Journal of Chromatography, 303 (1984) 39–51
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,975

PARAMETERS DETERMINING AFFINITY PARTITIONING OF YEAST ENZYMES USING POLYMER-BOUND TRIAZINE DYE LIGANDS

GÖTE JOHANSSON* and MONICA ANDERSSON
Department of Biochemistry, University of Lund, Lund (Sweden)
(Received June 18th, 1984)

SUMMARY

Triazine dyes, bound to polyethylene glycol, have been used to influence the partition of some enzymes within a dextran–polyethylene glycol–water two-phase system. The enzymes, present in a protein extract from baker's yeast, included glucose 6-phosphate dehydrogenase, glyceraldehyde phosphate dehydrogenase, 3-phosphoglycerate kinase and alcohol dehydrogenase. The partition coefficients of the enzymes could be changed by a factor of 10–500 in favour of the polyethylene glycol-rich phase, while the partition of bulk protein was much less affected. The influence of the concentration of polymer-bound dye and phase-forming polymers, temperature, pH, kind and concentration of salt and the presence of nucleotides on this affinity partitioning effect was studied. The extraction was effective even at high concentrations of dye and protein (40 g/l). A partial purification (32-fold) of glucose 6-phosphate dehydrogenase was carried out by an extraction in five steps.

INTRODUCTION

Aqueous two-phase systems have been used for the purification of enzymes and other proteins by taking advantage of the difference in partition of proteins between the two liquid phases. These systems are usually comprised of water and two polymers, *e.g.*, dextran and polyethylene glycol (PEG)[1–3]. Even at low concentrations of polymers, 3–8% of each, two phases are formed and the polymers occur in opposite phases. Because of the high water content of both phases, proteins can be included in the system and their partition between the phases is dependent on the concentration and molecular weight of the polymers, salt and buffer included in the system, pH and temperature[1,4,5]. The partition of proteins can be significantly and selectively influenced by covalently binding various chemical groups to one of the phase-forming polymers. This has been demonstrated by using charged groups[3,6], hydrophobic groups[6–9] or specific ligands[10–12], so-called affinity partitioning. The polymer most often used as carrier for these groups is PEG.

Triazine dyes can be effective ligands for dehydrogenases, kinases and a number of other enzymes and proteins. These dyes have found much use in affinity chromatography[13,14], but have also been used for affinity partitioning[5,15–20]. The param-

0021-9673/84/$03.00

eters affecting affinity partitioning have been studied for pure enzymes, especially phosphofructokinase and glucose 6-phosphate dehydrogenase[16,17]. In the present work, various parameters affecting the partition of enzymes present in an extract of baker's yeast have been studied using triazine dyes (Procion dyes) as polymer-bound ligands.

MATERIALS AND METHODS

Chemicals

Polyethylene glycols (Carbowax) with M_r = 6500–8000 (PEG 6000) and 35,000–40,000 (PEG 40,000) were purchased from Union Carbide (New York, NY, U.S.A.) and Serva (Heidelberg, F.R.G.), respectively. Dextrans, M_r = 70,000 (dextran 70) and 500,000 (dextran 500), were obtained from Pharmacia (Uppsala, Sweden). Triazine dye derivatives of PEG were prepared as described earlier[15,17] using Procion dyes donated by Swedish ICI (Procion Yellow HE-3G and Procion Olive MX-3G). Cibacron Blue F3G-A was purchased from Serva. Biochemicals and auxiliary enzymes were provided by Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Ligand–dextran derivatives

A 20-g amount of dextran was dissolved in 80 g water with heating and continuous stirring, followed by 8 g $Na_3PO_4 \cdot 12H_2O$ and 0.6 g triazine dye. The mixture was kept for 1 h on a water-bath at 90°C and was then neutralized with concentrated acetic acid, using a pH meter. The dextran was precipitated by addition of 150 ml ethanol with careful mixing. The liquid was decanted and the dextran was dissolved in 100 ml of warm water. It was again precipitated with 150 ml ethanol and the residue was dissolved in 50 ml water. This solution was treated with 5 g DEAE-cellulose (Whatman DE52, washed to neutrality) for 1 h to remove unbound dye. After filtration the solution was dialyzed against 2 × 5 l of distilled water. The dextran concentration of the solution was determined by a polarimeter, assuming an optical rotation of 199° per dm at a concentration of 1 g/ml[1]. The dye content was determined photometrically at suitable wavelengths in the visible region[17].

Two-phase systems

The systems were prepared from stock solutions of the polymers in water. The PEG solutions were 30 or 40% (w/w) and the dextran solutions 20% (w/w). The polymer solutions were weighed out and were mixed with salt, buffer, water and sample to the final weight, usually 4 g. After equilibration at 0°C in a water–ice mixture, the systems were carefully mixed by twenty inversions of the graduated centrifuge-tubes normally used. The contents were then centrifuged at 700 *g* at the same temperature for 3–5 min. The volumes of the phases were read and samples were withdrawn and analyzed for protein and enzyme activity. In the cases where the pH was varied this was determined in the mixed system using a glass electrode of combined type (Radiometer GK 2401C). The partition coefficient, K, of the enzyme and protein, respectively, is defined as the ratio between the concentrations of the component in the upper and lower phases.

