0273-2289/95/5302—0147$05.60

Horseradish Peroxidase Extraction and Purification by Aqueous Two-Phase Partition

MARÍA V. MIRANDA, HÉCTOR M. FERNÁNDEZ LAHORE, AND OSVALDO CASCONE*

Cátedra de Microbiología Industrial y Biotecnologia, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina

Received April 26, 1994; Accepted August 9, 1994

ABSTRACT

The effect of poly(ethyleneglycol) (PEG) molecular weight, system pH, and sodium chloride concentration on the partitioning behavior of horseradish peroxidase from *Armoracia rusticana* root extract was investigated in poly(ethyleneglycol)/sodium phosphate systems.

PEG molecular weight strongly affects the enzyme partition coefficient, whereas pH variation from 5.5 to 8.0 has little effect. The addition of sodium chloride (8% w/w) to a PEG 1540/phosphate system, pH 7.0, raises the peroxidase partition coefficient 13.5-fold without important changes in that of total horseradish root proteins. Moreover, these conditions allow direct homogenization of the *A. rusticana* roots with the selected aqueous two-phase system with the clear top phase containing over 90% of the enzyme and the purification factor being 4.8.

Index Entries: Horseradish peroxidase; purification; aqueous two-phase partition.

Abbreviations: ATPS, aqueous two-phase systems; PEG, poly(ethyleneglycol); PO, horseradish peroxidase; TP, total protein.

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

INTRODUCTION

Peroxidase (EC 1.11.1.7) is the most widely used enzyme in the manufacture of enzyme immunoassay kits and in immunohistochemistry. *Armoracia rusticana* (horseradish) roots are the main source of the enzyme.

Several methods have been reported for the purification of peroxidase from this raw material. They involve ammonium sulfate precipitation, size-exclusion, and ion-exchange chromatography (*1,2*), salting-out chromatography (*3,4*), and affinity chromatography with concanavalin A (*5,6*), monoclonal antibodies (*7,8*), or chelated metals (*9*). Tjissen (*10*) developed a simpler purification method based on ion-exchange chromatography, but starting from a raw material that must be bought from commercial manufacturers. When the cost is taken into account, only a few methods seem to be suitable for large-scale purification.

Aqueous two-phase partition is a technique that can be used efficiently for the separation and purification of proteins owing to its simplicity, mildness, and high yields (*11–13*). It is specially suited to enzyme extraction and purification from biological media, such as animal and vegetable tissues, since a clarification step is not required. Therefore, it appears to be an alternative method for peroxidase large-scale production.

In this article, we report the partition behavior of peroxidase from *A. rusticana* roots in different PEG/phosphate aqueous two-phase systems (ATPS), and its extraction and purification in a selected system up to an SA 60 guaiacol U/mg in just one step.

MATERIALS AND METHODS

Materials

PEG 600, 1540, 4000, and 6000 were obtained from Sigma Chemical Co., St. Louis, MO. PEG 20000 was from Fluka Chemie AG, Switzerland. Horseradish roots were bought from a local market in Buenos Aires. Guaiacol was from Mallinkrodt Chemical Works, St. Louis, MO. All other reagents were AR-grade.

Aqueous Two-Phase Systems

They were prepared from stock solutions: 80% (w/w) PEG 600, 60% (w/w) PEG 1540, 40% (w/w) PEG 4000 and 6000, 20% (w/w) PEG 20000 and 40% (w/w) phosphate buffer (as monosodium and dipotassium mixture). Solid NaCl was added when necessary.

In all experiments, the phase:volume ratio was maintained at approx 1:1, the total system weight 2 g, and the tie-line length (measured through the "distance to binodial"), constant at 0.25 g. The distance to binodial was obtained by measuring the amount of water (in g) to be added to a 1-g two-phase system to change it into a monophasic one (*14*).

Purification Procedure

Roots were cut into small cubes of approx 5 mm, homogenized in 1 vol of water in a Waring Blender, and the homogenate was centrifuged at 2000*g* for 15 min; 150-μL aliquots of the supernatant were used in trials for finding optimum partition conditions. Under such conditions, *A. rusticana* roots were homogenized directly with the components of the selected ATPS.

Analysis of the Phases

Total protein (TP) concentration was measured according to the method of Bradford (*15*).

Peroxidase activity was determined by the method of Tjissen (*10*): The assay mixture contained 3.0 mL of 100 m*M* potassium phosphate buffer, pH 7.0, 50 μL of guaiacol (2.45 mg/mL), and 10-μL aliquots of top or bottom phase. Reaction was started by the addition of 40 μL of 8 m*M* hydrogen peroxide, and absorbance at 436 nm was recorded for 5–10 min. Activity calculations were also made as described by Tjissen (*10*).

Partition coefficient *K* was calculated by dividing the enzyme activity or protein concentration in the top phase by the enzyme activity or protein concentration in the bottom one. All determinations were performed in triplicate.

The coefficient of variation for K_{PO} determination was 5.8%, whereas that for K_{TP} was 3.9%, both determined from 30 independent experiments in a system where *K* is approx 1.

RESULTS AND DISCUSSION

Effect of PEG Molecular Weight

Figure 1 shows the partition coefficient of peroxidase and TP of the horseradish root extract in various PEG/phosphate systems prepared with different PEG molecular weights, at pH 7.0. The values of K_{PO} strongly depend on the molecular weight of PEG. As it increases from 600 to 20,000, the partition coefficient decreases from 37.7 to 0.02. In contrast, the values of K_{TP} do not show this behavior. With PEG 600, K_{TP} is high (5.2), but when the molecular weight of PEG increases, K_{TP} remains approximately constant around 0.7.

Only with PEG 600, peroxidase prefers the top phase. However, it is known that, at a low molecular weight of PEG, many proteins have high *K* values, which leads to poor separation from the contaminating proteins (*12*). This is the case for contaminants in horseradish root; therefore, PEG 600 will not be selected for peroxidase purification.

With PEG 1540 and over, peroxidase prefers the bottom phase, thus preventing a successful extraction in the top phase, and therefore sepa-

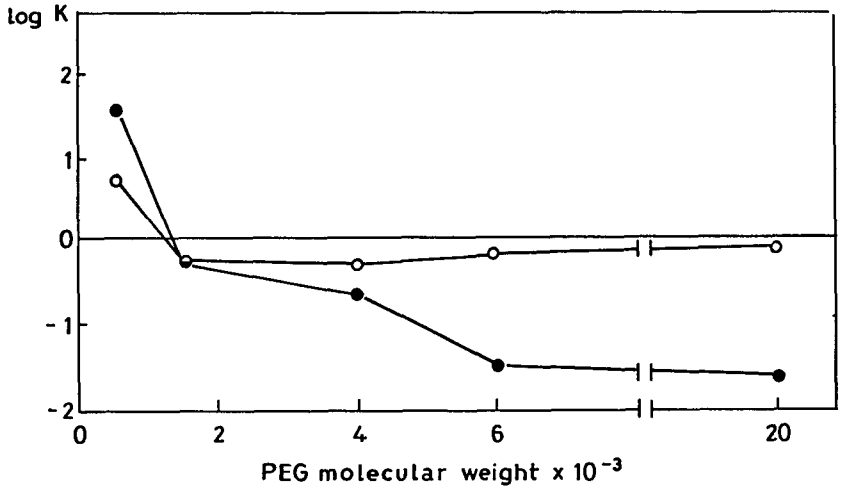

Fig. 1. Effect of PEG molecular weight on the partitioning behavior of PO (●) and total protein (○) in different PEG/phosphate systems, at pH 7.0. ATPS composition, expressed as % w/w: PEG 600, 18.5—phosphates, 15.1. PEG 1540, 10.0—phosphates, 14.8. PEG 4000, 7.7—phosphates, 14.6. PEG 6000, 6.3—phosphates, 14.0. PEG 20000, 4.9—phosphates, 13.2.

rating it from the bulk particulate material. Similar qualitative results were obtained at pH 5.5, 6.0, 6.5, and 8.0 (Table 1). At pHs under 5.0 and above 9.0, very low recoveries of enzymatic activity were found.

Effect of pH

Table 1 shows the effect of pH between 5.5 and 8.0 on K_{PO} and K_{TP} for PEG/phosphate systems prepared with PEG 600, 1540, 4000, 6000, and 20000. These data indicate that the pH does not have a definite influence on K_{TP} or K_{PO}. However, the pH was varied over a narrow range, too narrow to affect significantly the net charge of the basic isoenzyme of peroxidase, which is quantitatively the main isoenzyme in horseradish root.

Effect of Sodium Chloride Addition

Partition of horseradish peroxidase (PO) was highly dependent on the NaCl concentration. Figure 2 shows the effect of increasing NaCl concentration on the partition of PO in different PEG/phosphate systems at pH 7.0. Only with PEG 1540, peroxidase mainly stays in the bottom phase in the absence of NaCl and transfers to the top phase in its presence.

Figure 3 shows the behavior of the extract total protein under the above conditions. The influence of NaCl on K_{TP} is far less than on K_{PO}. With PEG 1540, the partition coefficient undergoes only very slight changes at a $K = 0.63$. Very similar results were obtained at pH 6 and 8 (not shown).

Table 1
Partition Coefficient of Horseradish Peroxidase and Total Protein of the Extract at Different pH Values in Various PEG/Phosphate ATPs

		PEG molecular weight				
	pH	600	1540	4000	6000	20,000
K_{PO}	5.5	30.0	0.67	0.12	0.09	0.01
	6.0	35.6	0.68	0.14	0.07	0.02
	6.5	35.0	0.65	0.25	0.05	0.02
	7.0	37.7	0.60	0.24	0.03	0.02
	8.0	35.3	0.56	0.27	0.03	0.03
K_{TP}	5.5	6.09	0.37	0.44	0.42	0.48
	6.0	6.25	0.45	0.37	0.58	0.86
	6.5	5.33	0.50	0.52	0.59	0.68
	7.0	5.20	0.63	0.50	0.63	0.79
	8.0	5.61	0.55	0.70	0.60	0.80

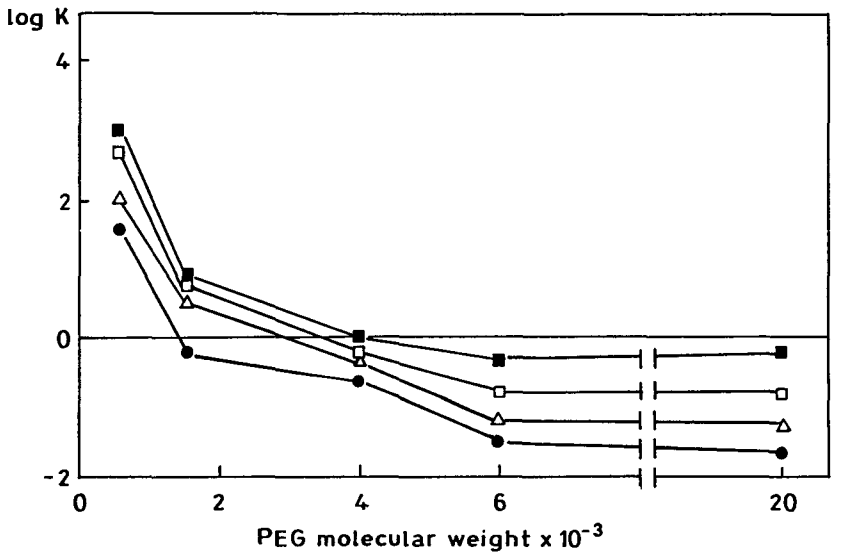

Fig. 2. Effect of the NaCl concentration on the PO partition coefficient in different PEG/phosphate systems, at pH 7.0. (●) Absence of NaCl; (△) NaCl 2.7% w/w; (□) NaCl 5.4% w/w; (■) NaCl 8.0% w/w. ATPS composition is the same as that in Fig. 1.

The enzyme partition coefficient rose 13.5-fold when the concentration of NaCl was increased from 0% (K_{PO} = 0.60) to 8% w/w salt (K_{PO} = 8.1) in PEG 1540/phosphate, pH 7.0. NaCl concentration over 8% w/w causes a shift of the total protein to the top phase, thus decreasing the purification factor (Fig. 4).

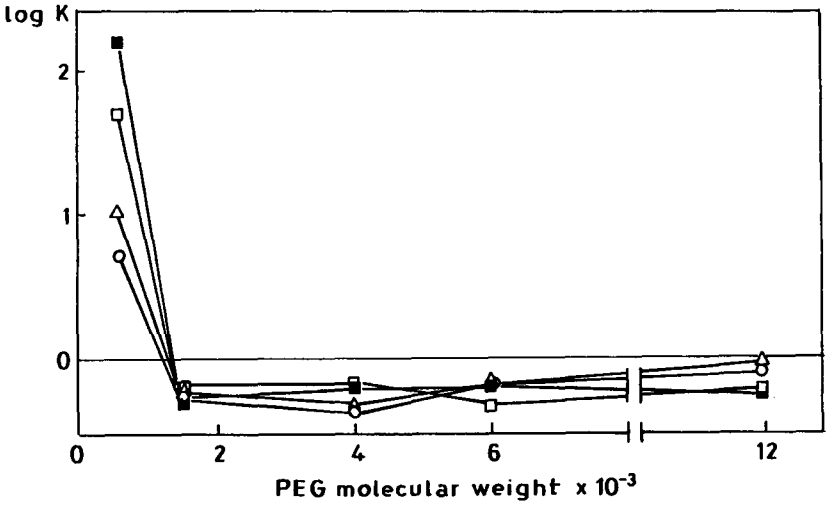

Fig. 3. Effect of the NaCl concentration on the total protein partition coefficient in different PEG/phosphate systems, at pH 7.0. (○) Absence of NaCl; (△) NaCl 2.7% w/w; (□) NaCl 5.4% w/w; (■) NaCl 8.0% w/w. ATPS composition is the same as that in Fig. 1.

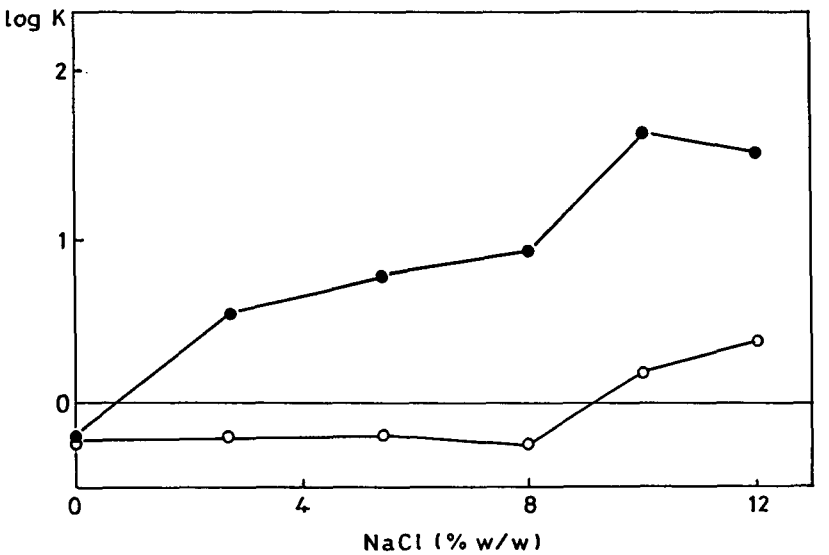

Fig. 4. Partition coefficients of peroxidase (●) and total protein (○) in PEG 1540, 10.0% w/w; phosphates, 14.8% w/w, at pH 7.0 without NaCl or with increasing concentrations of NaCl.

The high concentrations of NaCl can promote an improvement in the separations of bovine serum albumin, thaumatin (*16*), and other proteins (*12*) in PEG/phosphate systems. The "water-structure making salt" and the effect of the salt on the protein's double layer are two alternative hypotheses to explain this phenomenon. Distinction between them would

require experiments over a wider pH range, information on the electrokinetic properties of peroxidase, and a study of the effects of chaotropes, most of which is beyond the scope of this article.

An increase in the salt concentration will increase the partition coefficient of biomolecules with relatively hydrophobic surfaces (*17–19*). The more hydrophobic the biomolecule is, the more it will favor the PEG-rich top phase. Results obtained suggest that PO is more hydrophobic than the bulk of the proteins in the crude extract of *A. rusticana* roots.

Salting-out chromatography on unsubstituted Sepharose CL-6B was applied to the purification of peroxidase from horseradish root extracts by Lascu et al. (3), and the same method, but on substituted Sepharose, was reported by Chavez and Flurkey (4). According to their results, this kind of chromatography is a good alternative for the process scale. ATPS allow exploitation of the salting-out effect for PO purification, but without resorting to adsorbents, such as Sepharose or Phenyl Sepharose, improving in that way the cost–benefit ratio of the procedure.

Taking the results obtained into account, a system that allowed for a good recovery and enrichment of the enzyme in the top phase was designed:

System composition
PEG 1540 10.0% w/w
Phosphate pH 7 14.8% w/w
NaCl 8.0% w/w.
$V_{top\ phase}/V_{bottom\ phase} = 1$.
Enzyme represents 24% of total protein in top phase.
Peroxidase recovery = 90.8%.
Purification factor = 4.8.
SA = 60 guaiacol/U/mg.

This proposed ATPS is based on the limited number of tests performed in this research, and can change when scale-up and cost analysis are carried out.

The system provides the possibility of extracting the roots directly with the phase components, thus obtaining a clear top phase, without the need for resorting to a clarification step, and recovering a high percentage of enriched enzyme in a single step.

ACKNOWLEDGMENTS

This work was supported by grants from the Universidad de Buenos Aires and the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

REFERENCES

1. Shannon, L. M., Kay, E., and Lew, L. Y. (1966), *J. Biol. Chem.* **241,** 2166–2171.
2. Grodecka, B., Kowalski, S., and Szymanska, E. (1985), Pol. Patent PL 133652.
3. Lascu, I., Abrudan, I., Muresan, L., Presecan, E., Vonica, A., and Proinov, I. (1985), *J. Chromatogr.* **357,** 436–439.
4. Chavez, C. and Flurkey, W. H. (1984), *J. Chromatogr.* **298,** 169–171.
5. Casl, M. T. and Malnar, M. (1985), *Jugoslav. Med. Biokem.* **4,** 33–38.
6. Casl., M. T. and Kostrencic, C. (1987), *Ann. Clin. Biochem.* **24 suppl. 2,** 271.
7. Suh, P. G., Lee, Y. H., Lee, J. H., Chung, J. H., Kim, S. R., Tchai, B. S., and Chung, H. K. (1989), *Korean J. Biochem.* **21,** 33–40.
8. Behn, I., Hellthaler, G., Fiebig, H., and Appelt, S. (1985), DDR Patent 228824.
9. Chaga, G., Andersson, L., Ersson B., and Berg, M. (1992), *Biomed. Chromatogr.* **6,** 172–176.
10. Tjissen, P. (1985), in *Practice and Theory of Enzyme Immunoassays,* Burdon, R. H. and van Knippenberg, P. H., eds., Elsevier, New York, Ch. 10, p. 173.
11. Abbott, N. L., Blankschtein, D., and Hatton, T. A. (1990), *Bioseparation* **1,** 191–225.
12. Albertsson, P. A. (1986), in *Partition of Cells and Macromolecules,* Albertsson, P. A., ed., 3rd ed., Wiley, New York, Ch. 15, p. 529.
13. Kula, M. R. (1990), *Bioseparation* **1,** 181–189.
14. Miranda, M. V. and Cascone, O. (1994), *Biotech. Techniques* **8,** 275–280.
15. Bradford, M. (1976), *Anal. Biochem.* **72,** 248–254.
16. Cascone, O., Andrews, B. A., and Asenjo, J. A. (1991), *Enzyme Microb. Technol.* **13,** 629–635.
17. