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# PARTIAL PURIFICATION OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND PHOSPHOFRUCTOKINASE FROM RAT ERYTHROCYTE HAEMOLYSATE BY PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

CRISTINA DELGADO, M. CRISTINA TEJEDOR and JOSE LUQUE*
*Departamento de Bioquimica y Biologia Molecular, Universidad de Alcala de Henares, 28871 Alcala de Henares, Madrid (Spain)*
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## SUMMARY

Glucose 6-phosphate dehydrogenase shows a high partition coefficient in poly(ethylene glycol)–dextran aqueous two-phase systems in comparison with those for 6-phosphogluconate dehydrogenase, phosphofructokinase and the bulk of proteins present in rat erythrocyte haemolysates. As a consequence, fractions highly enriched in glucose 6-phosphate dehydrogenase can be obtained after multiple partitions in the above systems with a counter-current distribution procedure. Phosphofructokinase shows a high affinity for Cibacron Blue and, as a result, the enzyme can be extracted in the top phase of poly(ethylene glycol)–dextran systems containing Cibacron Blue–poly(ethylene glycol) (affinity systems). The efficiency for the purification of the enzymes by partitioning is increased up to 10-fold when enzyme-rich fractions, obtained by precipitation with poly(ethylene glycol), are used instead of original haemolysate. The recovery of enzyme activities is near 100% in both instances.

## INTRODUCTION

The principle of partitioning has been applied to the fractionation of a mixture of proteins with the development of aqueous two-phase systems formed by either two hydrophilic polymers, poly(ethylene glycol) (PEG) and dextran, or a polymer (PEG) and salt[1–3]. The high water content of the phases, the low interfacial tension between them and the protective effect exerted by the polymers make these systems very mild toward labile macromolecules[1–3]. The ease, speed and economy of the methodology involved in aqueous two-phase systems have been emphasized[4–6]. Further, as the partition coefficient of the protein and the properties which determine it share an exponential relationship, partitioning in aqueous two-phase systems allows fractionations of proteins which are not currently available by other methods[1,7].

Partitioning of proteins in standard biphasic systems depends on the concentration and molecular weight of the polymers, the concentration and type of salt and pH[1–3]. By manipulation of these parameters, single partition experiments can be used to direct a protein selectively towards the desired phase[1–3]. Using this approach,

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preparations of proteins free from nucleic acids[8] and the separation of proteins from nucleic acids and polysacharides[9] have been achieved.

The partition coefficient ($K$) of the target protein is not always different enough from that of the bulk proteins to allow an efficient separation with a single extraction. The separation may then be achieved by using a multiple partition procedure such as counter-current distribution[1,2]. Alternatively, the introduction in one of the phases of a ligand with a selective affinity for the target protein gives rise to affinity-partitioning systems, in which the protein is directed towards that phase[10–12]. The triazine dye Cibacron Blue F3GA (Cb) covalently linked to PEG (PEG–Cb) has been used as an affinity ligand for the selective extraction of phosphofructokinase from bakers' yeast into the top phase of a PEG–dextran system[13,14]. The use of these affinity systems for multiple partitions with the counter-current distribution procedure has provided a high-resolution methodology for the fractionation of glycolytic enzymes from yeast extract[15–16].

In this paper, we report the partitioning behaviour of glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49, G6PD), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44, 6PGD) and phosphofructokinase (E.C. 2.7.1.11, PFK) from rat erythrocytes in both standard and affinity PEG–dextran two-phase systems. The aim of the work was to design an experimental protocol for the extraction of these enzymes by using partitioning in aqueous two-phase systems which allows the extraction of enzymes under very mild conditions. Our group is interested in the study of regulatory enzymes in the glucolytic and pentose–phosphate pathways in eryhtrocytic cells[17,18]. A first extraction step for phosphofructokinase and glucose 6-phosphate dehydrogenase by precipitation with PEG[19] was employed to improve the efficiency of the partitioning step.