Yeast extract

Commercial baker's yeast (press yeast, jästbolaget; Sollentuna, Sweden) was homogenized with twice its weight of crushed solid CO_2 in a household 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* ethylenediaminetetraacetate (EDTA). After centrifugation at 5000 *g* for 15 min, the main part of the proteins of the supernatant was recovered by fractional precipitation with 6–12% (w/w) PEG (M_r = 6500–8000) as described in detail elsewhere[15]. The precipitate was dissolved in buffer (50 m*M* sodium phosphate, pH 7.0, 0.2 m*M* EDTA and 5 m*M* 2-mercaptoethanol) and stored at −30°C.

Protein determination

Protein was assayed according to Bradford[21] by using Coomassie Brilliant Blue G and measured at 595 nm using bovine serum albumin as standard.

Enzyme assay

Enzyme activities were determined photometrically at 340 nm using a Hitachi 100-60 double-beam spectrophotometer connected to a LKB 2210 potentiometric recorder. Alcohol dehydrogenase (E.C. 1.1.1.1) was measured according to Racker[22], glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) according to Noltmann *et al.*[23], 3-phosphoglycerate kinase (E.C. 2.7.2.3) according to Scopes[24] and glyceraldehyde phosphate dehydrogenase (E.C. 1.2.1.12) according to Bergmeyer[25].

RESULTS

The partition of enzymes between the two liquid phases has been studied by using a crude protein preparation from baker's yeast as described under Materials and Methods. By comparing the partition of the target enzymes with the partition of total protein the usefulness of affinity partitioning for enzyme purification can be evaluated. A number of parameters have been investigated in the search for the optimum conditions for extraction of enzymes by this affinity technique.

Concentration of polymer-bound ligand

As in the case of pure enzymes[17,18], the partition of enzymes present in the yeast extract shows a typical "saturation" curve when the logarithm of the partition coefficient, log *K*, is plotted *versus* the concentration of polymer-bound ligand, Fig. 1. The steepness of the extraction curves is related to the binding constant for the ligand–enzyme interaction. The relative binding constant and the maximum *K* values can be evaluated by using an inverse plot[17], Fig. 2. While pure enzyme in general seems to give rise to linear inverse plots[17,18], enzymes in the extract often exhibit curved lines. The extrapolated maximum values of Δ log *K* are, except for 3-phosphoglycerate kinase with Cibacron blue, close to 2, Fig. 2. For total protein the corresponding value is 0.9–1.

Concentration of phase-forming polymers

When no ligand is present in the system, an increase in the concentration of PEG and dextran results in a stronger preference of both total protein and enzymes

