0273-2289/03/105-108/0787/$20.00

Partition Behavior and Partial Purification of Hexokinase in Aqueous Two-Phase Polyethylene Glycol/Citrate Systems

George G. G. Oliveira,[1] Daniel P. Silva,[1] Inês Conceição Roberto,[2] Michele Vitolo,[1] and Adalberto Pessoa Jr.*[,1]

[1]Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmacy, University of São Paulo, Av. Prof. Lineu Prestes, 580, B.16. 05508-900, São Paulo, SP, Brazil, E-mail: pessoajr@usp.br; and [2]DEBIQ-FAENQUIL, Rod. Itanjuba - Lorena, Km 74.5, 12,600-000Lorena, SP, Brazil

Abstract

This study dealt with the partition behavior and partial purification of hexokinase (HK) from baker's yeast by liquid-liquid extraction using aqueous two-phase polyethylene glycol (PEG)/citrate systems. First, we investigated the effect of agitation type (vortex and 8 rpm rotation) on the stability of the system, and then the effects of sodium citrate concentration, PEG concentration, and molar mass of PEG on the partition coefficient of this enzyme by using a 2^5 factorial experimental design. The results of this factorial experiment showed the possibility of a partial purification of HK by using two extraction steps, since the enzyme preferentially migrated to the top phase and the total proteins (mainly contaminants) remained in the bottom phase. The purification factor (Pur_{TOP}) of the enzyme in the top phase was 1.87, and the partition coefficient of the total proteins (K_{Prot}) was 0.47.

Index Entries: Hexokinase; liquid-liquid extraction; aqueous two-phase systems; *Saccharomyces cerevisiae*.

Introduction

Hexokinase (HK) (EC 2.7.1.1) is the first enzyme of glycolysis to catalyze the phosphorylation of glucose into glucose 6-phosphate (G6P). G6P, in turn, is a key intermediate for several pathways such as gluconeogenesis, shunt of pentoses, and glycogen metabolism. The same enzyme is sensitive to the catabolic repression and plays an important role in the

*Author to whom all correspondence and reprint requests should be addressed.

glucose uptake mechanism through the plasma membrane *(1)*. Furthermore, HK is used in analytical methods to measure glucose, fructose, manose, adenosine triphosphate (ATP), and creatine-kinase activity *(2)*, in the phosphorylation of pyranose and furanose analogs of glucose *(3)*, and in wine and fruit juice industries for the detection of illegal sugar addition to the final products *(4)*.

Although the HK enzyme can be found in almost every animal tissue and in several microbial species, the use of *Saccharomyces cerevisiae* as an enzymatic source and other products *(5)* makes sense in Brazil because the large experience of the Brazilian chemical industry on handling this strain. In addition, coupling the yeast processing with ethanol production should probably have a positive effect on the profits of the distilleries. However, production of HK can only be viable if large quantities of this enzyme are available at competitive prices. The development of techniques for the separation and purification of enzymes has been an indispensable prerequisite for many of the advances made in the biotechnological industry *(6,7)*.

According to Diamond and Hsu *(8)*, 50–90% of the production costs of biologic products are determined by the purification strategy. Purification is troublesome because of the system complexity and the need to retain biologic activity. Aqueous two-phase systems (ATPS) are liquid-liquid extraction processes widely used for the extraction and purification of many biologic products. Their technical simplicity and ease of operation and scale-up make them very attractive for industrial applications *(9, 10)*. Moreover, aqueous two-phase systems such as polyethylene glycol (PEG)/citrate, PEG/phosphate and PEG/sulfate are adequate for continuous large-scale purification of materials of biological origin and allow the use of traditional liquid-liquid extraction equipment *(11–13)*.

