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LIQUID–LIQUID EXTRACTION OF GLYCOLYTIC ENZYMES FROM BAKERS' YEAST USING TRIAZINE DYE LIGANDS

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SUMMARY

Several glycolytic enzymes from an extract from Bakers' yeast have been partitioned between the two phases of an aqueous biphasic system comprising water, dextran and polyethylene glycol. The enzymes, which all showed high affinity for the lower, dextran-rich phase, in several cases could be extracted into the upper phase by using triazine dyes of the Procion type, bound to polyethylene glycol (enriched in this phase), as affinity ligand. The effectiveness of the extraction was determined by a nine-transfers counter-current distribution process, which made it possible to determine the enzyme partition also in cases when the polymer-bound ligand interfered with the enzyme assay. Seven dyes were tested and these showed large differences in their effect on the extraction of hexokinase (E.C. 2.7.1.1), glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49), aldolase (E.C. 4.1.2.13), glyceraldehyde 3-phosphate dehydrogenase (E.C. 1.2.1.12), phosphofructokinase (E.C. 2.7.1.11), 3-phosphoglycerate kinase (E.C. 2.7.2.3), phosphoglycerate mutase (E.C. 2.7.5.3), enolase (E.C. 4.2.1.11) and alcohol dehydrogenase (E.C. 1.1.1.1). The use of this kind of affinity partitioning for large scale extraction of enzymes is discussed.

INTRODUCTION

The two-phase systems comprising two polymers (usually dextran and polyethylene glycol) and water have been used for purification and studies of proteins[1–17]. The partition of proteins between the two liquid phases has been shown to depend on a number of factors, including the presence of salts and pH[2–4], the concentration and molecular weight of the two phase-forming polymers[4] and substituents bound to one of the polymers, *e.g.*, charged groups[5–7], hydrophobic groups[8–11] or specific protein ligands[12,13]. Attention has focused on the possibility of using triazine dyes as ligands for the extraction of enzymes[14–18]. By using the two-phase systems for affinity partitioning[12], it was possible to isolate phosphofructokinase from Bakers' yeast with Cibacron blue F3G-A as ligand[16]. The parameters determining the partition of this enzyme were studied by using the pure enzyme[17]. The effect of a number of triazine dyes on the partition of pure enzymes belonging to the classes of dehydrogenases and kinases has been studied by Kopperschläger *et al.*[18]. These enzymes

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are known to interact with some of the dyes, and this fact has been used for their purification by affinity chromatography[19–22].

In the present work we studied the possibility of isolating from a crude extract of Bakers' yeast certain enzymes involved in, or closely connected with, the process of glycolysis, by using triazine dyes bound to polyethylene glycol (PEG). The ligand polyethylene glycol is found almost completely in the upper phase, while proteins are normally enriched in the lower phase[17].

MATERIALS AND METHODS

Chemicals

Dextran (M_r = 500,000) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), polyethylene glycol (M_r = 6000–7500) from Union Carbide (New York, NY, U.S.A.). The Procion dyes were kindly donated by Swedish ICI Co. All chemicals were of analytical reagent grade. Biochemicals and enzymes were purchased from Sigma (St. Louis, MO, U.S.A.). The water was singly distilled and then passed through an ion exchanger.

Synthesis of dye–polyethylene glycol derivatives

The dyes were treated with polyethylene glycol in water under alkaline conditions and purified on a DEAE-cellulose bed as described elsewhere[17,23].

Yeast extract

Commercial Bakers' yeast (press yeast; Jästbolaget, Sollentuna, Sweden) was homogenized with crushed dry ice in an ordinary house-hold blender with rotating knives, and the major part of the protein was recovered by fractional precipitation with polyethylene glycol (M_r = 6000) as described earlier[16].

Counter-current distribution

Systems containing 10% (w/w) dextran and 7% (w/w) polyethylene glycol together with 25 m*M* sodium phosphate buffer (pH 7.1), 1 m*M* $MgSO_4$, 0.13 m*M* EDTA and 6.5 m*M* 2-mercaptoethanol were made up from stock solutions as described elsewhere[2,6,24]. A row of ten tubes were labelled from zero to nine. Tube 0 was used for the original phase system (2 g, 1.13 ml upper phase, 0.76 ml lower phase) containing the protein extract. Tubes 1–9 were each filled with 0.76 ml pure lower phase and 0.10 ml pure upper phase not containing any protein. All systems (with and without sample) were equilibrated at 0°C in an ice–water bath. The system in tube 0 was equilibrated by inverting the centrifuge tube 30 times, left in the ice-bath to settle for 3 min and then centrifuged at 800 *g* for 2 min in the cold. The main part of the upper phase, 1.03 ml, was transferred to tube 1, and the same volume of pure upper phase was then added to tube 0. After equilibration and separation as above, the upper phase (1.03 ml) of tube 1 was transferred to tube 2, the upper phase of tube 0 was transferred to tube 1 and fresh upper phase was added to tube 0. This procedure was repeated until nine transfers had been carried out and ten complete two-phase systems (0–9) obtained. Buffer, 10 m*M* sodium phosphate, pH 7.1 (5 ml) was added to each tube, yielding single phases. These solutions were analyzed for enzymes and protein. The solutions could be frozen and thawed with negligible loss

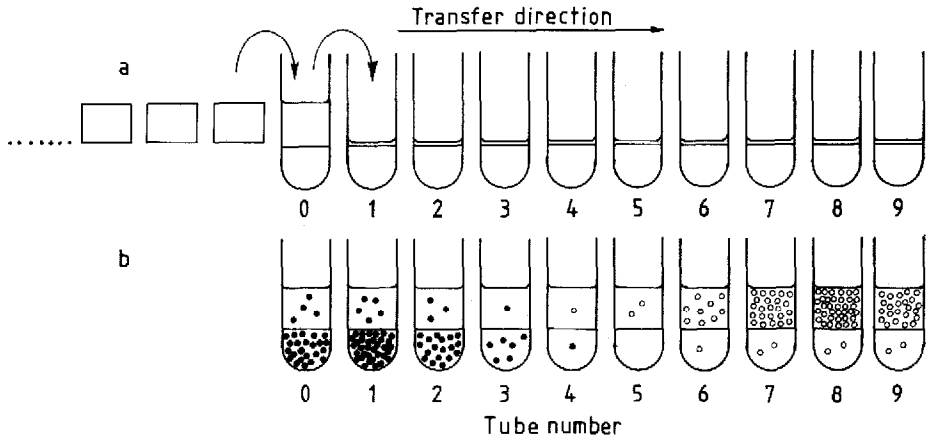

Fig. 1. Manual counter-current distribution. a, Arrangment for a nine-transfer counter-current distribution as described in the text. Sample is included in the system in tube 0. Curved arrows indicate the first transfer. b, The ten systems obtained after nine transfers. The dots show the distribution of homogeneous proteins with $K = 0.1$ (●) and $K = 10$ (○), respectively.

of enzymatic activity. Frozen fractions showed excellent enzyme activity also after several months at −30°C. The counter-current distribution process is illustrated in Fig. 1. The total amount of protein in each experiment was 9 mg.

Protein assay

Protein was determined according to Bradford[25] using a calibration curve obtained with bovine serum albumin.

Enzyme assays

All assays were carried out photometrically at 340 nm or at 240 nm (enolase and phosphoglycerate mutase) using a Hitachi 100-60 double beam spectrophotometer connected with a LKB 2210 potentiometric recorder. The assays used have been described for hexokinase[26], phosphofructokinase[27], aldolase[28], but without mercaptoethanol, 3-phosphoglycerate kinase[29], glucose 6-phosphate dehydrogenase[30], glyceraldehyde 3-phosphate dehydrogenase[31], enolase[32], phosphoglycerate mutase[33] and alcohol dehydrogenase[34], but with triethanolamine–HCl buffer.

