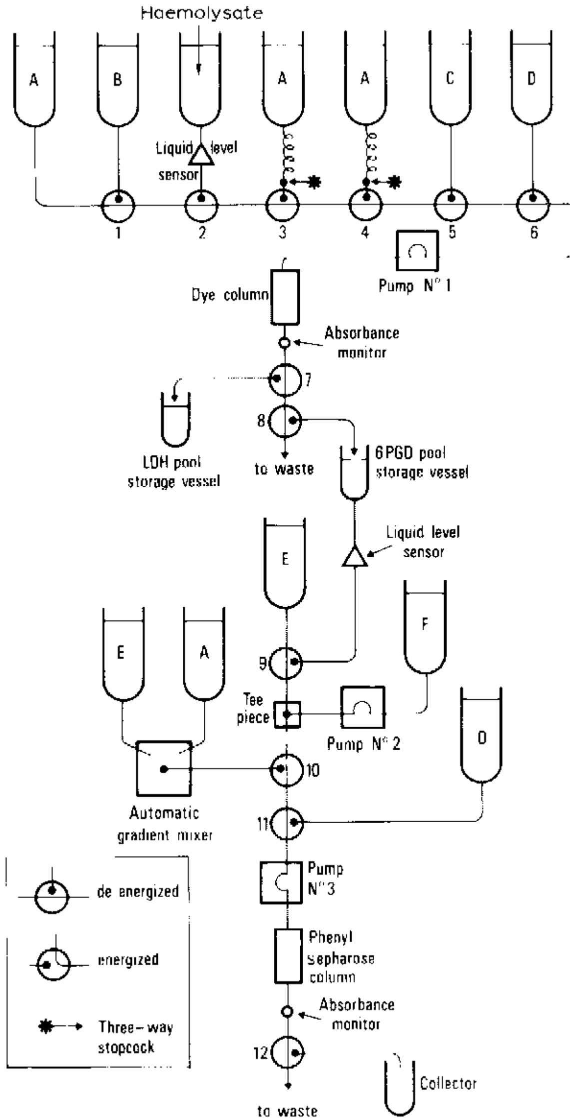

Fig. 1. Diagram of the automatic chromatographic system. The valves and pumps are numbered as in Fig. 2. A–F are buffers.

Overall procedure

Preparation of haemolysate. We use routinely as starting material either bags of outdated packed red cells or blood of patients undergoing therapeutic phlebotomy for primary polycythaemia (in this instance red cells are collected by centrifugation and washed once in phosphate-buffered saline). Red cells are lysed by 3-fold dilution with water and freezing and thawing. The lysate is then diluted twice with buffer A and cleared from membrane remnants by centrifugation at 10 000 g for 45 min.

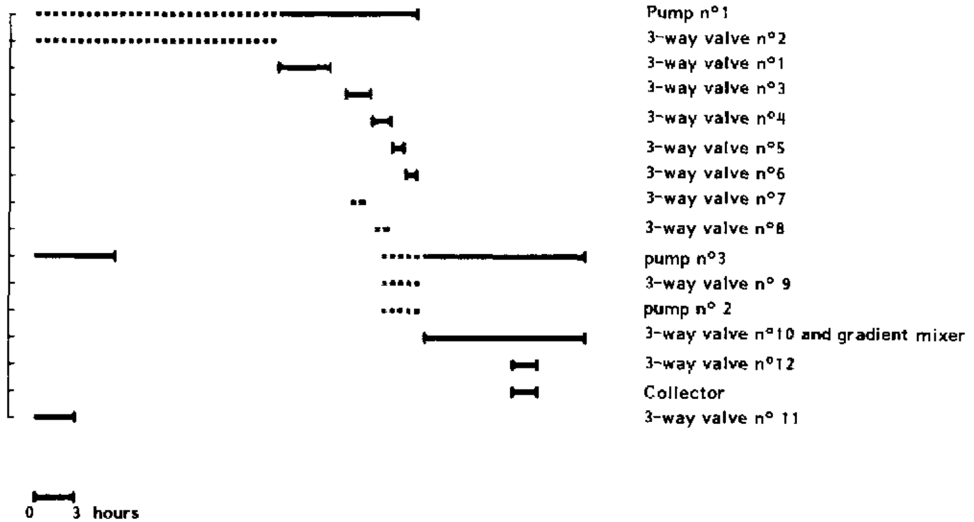

Fig. 2. Diagram of the programme routinely used for the automatic purification of 6PGD. The horizontal lines show when power is brought to the valves, pumps, gradient mixer and collector. Most of the programming relies on time counting (solid lines) but the end of starting material deposition and collection of LDH and 6PGD pools relies on sensing of liquid levels and voltage levels, respectively (dotted lines).