The systems are formed by the mixture of aqueous solutions of two polymers or a polymer and an electrolyte solution. With the concentration of each component of the system in one of the phases (top or bottom), there is partition of biomolecules such as proteins, cells, cell particles or nucleic acids. To achieve a significant extraction, the target product has to be preferentially partitioned in favor of one phase, whereas the interfering substances (other biomolecules) should be partitioned to the other phase. It is important to remember that higher water content in both phases avoids protein denaturation. 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) *(14)*. Factors and mechanisms that cause the uneven distribution of proteins between the two-phases are little understood, but empirical rules have been developed. In practice, the technique requires further experimentation to find an adequate system for each particular application, since many factors influence the partition and purification of proteins. For this reason, the aim of the present work was to verify the influence of some variables (PEG molar

mass, PEG concentration, and citrate concentration) on the partial purification of HK of *S. cerevisiae* in aqueous PEG/citrate systems.

Materials and Methods

Chemicals

Glucose-6-phosphate dehydrogenase (G6PDH), ATP, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate (NADP) and G6P, utilized in the enzymatic analysis, were obtained from Sigma (St. Louis, MO). PEG with a molar mass of 300, 400, 1500, and 4000 g/mol was purchased from Labsynth (São Paulo, Brazil) and of 600 and 1000 g/mol from Merck (Darmstadt, FRG). Sodium citrate was purchased from Grupo Química Industrial (São Paulo, Brazil). All other chemicals were of analytical grade.

Cell Homogenate

The solution containing disrupted cells (denominated cell homogenate) was prepared through disruption of commercial baker's yeast, by submission to a cell disruption in a mechanical grinder (bead mill) with glass beads (diameter = 0.5 mm). The cell suspension (wet cell cake and Tris-HCl buffer) and glass beads were mixed in the volumetric proportion of 1:1 (below 10°C). After disruption, cell debris and glass beads were removed by filtration. Before each extraction in aqueous two-phrase system, the cell homogenate, stored at –20°C, was thawed and centrifuged (2880*g*/10 min). The supernatant was collected with HK and other proteins.

Binodal Curves and Equilibrium of PEG/Citrate System

The binodal curves were built by the titulation method, as described by Albertsson *(15)*, using stock solutions made of 50% (w/w) PEG and 30% (w/w) citrate. Equilibrium studies of the PEG/citrate systems were conducted for the extractions containing cell homogenate. Binodal curves are present on the phase diagram and divide a region of component concentrations that will form two immiscible aqueous phases (i.e., above the curve) from those that will form one phase (i.e., at and below the curve).

Homogenization of System

The extraction conditions were the same as those employed in the study of the variation in pH, except only PEGs of 400, 600, and 1000 g/mol were used. The influence of agitation (vortex for 1 min, 8 rpm for 20 min or vortex/rotation) was evaluated in the centrifugal-phase volumes.

Liquid-Liquid Extraction

Aqueous two-phase systems were prepared with different PEGs (300, 400, 600, 1000, 1500, and 4000 g/mol) and sodium citrate ($Na_3C_6H_6O_7 \cdot 2H_2O$). In graduated centrifuge tubes (15 mL), 2.0 g of medium containing the

Table 1
Factors and Levels Used for Optimizing the Enzyme Partition Coefficient by Aqueous Two-Phase System

	Level of variables		
Factor	High (+)	Central (0)	Low (–)
MMPEG (g/mol)	1500	1000	400
PEG (% [w/w])	24	22	20
Citrate (% [w/w])	20	17.5	15

target enzyme (cell homogenate or pure enzyme; Sigma, St. Louis, MO) was mixed with PEG (stock solution) and sodium citrate (solid). Deionized water was then added to the mixture in order to adjust the final concentration desired for the components (system of 10 g). Next, the mixture was agitated in vortex (1 min), rotated (8 rpm/20 min), and centrifuged (1500*g*/10 min) to separate the phases from each other. Samples of the phases (top and bottom) were removed and analyzed to verify the enzymatic activity, total protein concentration, pH, and volume. During all the experiments, the temperature was maintained at 25°C.

The factors and levels used for the HK extraction are provided in Table 1 and were statistically analyzed by means of the program *Statgraphics Plus 6.0* (Statsoft) according to the method of experimental design proposed by Box et al. *(16)*. After all the experiments, which consisted of a 2^3 factorial experimental design with three repetitions in the central point (totaling 11 extractions), the effect of molar mass of PEG (MMPEG, g/mol), PEG concentration (% [w/w]), and citrate concentration (% [w/w]) were used to optimize the partition coefficient of the enzyme. For each of the three factors, high (coded value: +1), central (coded value: 0), and low (coded value: –1) set points were selected (Table 1).

