Journal of Chromatography, 298 (1984) 483–493
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,832

COUNTER-CURRENT DISTRIBUTION OF YEAST ENZYMES WITH POLYMER-BOUND TRIAZINE DYE AFFINITY LIGANDS

GÖTE JOHANSSON*, MONICA ANDERSSON and HANS-ERIK ÅKERLUND
Department of Biochemistry, Chemical Center, University of Lund, P.O. Box 740, S-220 07 Lund (Sweden)
(Received April 17th, 1984)

SUMMARY

A protein extract from bakers' yeast has been resolved into several of its enzyme components by counter-current distribution using aqueous polymeric two-phase systems comprising water, dextran and polyethylene glycol. The distribution of dehydrogenases and kinases has been effectively changed by binding triazine dyes (Cibacron Blue F3G-A, Procion Yellow HE-3G and Procion Olive MX-3G) to the polyethylene glycol, confined to the upper phase. The effect of the dye ligands on the enzymes decreased roughly in the order: phosphofructokinase, (EC 2.7.1.11), glucose 6-phosphate dehydrogenase, (EC 1.1.1.49), glyceraldehydephosphate dehydrogenase, (EC 1.2.1.12), alcohol dehydrogenase (EC 1.1.1.1), 3-phosphoglycerate kinase (EC 2.7.2.3), phosphoglycerate mutase (EC 2.7.5.3), hexokinase (EC 2.7.1.1) and enolase (EC 4.2.1.11). However, the distribution of specific enzymes was strongly dependent also on the dye concentration. By appropriate selection of conditions, purifications of up to 10-fold could be achieved for phosphofructokinase and 100-fold for one of the isoenzymes of hexokinase. Furthermore, the two isoenzymes of hexokinase could be almost completely separated from each other. The counter-current distributions were performed in a newly developed apparatus where the separation of phases was enhanced by centrifugation. This allowed 55 transfers to be completed within about 8 h.

INTRODUCTION

Most of the chromatographic methods used for purification of proteins, including ion-exchange, adsorption and affinity chromatography, work on the basis of salt-gradient (or ligand) resolvation. The binding of proteins to the solid phase is an "either-or" phenomenon, and the eluent can be adjusted so that the protein moves more slowly through the column than the liquid phase only in the case of small protein molecules. Proteins with higher molecular weights, $M_r > 25{,}000$, do not show any measurable partition between the solid and the liquid phase. Examples of partition of proteins between two phases are found in gel chromatography and in the so-called aqueous polymeric two-phase systems[1,2]. The two-phase systems may be used for separation of proteins by taking advantage of differences in their distribution

0021-9673/84/$03.00 © 1984 Elsevier Science Publishers B.V.

between the phases. Since many proteins show a similar partition it has been necessary to devise various means of directing the distribution of the proteins. Beside the choice of salt[2–4], the partition can be directed by binding of charged groups[4,5], hydrophobic groups[6] or specific ligands for the protein[7–9] to one of the polymers (polyethylene glycol) present in the upper phase. The latter method has been used for purification of phosphofructokinase from yeast[10]. For the extraction of kinases and dehydrogenases, triazine dyes have been used as ligands. These so-called reactive dyes are cheap, easy to bind to the polymers and are commercially available in several molecular structures which show great variety in binding to different enzymes[11]. By increasing the concentration of polymer-bound ligand the partition of the ligand-binding enzymes can be gradually changed[12]. In some cases it has been possible to alter the partition coefficient selectively by a factor of 10^4 by introducing a ligand into one of the phases[12]. Different proteins show different partition responses to the ligand[13,14], which suggests that it should be possible to resolve them in a multistep procedure.

In the present work we have investigated the potential for protein purification of ligand-containing aqueous two-phase systems when combined with a multistep extraction, *i.e.*, counter-current distribution. The results show that by appropriate selection of ligand and ligand concentration a powerful separation of different proteins can be obtained.