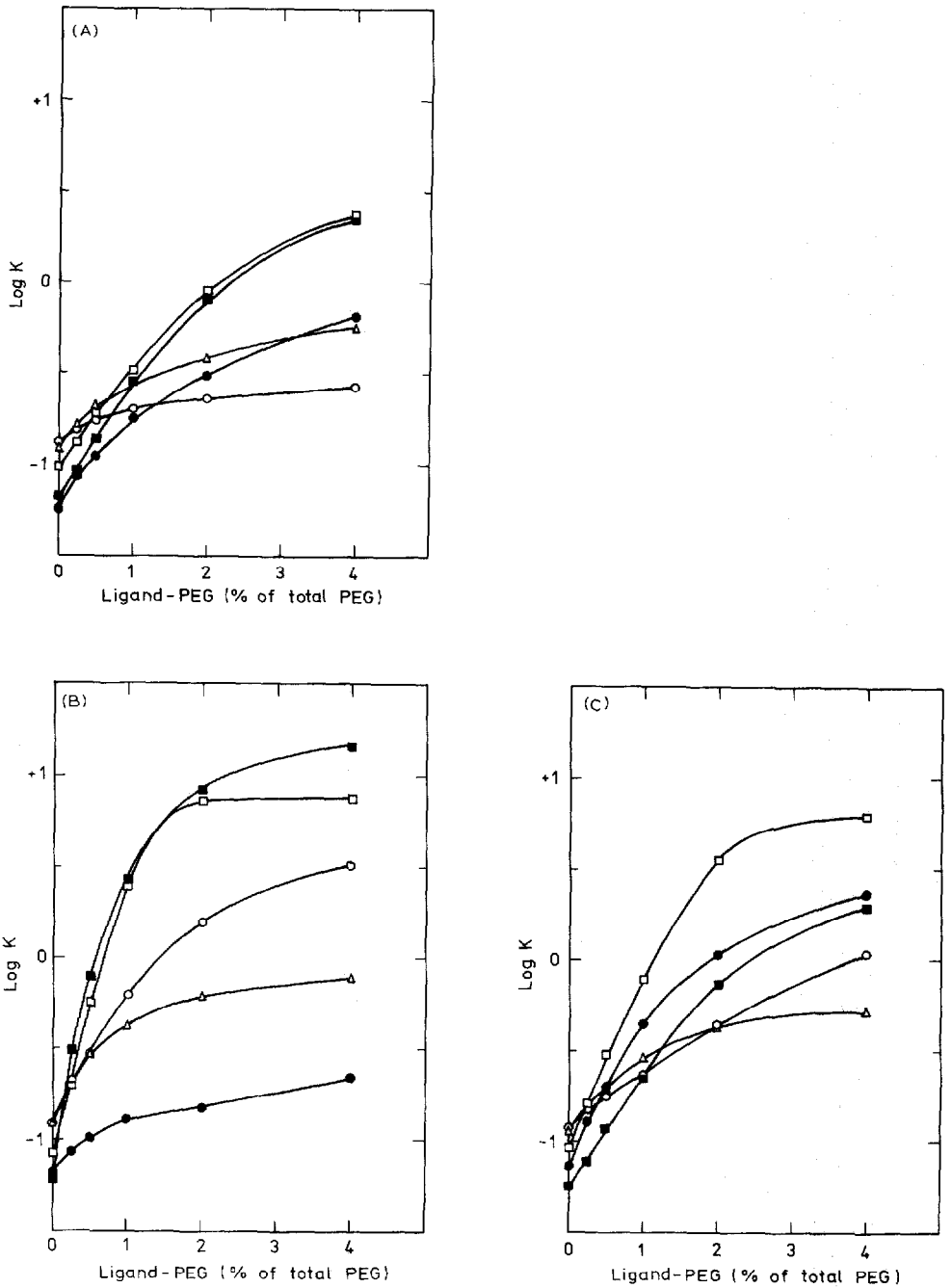

Fig. 1. Partition as function of the concentration of PEG-bound ligand. Ligands: A, Cibacron Blue F3G-A; B, Procion Yellow HE-3G; C, Procion Olive MX-3G. System: 7% dextran 500, 5% total PEG 6000, 25 m*M* sodium phosphate buffer pH 7.0 and yeast extract (10% of the system). Temperature: 0°C. Curves: △, protein; □, glucose 6-phosphate dehydrogenase; ○, 3-phosphoglycerate kinase; ●, alcohol dehydrogenase; ■, glyceraldehyde phosphate dehydrogenase.

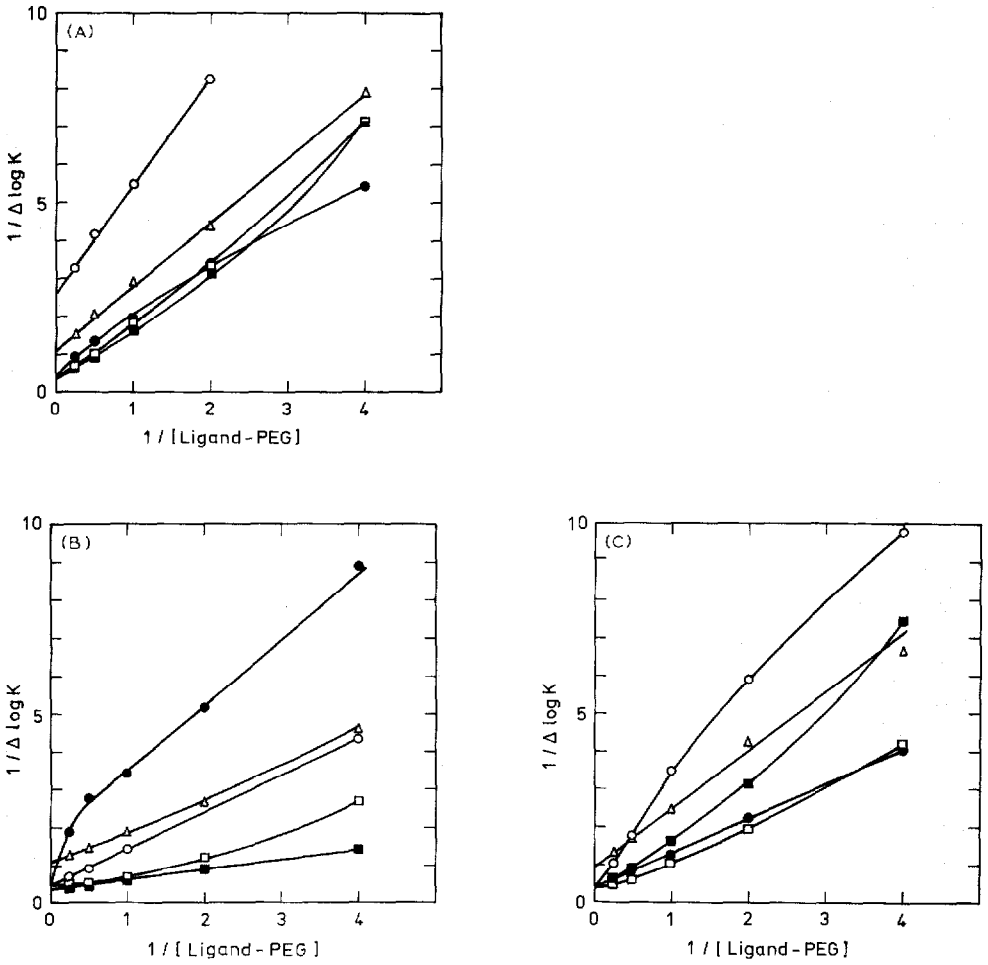

Fig. 2. Inverse plots of the data in Fig. 1 (same symbols).

for the lower, dextran-rich phase, *i.e.*, the K value decreases, Fig. 3. The addition of PEG-bound ligand (in excess) results in an extraction of the ligand-binding enzymes into the upper phase, the effectiveness of which, expressed as Δ log K, *i.e.*, log K (with ligand) − log K (without ligand), increases with increasing polymer concentration. The system used reverts to one phase if the concentrations of polymers are below 3% PEG and 4.2% dextran. Extrapolations towards this point in Fig. 3 indicate a limiting K value of 1 for all the enzymes and total protein, whether or not the ligand is present.

Two affinity ligands in opposite phases

Since the two phase-forming polymers are concentrated in opposite phases, dextran in the lower and PEG in the upper phase, one ligand can be anchored in each phase. If both ligands bind to the same enzyme the relative ligand concentration and the relative binding strength will determine the resulting partition coefficient.

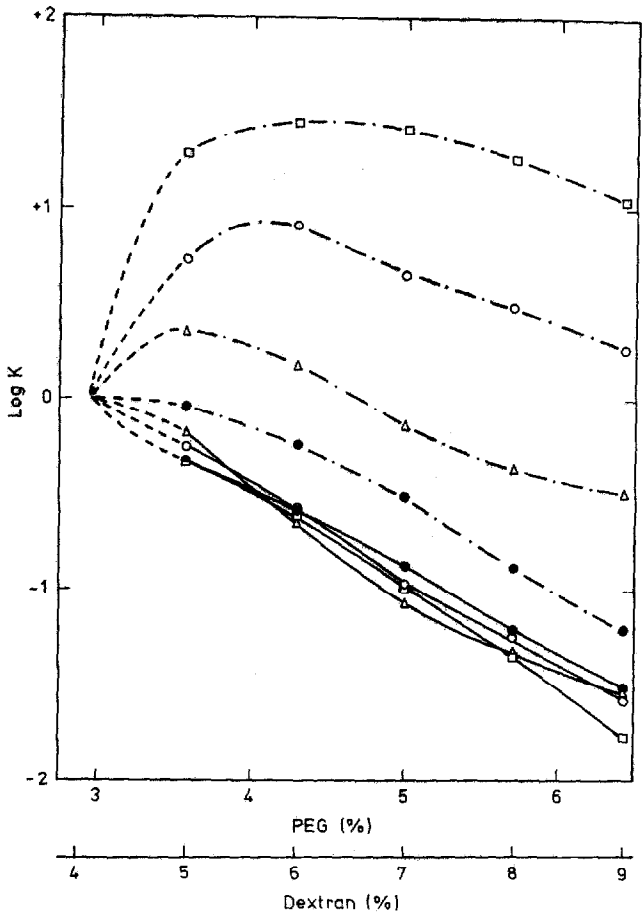

Fig. 3. Dependence on the concentration of phase-forming polymers (dextran and PEG). System: dextran 500, total PEG 6000, 25 m*M* sodium phosphate buffer pH 7.0 and yeast extract (10%). Temperature: 0°C. Curves: ——, without PEG-bound ligand; — · —, with PEG-bound Procion Yellow HE-3G. △, Protein; □, glucose 6-phosphate dehydrogenase; ○, 3-phosphoglycerate kinase; ●, alcohol dehydrogenase.

This is shown in Fig. 4 where the concentration of ligand in the upper phase is kept constant while the concentration of a second ligand in the lower phase is varied. In this way, glucose 6-phosphate dehydrogenase can be effectively extracted into the lower phase and the partition coefficient is changed by a factor of 10,000. The partition of total protein also changes, but to a lesser degree.

By using the same ligand in both phases (not shown) it was found that the dextran-bound ligand can have a greater affinity partitioning effect, *i.e.*, result in a larger change in log *K*, than can the PEG-bound ligand. This might be related to the fact that PEG does not bind more than one ligand per molecule, while the large dextran molecule may bind several ligand molecules which permits a more effective binding of the chelate type.

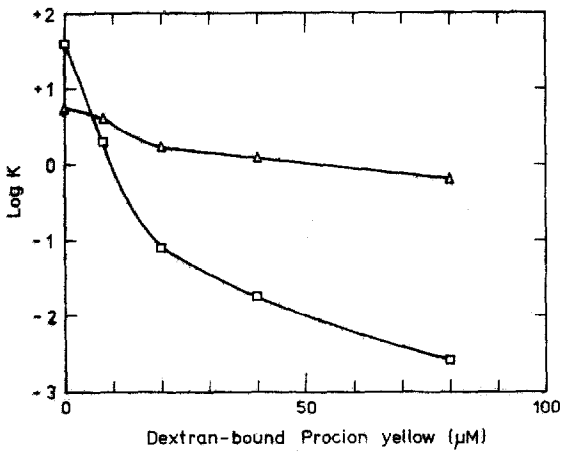

Fig. 4. Effect of dextran-bound Procion Yellow HE-3G. System: 10% dextran 500, 7% total PEG 6000 containing Cibacron Blue F3G-A (1:10) and 50 m*M* sodium phosphate buffer pH 7.0. Temperature: 0°C. Curves: △, protein; □, glucose 6-phosphate dehydrogenase.

TABLE I

EFFECT OF TEMPERATURE ON THE PARTITION OF PROTEIN, GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND 3-PHOSPHOGLYCERATE KINASE

The increase in log *K* due to PEG-bound ligand = *Δ* log *K*. System composition: 7% dextran 500, 5% PEG 6000, 25 m*M* sodium phosphate buffer pH 7.0 and 10% yeast extract. Concentration of PEG-bound ligand = 4% of total PEG.

Measured activity	*Temperature (°C)*	*log K in system with no ligand*	*Δ log K*	
			With Procion Yellow HE-3G	*With Procion Olive MX-3G*
Protein	0	−1.07	0.93	0.78
	10	−0.71	0.73	0.64
	20	−0.35	0.48	0.43
	30	−0.14	0.33	0.31
	40	0.04	0.21	0.19
Glucose 6-phosphate dehydrogenase	0	−0.99	2.40	1.81
	10	−0.81	2.00	1.72
	20	−0.52	1.56	1.16
	30	−0.35	0.98	0.74
	40	−0.30	0.61	0.34
3-Phosphoglycerate kinase	0	−0.97	1.62	0.84
	10	−0.82	1.48	0.80
	20	−0.59	1.18	0.56
	30	−0.33	0.50	0.28
	40	−0.23	0.17	−0.01

TABLE II

pH DEPENDENCE

Yeast extract (2.5%) was partitioned in a system containing 7% dextran 500, 5% PEG 6000 and 25 m*M* sodium phosphate buffer at 0°C. The increase in log *K*, *Δ* log *K*, was achieved with PEG-bound Cibacron Blue F3G-A.

Activity	*Concentration of Cibacron Blue PEG (% of total PEG)*	*Δ log K*		
		pH 6.9	*pH 7.7*	*pH 8.1*
Glucose 6-Phosphate dehydrogenase	0.1	0.46	0.22	0.15
	0.5	1.52	0.86	0.64
	2.0	2.27	1.47	1.35
Alcohol dehydrogenase	0.1	0.48	0.22	0.13
	0.5	0.98	0.48	0.33
	2.0	1.65	1.01	0.75

Temperature

The partition of the enzymes and total protein depends on the temperature. Table I. The *K* values increase significantly, 5–10 times, when the temperature is increased from 0 to 40°C. The ligand partition effect, as measured by *Δ* log *K*, also decreases strongly with increasing temperature.

pH effects

Even in a moderate pH range, 6.9–8.1, Table II, the affinity partitioning effect is strongly reduced by increasing pH.

TABLE III

INFLUENCE OF NUCLEOTIDES ON THE AFFINITY PARTITIONING OF ENZYMES PRESENT IN YEAST EXTRACT

Δ log *K* = log *K* (with PEG-bound Procion Yellow HE-3G, 5% of total PEG, with or without nucleotide) − log *K* (with no PEG-bound ligand and without nucleotide). Systems contained 7% dextran 500, 5% PEG 6000, 25 m*M* sodium phosphate buffer pH 7.0, 10% yeast extract, 1.6 m*M* $MgSO_4$ and 0.8 m*M* EDTA. Values in parentheses show the remaining affinity partitioning effect relative to the case with no nucleotide.

Enzyme	*Δ log K*			
	No nucleotide	*+ NAD (1 mM)*	*+ NADP (1 mM)*	*+ ATP (1 mM)*
Glucose 6-phosphate dehydrogenase	2.67 (100%)	2.62 (98%)	1.39 (52%)	2.32 (87%)
Alcohol dehydrogenase	0.82 (100%)	0.05 (6%)	0.52 (63%)	0.42 (52%)
3-Phosphoglycerate kinase	1.73 (100%)	1.54 (89%)	1.62 (94%)	1.38 (79%)
Glyceraldehyde phosphate dehydrogenase	2.48 (100%)	2.16 (87%)	2.48 (100%)	2.30 (93%)