## EXPERIMENTAL

### *Chemicals*

PEG ($M_r$ 6000) and Cibacron Blue F3GA were obtained from Serva (Heidelberg, F.R.G.), dextran T-500 from Pharmacia (Uppsala, Sweden), nucleotides (sodium salts of ATP, NADH and NADP), dithioerythritol, glucose 6-phosphate, 6-phosphogluconate, fructose 6-phosphate and coupling enzymes from Boehringer (Mannheim, F.R.G.), EDTA (disodium salt) from Sigma (St. Louis, MO, U.S.A.) and Tris, 2-mercaptoethanol and all other chemicals (analytical-reagent grade) from Merck (Darmstadt, F.R.G.).

### *Preparation of the haemolysate*

Anesthetized male Wistar rats weighing 180–200 g were decapitated and whole blood was collected in heparinized tubes and centrifuged (400 $g$, 10 min) to separate the plasma. Red cells were washed three times with 0.9% sodium chloride solution and freed from leucocytes and platelets by removing the top layer of cells after centrifugation. Subsequently, the red cells were lysed by mixing with an equal volume of a hypotonic solution containing 2.7 m$M$ EDTA and 0.7 m$M$ 2-mercaptoethanol. The mixture was frozen and thawed twice and finally centrifuged (15000 $g$, 30 min) to remove stroma. All these steps were carried out at 4°C.

*Preparation of fractions enriched in phosphofructokinase and glucose 6-phosphate dehydrogenase*

Enriched fractions were obtained by mixing one volume of haemolysate (total protein about 20 mg/ml) with one volume of a solution of 12% (w/w) PEG made up in 96 m*M* potassium phosphate buffer (pH 6) (for the precipitation of phosphofructokinase) and 170 m*M* sodium acetate buffer (pH 5) (for the precipitation of glucose 6-phosphate dehydrogenase). The buffers contained 10 m*M* EDTA and 1 m*M* 2-mercaptoethanol. The mixtures of PEG and haemolysate were kept in an ice-bath for 30 min and then centrifuged (5000 *g*, 10 min). The supernatants were discarded and the pellets redissolved in 1 volume of 25 m*M* sodium phosphate buffer (pH 7) containing 5 m*M* EDTA and 0.5 m*M* 2-mercaptoethanol[17].

*Preparation of Cibacron Blue–poly(ethylene glycol)*

This substituted polymer was prepared as described by Johansson[12]. In brief, 50 g of PEG were dissolved in 100 ml of water and the solution was heated in a water-bath at 80°C, then 5 g of Cibacron Blue F3GA and 2 g of sodium hydroxide were added and the mixture was stirred for 2 h at 80°C. Solid sodium phosphate was added to adjust the pH of the mixture to 7. The PEG–Cb was extracted with five portions of 150 ml of chloroform and the pooled organic extracts were dried with anhydrous sodium sulphate and filtered through a Whatman GF/A paper. The dark-blue solid was recovered by evaporation of the solvent under reduced pressure. The excess of Cibacron Blue was eliminated by ion-exchange chromatography with DEAE-cellulose (DEAE-52; Servacel, Heidelberg, F.R.G.). The PEG–Cb was eluted from the column with 2 *M* potasium chloride. The dye–polymer was finally extracted with chloroform and the solvent removed by evaporation.

*Single partitioning*

Systems of 4 g were prepared in 10-ml graduated tubes by weighing 0.4 g of sample and appropriate amounts of distilled water and the following stock solutions: 40% (w/w) PEG, 20% (w/w) dextran (standardized by polarimetry) and 0.25 *M* sodium phosphate buffer (pH 7) containing 2.5 m*M* EDTA and 25 m*M* 2-mercaptoethanol. A ratio of 1.5:1 (w/w) of dextran to PEG was maintained in all the systems to obtain two-phase systems in which the volume ration of the top to the bottom phases was close to 1. After mixing by 30–40 inversions the mixtures were left to settle at 4°C until complete separation of the phases was achieved. Aliquots from the top and bottom phases were then analysed for protein concentration and enzymic activities.

The affinity systems were prepared as above but using a 40% (w/w) stock solution of PEG containing 6% of PEG–Cb. This substituted polymer was prepared as described by Johansson and Joelsson[6].

The partition coefficient, *K*, is defined as the ratio between the enzyme activities or total protein concentrations in the top and bottom phases of the systems. The percentage of protein in the top phase of the systems was calculated from the partition coefficient and volume ratio values as described by Johansson[12]. The concentration of polymers in the systems is always expressed by weight.