MATERIALS AND METHODS

Chemicals

Polyethylene glycol (PEG) with M_r = 6500–8000 was purchased from Union Carbide (New York, NY, U.S.A.). Dextran, M_r = 500,000, was obtained from Pharmacia (Uppsala, Sweden). Triazine dye derivatives of PEG were prepared as described earlier[12,15] using Procion dyes donated by Swedish ICI. Biochemicals and auxiliary enzymes were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Two-phase systems

The systems were prepared from stock solutions of the polymers in water, 40% (w/w) PEG and 20% (w/w) dextran. The polymer solutions were weighed out and were mixed with buffer, water and sample to give the following final concentrations: 7% (w/w) dextran and 5% (w/w) polyethylene glycol; 50 m*M* sodium phosphate buffer, pH 7.0; 0.2 m*M* EDTA and 5 m*M* 2-mercaptoethanol. The system was equilibrated at 3°C.

Yeast extract

Commercial bakers' yeast (press yeast, jästbolaget; Sollentuna, Sweden) was homogenized with twice its weight of crushed solid CO_2 in a house-hold blender equipped with rotating knives. After evaporation of the carbon dioxide, the mixture was diluted in 50 m*M* sodium phosphate buffer, pH 7.0, containing 5 m*M* 2-mercaptoethanol and 0.2 m*M* EDTA. After centrifugation at 5000 *g* for 15 min, the main part of the proteins in the supernatant was recovered by fractional precipitation with PEG as described in detail elsewhere[10]. The precipitate obtained between 6 and 12%

(w/w) PEG was dissolved in buffer (50 m*M* sodium phosphate, pH 7.0, 5 m*M* 2-mercaptoethanol and 0.2 m*M* EDTA) and stored at −30°C.

Protein determination

Protein was assayed according to Bradford[16] by using Coomassie Brilliant Blue G and its absorption measured at 595 nm using bovine serum albumin as standard.

Enzyme assay

Enzyme activities were determined photometrically at 340 or 240 nm (enolase and phosphoglycerate mutase) using a Hitachi 100-60 double beam spectrophotometer connected to an LKB 2210 potentiometric recorder. Descriptions of the assays are found in the following references: phosphofructokinase[17], glucose 6-phosphate dehydrogenase[18], 3-phosphoglycerate kinase[19], hexokinase[20], glyceraldehydephosphate dehydrogenase[21], phosphoglycerate mutase[22], enolase[23] and alcohol dehydrogenase[24].

Centrifugal counter-current distribution

The apparatus has been described in detail elsewhere[25]. The operating unit consists of an inner plate with 60 cavities for the upper phases surrounded by an outer ring containing corresponding cavities for the lower phases. The volume of each of the outer cavities was 0.96 ml. All operations were carried out at 3°C. The apparatus was loaded with 1.2 ml of upper phase and 0.8 ml of lower phase in each chamber formed by the disk and the ring. The systems in chambers 0–2 were loaded with yeast extract in the phase system, 25% or 17 mg protein per ml. Fifty-five transfers were carried out automatically with shaking for 5 min followed by centrifugation (80 *g*) for 3 min after each transfer. When the distribution was complete, 1.8 ml of 10 m*M* sodium phosphate buffer, pH 7.0, were added to each chamber giving one-phase systems which were analyzed for protein and enzyme activities.

Thin-layer counter-current distribution

The apparatus used was of the standard type for aqueous polymeric two-phase systems[1,2,26]. The separation of the phases occurred on standing and the heights of the systems were only a few millimetres to minimize the separation time. A set of plates with 60 chambers was used. The volume of each cavity in the lower plate was 0.79 ml. One ml of the upper and 0.65 ml of the lower phase were used. The number of transfers was 55. The shaking time was 2 min and the separation time 15 min per transfer cycle. All steps were carried out at 3°C. The systems were converted into one phase by addition of 1.5 ml of 10 m*M* sodium phosphate buffer after each separation.

