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Liquid–liquid partitioning of some enzymes, especially phosphofructokinase, from *Saccharomyces cerevisiae* at sub-zero temperature

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Abstract

The effects of low temperature (−18°C) on the stability and partitioning of some glycolytic enzymes within an aqueous two-phase system were studied. The enzymes were phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase present in a crude extract of bakers' yeast. The partitioning of pure phosphofructokinase, isolated from bakers' yeast, was also examined. The two-phase systems were composed of water, poly(ethylene glycol), dextran, and ethylene glycol and buffer. The influence on the partitioning of the presence of ethylene glycol, phenylmethylsulfonyl fluoride and poly(ethylene glycol)-bound Cibacron Blue F3G-A was investigated at −18, 0 and (in some cases) 20°C. The presence of ethylene glycol, phase polymers and low temperature stabilized all three enzyme activities. Cibacron Blue, an affinity ligand for phosphofructokinase, increased its partitioning into the upper phase with decreasing temperature. Depending on the conditions, various amounts of the enzymes were recovered at the interface, also in systems not containing ethylene glycol. The implications of the observed effects on the use of aqueous two-phase systems for the extraction and fractionation of proteins are discussed.

Keywords: *Saccharomyces cerevisiae*; Partitioning; Aqueous two-phase systems; Bakers' yeast; Enzymes; Phosphofructokinase; Glyceraldehyde-3-phosphate dehydrogenase; Alcohol dehydrogenase; Poly(ethylene glycol)

1. Introduction

Aqueous two-phase systems obtained by dissolving two polymeric substances in water have been widely used for the fractionation and studies of cellular components, e.g., enzymes, by partitioning of the biomaterial between the two liquid phases [1–3]. The phases normally freeze when the temperature is below −1°C. However, the two-phase systems can be used for partitioning at much lower temperatures if part of the water is replaced by a (water-miscible) solvent, e.g., ethylene glycol or glycerol, which lowers the freezing point. Such two-phase systems for partitioning at sub-zero temperatures (cryopartitioning) have been studied in a few cases [4–6].

It may be assumed that by using this type of system at very low temperatures, enzymes might be kept in "frozen" conformational states which could even be separated from each other by

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partitioning. It has been shown earlier that a decrease in temperature from 0 to −10°C has a stabilizing effect on synaptic membrane structures [6].

In this work, the partitioning of enzymes and bulk proteins in an extract of bakers' yeast, and also pure phosphofructokinase, within aqueous two-phase systems was studied at sub-zero temperature (−18°C) and also, for comparison, at 0°C and in some cases at 20°C. In addition to the influence on the partitioning and stability of the enzymes at these temperatures, the effects of a polymer-bound affinity ligand, Cibacron Blue F3G-A, and of a protease inhibitor, phenylmethylsulfonyl fluoride, were investigated.

2. Experimental

2.1. Chemicals

Dextran, M_r 40 000, was purchased from Pharmacia (Uppsala, Sweden), poly(ethylene glycols) (PEG), M_r 3350 and 8000, from Union Carbide (New York, NY, USA), Cibacron Blue F3G-A from Serva (Heidelberg, Germany) and ethylene glycol (for analysis grade) from Merck (Darmstadt, Germany). Auxiliary enzymes, substrates and coenzymes were all obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical-reagent grade. Ultra-pure water (Amicon filtered) was used. Cibacron Blue–poly(ethylene glycol), M_r 8000 and containing 0.158 mmol/g of dye, was prepared according to a method described previously [7]. The phosphofructokinase (PFK) we obtained was prepared from bakers' yeast according to Hofmann and Kopperschläger [8] and was a kind gift from the latter. Most of the salt, $(NH_4)_2SO_4$, in which the enzyme was stored in precipitated form, was removed before adding it to the two-phase systems, by pelleting the PFK and resuspending it in 1 *M* triethanolamine (TEA)–HCl buffer.