*Counter-current distribution*

Counter-current distribution (CCD) was carried out in a two-phase system formed by 5% PEG–7.5% dextran, 50 m*M* sodium phosphate buffer (pH 7), 0.5 m*M* EDTA and 5 m*M* 2-mercaptoethanol. The two-phase system (normally 250 g) was prepared by mixing the required amounts of the above stock solutions and distilled water. Once the top and bottom phases had separated at 4°C, they were stored at 4°C.

An automatic thin-layer counter-current distribution apparatus (Bioshef TLCCD MK3; University of Sheffield, Sheffield, U.K.) with a distribution rotor formed by two circular plates (60 cavities) was used[20]. Cavities 4–60 each received an equal volume (0.765 ml) of the bottom and top phases. Cavities 1–3 each received an equal volume (0.765 ml) of the bottom and top phases of a "biphasic system containing sample" prepared immediately before use from the same stock solutions as the bulk system but replacing the distilled water for the sample. A distribution run consisted of 57 partitions. Each partition step was composed of a 20-s shaking time followed by a 6-min settling time and a transfer of the top phase to the next right bottom phase by means of a displacement of the top rotor plate. The experiment was carried out at 4°C. After completion, the contents of each cavity were diluted with 1 ml of 25 m*M* sodium phosphate (pH 7), 0.25 m*M* EDTA and 5 m*M* 2-mercaptoethanol (to convert the system into one phase) and collected in plastic tubes. Aliquots from each alternate cavity were assayed for total protein or enzymic activities.

*Enzyme and total protein assays*

Enzymic activities were measured in a double-beam spectrophotometer (Kontron Uvikon 810) at 37°C.

Glucose 6-phosphate and 6-phosphogluconate dehydrogenases were measured by using a double-step assay. The first cuvette contained 50 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 0.5 m*M* NADP, 0.6 m*M* glucose 6-phosphate and 0.6 m*M* 6-phosphogluconate. From the linear increase in the absorbance due to NADPH (340 nm), activities of both enzymes were obtained. In the second step, glucose 6-phosphate was omitted and the activity of only 6-phosphogluconate dehydrogenase was obtained. The activity of glucose 6-phosphate dehydrogenase was then calculated by the difference in activities between the first and second steps.

Phosphofructokinase activity was measured by the linear decrease in NADH absorbance (340 nm) in a test mixture containing 100 m*M* Tris–HCl (pH 7.1), 0.3 m*M* EDTA, 3 m*M* magnesium chloride, 90 m*M* potassium chloride, 0.15 m*M* NADH, 1.5 m*M* dithiothreitol, 1 m*M* phosphate, 3 m*M* fructose 6-phosphate, 9 m*M* glucose 6-phosphate, 1.5 m*M* ATP, 0.1 U/ml fructose diphosphate aldolase, 6 U/ml triose phosphate isomerase and 0.5 U/ml glycerol 3-phosphate dehydrogenase.

Total protein concentration was measured by the Coomassie Brilliant Blue assay[21], which is not subject to any interference from either PEG or dextran in the samples[2].

## RESULTS AND DISCUSSION

*Partitioning in standard systems*

Partition coefficients of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphofructokinase and total protein from rat erythrocyte

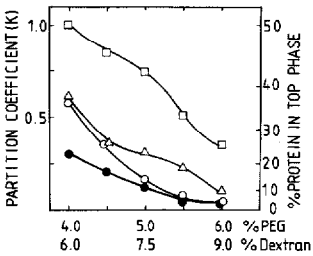


Fig. 1. Influence of the concentration of polymers on the partition coefficient of (□) glucose 6-phosphate dehydrogenase, (△) 6-phosphogluconate dehydrogenase, (○) phosphofructokinase and (●) total proteins from rat erythrocyte haemolysate in PEG–dextran aqueous two-phase systems. All systems were buffered with 50 m*M* sodium phosphate (pH 7) containing 0.5 m*M* EDTA and 5 m*M* 2-mercaptoethanol. The percentage of protein in the top phase is also shown for a system with phases of equal volumes.

haemolysate in two-phase systems with concentrations varying from 4% to 6% PEG and from 6% to 9% dextran have values lower than one (Fig. 1), *i.e.*, partition favours the dextran-rich bottom phase. In all instances, the increase in the concentration of both polymers in the system leads to a decrease in *K*. These two features have been described as a general behaviour for the partitioning of proteins[1,2].