RESULTS

Counter-current distribution of a protein extract from bakers' yeast, in a phase system containing Cibacron Blue PEG, resulted in a broad protein peak (Fig. 1a). Assays of the enzyme activities revealed drastic differences in the partition behaviour of individual enzymes. Thus, phosphofructokinase could be almost completely separated from glucose 6-phosphate dehydrogenase and hexokinase. Furthermore, hex-

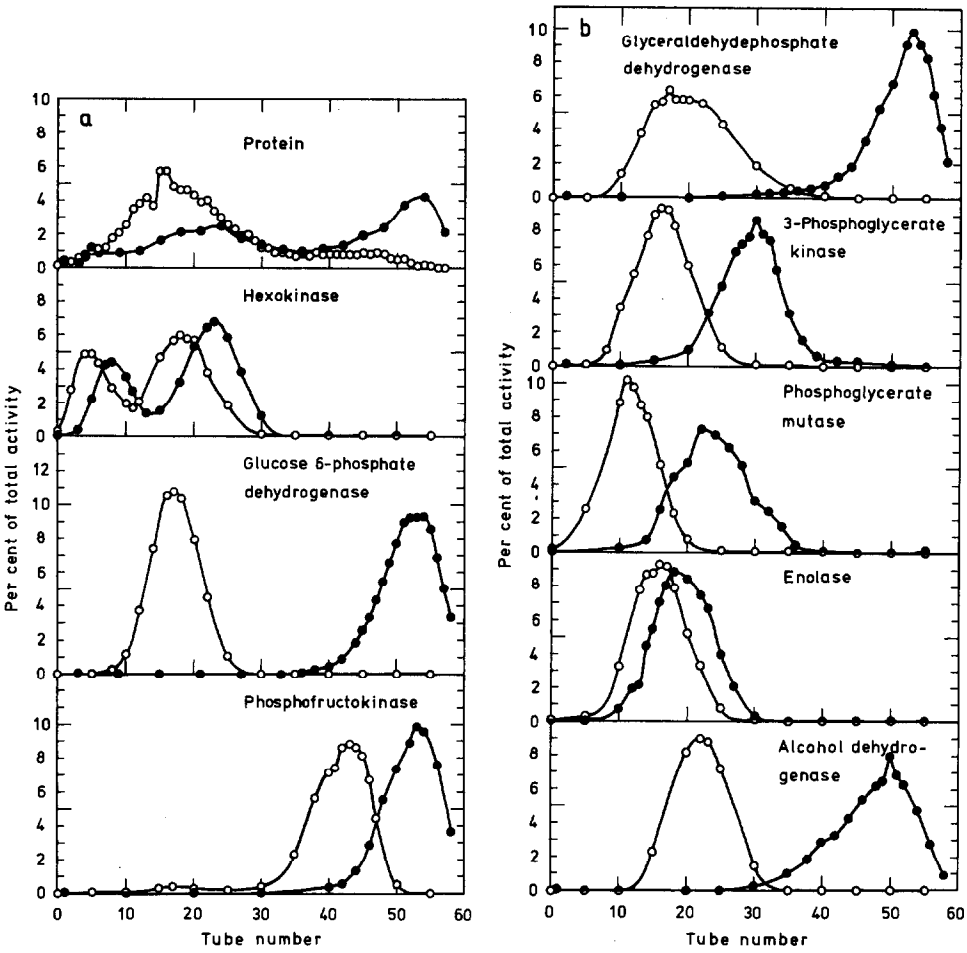

Fig. 1. C-CCD of yeast extract with 0.5% (○) and 5% (●) of total PEG in the form of Cibacron Blue–PEG. The sample was loaded in tubes 0–2.

okinase showed two peaks, probably representing the isoenzymes[27]. When the same experiments were performed in the absence of ligand–PEG, most of the material was retained within the first 20 tubes, Fig. 3, except for part of the second peak of hexokinase. The phase composition was actually chosen so that most of the proteins were partitioned into the lower phase in the absence of ligand–PEG. Thus, the separation between the enzyme activities seen in Fig. 1 can be ascribed to the various degrees of interaction between the enzymes and the ligand–PEG.

