INFLUENCE OF pH ON THE PARTITION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HEXOKINASE IN AQUEOUS TWO-PHASE SYSTEM

Daniel Pereira da Silva[1]; Marcela Zanella Ribeiro Pontes[1]; Maria Aparecida de Souza[1]; Michele Vitolo[1]; João Batista de Almeida e Silva[2]; Adalberto Pessoa-Junior[1]*

[1]Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brasil; [2]Departamento de Biotecnologia – FAENQUIL, Lorena, SP, Brasil

ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PDH) and hexokinase (HK) are important enzymes used in biochemical and medical studies and in several analytical methods. Aqueous two-phase system (ATPS) formed by a polymer solution and an electrolyte solution provides a method for the separation and purification of enzymes with several advantages, including biocompatibility and easy scale up of the process. In this work, the effects of different pH values on the storage stability and partitioning behavior (K, partition coefficient) of the enzymes G6PDH and HK from baker's yeast extract were investigated in ATPS. The results, obtained from the 17.5% PEG 400 : 15.0% phosphate system, showed that when the pH was increased from 5.0 to 8.8, the K_{HK} increased 26-fold and the K_{G6PDH} 2.2-fold. In the 20.0% PEG 1500 : 17.5% phosphate system, the K_{HK} and K_{G6PDH} increased 13 and 1.2-fold, when the pH value was increased from 3.8 to 8.8, respectively. This leads to the conclusion that the partition coefficient for both enzymes is favored by high pH values. A statistical analysis of the results was conducted to confirm this conclusion.

Key words: aqueous two-phase systems, partition coefficient, protein purification, hexokinase, glucose 6-phosphate dehydrogenase.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), the first enzyme of the pentose phosphate pathway, catalyzes the oxidation of glucose 6-phosphate (G6P) into 6-phosphogluconate. Hexokinase (EC 2.7.1.1), an important enzyme for the carbohydrate metabolism, is the first enzyme of glycolisis, catalyzes the phosphorylation of glucose into glucose 6-phosphate (G6P), and is an intermediary between several metabolic pathways such as gluconeogenesis, shunt of pentoses, glycogen metabolism (3,23).

HK and G6PDH enzymes, essential to the cell metabolism, are used in biochemical and medical studies, as analytical reagents to measure creatin-kinase activity as well as ATP and hexoses concentrations (3). Using these enzymes to measure glucose mixed with fructose allows one to detect illegal addition of sugar to the final product in wine and fruit juice industries, both highly developed in Brazil (25). However, industrial production of G6PDH and/or HK can only be viable if large quantities of these enzymes are available at competitive prices. Although these enzymes can be found in almost every animal tissue and in several microbial species, the use of *S. cerevisiae* as a source of enzymes (11) makes sense in Brazil, due to the large experience of the Brazilian chemical industry on handling this strain. Besides, the simultaneous processing of yeast and ethanol should have a positive effect on the profits of the distilleries.

The development of techniques and methods for the separation and purification of enzymes has been an indispensable prerequisite for many of the advances made in the biotechnological industry (18,19). Aqueous two-phase

* Corresponding author. Mailing address: Departamento de Tecnologia Bioquímico-Farmacêutica, FCF – USP, Av. Prof. Lineu Prestes, 580, Bloco 16, Cidade Universitária, 05508-900, São Paulo, SP, Brasil. Tel.: (+5511) 3091-3862. Fax: (+5511) 3815-6386. E-mail: pessoajr@usp.br

system (ATPS) formed by the addition of aqueous solutions of two polymers, such as PEG and dextran, or a polymer and an electrolyte solution, such as PEG and phosphate salt, is a method based on the partition of proteins between two liquid phases whose advantages include biocompatibility and possibility of application on industrial scale (8,10). The partitioning in aqueous two-phase systems (ATPS) has proved to be an effective method for extraction and separation of soluble proteins from cell homogenates. These systems have important advantages over other techniques in their capacity and high activity yields at low labour, energy and capital cost, and handling of large volumes in a continuous process with relatively short contact times (13). Differences in enzyme partitioning can be ascribed to the interaction of the factors or mechanisms inherent in the system itself (such as choice of system components, polymer molecular weight, concentration of polymers and salts, ionic strength, and pH values) with those of the target protein (such as hydrophobicity, charge, and molecular weight) (15). According to Asenjo (2) the pH can alter the charge of chemical groups located in the side chain of protein amino acids leading to a net charge modification of the overall macromolecule, changing its solubility properties, at the end. Moreover, the enzyme can lose the catalytic capability either reversibly (precipitation at isoelectric pH) or irreversibly (denaturation at pH values out of the stability pH interval) (15). Then the evaluation of the enzyme stability becomes indispensable, since during the downstream procedures it can be denaturated.