The partition coefficient of glucose 6-phosphate dehydrogenase is higher than those for 6-phosphogluconate dehydrogenase, phosphofructokinase and the bulk of proteins, all of which show a similar behaviour in any of the biphasic systems used (Fig. 1). This fact will be exploited partially to purify the enzyme by counter-current distribution.

*Partitioning in affinity systems*

Partition coefficients of phosphofructokinase, glucose 6-phosphate and 6-phosphogluconate dehydrogenases in affinity PEG (PEG–Cb)–dextran systems are, in general, higher (Fig. 2a) than in the relevant standard systems (Fig. 1). This fact reflects an interaction between the Cb molcule and each of the three enzymes. The total protein shows similar partition coefficients in affinity (Fig. 2a) and standard

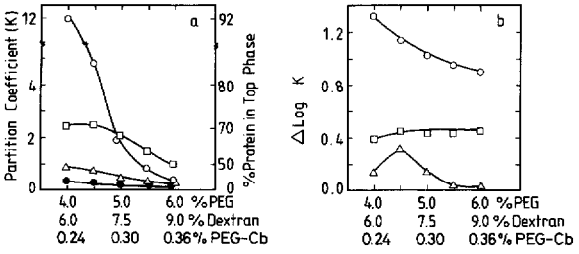


Fig. 2. (a) Partition coefficient of (□) glucose 6-phosphate dehydrogenase, (△) 6-phosphogluconate dehydrogenase, (○) phosphofructokinase and (●) total protein from rat erythrocyte haemolysate in aqueous two-phase systems with increasing concentrations of PEG, dextran and PEG–Cb. Other conditions as in Fig. 1. (b) Change in the logarithm of the partition coefficient of (□) glucose 6-phosphate dehydrogenase, (△) 6-phosphogluconate dehydrogenase and (○) phosphofructokinase in whole haemolysate between affinity and standard systems.

biphasic systems (Fig. 1). In all instances, the partition coefficient decreases with increase in the concentration of polymers.

The affinity partitioning effect due to the presence of Cb in the system is given by the increase in the logarithm of the partition coefficient in affinity systems (Fig. 2a) with respect to that in standard systems (Fig. 1). Results thus calculated ($\Delta \log K$) are shown in Fig. 2b. 6-Phosphogluconate dehydrogenase shows the lowest affinity for the ligand, the affinity being totally prevented when the concentration of polymers is increased above 5.5% (0.33%) PEG (PEG–Cb)–8.25% dextran. Glucose 6-phosphate dehydrogenase shows an intermediate affinity for Cibacron Blue with a $\Delta \log K$ value of about 0.45 in any of the systems (Fig. 2b). Phosphofructokinase shows the highest value of $\Delta \log K$ in all the systems, reflecting a higher affinity of the phosphofructokinase for Cibacron Blue. In spite of the concentration of PEG–Cb being raised, an increase in the concentration of polymers in the systems leads to a decrease in $\Delta \log K$ for phosphofructokinase. It seems, therefore, that the effect due to the increase in the concentration of PEG and dextran which directs the protein towards the bottom phase[2,12] is stronger than the affinity effect promoted by PEG–Cb which should direct the protein to the top phase.