These counter-current distribution experiments were performed in a newly developed apparatus where the phase separation was facilitated by centrifugation (C-CCD). When the counter-current distribution was carried out in a conventional apparatus[26], where phase separation was facilitated by the use of chambers which allow the systems to form thin layers (TL-CCD), a separation effect could also be demonstrated (Fig. 2). However, the peaks obtained were much broader and considerable

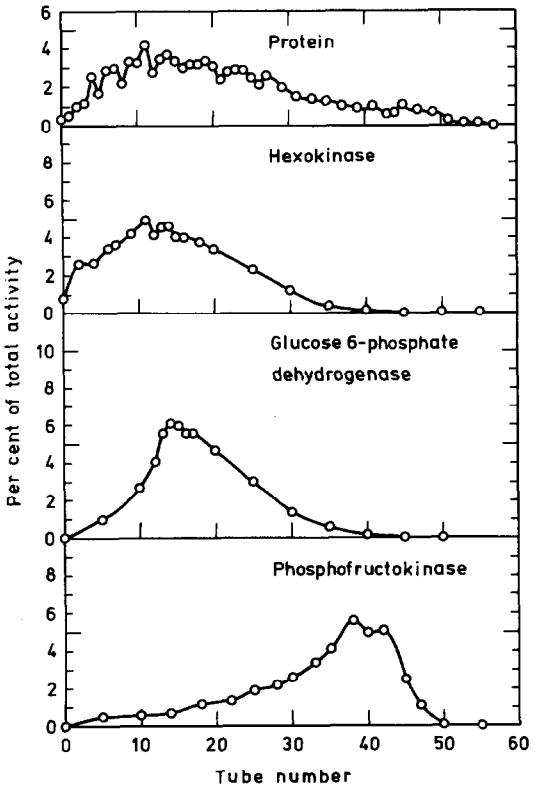

Fig. 2. TL-CCD of yeast extract with 0.5% of total PEG in form of Cibacron Blue–PEG.

overlap was seen between phosphofructokinase and glucose 6-phosphate dehydrogenase. The difference between C-CCD and TL-CCD was especially pronounced for hexokinase, which could be resolved into two peaks by C-CCD while it showed only one broad peak in TL-CCD. The basis for these differences can be found in the properties of the phase systems used. The combination of high molecular weights and high concentrations of the phase-forming polymers results in a high viscosity of the lower phase, which is accentuated at the low temperature (3°C) used. Also the dye–PEG and the proteins from the sample make the phases more viscous compared to normal phase systems without sample. It should in principle be possible to overcome this problem by just increasing the time for phase separation in each step. However, the 15 min used here for TL-CCD is already inconveniently long. The separation time required for C-CCD was only 3 min. Due to differences in shaking intensity, the required mixing time in each step was 5 min for the C-CCD machine but only 2 min for the TL-CCD machine. Despite this, the C-CCD gave a better resolution within about half the time required for the TL-CCD. We therefore used the C-CCD technique in the following work.

As the C-CCD machine utilizes centrifugation (80 *g*) to facilitate phase separation it is possible that some large particles, with associated proteins, would sediment during a run. This possibility could, however, be ruled out for the double peak

TABLE I

G VALUES OF THE ENZYMES CALCULATED FROM THE C-CCD EXPERIMENTS IN FIGS. 1–4

The separation factor, $\beta_{HK(1)}$ is the ratio between the *G* values of the enzyme and of hexokinase (left peak), respectively. The percentages refer to the fraction of polyethylene glycol bound ligand.

Enzyme	*No ligand*		*Cibacron Blue F3-GA*				*Procion Yellow HE-3G, 1%*		*Procion Olive MX-3G, 1%*	
	G	$\beta_{HK(1)}$	*0.5%*		*5%*		*G*	$\beta_{HK(1)}$	*G*	$\beta_{HK(1)}$
			G	$\beta_{HK(1)}$	*G*	$\beta_{HK(1)}$				
Hexokinase 1	0.02	1	0.07	1	0.15	1	0.15	1	0.02	1
Hexokinase 2	0.45	22	0.45	6.3	0.67	4.4	1.3	8.6	0.45	22
Glucose 6-phosphate dehydrogenase	0.17	8.5	0.41	5.9	17.3	115	6.9	46	2.44	122
Phosphofructokinase	0.08	4	3.2	46	17.3	115	5.1	34	4	200
Glyceraldehydephosphate dehydrogenase	0.17	8.5	0.4–0.6	5.7–8.3	17.3	115	8.2	54	0.77	39
3-Phosphoglycerate kinase	0.29	15	0.38	5.4	1.12	7.4	3.4	23	0.62	31
Phosphoglycerate mutase	0.17	8.5	0.22	3.2	0.62	4.1	1.9	13	0.93	27
Enolase	0.32	16	0.38	5.4	0.45	3.0	0.57	3.8	0.34	17
Alcohol dehydrogenase	0.29	15	0.62	8.8	8.2	54	0.67	4.4	1.04	52