Analytical Methods

HK activity was determined by spectrophotometric analysis (340 nm) of reduced $NADP^+$ at 30°C, according to the method described by Bergmeyer *(2)*. One unit of HK was defined as the amount of enzyme that catalyzes the reduction of 1 mmol of $NADP^+$/min in the conditions of the experiment. The concentration of total proteins was determined according to Bradford *(17)*, using patterns of bovine serum albumin (Sigma). The partition coefficient of the enzyme (K) was calculated as the ratio between the HK enzymatic activities of the top and bottom phases (Eq. 1), and the partition coefficient of the proteins (K_{Prot}) was calculated as the ratio between the total protein concentration of the top and bottom phases (Eq. 2). Selectivity (S) was calculated as the ratio between the partition coefficient of HK and the partition coefficient of the total proteins (Eq. 3). The increase in purity in the top phase (Pur_{TOP}) was obtained from the relationship between the specific activity of the enzyme (U/mg_{Prot}) before extraction and after extraction (Eq. 4). The yield

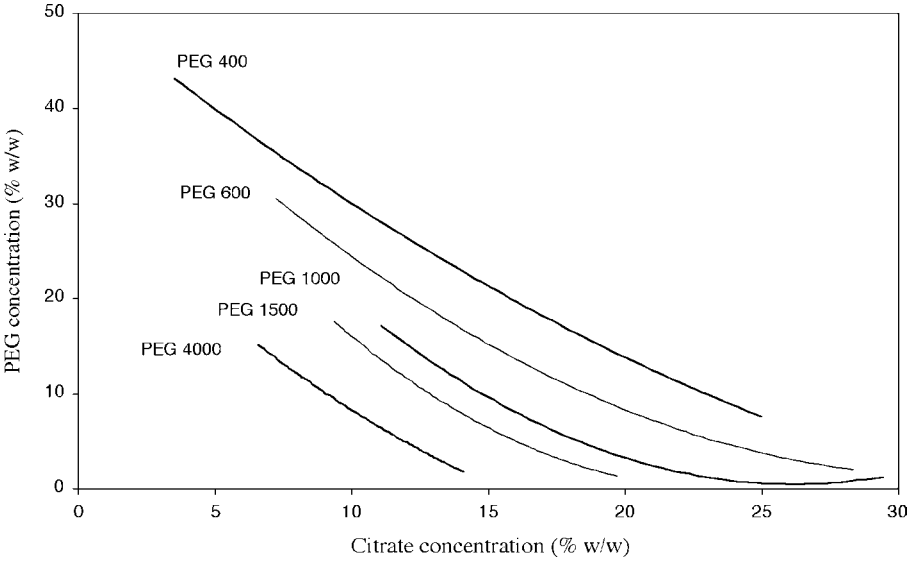

Fig. 1. Binodal curves obtained in PEG 400, 600, 1000, 1500, and 4000, and citrate (pH 8.5, 25°C) systems.

of extraction (%R) was obtained from the relationship between the total proteins (mg) or total activity of the enzyme (U) in the phases obtained after and before the extraction, using the volume of each phase:

$$K = \frac{\mathrm{HK_{TOP}}}{\mathrm{HK_{BOT}}} \tag{1}$$

$$K_{Prot} = \frac{Prot_{\mathrm{TOP}}}{Prot_{\mathrm{BOT}}} \tag{2}$$

$$S = \frac{K}{K_{Prot}} \tag{3}$$

$$Pur_{\mathrm{TOP}} = \frac{\mathrm{U_{TOP}/mg}_{Prot}}{\mathrm{U_{initial}/mg}_{Prot}} \tag{4}$$

Results and Discussion

Binodal Curves: PEG/Citrate System

The curves of the PEG/citrate system (Fig. 1) were obtained with the intention of establishing extraction conditions (%PEG and %citrate). These curves present a significant variation as a function of the molar mass of PEG (MMPEG). It was observed that there was no formation of a two-phase system with PEG 300. The binodal curves built with PEG 400, 600, 1000, 1500, and 4000 g/mol were close to those presented by Vernau and Kula *(18)*.