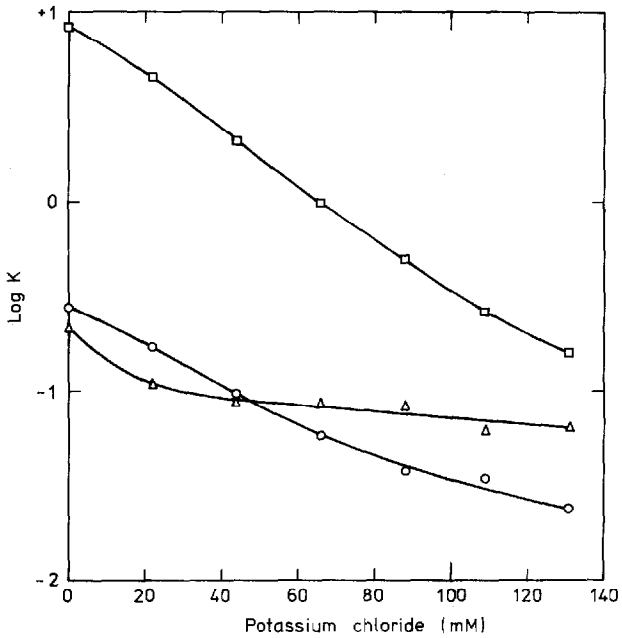

Fig. 5. Effect of KCl on the partition of yeast extract. System: 9% dextran 70, 5.5% PEG 40,000, 25 m*M* sodium phosphate buffer pH 7.0 and yeast extract (6%). Temperature: 0°C. Curves: △, protein; □, glucose 6-phosphate dehydrogenase; ○, 3-phosphoglycerate kinase.

Competition with free nucleotide ligands

The triazine dyes are assumed to bind mainly to the same sites as nucleotides such as NAD, NADP or ATP[26]. In some cases this reduces the affinity partitioning, Table III. The effect is small for 3-phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase, but a selectivity for the natural coenzyme (ATP and NAD, respectively) can be seen. Large effects are observed in the case of glucose 6-phosphate dehydrogenase with NADP and alcohol dehydrogenase with NAD.

TABLE IV

EFFECT OF SALTS ON THE PARTITION OF YEAST EXTRACT

System: 10% dextran 500, 7% PEG 6000, 25 m*M* sodium phosphate buffer pH 7.0, 10% yeast extract and various amounts of salt; 1% PEG was in form of PEG-bound Procion Yellow HE-3G. Temperature: 0°C. The partition coefficients (log *K*) of total protein and glucose 6-phosphate dehydrogenase (G6PDH) were determined.

Concentration of salt (mM)	*With sodium acetate*		*With KCl*		*With potassium phthalate*		*With benzyl-ammonium acetate*	
	Protein	*G6PDH*	*Protein*	*G6PDH*	*Protein*	*G6PDH*	*Protein*	*G6PDH*
0	0.06	0.50	0.06	0.50	0.06	0.50	0.06	0.50
5	–	–	–	–	−0.12	0.10	0.00	0.33
10	−0.04	0.24	−0.07	0.01	−0.30	−0.14	−0.06	0.18
30	−0.14	−0.06	−0.26	−0.33	–	–	−0.22	−0.29
50	−0.21	−0.23	−0.45	−0.70	–	–	−0.31	−0.66
100	−0.32	−0.46	−0.76	−1.28	–	–	–	–

Salts

The affinity partitioning can be reduced by using salts of various kinds. This effect depends on the salt concentration, Fig. 5. Acetate has a more moderate reducing effect than chloride on the affinity partitioning of glucose 6-phosphate dehydrogenase, Table IV. Even aromatic ions show a strong effect. The benzylammonium ion, Table IV, is comparable to KCl even when acetate is chosen as counter ion; phthalate is even more potent. The strengthening of the dye–enzyme interaction caused by low concentrations of divalent cations, *e.g.*, Ni^{2+}, Co^{2+} or Zn^{2+}, that has been demonstrated for affinity chromatography could not be detected in affinity partitioning. This was true even when the concentration of the divalent ion was ten times higher than that of EDTA.

High protein concentration

The affinity partitioning works well even when relatively large quantities of protein are included in the systems. This is illustrated in Fig. 6 where 60% of the system is in the form of yeast extract, corresponding to about 4% protein. In this case all the PEG carries ligand, which makes the systems very viscous. To obtain phase separation the systems must be centrifuged. As in Fig. 3 the partition is dependent on the concentration of phase-forming polymers. The partition coefficients have been plotted *versus* the length of the tie-line of the system found in the phase diagram[1]. By definition the square of the length of the tie-line is equal to the sum of the squares of the difference between the concentrations in the upper and lower phase of dextran and PEG, respectively. This parameter has been found to give a relatively linear dependence on the K values of proteins[6]. The purification factors (enzyme/protein) obtained in the upper phase are 3.4–3.8 with more than 95% recovery of glucose 6-phosphate dehydrogenase in a single partition step.