of hexokinase, since none of these enzyme fractions sedimented during a 30-min centrifugation at 34,000 *g* when diluted five-fold in water.

Fig. 1b shows the distribution of some other enzymes present in the yeast extract (same experiment as in Fig. 1a). Only phosphofructokinase, of the measured enzymes (Figs. 1a and 2), showed high affinity for the upper phase when 0.5% of the polyethylene glycol was in the form of the Cibacron Blue F3G-A derivative. The other enzymes, however, did not appear in identical positions, thereby showing differences in their partition ratio, *G*. The *G* value is defined as the percentage of the enzyme (at equilibrium) in the mobile part of the system (main part of the upper phase) divided by the percentage in the stationary part (lower phase + small part of the upper phase). From the positions of the peaks in the C-CCD diagram, the *G* values have been calculated, Table I, using the approximative relationship[28] $G = \hat{\imath}/(n - \hat{\imath})$, where $\hat{\imath}$ is the distance in number of tubes that the enzyme (peak position) has moved and *n* is the number of transfers. Corrections have been made for the fact that the sample was loaded in three cavities.

By increasing the bound ligand concentration ten times, from 0.5 to 5% of the total polyethylene glycol, several of the enzymes together with a considerable part of the protein are moved further to the right, Fig. 1, because of increased affinity for the upper, ligand-containing phase. The most drastic shifts were found for alcohol dehydrogenase, glucose 6-phosphate dehydrogenase and glyceraldehydephosphate dehydrogenase. The effect on 3-phosphoglycerate kinase was less marked. The *G*

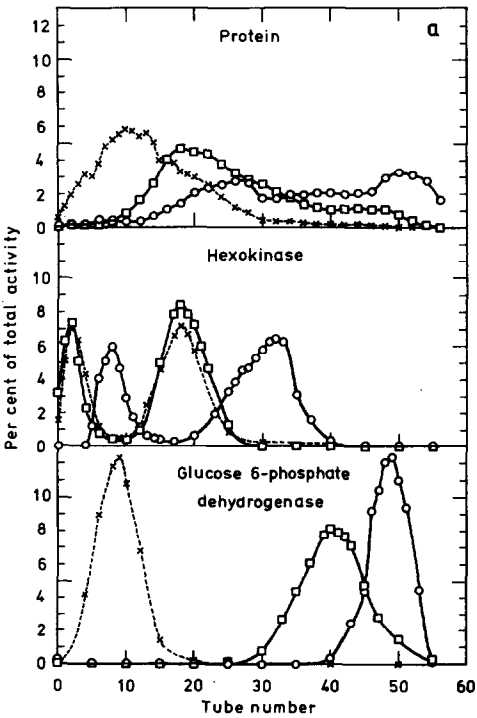

Fig. 3.

(Continued on p. 490)