Chromatography on immobilized Procion Blue HE-GN. Centrifuged haemolysate (the volume is usually *ca.* 1400 ml) is poured into the appropriate reservoir of the machine as shown in Fig. 1 and the instrument is turned on. The haemolysate will therefore flow to the immobilized dye column (I.D. 5 cm, height 5 cm, flow-rate 150 ml/h).

When the haemolysate reservoir becomes empty the column is rinsed for 1 h with buffer B, then the flow-rate is automatically increased to 300 ml/h and the dye column is rinsed for a further 3 h with buffer B (the higher pH of buffer B allows some unwanted proteins to be eluted). The column is thereafter rinsed for 1 h with buffer A, then 300 ml of 5 m*M* AMP dissolved in buffer A (contained in a storage loop fixed under a buffer A reservoir, see Fig. 1) are pumped on to the column. When AMP begins to leave the column (LDH being eluted at the same time), the effluent from the column is adequately collected in the LDH pool storage vessel . The column is then developed with buffer A for 1 h. The column is thereafter developed with 300 ml of 2 m*M* hydrolysed NADP. The eluate is diverted to the 6PGD pool storage vessel. The immobilized dye column will then be rinsed with buffer C and D and subsequently re-equilibrated with buffer A in order to be ready for the next use.

Phenyl-Sepharose chromatography for 6PGD purification. The 6PGD-containing pool is automatically mixed with buffer F in such a way as to make it 20% saturated in ammonium sulphate. It is deposited on to the Phenyl-Sepharose column (I.D. 2.2 cm, height 26 cm) at 72 ml/h. The column is then rinsed for 3 h with buffer E and developed with a linear gradient between buffer E and buffer A for 6 h. The effluent from the Phenyl-Sepharose column is adequately collected in fractions. The Phenyl-Sepharose column will then be rinsed by buffer D and re-equilibrated with buffer

TABLE II

TYPICAL PURIFICATION OF HUMAN ERYTHROCYTIC 6PGD AND LDH FROM A GIVEN STARTING MATERIAL

On a routine basis specific activities are checked in the starting material and final products only.

Enzyme	*Sample or collected peak*	*Applied or collected volume (ml)*	*Activity (IU/ml)*	*Yield (%)*	*Protein concentration (mg/ml)*	*Specific activity (IU/mg)*	*Purification factor*
6PGD	Haemolysate	1400	0.28	100	63	0.0045	1
	Procion Blue HE-GN	235	1.57	94	0.080	19.6	4400
	Phenyl-Sepharose	115	2.31	68	0.092	25	5600
LDH	Haemolysate	1400	3.0	100	63	0.047	1
	Procion Blue HE-GN	400	7.25	69	0.036	201.4	4285
	Phenyl-Sepharose	220	10.6	55.5	0.026	407	8659

E during the early stages of the next automatic purification cycle so as to be ready for the next use.

Pure 6PGD is eluted from the Phenyl-Sepharose column as a symmetrical optical density peak after 3.5 h of the beginning of the linear gradient between buffers E and A.