In practice, the technique of extraction in ATPS requires further experimentation to find an adequate system for each particular application (16). The aim of this work was to verify the effects of different pH values (3.8, 5.0, 6.0, 7.0, 8.0 and 8.8) on the partition coefficients (K) of the enzymes G6PDH and HK in aqueous two-phase systems (17.5% w/w PEG 400 : 15.0% w/w phosphate, and 20.0% w/w PEG 1500 : 17.5% w/w phosphate). This work also deals with the effect of pH (4.0 to 9.5) on the storage stability of HK and G6PDH.

MATERIALS AND METHODS

Chemicals

Hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PDH), adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP) and glucose 6-phosphate (G6P) utilized in the enzymatic analysis were obtained from Sigma Chemical Co. (St. Louis, USA). Poly(ethyleneglycol), samples with nominal molecular weights of 400 and 1500 g/mol were purchased from Merck (Darmstadt, FRG). Sodium monohydrogen phosphate and potassium dihydrogen phosphate were purchase from CAAL (São Paulo, Brazil). All the other chemicals were of analytical grade.

Cell Homogenate

The cell homogenate, was prepared through disruption of commercial baker's yeast. The baker's yeast (45 g) was previously washed with 300 mL solution buffer (Tris-HCl 50 mM) pH 7.5 and suspended in 50 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 2 mM aminocaproic acid, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 0.2 mM EDTA (ethylenediaminetetraacetic acid). Then, cells were disrupted with glass beads (diameter = 0.5 mm). The mixture was maintained below 10ºC all the time. The cell suspension (concentration of 250 g/L) and glass beads were mixed in a volumetric proportion of 1:1. After disruption, cell debris and glass beads were removed by filtration from the cell homogenate solution, which was then stored at 4ºC. Before the extraction in aqueous two-phase system, this cell homogenate was centrifuged (2880 g, 10 min) and the supernatant containing G6PDH, HK and other enzymes were collected.

Preparation of the storage solution

The supernatant and an appropriate 0.1 M buffer solution (acetic acid- acetate buffer pH 4.0, 4.5, 5.0 or 5.5; phosphate buffer pH 6.0, 6.5 or 7.0 and Tris-HCl pH 7.5, 8.0, 8.5, 9.0 or 9.5) were mixed in a volumetric proportion of 1:9. In all tests the pH was checked after homogenizing the mixture. Samples of the storage solutions were taken for residual enzyme activity determination after 0, 4h, 8h, 12h, 24h and 51h of cell free extract-buffer contact.

Aqueous Two-Phase Systems (ATPS)

Systems were prepared with stock solutions: 50% w/w PEG and 40% w/w phosphate salts (as monosodium and dipotassium mixture - NaH_2PO_4 and K_2HPO_4) utilizing deionized water. The systems were made up in the final concentration from 17.5% w/w PEG 400 : 15.0% w/w phosphate, and from 20.0% w/w PEG 1500 : 17.5% w/w phosphate. In a graduated centrifuge tube (15 mL) two grams of the medium containing cell homogenate were mixed with PEG and phosphate solutions, the final ATPS component concentrations being adjusted with deionized water. The pH values of the systems were adjusted to 3.8, 5.0, 6.0, 7.0, 8.0 and 8.8 by varying the proportion between NaH_2PO_4 and K_2HPO_4 adequately. After that, the mixture was submitted to rotation (8 rpm, 20 min) followed by centrifugation (1500 g, 10 min) and rest for 3 h. During all the experiments the temperature was kept at about 4ºC. Samples of the top and bottom phases were then assayed to determine enzyme activities, total protein concentration, volumes and pH of the system phases.