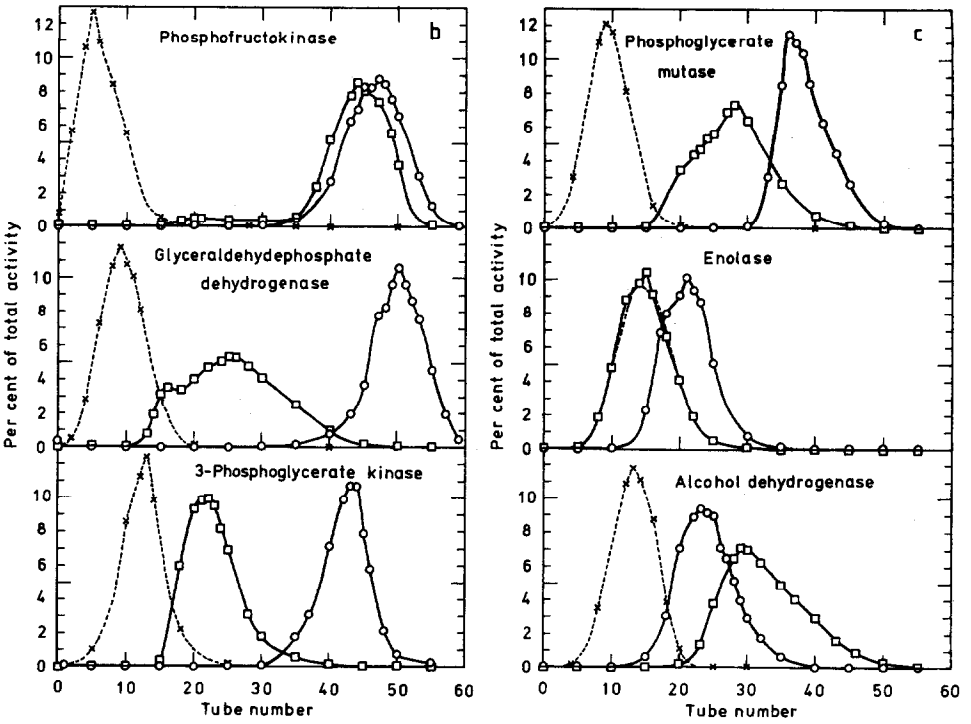

Fig. 3. C-CCD of yeast extract with 1% Procion Yellow–PEG (○), 1% Procion Olive–PEG (□) (of total PEG) or with no ligand (× -- ×).

values are given in Table I. The separation ability of counter-current distribution depends on the volumes of the two phases and the volumes of the stationary and mobile phase. However, the ratio between the *G* values for two components can be used as a general measure of the separation capacity. This ratio has been calculated for the enzymes relative to the left peak of hexokinase, Table I. Practically all the measured enzymes showed increased *G* values at higher ligand concentrations, but to various extents.

Two other ligands, Procion Yellow HE-3G and Procion Olive MX-3G, have been used. The results of the C-CCD using 1% ligand–polyethylene glycol as well as with no ligand are shown in Fig. 3. Procion Yellow in general showed greater separation effects on most of the enzymes than did Procion Olive, even if the increase in the *G* values varied from one enzyme to another (from a factor of 1.3 for phosphofructokinase to one of 11 for glyceraldehydephosphate dehydrogenase). In contrast, alcohol dehydrogenase was more effectively extracted with Procion Olive. Two enzymes, hexokinase and enolase, did not change their position, Fig. 3, when Procion Olive was used compared to the case without ligand. The ratio between the *G* values obtained with the two dyes and with one dye relative to no ligand are given in Table II.

TABLE II

RELATIVE *G* VALUES FOR THE ENZYME PEAKS IN FIG. 3

G_n, with no ligand; G_y, with Procion Yellow HE-3G and G_o, with Procion Olive MX-3G.

Enzyme	G_y/G_n	G_o/G_n	G_y/G_o
Hexokinase 2	2.9	1.0	2.9
Glucose 6-phosphate dehydrogenase	41	14	2.8
Phosphofructokinase	64	50	1.3
Glyceraldehydephosphate dehydrogenase	48	4.5	10.6
3-Phosphoglycerate kinase	12	2.1	5.5
Phosphoglycerate mutase	48	4.5	2.0
Enolase	1.8	1.1	1.7
Alcohol dehydrogenase	12	2.1	0.64