2.2. Extract of bakers' yeast

A 100-g sample of bakers' yeast (Jästbolaget, Sollentuna, Sweden) was treated in a blender with rotating knives together with 500 g crushed dry-ice for 3 × 2 min. The powder obtained was spread in a 1-cm thick layer on a tray to let the carbon dioxide evaporate (ca. 30 min). The liquified material was mixed with 100 ml of cold water and centrifuged for 15 min at 3600 *g* at 19°C. The supernatant was recovered and traces of fat were removed by filtration. This protein extract, of pH ca. 6, was used for the stability and partitioning experiments. For some experiments the carbon dioxide was evaporated at −20°C and 40% ethylene glycol was used instead of water and added at −18°C. In this case the centrifugation was carried out at −9°C.

2.3. Protein analysis

Protein concentration was determined by using Coomassie Brilliant Blue G according to Bradford [9] and measuring the absorbance at 595 nm. Bovine serum albumin was used as a standard.

2.4. Enzyme assays

Glyceraldehyde-3-phosphate dehydrogenase, GAPDH (EC 1.2.1.12), and alcohol dehydrogenase, ADH (EC 1.1.1.1), were assayed at 340 nm as described by Bergmeyer [10,11]. Phosphofructokinase, PFK (EC 2.7.1.11), was measured according to Hofmann and Kopperschläger [8]. In both cases a Hitachi Model 100-60 spectrophotometer connected with a potentiometric recorder was used. All measurements were carried out at room temperature (23 ± 0.5°C).

2.5. Stability of enzyme activity

The crude extract of bakers' yeast was used for enzyme assays either directly or after addition of ethylene glycol to a final concentration of 40% (v/v). The solutions were incubated at three different temperatures (+20, 0, −18°C) and analysed after various times for enzyme activity.

2.6. Preparation of two-phase systems

The two-phase systems were prepared from concentrated solutions [50% PEG, 40% dextran,

Table 1
Two-phase systems, consisting of water, dextran 40, PEG 3450, 25 m*M* triethanolamine–HCl buffer (pH 7.4) and 40% (w/w) ethylene glycol, used for the partitioning experiments at different temperatures together with their volume ratio (top to bottom) of systems with or without 0.3% Cibacron Blue F3GA–PEG 8000 (Cb–PEG)

Temperature (°C)	System composition		Volume ratio	
	Dextran (% w/w)	PEG (% w/w)	Without Cb–PEG	With Cb–PEG
+20	10.7	7.3	1.03	1.03
0	10.1	6.9	1.27	1.20
−18	9.3	6.3	1.36	1.13

0.1 *M* TEA–HCl buffer (pH 7.4)] and ethylene glycol. The compositions of the phase systems are given in Table 1 (all percentages are w/w). The two-phase systems chosen were at the same distance from the transition point, which made it easier to compare the results. The system compositions (Table 1) were chosen based on an earlier study on the dependence of the transition point (on the binodal curve) on temperature [6]. The volume ratio for the systems, with or without a PEG-bound affinity ligand, are shown in Table 1. The ligand showed an effect on the volume ratio at −18°C but not at +20°C.

The size of the systems was typically 4 g and they were weighed out in a 10-ml graduated glass tube leaving space for the sample. Yeast extract was added to the system placed in a thermostated bath to a final mass of 4 g. In some cases the two-phase systems also contained phenylmethylsulfonyl fluoride and/or ligand–PEG. The systems were carefully mixed for 5 × 10 s and left to equilibrate at the temperature at which partitioning was performed. When the systems were used at 20 and 0°C, a thermostated bath and a bath with ice, respectively, were used. For the experiments at −18°C a well insulated cold bath was set up. In this bath, the cooling helix fed from a cooling machine (C2G; Grant, Cambridge, UK) was dipped into an antifreeze solution [ethylene glycol–water (1:1)]. When the two-phase systems were well settled, aliquots of 20–100 μl were withdrawn from each phase for analysis. From the concentration of enzyme activity the partition coefficient was determined. The partition coefficient, *K*, is defined as the ratio between the concentrations in the upper and lower phases.

By using this kind of system, the time needed for separation differs with temperature. The separation time was estimated by visual inspection to be around 30 min at +20°C, 1.5 h at 0°C and 2.5 h at −18°C. The increase in separation time seems to be mainly due to the strongly increasing viscosity of ethylene glycol–water mixture with decrease in temperature.

Ethylene glycol (EG) was chosen as the antifreeze solvent for the following reasons. The lowest temperature reached with our cooling equipment was −18°C and 40% EG freezes at −23.8°C [12], and at this temperature and concentration the viscosity (twelve times that of water at 0°C) is not too high [12]. Further, according to previous work, 40% EG does not have a negative effect on the studied enzymatic activities (membrane-bound acetylcholinesterase and succinate dehydrogenase) [6].

2.7. Volume ratio

The ratio between the volumes of the upper and lower phases was determined by equilibrating the systems (4 g) in graduated test-tubes (10 ml) and reading the volume of the two phases when the settling was complete. The volume ratio was used for calculating the amount of enzyme and protein in the phases and, by comparison with the total amount (from measurements of mixed system), for the determination of the amount recovered at the interface between the two phases.

3. Results

3.1. Effect of EG on enzyme activity

EG showed an activation of some enzymes present in the crude extract of bakers' yeast; GAPDH was 1.55 times and PFK 1.30 times more active in 40% EG than in water. The activity of ADH was not affected by the presence of EG.

The variation of enzyme activity with time is shown in Table 2. The enzymes are more stable with time in the presence of EG than when stored in the extract. GAPDH, however, was more stable than PFK in crude extract and the latter was totally deactivated after 68 h. PFK was stabilized by EG at all three temperatures (+20, 0 and −18°C). GAPDH was stabilized mainly at the two lower temperatures in the presence of EG.

3.2. Partitioning of total protein of yeast extract

The partitioning of protein between the phases when the yeast extract was included in the phase systems at various temperatures is shown in Table 3. The temperature has virtually no influence on the partitioning. Also, addition Cibacron Blue F3GA–PEG, known to affect the partitioning of several proteins, had only a slight effect on the partition coefficient of protein.

3.3. Partitioning of enzymes present in yeast extract

The partition coefficients, K, of ADH and PFK in aqueous two-phase systems were determined at the same three temperatures as the relative enzyme activities in the upper and lower phases. The systems contained EG (40%, w/w) and yeast extract as an enzyme source. Further, the effect of a polymer-bound ligand for PFK was investigated by including Cibacron Blue F3GA–PEG (0.1%) in some systems (Table 3). The K values of GAPDH were also determined but only at −18°C.

In the case of ADH (Table 3), the ligand has no effect on the partitioning at −18°C and marginal positive or negative effects were observed at 0 and +20°C, respectively. Also, the partitioning of GAPDH at −18°C was not markedly affected by Cibacron Blue F3GA–PEG (Table 3).

The partitioning of PFK at different temperatures shows that this enzyme partitions strongly

Table 2
Stability of PFK and GAPDH, present in yeast extract, when mixed with 40% of its mass of either water or EG and incubated at various temperatures and times, and data for activities when the yeast extract (25%) was included in the two-phase system with 40% EG and kept at −18°C

Time (h)	Enzyme activity (% of initial value)[a]									
	PFK					GAPDH				
	In water		In 40% EG			In water		In 40% EG		
	20°C	0°C	20°C	0°C	−18°C	20°C	0°C	20°C	0°C	−18°C
In solution										
18	22	100	89	101	102	160	122	102	104	97
68	0	0	72	88	102	67	96	77	122	126
104	0	0	61	86	86	43	30	49	120	117
In two-phase system										
18	–	–	–	–	100	–	–	–	–	115
80	–	–	–	–	94	–	–	–	–	115

[a] All activities are given as a percentage relative to the value observed at zero time (just after mixing).