Enzyme Activities

The G6PDH and HK activities were measured by spectrophotometry. The rate of increase in absorbance at 340 nm (caused by the increase in reduced $NADP^+$ as a function

of time) was measured at 30ºC and pH 7.5, according to Bergmeyer (3). For both enzymes, one activity unit (U) was defined as the amount of enzyme catalyzing the reduction of 1 μmol of $NADP^+$ per minute under the assay conditions. Enzyme activity degradation during storage of enzymes and accuracy of the determinations were checked in separate experiments.

Protein Concentration

The amount of total protein was determined according to the Coomassie Blue method described by Bradford (4), using bovine serum albumin (BSA) as standard.

Partition coefficient (K)

It was calculated by the ratio of the enzyme activity or the protein concentration in the top phase by the enzyme activity or protein concentration in the bottom phase. All determinations were performed in duplicate.

RESULTS AND DISCUSSION

Hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) were extracted in the systems presented in Fig. 1. This Figure shows the partition coefficient (K), expressed as Log K, obtained for both enzymes (K_{HK} and K_{G6PDH}), and total protein (K_{TProt}) as a function of pH. It was observed that the best partition coefficients are obtained at higher pH values.

The increase in the partition coefficient as a function of pH has been observed for other proteins, like α-amylase, taumatin, L-aspartase and fumarase (5,12,20). According to these authors, the negatively charged protein should prefer the top phase of the aqueous two-phase system. This fact, probably explains the increase in the HK partition coefficient (K_{HK}), as compared to the G6PDH partition coefficient (K_{G6PDH}), since the G6PDH isoelectric point is approximately 6.5 and HK isoelectric point is 4.7-5.0 (6,26).

As can be seen in Fig. 1, the extractions performed with PEG 1500 provided lower partition coefficients, as compared to PEG 400, independent on pH value, for both enzymes. But, extractions conducted with PEG 1500 are, particularly, promising, since the significant differences in partition between both enzymes can be used to selectively separate them. For all studied pH values, the enzyme HK goes, preferentially, to the top phase (K value higher than 1), while G6PDH goes to the bottom phase (K value lower than 1).

The results, presented in Fig. 1, were statistically analyzed by the method described as Polynomial Regression Analysis, and are displayed in Fig. 2. The mathematical equation obtained for the system formed with PEG 400 showed that the relationship between the K_{HK} and pH value was a third-order polynomial model (model A - Fig. 2-A). On the other hand, the mathematical model that describes the relationship between
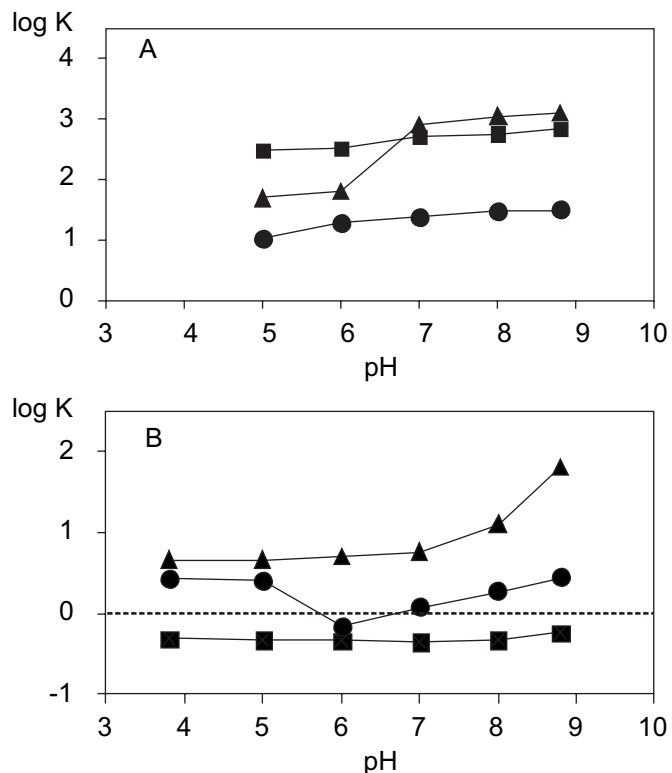

Figure 1. Effect of pH on the partitioning behavior (log K) of HK (▲), G6PDH (■) and total protein (●) in different PEG/ phosphate systems: 17.5% w/w PEG 400 with 15.0% w/w phosphate (A) and 20.0% w/w PEG 1500 with 17.5% w/w phosphate (B).