Table 2
Influence of Homogenization Type on Volume of Interface (Occupied by Cellular Fragments) as Function of MMPEG

	Type of homogenization		
	Vortex 1 min	Rotation 8 rpm/20 min	Vortex (1 min) + rotation (8 rpm/20 min)
MMPEG	Volume of interface (mL)		
400	1.1	0.3	0.3
600	1.7	1.5[b]	1.0
1000	2.0	1.8[b]	1.2

[a] Total volume of system was 8.5 mL.
[b] Citrate salt was not totally dissolved.

Studies of Equilibrium

Extractions in PEG/citrate systems with cell homogenate presented cellular fragments occupying a large space between the top and bottom phases of aqueous two-phase systems. This volume, according to Geneviève et al. *(19)*, can be called interface volume or be considered as a third phase of the system, harming not only the transfer of proteins between the phases *(20)*, but also the total solubility of the citrate salt in the system. For this reason, studies of equilibrium of the PEG/citrate system were carried out in order to define extraction conditions precisely with small or no interface volume. The results (Table 2) showed that the association between vortex and rotation (vortex for 1 min + 8 rpm/20 min) makes it possible to decrease the interface volume as well as the total dissolution of the citrate salt.

Experiments were conducted with another enzyme (G6PDH) to verify the partition coefficient as a function of different times of rest of the PEG/citrate system after its homogenization and centrifugation. The results showed that only after 6 h of rest was the PEG/citrate system stable, which is in agreement with the findings of Jain and Johri *(21)* in relation to the use of different times of rest after the homogenization of the aqueous two-phrase system.

Liquid-Liquid Extraction in PEG/Citrate System: HK

After obtaining the binodal curves and evaluating the equilibrium of the PEG/citrate system, we conducted experiments to verify the effects of citrate concentration (%citrate), PEG concentration (%PEG), and MMPEG on the partition coefficient (K) of pure HK enzyme (Sigma). The statistical analysis of the results employing t-tests and variance analysis showed that MMPEG and %citrate were significant variables in the extraction process, with 95% confidence. A negative effect of MMPEG and a positive effect of %citrate on the response variable (K) were observed. This means

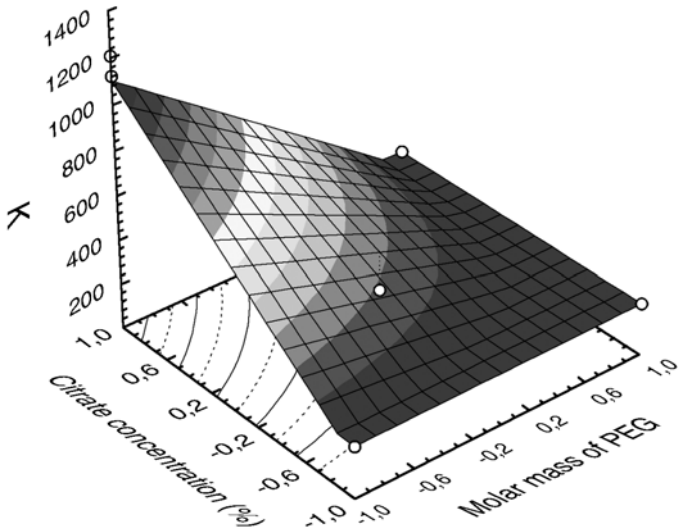

Fig. 2. Response surface obtained in experimental design (2^3 factorial + central point) with variables MMPEG and %citrate, and coefficient of partition (K) of HK as response (22% [w/w] PEG, pH 8.5, and 25°C).