Phosphofructokinase was partially extracted by precipitation with PEG and its partition coefficient was measured in standard and affinity two-phase systems (Fig. 3a). Again, the partition coefficient of the phosphofructokinase is higher in affinity than in standard systems. Moreover, in all the affinity systems the $K$ values for the phosphofructokinase in the enriched fraction are higher than those for the enzyme in the whole haemolysate. This result indicates than the interaction of phosphofructokinase with Cibacron Blue is reduced by a higher amount of other proteins in the haemolysate which, in competing for the ligand, decreases the concentration of free ligand. This result emphasizes the importance that the presence of other proteins may have in the affinity partitioning effect of a selected protein. Therefore, when applying affinity partitioning to the purification of a protein from a crude extract, it should be

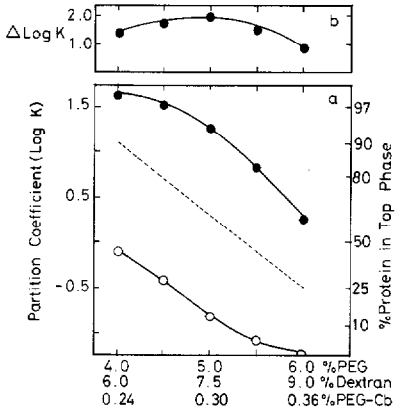


Fig. 3. (a) Partition coefficient of phosphofructokinase from a PFK-enriched fraction in (○) standard and (●) affinity systems with increasing concentrations of PEG, dextran and PEG–Cb. The broken line represents the partition coefficient of phosphofructokinase from the whole haemolysate in affinity systems. Other conditions as in Fig. 1. (b) Change in the logarithm of the partition coefficient of phosphofructokinase in the enriched fraction between affinity and standard systems.

taken into account that the presence of other proteins that interact with the ligand may reduce the yield of protein in the affinity phase.

In contrast to the $\Delta \log K$ observed for phosphofructokinase in the whole haemolysate, that for phosphofructokinase in the enriched fraction (Fig. 3b) increases when the concentration of the polymers in the system is increased to 5% (0.3%) PEG (PEG–Cb)–7.5% dextran. Such an increase in $\Delta \log K$ (Fig. 3b) seems to be consistent with the increase in $\Delta \log K$ found by Johansson[2,12] when purified bakers' yeast phosphofructokinase was partitioned in biphasic systems containing increasing concentrations of PEG, PEG–Cb and dextran. Further increases in the concentration of the polymers lead to reductions in this affinity effect (Fig. 3b), as observed in the whole haemolysate.

*Application of partitioning in aqueous two-phase systems in purification schedules for phosphofructokinase and glucose 6-phosphate dehydrogenase*

The highest affinity of phosphofructokinase for PEG–Cb and the high partition coefficient of glucose 6-phosphate dehydrogenase in standard systems allow experimental protocols for their partial purification to be designed. The similar behaviour of 6-phosphogluconate dehydrogenase and the total proteins in either standard or affinity systems does not lead to an enrichment of this enzyme under any of the conditions studied.

TABLE I

TWO-STEP PROCEDURES FOR THE EXTRACTION OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND PHOSPHOFRUCTOKINASE FROM RAT ERYTHROCYTE HAEMOLYSATE INVOLVING PRECIPITATION WITH POLY(ETHYLENE GLYCOL) AND PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

| *Enzyme* | *Step* | *Procedure* | *Fraction Collected* | *Yield (%)*[a] | *Purification Factor*[b] |
|---|---|---|---|---|---|
| Phosphofructokinase | 1 | Precipitation, 6% PEG, pH 6 | Pellet | 80 | 8 |
| | 2 | Affinity partitioning[c] | Top phase | 95 | 10 |
| | | Overall result | | 75 | 80 |
| Glucose 6-phosphate dehydrogenase | 1 | Precipitation, 6% PEG, pH 5 | Pellet | 90 | 5 |
| | 2 | Counter-current distribution[d] | Tubes 22–36 | 70 | 8 |
| | | Overall result | | 60 | 40 |

[a] Percentage of enzymic units recovered in each step with respect to those in the fraction subjected to the procedure.

[b] Ratio between specific activities in the fraction collected and the fraction subjected to the procedure.

[c] The system used is formed by 5% (w/w) PEG, 7.5% (w/w) dextran in 25 m*M* sodium phosphate buffer (pH 7) containing 0.25 m*M* EDTA and 2.5 m*M* 2-mercaptoethanol. 6% of the total PEG is PEG–Cb.

[d] The system used is formed by 5% (w/w) PEG, 7.5% (w/w) dextran in 50 m*M* sodium phosphate buffer (pH 7) containing 0.5 m*M* EDTA and 5 m*M* 2-mercaptoethanol.