K_{G6PDH} and pH value was polynomial of a first-order (model B – Fig. 2-B). The system formed with PEG 1500, provided a fourth-order polynomial model to describe the relationship between the K_{HK} and pH (Fig. 2-C). But, although the relationship between K_{G6PDH} and pH generated a model of second-order (Fig. 2-D), it can be seen that the pH did not influence the G6PDH partition coefficient. The Analysis of Variance of the results showed a P-value (probability value) less than 0.01, for the models obtained for K_{HK} and K_{G6PDH} in the system formed with PEG 400 (17.5% w/w and 15.0% w/w phosphate) and for K_{HK} in the system formed with PEG 1500 (20.0% w/w with 7.5% w/w phosphate). This fact indicates a statistically significant relationship between partition coefficient and pH value, at 99% confidence level. But, for K_{G6PDH} in the system formed for PEG 1500 (20.0% w/w with 7.5% w/w phosphate) the P-value was greater than 0.10, and was not statistically significant at 90% confidence level.

The pH values, determined after the partition of the phases (Table 1), varied for both PEG 400 and PEG 1500 during the extraction process. These variations can influence the
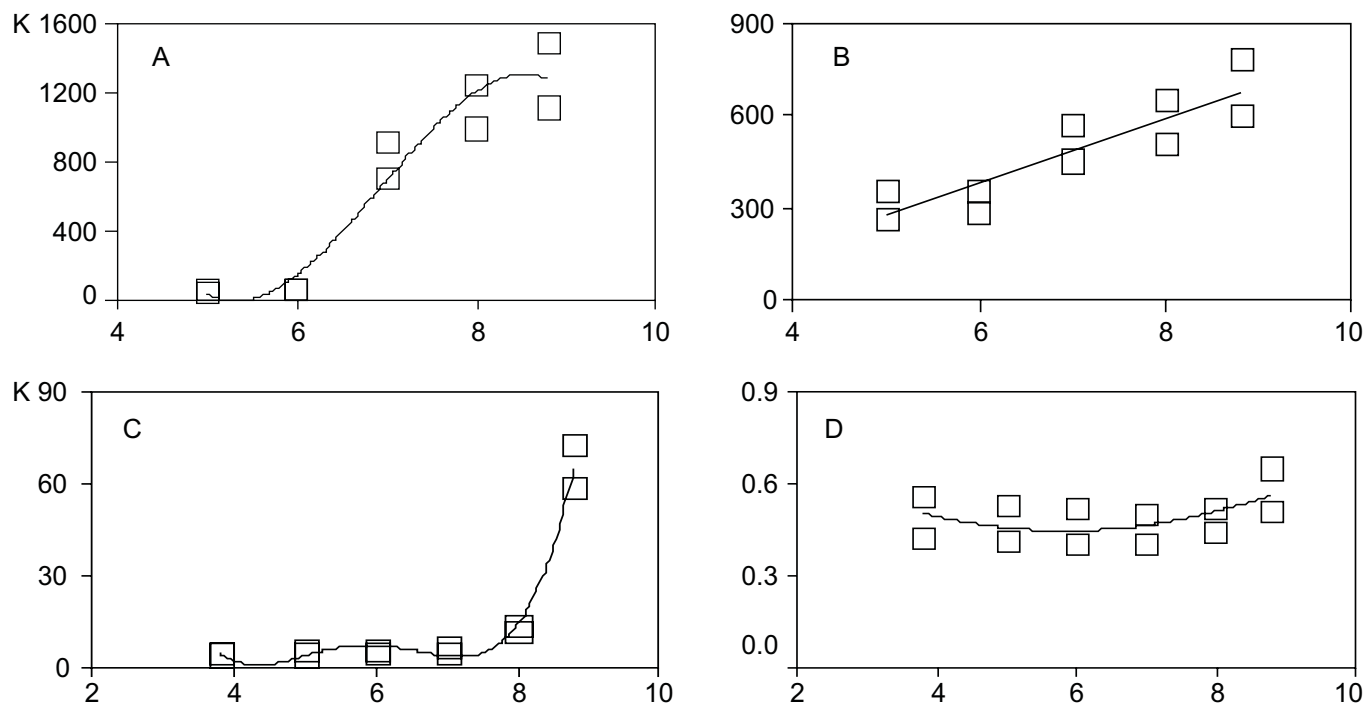
Figure 2. Effect of the pH on the partitioning coefficient (K) of HK (A and C) and G6PDH (B and D) in different PEG/phosphate systems: 17.5% w/w PEG 400 with 15.0% w/w phosphate (A and B) and 20.0% w/w PEG 1500 with 17.5% w/w phosphate (C and D). The mathematical models that correlate pH value with partition coefficients (K) are:

A - model (PEG 400, HK) .... $K = 22871.8 - 10852.2(pH) + 1654.7(pH)^2 - 79.6(pH)^3$;

B - model (PEG 400, G6PDH) .... $K = -256.3 + 106.1(pH)$;

C - model (PEG 1500, HK) .... $K = 1199.5 - 883.4(pH) + 238.9(pH)^2 - 28.1(pH)^3 + 1.2(pH)^4$;

D - model (PEG 1500, G6PDH) .... $K = 0,9 - 0.16(pH) + 0.01(pH)^2$.

Table 1. pH values of the phases after extractions obtained with different pH values.

pH	17.5% w/w PEG 400 with 15.0% w/w phosphate		20.0% w/w PEG 1500 with 17.5% w/w phosphate	
	Top phase	Bottom phase	Top phase	Bottom phase
3.8	NP	NP	3.5	3.4
5.0	5.4	5.2	4.6	4.5
6.0	6.3	6.2	5.3	5.2
7.0	7.1	7.0	6.1	6.1
8.0	7.9	7.8	6.9	7.0
8.8	8.7	8.7	7.3	7.6

NP = no partition between the phases

enzymatic stability of the product, and so it is important to verify the interval of stability of the target enzyme, as follow. The data related to the stability of hexokinase and glucose-6-phosphate dehydrogenase, after leaving the cell-free extract (supernatant) in buffer solutions at different pH by 51h are presented in Fig. 3. As can be seen, both enzymes, present in the cell-free extract, were relatively stable (remaining activity >80%) at the beginning (about 5 hours) of the storage time at pH interval between 4.0 and 9.5. However, both enzymes were markedly unstable at pH 4.0. It can also be seen that the G6PDH is quite stable at pH interval from 5.0 to 9.5, even after 51h of buffer-enzyme contact. The hexokinase, in turn, was sensitive to pH values higher than 7.5. These results are quite similar to those found by Silva et al. (21) for the purified forms of HK (SIGMA[R]-H5375) and G6PDH (SIGMA[R]-G8529). Probably, the macromolecular structures of these proteins are well adapted to less acidic pH values, because the pH of the yeast cell cytoplasm, their natural location, is normally buffered at pH of 6.5-7.5 (24). Therefore, to avoid decrease in yield extraction, it is recommended to perform the extractions at pH values higher than 5.0 and lower than 9.5.