Quantitative data on one typical purification procedure are given in Table II. The purity of the enzyme pool is routinely checked by SDS-PAGE. Fig. 3 shows the results of such an electrophoresis. This photograph and also Table II demonstrate the high efficiency of the immobilized dye chromatography step. The specific activity of 6PGD routinely obtained at the end of the purification procedure is 25 IU/mg, which corresponds to a 5600-fold purification. The overall yield of the purification procedure, which was repeated many times, usually lies between 60 and 70%. These

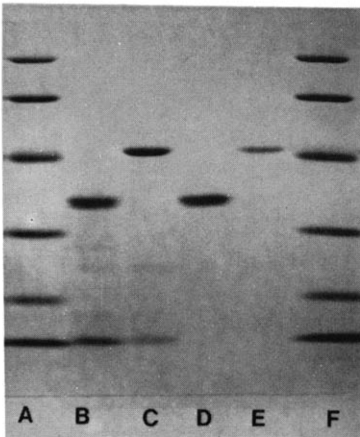

Fig. 3. Electrophoresis (SDS-PAGE) of the products. Lanes A and F, molecular weight markers (from top to bottom 94, 67, 43, 30, 20 and 14.4 kDa); lanes B and C, aliquots of the LDH and 6PGD pools, respectively, obtained after the immobilized Procion Blue. HE-GN chromatography step; lanes D and E, aliquots of purified LDH and 6PGD, respectively, obtained after Phenyl-Sepharose chromatography.

results seem as good as (with regard to specific activity) or better than (with regard to yield) than those produced by the technique formerly used[6], based on a much more expensive affinity chromatography support (2′,5′-ADP agarose). If one is not interested in simultaneous LDH purification, it is possible to elute 6PGD directly from the Procion Blue HE-GN column directly with hydrolysed NADP, without prior use of AMP. It was checked also on a large scale that no LDH was eluted by hydrolysed NADP, the 6PGD obtained at the end of the purification procedure is of same purity as that produced by the complete procedure.

Phenyl-Sepharose chromatography for LDH purification. The dimensions of the Phenyl-Sepharose column used for LDH purification are 3.2 cm I.D. and height 23 cm. Phenyl-Sepharose chromatography is conducted manually. Solid ammonium sulphate is added up to 10% saturation to the LDH pool obtained in the Procion Blue HE-GN chromatographic step. The LDH pool is then deposited at a flow-rate of 110 ml/h on to the column equilibrated in buffer E containing ammonium sulphate at 10% saturation. After completion of sample deposition, the column is developed with a non-linear gradient obtained by adequately mixing in an automatic gradient former (Gilson) initial buffer and buffer E. LDH is obtained in pure form (see Fig. 3). Quantitative data on one typical purification procedure are given in Table II.

General comments

It has already been described by others that it was possible to elute enzymes from dye columns with substances not known to interact with them (for instance, 2,3-diphosphoglycerate can elute glucose phosphate isomerase from a dye column as shown by Prehu *et al.*[13], who also gave several other examples of the elution of enzymes with non-natural ligands). It is tempting to speculate that every substance which in some way mimics a dye (*i.e.*, which associates a high density of charged groups with hydrophobic parts) has the potential to elute a protein from a dye column. It has even been demonstrated that metallic complexes could elute with some specificity enzymes from dye columns[14]. Hence we can hypothesize that to find for a given protein a more or less specific eluent that is readily available at a low price will be mostly a matter of systematic trial.

It has to be stressed, however, that another approach does exist to reducing the costs of affinity elution. Scopes[15] has demonstrated the usefulness of associating non-specific elution modes with affinity elution: a specific eluent has to be applied to the column only when by non-specific means (*e.g.*, by increasing the ionic strength or pH and/or possibly by using a buffer with no divalent cation added) the protein of interest "is just beginning to move down the column"; in this way it is possible to use low concentrations of specific eluents[16,17]. Obviously it could be interesting to combine both approaches.

Our work on erythrocytic 6PGD and LDH purification is in accordance with the now generally accepted notion that immobilized dye columns can compete in terms of efficiency with more expensive affinity chromatography matrices. Immobilized dye chromatography media are often used in the later steps of purification protocols for obtaining enzymes from haemolysate[18–20]. Our results confirm work by others[21,22] demonstrating that immobilized dye chromatography can also be used as the first step in purification from crude haemolysate. Screening procedures such as those described in the preceding paper can be very useful in choosing a dye well adapted to a specific purification problem.

ACKNOWLEDGEMENTS

The authors are grateful to ICI France for the generous gift of the dye used in this study and to the Association pour la Recherche en Structure Protéique and the Conseil Scientifique de l'UER Xavier-Bichat (Université Paris 7) for financial support. This work was also supported by the Ministère de l'Industrie et de la Recherche (contrat 85.T.0318).

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