RESULTS

Since in several cases the enzyme assays were affected by the presence of polymer-bound ligand, the direct determination of the partition coefficient by analysis of samples from the upper (ligand-rich) and lower phases was unsatisfactory. Instead the two-phase systems were used for a counter-current distribution (CCD), Fig. 1. The protein mixture was placed in tube 0, and the upper phase of this system was systematically (see Materials and Methods) equilibrated with pure lower phase as first described by Craig[35]. Material with high affinity for the lower phase will mainly remain in tube 0, while material with more or less pronounced affinity for the upper

phase will move to higher-numbered tubes (Fig. 1). In the described CCD experiments, 1.03 ml of the upper phase were mobile, while the lower phase (0.76 ml) and 0.1 ml of the upper phase were stationary.

The partition coefficient, K, of an enzyme is defined as the ratio between its concentrations in the upper and lower phases. The fraction of material, T_i, in tube i (i = 0–9) can be calculated from eqn. 1:

$$T_i = \frac{9!}{i!\,(9-i)!} \cdot \frac{G^i}{(1+G)^9} \tag{1}$$

if the K value is known[36]. The distribution ratio, G, is related to K[36] and the volumes of the lower phase, and mobile and stationary upper phases (given above), via eqn. 2:

$$G = \frac{K \cdot 1.03}{K \cdot 0.10 + 0.76} \tag{2}$$

Since the two-phase systems after CCD are broken down by addition of water, the total (apparent) enzyme activity can be determined. If it is assumed that an enzyme has a partition coefficient equal to one, the above equations indicate that the amount of enzyme is at a maximum in tube 5. On the other hand, an enzyme appearing completely in the upper phase, $K = \infty$ (at equilibrium), will have nearly equal amounts in tubes 8 and 9 (1:1.09) since the G value is 10.3. This is due to incomplete transferred of the upper phase.

CCD of yeast extract using nine transfers is shown in Fig. 2. When no ligand is attached to PEG the material is retained by the lower phase and appears mainly in tubes 0 and 1. Upon introducing the PEG-bound ligand (Procion dye), some enzymes move to the right in the diagram, Fig. 2. Most dyes effect the partition of glucose 6-phosphate dehydrogenase, while other enzymes are more selectively extracted. The approximate partition coefficients of the enzymes, calculated from the CCD, are shown in Table I.

DISCUSSION

One of the problems in the affinity partition of enzymes is the influence of the polymer ligand on the enzyme analysis. Triazine dyes bind to parts of the active and/or regulatory sites of kinases and dehydrogenases and they therefore influence the assay. Because of the high ligand concentration in the upper, polyethylene glycol-rich phase and very low concentration in the lower phase, for several enzymes it may be difficult to determine directly the partition coefficient using activity measurements. Thus, time consuming studies of the effect of the ligand polyethylene glycol on the assay are required. Another possibility, employed in this work, is the use of counter-current distributions followed by dilution of the two-phase systems in water after the distribution. The CCD train will then consist of a series of solutions, all containing the same concentration of the ligand polyethylene glycol. The extent of partition of the enzyme between the two phases is reflected in how far the enzyme "peak" travels along the series of tubes. Its position can easily be detected even if