DISCUSSION

The results demonstrate that aqueous two-phase systems containing PEG-bound triazine dyes, in combination with counter-current distribution, can be used for fractionation of a complex protein mixture. The triazine ligands on PEG not only result in the extraction of specific enzymes but different enzymes were extracted to various degrees. Thus a separation could be obtained based on the strength of binding. Furthermore, the partition of the proteins could be varied by choice of ligand and ligand concentration, Table I. In several cases, the CCD diagram also show symmetrical peaks which indicate a true partition equilibrium. Unsymmetrical or broad peaks (alcohol dehydrogenase, Figs. 1b and 3c; glyceraldehydephosphate dehydrogenase, Figs. 1b and 3b and phosphoglycerate mutase, Figs. 1b and 3c), present in the same C-CCD diagram as symmetrical peaks, indicate that the enzymes seem to exist in multiple forms. These enzymes are known to exist in several isoenzymic forms[29,30]. The unsymmetrical peaks may also be due to interactions with other proteins[31]

The C-CCD technique can be used for purification of enzymes and it has the advantage compared with batchwise extraction that no enzyme is lost. This is of special importance when the enzyme activity can be split up into several components, *i.e.*, isoenzymes. The resolution of the hexokinase activity into two peaks, Figs. 1a and 3a, is an example. The importance of the choice of the ligand so as to obtain a high degree of purification can be seen in the case of phophofructokinase, Figs. 1a and 3b. The lower concentration used (0.5% bound ligand) resulted in a high degree of purification with 8–10 times higher specific activity, while at higher concentrations of ligand the specific activity was lower due to co-extraction of several other enzymes having lower binding constants. The fact that most proteins have a low but significant affinity for the triazine dyes can also be used for the purification of enzymes which do not interact at all. An example is hexokinase I (left peak) which was purified more than 100-fold in the case of Procion Olive, Fig. 3a.

The results in Fig. 3 have been compared with those from a manual CCD with only nine transfers[14]. Large differences are noticed for some enzymes on the basis of their R_F values, *i.e.*, distance of travel of the peak divided with the number of transfers, Table III. The nine-transfer CCD differs from the reported method in having

TABLE III

R_F VALUES FOR THE ENZYMES WITH (FIG. 3) AND WITHOUT LIGAND-PEG

n = Number of transfers used in the counter-current distribution. The values for the manual CCD with $n = 9$ have been calculated from data in ref. 14.

Enzyme	*Procion Yellow HE-3G*		*Procion Olive MX-3G*		*No ligand*	
					n = 9	*n = 55*
	n = 9	*n = 55*	*n = 9*	*n = 55*		
Hexokinase	0.11	0.13 0.56	0.11	0.02 0.31	0	0.02 0.31
Glucose 6-phosphate dehydrogenase	0.89	0.87	0.89	0.71	0	0.15
Phosphofructokinase	0.78	0.84	0.67	0.80	0	0.07
Glyceraldehydephosphate dehydrogenase	0.11	0.89	0.06	0.44	0	0.15
3-Phosphoglycerate kinase	0.28	0.77	0.22	0.38	0	0.22
Phosphoglycerate mutase	–	0.65	0.72	0.49	0	0.15
Enolase	0	0.36	0	0.25	0	0.24
Alcohol dehydrogenase	0	0.40	0.72	0.51	0	0.22

higher polymer concentrations (10% dextran and 7% PEG), a 5.6 times higher concentration of bound ligand and a somewhat lower temperature (0°C). Despite this, both glucose 6-phosphate dehydrogenase and phosphofructokinase have comparable R_F values in the two systems. Glyceraldehydephosphate dehydrogenase and 3-phosphoglycerate kinase show, however, much higher R_F values for the 55-transfers CCD. This may be due to a relatively strong interaction of the enzymes with other proteins favoured by the high polymer concentrations. With the higher number of transfers (and with lower polymer concentrations), the assumed protein–protein interactions are successively decreased due to dilution. Furthermore, as the ligand-interacting protein moves along the CCD train leaving the competing protein behind, the partition coefficient will gradually increase. In some other cases, alcohol dehydrogenase and phosphoglycerate mutase with Procion Olive, the enzymes show lower mobility upon 55 transfers, indicating that the ligand concentration was not high enough for saturation of the binding.