Table 3
Logarithmic partition coefficients, log K, of total protein and of the enzymes ADH, PFK and GAPDH when yeast extract was included in the two-phase systems and of PFK when it was added in pure form

Measured substance	Partitioned material	Temperature (°C)	Log K		ΔLog K
			With Cb–PEG	Without Cb–PEG	
Total protein	Yeast extract	+20	−0.50	−0.64	0.14
		0	−0.50	−0.52	0.02
		−18	−0.52	−0.57	0.05
ADH	Yeast extract	+20	−0.68	−0.58	−0.10
		0	−0.44	−0.51	0.07
		−18	−0.47	−0.47	0.00
GAPDH	Yeast extract	−18	−0.85	−0.92	0.07
PFK	Yeast extract	+20	−0.66	−1.57	0.91
		0	−0.86	−1.92	1.06
		−18	−0.50	−2.00	1.50
PFK	Purified	0	0.70	−0.32	1.02
		−18	0.52	−0.75	1.43

Log K values were determined in systems with and without Cb–PEG and the difference is given as Δlog K. All systems contained 40% EG. In the case of GAPDH, the extraction of protein was performed with 40% EG solution.

to the lower phase with K values of 0.01 (log K = −2) at −18 and 0°C and higher at +20°C (Table 3). The influence of ligand–PEG on the partitioning of PFK measured as the increase in the logarithmic partition coefficient, Δlog K, is greater when the temperature is lower. From 0.91 at 20°C it increases to as much as 1.50 at −18°C. Since Δlog K has a higher value at −18°C than at 0 and +20°C, the experiments clearly show that the ligand is more effective in changing the partitioning of PFK towards the upper phase at sub-zero temperature than at higher temperature.

These results are in sharp contrast to those obtained earlier with pure PFK. It was reported [4] that the Δlog K value decreased with decrease in temperature and was totally eliminated around −10°C, whereas at lower temperatures the ligand–PEG caused exclusion of the enzyme from the upper phase. The stabilities of both PFK (without Cibacron Blue F3GA–PEG) and GAPDH (both with and without Cibacron Blue F3GA–PAG) in the two-phase systems were very good, with more than 90% recovery after 80 h. In the case of PFK in the presence of Cibacron Blue F3GA–PEG, 75% remained after 80 h.

3.4. Partitioning of pure PFK in aqueous two-phase systems at 0 and −18°C

Pure yeast PFK was partitioned in systems at 0 and −18°C in order to verify previously reported results [4]. The Δlog K values for pure PFK (Table 3), are roughly the same as for PFK in yeast extract at both temperatures. However, the K values themselves show great differences on comparing the PFK in pure form and in extract form. The stability in the systems was good at −18°C with a yield of 82% after 20 h, while 65% remained after the same time when incubated at 0°C.

3.5. Accumulation at the interface of enzymes present in yeast extract

Yeast extract, obtained by using 40% ethylene glycol, was partitioned in systems containing a final concentration of 40% ethylene glycol and at various temperatures. The mass balance between the enzyme activities found in the settled phases and that of the mixed system, monitored both before and after the sampling of the phases,

Table 4
Percentage of enzyme activities recovered at the interface when systems containing either yeast extract or pure PFK were incubated at −18°C, and PFK interfacial recovery at 0°C

Enzyme measured	Partitioned material	Cb–PEG (%)	Temperature (°C)	Recovery at the interface (%)		
				2–2.5 h	18–20 h	80 h
PFK	Pure enzyme	0.1	−18	12	21	N.D.[a]
		0	−18	33	39	N.D.
		0.1	0	36	53	N.D.
		0	0	41	60	N.D.
PFK	Yeast extract	0.3	−18	0	29	28
		0	−18	0	16	20
GAPDH	Yeast extract	0.3	−18	0	17	21
		0	−18	0	20	32
ADH	Yeast extract	0.3	−18	0	0	12
		0	−18	0	0	0

All systems contained 40% EG and the indicated concentrations of Cb–PEG.
[a] N.D., not determined.

indicates that a considerable amount of enzyme is present at the interface (Table 4).