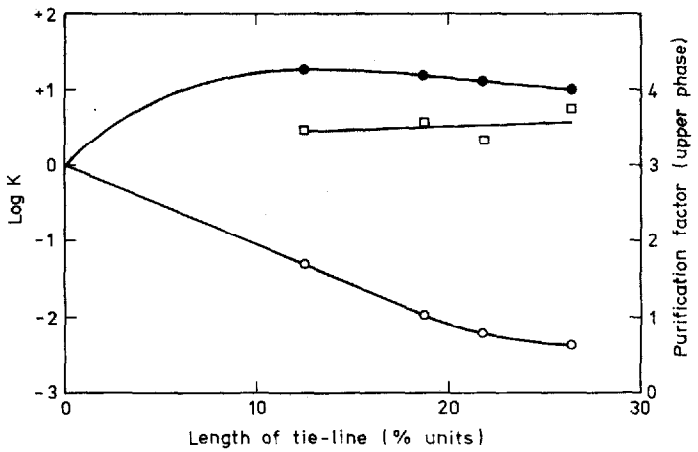

Fig. 6. Partition of glucose 6-phosphate dehydrogenase at high concentration of extract and various polymer concentrations. System: dextran 500, PEG 6000, 30 mM sodium phosphate buffer pH 7.0 and yeast extract (60%). Curves: ○, no ligand; ●, PEG-bound Cibacron Blue F3G-A (100%). Temperature: 0°C. □, Purification factor for the enzyme in the upper phase in the presence of PEG-bound ligand.

TABLE V

MULTISTEP EXTRACTION

Systems were made up from phases of 9% dextran 70, 5.5% PEG 40000 and 45 m*M* sodium phosphate buffer pH 7.0. Temperature: 0°C. System 1 contained yeast extract (14 mg protein and 28 units of glucose 6-phosphate dehydrogenase).

Extraction No.	*Polymer-bound ligand*		*Volume (ml)*		*Phase transferred to next extraction step*	*Enzyme recovery in transferred phase (% of original activity)*	*Purification factor*
	Upper phase	*Lower phase*	*Upper phase*	*Lower phase*			
1	Procion olive	–	4.2	1.8	Upper	92.1	4.4
2	Procion olive	–	3.8	1.7	Upper	78.6	6.6
3	Procion olive	–	3.8	1.8	Upper	73.6	7.5
4	Procion olive	Procion yellow	3.6	1.9	Lower	60.0	20.1
5	Procion olive	Procion yellow	2.1	1.8	Lower	55.7	32.0

Extraction of enzymes in several steps

By partitioning of the protein extract from baker's yeast in a system containing ligand only in one phase, followed by repeated "washing" of the phase containing the target enzyme with the opposite phase, with or without ligand, a considerable degree of purification could be achieved for glucose 6-phosphate dehydrogenase, Table V.

DISCUSSION

The affinity partitioning of enzymes is an interesting method for their purification since even crude protein extracts can be used as enzyme sources as has been demonstrated in this work. To be able to optimize the separationing power of the two-phase aqueous systems it is necessary to know the influence of the possible factors on the partition, both for the target enzyme and protein in general. The protein extract of baker's yeast used here was obtained by fractional precipitation with PEG, in order to have a low contamination of membranes, high-molecular-weight nucleic acids and salts.

The binding curves, Fig. 1, show that affinity partitioning can be very effective, with an up to 500 times increase in the partition coefficient even when the enzyme has only two binding sites per molecule, as is the case for glucose 6-phosphate dehydrogenase. In some cases, as for alcohol dehydrogenase, the extraction curves and the inverse plots, Fig. 2, indicate that at least two types of binding sites are present. The concentration of ligands necessary to reach 50% of saturation is usually more than 50 times the concentration of binding sites of the enzymes studied. Since no sigmoidal extraction curves have been found, it can be assumed that no components with acceptionally strong binding of the ligand are present in noticeable amount.

The effect of the increasing concentration of polymers is the result of a more

and more effective exclusion of the polymer present in low concentration, *e.g.*, dextran from the upper phase. The polymer-bound ligand will consequently get a more extreme partition coefficient, since binding of a ligand has only little or no effect on the partition of the polymer.

The experiments in Fig. 4 show that both polymers can be used as carriers for the ligand, and the choice depends on whether the target enzyme is to be extracted into the upper or the lower phase. This offers several interesting possibilities. By using a second ligand bound to the lower phase the upper phase, from a preliminary extraction, can be further depleated of protein or the target enzyme may be extracted back into the lower phase, all depending on the relative binding to the two ligands. Systems with two ligands in opposite phases should also be an effective way of separating two proteins each having exclusive binding to one of the ligands. The ratio between the partition coefficients of the two proteins (containing at least two binding sites per molecule) could be of the order 10^4–10^8.