The effectiveness of the polymer-bound ligand, in changing the partition coefficient of an enzyme, increases with the concentration of the two polymers necessary to form the two phases[12]. High polymer concentrations, however, also make the phases very viscous which may cause problems in equilibration and separation of the two liquid layers. The results obtained here show that the C-CCD technique is superior to TL-CCD under such conditions. The time required for C-CCD was less than half that for TL-CCD. With equally good separation of the phases, TL-CCD might need 30–60 min per step using the same two-phase system as employed here. In an attempt to increase even further the effectiveness of the ligand-specific extraction, higher polymer concentrations were tested (10% dextran and 7% polyethylene glycol). A good separation was still achieved but the mixing step was not satisfactory (not shown). This is, however, a minor technical problem and adjustments of the C-CCD apparatus will in the near future make such experiments possible too.

ACKNOWLEDGEMENTS

We thank Professor Per-Åke Albertsson for valuable discussions and Swedish ICI for the generous gift of the Procion dye samples. This work was supported by a grant (to G.J.) from STU-National Swedish Board for Technical Development.

REFERENCES

1 P.-Å. Albertsson, B. Andersson, C. Larsson and H.-E. Åkerlund, *Methods Biochem. Anal.*, 28 (1981) 115.
2 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist & Wiksell, Stockholm, 2nd ed., 1971.
3 G. Johansson, *Acta Chem. Scand., Ser. B*, 28 (1974) 873.
4 G. Johansson, *J. Chromatogr.*, 150 (1978) 63.
5 G. Johansson, A. Hartman and P.-Å. Albertsson, *Eur. J. Biochem.*, 33 (1973) 379.
6 V. P. Shanbhag and G. Johansson, *Biochem. Biophys. Res. Commun.*, 61 (1974) 1141.
7 S. D. Flanagan and S. H. Barondes, *J. Biol. Chem.*, 250 (1975) 1484.
8 P. Hubert, E. Dellacherie, J. Neel and E.-E. Baulieu, *FEBS Lett.*, 65 (1976) 169.
9 M.-R. Kula, G. Johansson and A. F. Bückman, *Biochem. Soc. Trans.*, 7 (1979) 1.
10 G. Kopperschläger and G. Johansson, *Anal. Biochem.*, 124 (1982) 117.
11 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301.
12 G. Johansson, G. Kopperschläger and P.-Å. Albertsson, *Eur. J. Biochem.*, 131 (1983) 589.
13 G. Kopperschläger, G. Lorenz and E. Usbeck, *J. Chromatogr.*, 259 (1983) 97.
14 G. Johansson and M. Andersson, *J. Chromatogr.*, 291 (1984) 175.
15 G. Johansson, *Methods Enzymol.*, 104 (1984) 356.
16 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
17 E. Hofmann and G. Kopperschläger, *Methods Enzymol.*, 90 (1982) 49.
18 E. A. Noltmann, C. J. Gubler and S. A. Kuby, *J. Biol. Chem.*, 236 (1961) 1225.
19 R. K. Scopes, *Methods Enzymol.*, 42 (1975) 127.
20 P. K. Maitra, *Methods Enzymol.*, 42 (1975) 25.
21 H. U. Bergmeyer, *Methoden der Enzymatischen Analyse*, Vol. I, Verlag Chemie, Weinheim/Bergstrasse, 2nd ed., 1970, p. 425.
22 S. Grisolia and J. Carreras, *Methods Enzymol.*, 42 (1975) 435.
23 E. W. Westhead, *Methods Enzymol.*, 9 (1966) 670.
24 E. Racker, *Methods Enzymol.*, 1 (1955) 500.
25 H.-E. Åkerlund, *J. Biochem. Biophys. Methods*, 9 (1984) 133.
26 P.-Å. Albertsson, *Anal. Biochem.*, 11 (1965) 121.
27 E. A. Barnard, *Methods Enzymol.*, 42 (1975) 6.
28 C. J. O. R. Morris and P. Morris, *Separation Methods in Biochemistry*, Pitman, London, 1964, p. 559.
29 W. B. Stallcup, S. C. Mockrin and D. E. Koshland, Jr., *J. Biol. Chem.*, 247 (1972) 6277.
30 H. Edelhoch, V. W. Rodwell and S. Grisolia, *J. Biol. Chem.*, 228 (1957) 891.
31 L. Backman and G. Johansson, *FEBS Lett.*, 65 (1976) 39.