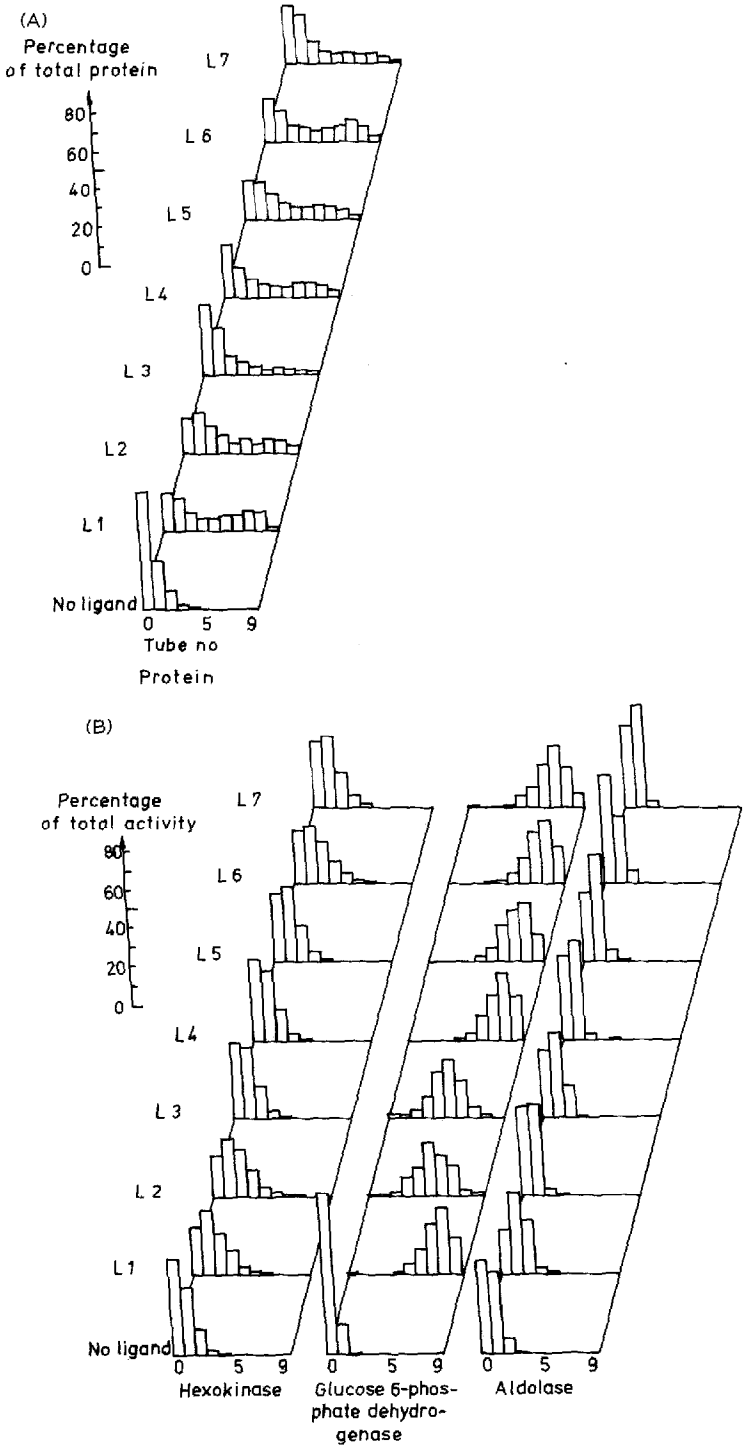

Fig. 2.

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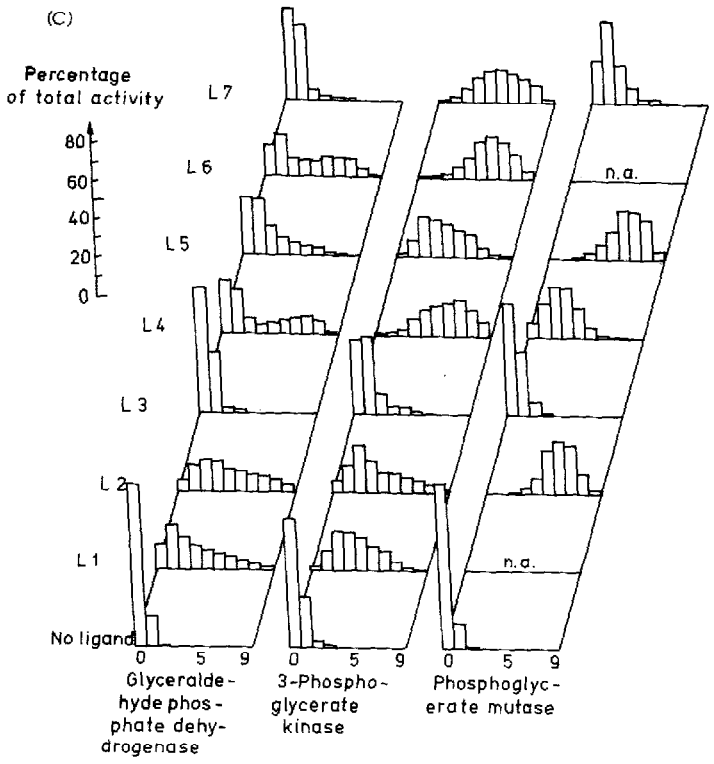
(C)
Percentage of total activity
80
60
40
20
0
L7
L6
L5
L4
L3
L2
L1
No ligand
n.a.
n.a.
0 5 9
0 5 9
0 5 9
Glyceralde-hyde phos-phate dehy-drogenase
3-Phospho-glycerate kinase
Phosphoglyc-erate mutase