The effect of salts on the affinity partitioning is mainly due to shielding effects on the electrostatic part of the ligand–enzyme interaction. It could be used to reduce weak and unwanted interactions between the ligand and the bulk proteins. Since the effect of ions depends both on their type and concentration, on the ligand dye and the enzyme, the influence of different salts on the affinity partitioning must be tested in every case. Nucleotides, which are biologically active ligands, *e.g.*, NADP for glucose 6-phosphate dehydrogenase, are of special interest. By using the appropriate nucleotides it should be possible selectively to eliminate enzyme–dye interactions and so to "strip off" enzymes with preferential binding to a certain nucleotide from the dye-containing phase. The effect of NAD and NADP can be strongly enhanced by addition of low concentrations of sulphite[18].

The conditions for a successful purification of an enzyme by using affinity partitioning with the inexpensive triazine dyes cannot be generalized. It is very useful if several dyes can be found which all bind to the target enzyme. By choosing a concentration of the base polymers which gives an acceptably high $\Delta \log K$ value the ligand concentration of the first dye used is adjusted so as to extract 75–95% of the target enzyme. If the ligands are arranged in a series of increasing binding constants the target enzyme can then successively be extracted into opposite phases. In order to increase the degree of purification, certain salts or nucleotides can be added to the enzyme which reduce co-extraction. The selectivity can be improved by increasing the pH value. Higher temperatures may also reduce "unspecific" binding.

Since the systems give good fractionation even with fully substituted PEG and the protein concentration is as high as 4%, Fig. 6, affinity partitioning should be useful for large-scale preparation of enzymes. The maximum extraction capacity corresponds to around 70 g protein per litre of system. This is the amount of protein obtained in our experiments from 14 kg of fresh press yeast.

ACKNOWLEDGEMENTS

We thank Professor G. Kopperschläger for valuable discussions concerning affinity partitioning and Swedish ICI Co, for the gift of triazine dyes. This work has been supported by grants from STU, The National Swedish Board for Technical Development.

REFERENCES

1 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist and Wiksell, Stockholm, 2nd edn., 1971.
2 G. Johansson, A. Hartman and P.-Å. Albertsson, *Eur. J. Biochem.*, 33 (1973) 379.
3 P.-Å. Albertsson, B. Andersson, C. Larsson and H.-E. Åkerlund, *Methods Biochem. Anal.*, 28 (1981) 115.
4 G. Johansson, *Acta Chem. Scand., Ser. B*, 28 (1974) 873.
5 G. Johansson, *Methods Enzymol.*, 104 (1984) 356.
6 G. Johansson, *J. Chromatogr.*, 150 (1978) 63.
7 V. P. Shanbhag and G. Johansson, *Biochem. Biophys. Res. Commun.*, 61 (1974) 1141.
8 C.-G. Axelsson and V. P. Shanbhag, *Eur. J. Biochem.*, 71 (1976) 419.
9 G. Johansson and V. P. Shanbhag, *J. Chromatogr.*, 284 (1984) 63.
10 S. D. Flanagan and S. H. Barondes, *J. Biol. Chem.*, 250 (1975) 1484.
11 P. Hubert, E. Dellacherie, J. Neel and E.-E. Baulieu, *FEBS Lett.*, 65 (1976) 169.
12 M.-R. Kula, G. Johansson and A. F. Bückmann, *Biochem. Soc. Trans.*, 7 (1979) 1.
13 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301.
14 G. Kopperschläger, H. J. Böhme and E. Hofmann, in T. K. Ghose, A. Flechter and N. Blakebrough (Editors), *Advances in Biochemical Engineering*, Vol. 25, Springer, Berlin, New York, 1982, p. 101.
15 G. Kopperschläger and G. Johansson, *Anal. Biochem.*, 124 (1982) 117.
16 K. H. Kroner, A. Cordes, A. Schelper, M. Morr, A. F. Bückmann and M.-R. Kula, in T. C. J. Gribnau, J. Visser and R. J. F. Nivard (Editors), *Affinity Chromatography and Related Techniques*, Elsevier, Amsterdam, 1982, p. 491.
17 G. Johansson, G. Kopperschläger and P.-Å. Albertsson, *Eur. J. Biochem.*, 131 (1983) 589.
18 G. Kopperschläger, G. Lorenz and E. Usbeck, *J. Chromatogr.*, 259 (1983) 97.
19 G. Johansson and M. Andersson, *J. Chromatogr.*, 291 (1984) 175.
20 G. Johansson, M. Andersson and H.-E. Åkerlund, *J. Chromatogr.*, 298 (1984) 483.
21 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
22 E. Racker, *Methods Enzymol.*, 1 (1955) 500.
23 E. A. Noltmann, C. J. Gubler and S. A. Kuby, *J. Biol. Chem.*, 236 (1961) 1225.
24 R. K. Scopes, *Methods Enzymol.*, 42 (1975) 127.
25 H. U. Bergmeyer, *Methoden der Enzymatischen Analyse*, Vol. I, Verlag Chemie, Weinheim, Bergstrasse, 2nd ed., 1970, p. 425.
26 A. J. Turner, *Trends Biochem. Sci.*, 6 (1981) 171.