With time, both PFK and GAPDH are collected at the interface in fairly large amounts (up to 32%). This phenomenon is only observed after several hours of settling. ADH does not show the same behaviour.

3.6. Accumulation at the interface pure yeast PFK

Also when pure PFK was partitioned, some enzyme was found at the interface when the systems contained 40% ethylene glycol (Table 4). The results show that, at both 0 and −18°C, the PFK recovered at the interface increased with time: 12–33% after 2.5 h and 31–39% after 28 h. The presence of Cibacron Blue F3GA–PEG reduced the amount at the interface in contrast to what was found in the extract (Table 4). An increase in temperature from −18 to 0°C increased the amount of enzyme calculated to be at the interface by 1.5 (with Cibacron Blue F3GA–PEG) or 2.5 times (without Cibacron Blue F3GA–PEG).

3.7. Influence of a protease inhibitor: PMSF

Since the presence of proteases in the yeast extract can affect the enzyme activities, PMSF (30 μl of 5% solution in 2-propanol for 4-g systems) was incorporated in some cases. This was considered as a blank to check if the proteases would act. Partitioning of pure PFK was therefore studied in systems with and without PMSF.

The accumulation of PFK at the interface seems to depend on several factors, as shown in Table 5. PMSF generally induced a higher affinity of PFK for the interface. With Cibacron Blue F3GA–PEG, less enzyme was found at the interface and this difference was more pronounced in the presence of PMSF. When yeast extract was used, both PFK and GAPDH (data not shown) were also affected by PMSF.

The partitioning of PFK was not affected by PMSF without ligand–PEG whatever the temperature (−18 or 0°C). The affinity partitioning effect, $\Delta \log K$, was weakened (40% decrease) at both temperatures.

4. Discussion

4.1. Enzyme stability

The experiments in Table 2 show that the presence of EG in combination with low tem-

Table 5
Effect of PMSF on the partitioning of pure yeast PFK in systems with 40% EG

Temperature (°C)	Cb–PEG present	Recovery interface (%)		Log K		Δlog K	
		No PMSF	With PMSF	No PMSF	With PMSF	No PMSF	With PMSF
0	No	60	76	−0.32	−0.24	1.02	0.64
	Yes	53	42	0.70	0.40		
−18	No	39	60	−0.75	−0.77	1.44	0.82
	Yes	21	33	0.69	0.05		

The systems (Table 1) were with or without PMSF (2 mM) and with or without 0.1% ligand–PEG. The systems also contain 0.06 μM PFK. The partition coefficient, K, was determined after 2 h and the percentage of enzyme at the interface after 20 h.

perature and in polymeric two-phase systems has a stabilizing effect on the enzymes present in the yeast extract. This, together with the fact that ethylene glycol can be used to lower the freezing point of aqueous two-phase systems to −20°C, makes it possible to fractionate proteins by liquid–liquid extraction under stabilizing sub-zero conditions.

4.2. Bulk protein partitioning

In previous work [4], only pure enzymes were partitioned at sub-zero temperatures. Here, a natural protein mixture in form of a yeast extract was used. This offers a more realistic situation regarding the future use of these systems for the isolation of enzymes from a protein mixture. The replacement of part of the water in the systems with EG did not change the overall partitioning of bulk proteins. Log K values for bulk proteins of ca. −0.5 (Table 3) are obtained when yeast protein extract is partitioned in two-phase systems without EG [13], but at the same distance from the binodal curve (1.26 in the percentage scale used for the PEG and dextran concentrations). EG therefore does not influence markedly the relative solubilization of bulk proteins in the two-phase system compared with the water-based system.