*Phosphofructokinase.* The enzyme is first precipitated from the haemolysate by addition of 6% PEG at pH 6. As previously reported[19], the pellet thus obtained contains 80% of the original phosphofructokinase with a specific activity eight times higher (Table I).

The second step involves extraction of the phosphofructokinase in the top phase of the system formed by 5% (0.3%) PEG (PEG–Cb)–7.5% dextran. In this system the highest value for the log $K$ is obtained (Fig. 3b) and it provides the best compromise between yield and purification factor. The top phase of this system contains 95% of the enzyme present in the enriched pellet (Fig. 3a) with a specific activity ten times higher (Table I). The net result is 75% of the original phosphofructokinase (95% of 80%) with a specific activity 80 times higher (10 times 8) (Table I).

This extraction of phosphofructokinase can be completed within 2 h. Partitioning in affinity two-phase systems appears to be a suitable purification method as the activity of the enzyme is not destroyed during the procedure. The preliminary precipitation of the phosphofructokinase with PEG leads to a higher yield of the enzyme in the top phase (see Fig. 3a) and also a higher enrichment, *i.e.*, a higher efficiency of the affinity systems.

*Glucose 6-phosphate dehydrogenase.* In the biphasic system formed by 5% PEG–7.5% dextran, in which the greatest difference between the partition coefficient for glucose 6-phosphate dehydrogenase and the total proteins is found, 43% of the enzyme appears in the top phase with a content of only 10% of the total protein (Fig. 1), *i.e.*, 4-fold enrichment. To improve such a low enrichment in glucose 6-phosphate dehydrogenase, multiple partition steps with Albertsson's counter-current distribution procedure were then analysed (Fig. 4). A recovery of more than 90% of the total activity introduced in the distribution rotor is obtained for the three enzymes.

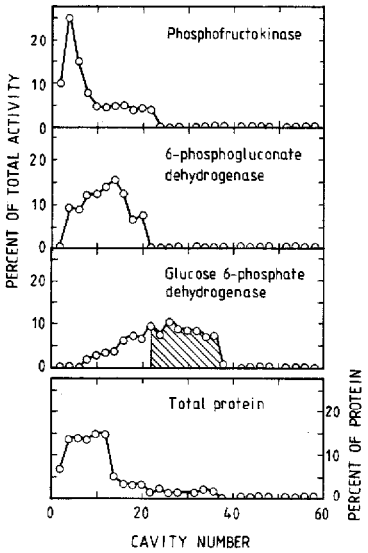


Fig. 4. Distribution profiles of total protein, phosphofructokinase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase obtained by counter-current distribution of rat erythrocyte haemolysate. The fractions highly enriched in G6PD are emphasized.

Distribution profiles for phosphofructokinase, 6-phosphogluconate dehydrogenase and total proteins are located towards the left-hand side of the distribution train, between cavities 1 and 20, in agreement with the low partition coefficients found in single-tube experiments (Fig. 1). Glucose 6-phosphate dehydrogenase shows a distribution profile between cavities 10 and 36. This location to the right of the other proteins was expected from the higher partition coefficient found for this enzyme in single-tube experiments (Fig. 1). The spreading of the peak could be due to the presence of isoenzymes differing in their $K$ values.

For purification purposes, fractions 22–36 contain 70% of the original glucose 6-phosphate dehydrogenase and only 10% of the total protein. This means that a 7-fold enrichment in glucose 6-phosphate dehydrogenase with a yield of 70% has been achieved.

Glucose 6-phosphate dehydrogenase present in the enriched fraction (see Experimental) has a distribution profile located similarly (data not shown) to that for the enzyme in the total haemolysate (Fig. 4). Again, fractions 22–36 contains 70% of the enzyme introduced in the distribution rotor with a specific activity eight times higher. As the enriched fraction had 90% of the enzyme present in the haemolysate with an activity five times higher[19] (Table I), the net result of the whole procedure is 60% (70% of 90%) of the original enzyme with an activity increased 50-fold (8 times 5) (Table I).

This two-step procedure can be completed within 8 h and the enzyme is not inactivated during the manipulation. This is a further example of the ease and speed of the methodology involved in partitioning in aqueous two-phase systems.

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