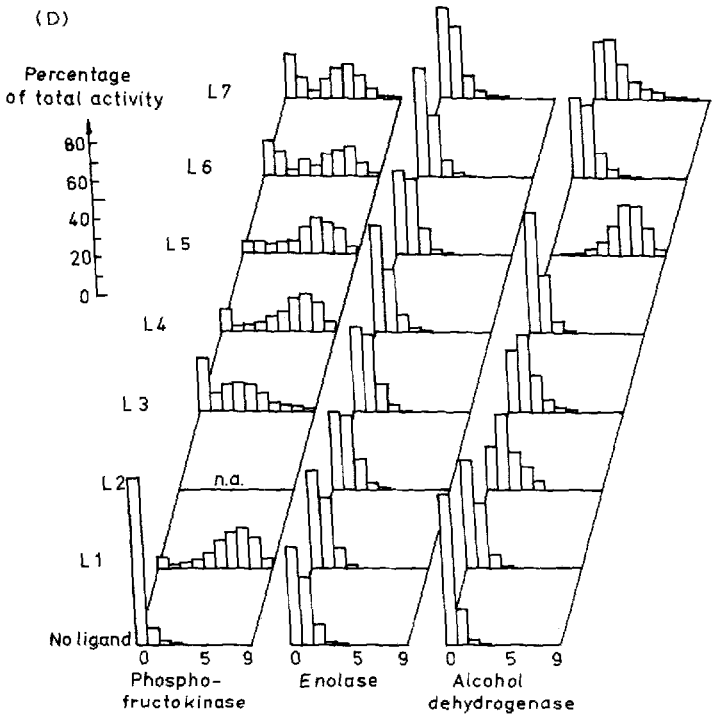
(D)
Percentage of total activity
80
60
40
20
0
L7
L6
L5
L4
L3
L2
L1
No ligand
n.a.
0 5 9
0 5 9
0 5 9
Phospho-fructokinase
Enolase
Alcohol dehydrogenase

TABLE I

PARTITION COEFFICIENT, K, OF ENZYMES AND TOTAL PROTEIN CALCULATED FROM THE COUNTER-CURRENT DISTRIBUTION EXPERIMENTS IN FIG. 2

HK = Hexokinase; G6PDH = glucose 6-phosphate dehydrogenase; PFK = phosphofructokinase; ALD = aldolase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; PGK = 3-phosphoglycerate kinase; PGM = phosphoglycerate mutase; EN = enolase; ADH = alcohol dehydrogenase.