Another point to be borne out is that the best pH values for stability are higher than the correspondent isoelectric points of the enzymes, which are 4.8 and 6.0, respectively, for HK and G6PDH (6,26). Then, for pH ≥ 6.5 we can assume that the effective
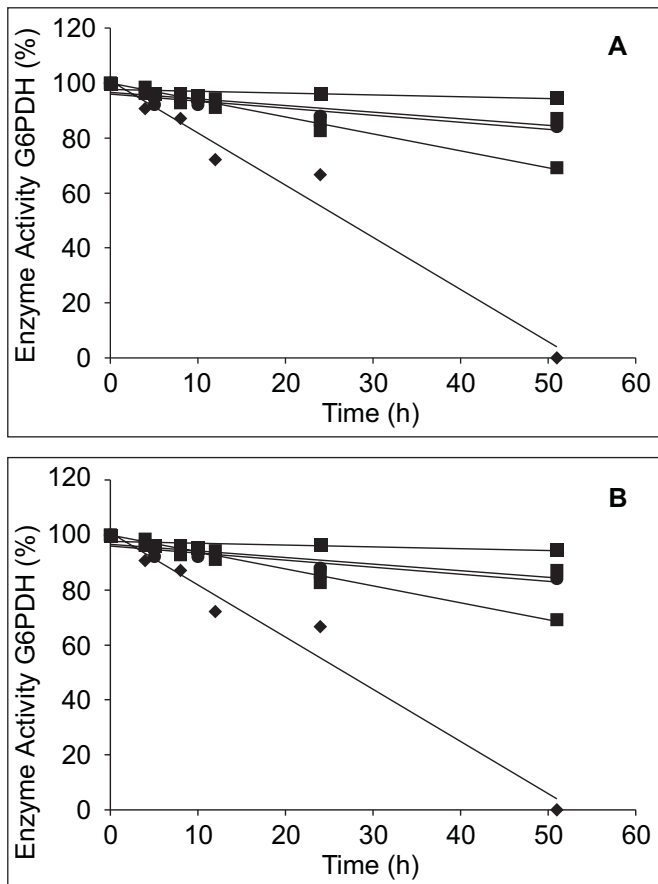
Figure 3. Remaining enzyme activities as function of pH and storage time at 4ºC. Enzyme G6PDH (Fig. 3-A): (¾¨¾ pH 4.0; ¾n¾ pH 4.5; ¾l¾ pH 5.0-5.5; ¾l¾ pH 6.0-6.5; ¾x¾ pH 7.0-9.5). Enzyme HK (Fig. 3-B): (¾¨¾ pH 4.0; ¾n¾ pH 4.5-5.5; ¾p¾ pH 6.0-7.5; ¾p¾ pH 8.0-8.5; ¾l¾ pH 9.0-9.5).

electrical charge of both proteins is negative, a favorable condition for an extraction of HK and G6PDH from yeast cell-free extract through an aqueous two-phase system.

CONCLUSIONS

From a preliminary study it can be concluded that the partition coefficient strongly depends on the pH value, in other words, the enzymatic partition coefficient of the enzymes HK and G6PDH (K_{HK} and K_{G6PDH}) was favored by high pH values. The statistical analysis indicated that only the model obtained for the partition coefficient of the enzyme glucose-6-phosphate dehydrogenase (K_{G6PDH}) was not significant at 90% or higher confidence level. Besides, during the extraction utilizing the system formed with PEG 1500 (20.0% w/w) and phosphate salts (17.5% w/w), there is a possibility of partition between HK and G6PDH enzymes, and also between HK enzyme and other

contaminant proteins. This is due to a significant difference in the partition coefficient values between K_{HK} with K_{G6PDH} and K_{Tprot}, when the pH value of the system is about 6.0. It is important to point out that the variations of the pH values during the extractions can denaturate both enzymes, and decrease the extraction yield.

RESUMO

Influência do pH na partição da glicose 6-fosfato desidrogenase e hexoquinase em sistema de duas fases aquosas

Glicose-6-fosfato desidrogenase (G6PDH) e hexoquinase (HK) são importantes enzimas usadas em estudos bioquímicos e médicos e em diversos métodos analíticos. Sistema de duas fases aquosas (SDFA) formado por uma solução polimérica e uma solução eletrolítica proporciona um método para separação e purificação de enzimas com diversas vantagens, incluindo biocompatibilidade, que pode ser facilmente escalonado para nível industrial. Neste trabalho, os efeitos de diferentes valores de pH na estabilidade e na partição (K, coeficiente de partição) por SDFA das enzimas G6PDH e HK, obtidas através de levedura de panificação, foram investigados. Os resultados, obtidos do sistema constituído por 17,5% de PEG 400 e 15,0% de fosfato, mostraram que com a elevação do pH de 5,0 para 8,8, o K_{HK} aumentou 26 vezes e o K_{G6PDH} 2,2 vezes. No sistema 20,0% PEG 1500 : 17,5% fosfato, quando o valor do pH variou de 3,8 para 8,8. os valores de K_{HK} e K_{G6PDH} aumentaram e 13 vezes e 1,2 vez, respectivamente. Isto permite concluir que o coeficiente de partição, para ambas as enzimas, é favorecido pela elevação do pH. Uma análise estatística dos resultados foi conduzida para confirmar esta conclusão.

Palavras-chave: sistema de duas fases aquosas, coeficiente de partição, purificação de proteína, hexoquinase, glicose 6-fosfato desidrogenase.

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