4.3. Enzyme partitioning

The partition coefficients of PFK (Table 3) in the systems without Cibacron Blue F3GA–PEG increase only slightly with temperature in the same way as observed for the water-based systems [14]. The change in the log K value of PFK in pure form, obtained by including Cibacron Blue F3GA–PEG in the two-phase system with EG, is more temperature dependent. This affinity extraction effect, measured by Δlog K, increased by more than 50% when the temperature was lowered from 20 to −18°C. The same qualitative behaviour has been found for systems without EG [14] between 40 and 0°C. In the water-based systems, Δlog K is, however, considerably greater, 2–2.5 times in comparable systems. These effects of temperature and ethylene glycol on Δlog K are probably a result of changes in the ratio of binding constants in the upper and lower phases. According to the model for affinity partitioning [15,16],

$$\Delta\log K = n \log K_{\text{Cb-PEG}} + \log(K_{\text{ass,T}}/K_{\text{ass,B}})$$

where n is the number of binding sites involved, $K_{\text{Cb-PEG}}$ is the partition coefficient of Cibacron Blue F3GA–PEG and $K_{\text{ass,T}}$ and $K_{\text{ass,B}}$ are the total association constants in the top and bottom phase, respectively. Whereas log $K_{\text{Cb-PEG}}$ is relative constant, the model predicts a decrease in $K_{\text{ass,T}}/K_{\text{ass,B}}$ by including EG in the system and an increase in $K_{\text{ass,T}}/K_{\text{ass,B}}$ with decreasing temperature. The discrepancy between the present and previous results (unexpected lack of affinity partitioning for pure PFK at low temperature [4]) is difficult to explain, since in both cases the results were well reproducible within the actual

enzyme preparation. It may possibly be caused by differences in the batches of enzymes due to uncontrolled variations in the treatment of the enzyme, e.g., in the addition of PMSF, when PFK was isolated and stored. In the earlier work the systems also contained 2-mercaptoethanol and EDTA, which were not used in the present experiments. As shown in Table 5, PMSF has a reducing effect on $\Delta \log K$. The results obtained here for pure PFK fit well with the $\Delta \log K$ obtained for PFK present in yeast extract (Table 3). However, the log K values differ strongly between pure PFK and PFK in yeast extract, which may indicate an interaction of the enzyme with other components present in the extract.

4.4. Interfacial phenomenon

The recovery of large amounts of enzymes at the interface is partly due to the presence of EG, but it was also found (results not shown), but to a lesser extent, in water-based systems at 0°C after 20 h of incubation. Normally the partitioning is determined by removing samples from the phases a relatively short time (2–20 min) after introducing the enzyme sample into the system, which explains why this phenomenon normally is not observed. The formation of enzyme-containing protein complexes is definitely a slow process, hardly detectable within 2 h. It is not related to the molecular mass of the proteins, since both PFK with M_r 835 000 [8] and GAPDH with M_r 144 000 [17] are collected to corresponding degrees at the interface. The effect observed for pure PFK, that the presence of Cibacron Blue F3GA–PEG in the system reduced the recovery of protein at the interface (Tables 4 and 5), may be explained by a protecting atmosphere of affinity-bound PEG around the enzyme molecule which reduces contacts between PFK molecules. In the case of yeast extract incubated for longer times in the glycerol-containing system at −18°C, preliminary results obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (data not shown) indicate that a large number of different proteins are collected at the interface, showing that this protein aggregation in this type of system is a general process.

4.5. PMSF effect

The strong negative effect of PMSF on $\Delta \log K$ may be the result of a reduced interaction between the Cibacron Blue F3GA–PEG and PFK. A weaker binding (ligand–enzyme) and/or a decrease in the number of binding sites may possibly be the result of chemical modification of the enzyme by the PMSF. It is also possible that the ligand is modified by reaction with PMSF.

4.6. General discussion

It has been shown that the proteins of a yeast extract can be included and partitioned in polymeric two-phase systems at sub-zero temperatures offering activity-stabilizing conditions. However, the observed continuing accumulation of proteins at the interface may cause technical problems in time-consuming multi-step procedures, e.g., counter-current distribution at low temperatures. The strong variations of the partitioning and extractability of PFK, depending on enzyme batch, presence of extract components and additives of PMSF in low concentrations, show that minor variations in the partitioning conditions may also introduce strong deviations in the extraction process. This has to be taken into account when the systems are (even slightly) modified.

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