Procion dye	*Protein*	*HK*	*G6PDH*	*PFK*	*ALD*	*GAPDH*	*PGK*	*PGM*	*EN*	*ADH*
–	0.04	0.06	0.014	0.01	0.07	0.015	0.03	0.013	0.07	0.02
Yellow HE-3G	0.07 5*	0.12	7.4	2.9	0.14	0.14	0.35	–	0.06	0.05
Navy H-4R	0.09 3*	0.13	1.3	–	0.08	0.3	0.3	1.8	0.08	0.11
Green H-E4BD	0.06	0.08	0.9	0.03 0.5*	0.09	0.04	0.07	0.05	0.08	0.09
Orange H-ER	0.05	0.07	10	3.0 0.02*	0.05	0.07 2.7	2.7	0.3	0.05	0.04
Olive MX-3G	0.08	0.09	5.6	1.9	0.09	0.08	0.4	2.3	0.07	2.3
Turquoise MX-G	0.06 3*	0.12	7.5	0.06 2.1	0.05	0.1 1.1	1.7	–	0.05	0.08
Scarlet MX-G	0.07	0.09	3.2	0.04 0.9*	0.09	0.07	1.0	0.12	0.07	0.09

* Minor peak.

the assay is strongly influenced by the ligand and if the sensitivity of the method is low. The CCD method also shows whether the enzyme activity behaves as a single component or whether several fractions exist as in the case of glyceraldehyde phosphate dehydrogenase (Fig. 2C) or phosphofructokinase (Fig. 2D). This heterogeneity may be due to isoenzymes, complex formation with other enzymes, etc. Such fractions can of course not be detected by a single partition experiment which will only show a mean of the partition of the components.

The CCD experiments with yeast proteins show that several kinds of enzymes can be extracted from a crude protein mixture using aqueous biphasic systems with an affinity ligand restricted to one phase. In contrast to affinity chromatography, where the binding in most cases is of all-or-none type, the present use of two liquid phases (affinity partitioning) is a clear-cut partition which can easily be changed by adjustment of pH[17,23], changes of ligand (Fig. 2) or the amounts of polymers[17].

The Procion dyes show a remarkable difference in their extraction properties. While all those tested here can be used for extraction of glucose 6-phosphate dehydrogenase (at pH 7), a higher specificity was found for the other enzymes. For an enzyme of interest there is a fair chance of finding a triazine dye which is effective, at least when the enzyme belongs to the classes of kinases or dehydrogenases. The

Fig. 2. Counter-current distribution of yeast extract and the distribution of total protein (A) and enzymes (B–D). The activities are expressed in per cent of total (recovered) activity. The abscissa shows the tube number (0–9). The following polymer-bound ligands have been used: L1 = Procion yellow HE-3G; L2 = Procion navy H-4R; L3 = Procion green H-E4BD; L4 = Procion orange H-ER; L5 = Procion olive MX-3G; L6 = Procion turquoise MX-G; L7 = Procion scarlet MX-G. 1/25 of the total polyethylene glycol was carrying the ligand. n.a. = No activity detectable.

described method for studying the dye–enzyme interaction may be useful for scanning the vast number of triazine dyes of which only a limited number are commercially available. The selectivity can be further enhanced by using two or more extraction steps combined with exchange of the upper or lower phase and change of ligand. Since the ligands have been chosen because of their low cost and ease of attachment to polyethylene glycol, this type of selective extraction should be useful for enzyme purification on a large scale.

The technical use of affinity partitioning is also favoured by the excellent volume capacity of the two-phase systems, first pointed out by Kroner *et al.*[15]. This is a consequence of the very high ligand concentration that can be used without the problems of non-reversible binding, *i.e.*, multiple-attachment phenomena, which frequently occur with matrix bound ligands of high concentration. The capacity of the systems used here is based on the concentration of polyethylene glycol, *i.e.*, 7% or 11 m*M*. If polyethylene glycol is fully monosubstituted with ligand and 10% of the ligands are bound (at equilibrium) to the enzyme the effective capacity should be 1.1 m*M*. For an enzyme with a molecular weight of 80,000 and two binding sites, the capacity will be 44 g ligand-binding enzyme per litre of two-phase system. The binding is fully reversible and the affinity-partitioning effect can be completely abolished by addition of excess of salt or coenzyme (NAD, NADP or ATP together with Mg^{2+} or SO_3^{2-})[17]. Preliminary results in our laboratory have also shown that the affinity partition works equally well when all the polyethylene glycol is monosubstituted.

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