Table 3
Partition Coefficient (K) of HK as Function of MM PEG, %PEG, and %citrate, at pH 8.5 and 25ºC

Experiment	MMPEG	%PEG	%Citrate	K^b
1	1500	24	20.0	1.46
2	400	24	20.0	1067.80
3	1500	20	20.0	0.36
4	400	20	20.0	1171.40
5	1500	24	15.0	0.03
6	400	24	15.0	NS
7	1500	20	15.0	0
8	400	20	15.0	NS
9	1000	22	17.5	1.87
10	1000	22	17.5	0.91
11	1000	22	17.5	1.78

[a] Agitation in vortex (1 min) Followed by Rotation (8 rpm/20 min) and centrifugation (1500*g*/10 min) with time of rest of 3 h for PEGs 1000 and 1500, and 1 h for PEG 400.

[b] NS, no phase separation.

that the lowest MMPEG and the highest citrate concentration provided the highest values of K, as observed in the response surface (Fig. 2). For the purpose of statistical analysis, the values of the partition coefficients obtained in experiments 6 and 8 (Table 3), which did not form two-phase systems, were considered null ($K = 0$).

Table 4
Partition Coefficient (*K*)of HK as Function of Growing Concentrations of citrate at pH 8.5 and 25°C[a]

Experiment	MMPEG	%PEG	%Citrate	*K*[b]
1	400	22.0	15	NS
2	400	22.0	17.5	245
3	400	22.0	20	1000

[a] Agitation in vortex (1 min) followed by rotation (8 rpm/20 min) and centrifugation (1500*g*/10 min) with time of rest of 1.0 h after centrifugation.
[b] NS, no phase separation.

Owing to these results and to the absence of two-phase systems, when MMPEG <400 g/mol was used, new experiments to evaluate the influence of %citrate on the *K* value of HK were performed. The conditions were 22% (w/w) PEG 400 and 15–20% (w/w) %citrate (Table 4). The highest *K* value (1000) was attained when cell homogenate, 22% (w/w) PEG 400, and 20% (w/w) citrate were employed. Experiments using higher values of %citrate were not possible, because little or no enzyme was detected in the bottom phase of the system.

Of all the variables tested, the low molar mass of PEG associated with a high concentration of citrate salt mostly favored the transfer of the HK enzyme to the top phase of the system. The high molar mass of this enzyme indicates that the volume exclusion effect by the PEGs of higher molar mass might have occurred. This means that in the top phase of the system there was no space available for the enzyme when PEGs with a molar mass >400 g/mol were used. Since it was not possible to obtain a two-phase system with PEGs of lower molar mass, it was concluded that it would not be possible to improve the partition coefficient as a function of the MMPEG. However, in the case of the citrate, the transfer of the enzyme to the top phase of the system can be attributed to the effect of "salting out," because under high concentrations of this salt the enzyme is expelled from the bottom phase. The combination of these two variables favored the extraction of the enzyme, since the enzyme excluded from the bottom phase was transferred to the top phase.

Liquid-Liquid Extraction for ATPS PEG/Citrate

Cell Homogenate

The conditions that furnished the best results of pure enzyme extraction, 22% (w/w) PEG 400 and 20% (w/w) citrate, were also used for extractions with HK of cell homogenate. However, the literature reports that when cell homogenate is used, the substances that can interfere with the characteristics of the system increase in number *(22,23)*. This explains not only the nonformation of the two-phase system during the preparation of the systems with 22% (w/w) PEG 400, 20% (w/w) citrate, and cell homo-

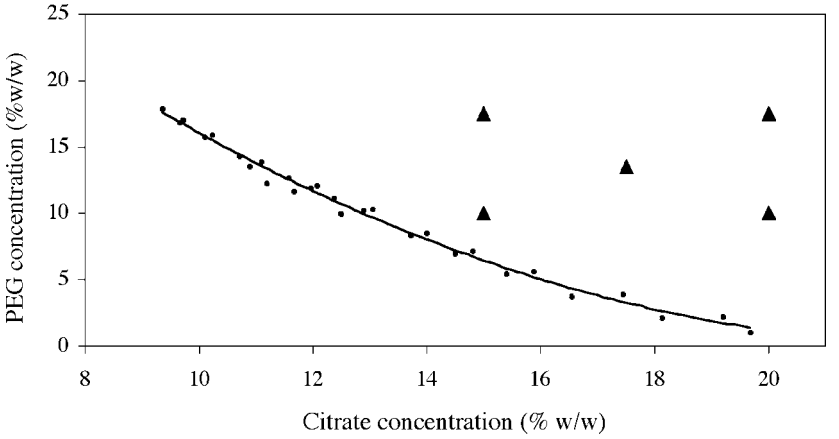

Fig. 3. Binodal curve built with PEG 1500 and citrate salt (pH 8.5 and 25°C). The points represented by the triangles were used for the initial extractions of the system PEG 1500/citrate in two stages.

genate, but also the formation of such a system when pure enzyme was used (Table 4, experiment 3).

Experiments aiming the reduction of the system viscosity were performed with a higher %citrate (significant variable on the value of *K*) and a lower PEG concentration, until a close point to the binodal curve, because, according to the results of the experimental design, this variable (%PEG) does not interfere with the response of the system. These extraction conditions and the systems in which there were separations of the phases provided a high value of enzymatic partition coefficient. However, the values of protein partition coefficient were high, and the purification factor was low. From this result it is possible to conclude that the system (12% [w/w] PEG 400 and 25% [w/w] citrate, pH 8.5, and 25ºC) prepared with cell homogenate and following the homogenization procedures can be used to prepurify the enzyme. However, to improve the purification factor of the enzyme, new experiments were conducted. A second extraction stage was performed and the top phase obtained in the first stage was used as the initial medium and source of HK.

Second Stage of Extraction

The top phase obtained after the first extraction was added to the second stage (PEG 1500/Citrate). This new stage of HK extraction was performed in different concentrations of PEG and citrate, and these concentrations were defined as a function of the binodal (Fig. 3). The extractions were performed with the lowest concentrations of PEG and citrate, but above the binodal curve. The results of the experiments are presented in Table 5.

The results showed that to reach higher enzyme purity (Pur_{TOP}) and partition coefficients (*K*), additional experiments should be performed.

Table 5
Extraction of HK in Aqueous Two-Phase System
Using Cell Homogenate in PEG 400/Citrate (Experiment 1)
and PEG 1500/Citrate (Experiments 2–9) at pH 8.5 and 25ºC

Experiment	%PEG (w/w)	%citrate (w/w)	K	K_{Prot}	Pur_{TOP}	S	Interface volume (mL)
1	12	25	1000	54.15	1.25	0.00	0.0
2	17	15	1.01	1.07	1.57	0.94	0.3
3	10	20	0.66	1.80	0.83	0.37	0.5
4	10	15	0.13	0.34	0.39	0.38	0.4
5	17	20	19.47	7.53	1.32	2.58	1.5
6	13.5	17.5	0.51	0.47	1.87	1.09	0.4
7	13.5	20.0	1.76	2.12	1.28	0.83	1.0
8	13.5	22.5	11.07	2.59	0.82	4.28	1.5
9	13.5	25.0	79.07	16.38	1.02	4.83	1.7

[a] Agitation in vortex (1 min) followed by rotation (8 rpm/20 min) and centrifugation (1500g/10 min).

These new extractions were performed with higher concentrations of citrate (20, 22.5, and 25 % [w/w]). However, the concentration of PEG should be constant (13.5% [w/w]), because high concentrations of this polymer can saturate the system, and low concentrations decrease the partition coefficients and purification factors. The results (experiments 7–9) showed that the augmentation of the citrate concentration (from 20 to 25%) improved the values of K and Pur_{TOP}. On the other hand, the extraction performed at 25% citrate provided a high interface volume and incomplete dissolution of the salt.

Conclusion

The extraction of proteins by the aqueous PEG/citrate system was effective, with quite simple reagents and stages of process. Its use in the partial purification of HK in two extraction steps is viable. In the first step, using 12% (w/w) PEG 400 and 25% (w/w) citrate at pH 8.5 and 25ºC, high enzymatic recovery was obtained in the top phase of the system with high values of partition coefficient. In the second step of extraction, performed with 13.5% (w/w) PEG 1500 and 17.5% (w/w) citrate, a good purification factor was obtained in the top phase (Pur_{TOP} = 1.87). This indicates a tendency of the HK to stay in this phase in comparison with the undesirable proteins (K_{Prot} = 0.47), which show a higher migration to the bottom phase of the system. The exclusion effect, caused by the PEG with high molar mass, did not allow the transference of the enzyme to the top phase. Furthermore, the effect of salting out can also explain the transference of the enzyme to the top phase of the system, because under high concentrations of citrate the enzyme is expelled from the bottom phase. The results also

showed that the response surface methodology is adequate to optimize the enzyme partition coefficient by the aqueous two-phase system.

Acknowledgments

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support, and Maria Eunice M. Coelho for assistance in writing the manuscript.

References

1. Kovák, L., Nelson, B. D., and Ernster, L. (1986), *Biochem. Biophys. Res. Commum.* **134(1),** 285–291.
2. Bergmeyer, H. (1984), *Methods of Enzymatic Analysis*, 3nd ed., Verlag Chimie, Weinheim, Germany.
3. Chenault, H. K., Mandes, R. F., and Hornberger, K. R. (1997), *J. Org. Chem.* **62,** 331–336.
4. Whitaker, J. R. (1991), in *Food Enzymology*, vol. 2, Fox, P. F., ed., Elsevier, New York, NY.
5. Godfrey, T. and West, S. (1996) *Industrial Enzymology*, MacMillan, London, England, UK.
6. Rodrigues, E. M. G., Pessoa-Jr., A., and Milagres, A. M. F. (1999), *Appl. Biochem. Biotechnol.* **78,** 779–788.
7. Rodrigues, E.M.G., Milagres, A.M.F., and Pessoa-Jr., A. (1999), *Process Biochem.* **34,** 121–125.
8. Diamond, A. D. and Hsu, J. T. (1992), *Adv. Biochem. Eng. Biotechnol.* **47,** 89–135.
9. Costa, S. A., Pessoa Jr., A., and Roberto, I. C. (1998), *Appl. Biochem. Biotechnol.* **70/72,** 629–639.
10. Gaikar, G. V., Bodhankar, S. S., and Latha, V. J. (1996), *J. Chem. Technol. Biotechnol.* **67,** 329–332.
11. Coimbra, J. S. R., Thommes, J., Meirelles, A. J. A., and Kula, M. R. (1995), *Bioseparation* **5,** 259–268.
12. Coimbra, J. S. R., Thommes, J., and Kula, M. R. (1994), *J. Chromatogr.* **668,** 85–94.
13. Coimbra, J. S. R., Mojola, F., and Meirelles, A. J. A. (1998), *J. Chem. Eng. Japan* **31,** 277–285.
14. Schmidt, A. S., Venton, A. M., and Asenjo, J. A. (1994), *Enzyme Microb. Technol.* **16,** 131–142.
15. Albertsson, P. A. (1986), *Partition of Cell Particles and Macromolecules*, John Wiley, New York, NY.
16. Box, G. P., Hunter, W. G., and Hunter, J. S. (1978) *Statistics for Experimenters*, John Wiley, New York, NY.
17. Bradford, M. A. (1976), *Anal. Biochem.* **72,** 248–254.
18. Vernau, J. and Kula, M. R. (1990), *Biotechnol. Appl. Biochem.* **12,** 397–404.
19. Geneviève, M. F., Walker, S. G., and Lyddiatt, A. (2000), *J. Chromatogr. B* **743(1),** 409–419.
20. Cascone, O., Andrews, B. A., and Asenjo, J. A. (1991), *Enzyme Microb. Technol.* **13,** 629–635.
21. Jain, A and Johri, B. N. (1999), *Bioresour. Technol.* **67,** 205–207.
22. Morré, D. J., Morré, D. M. (2000), *J. Chromatography B* **743,** 369–376.
23. Rito-Palomares, M. and Cueto, L. (2000), *J. Chromatogr. B* **743,** 5–12.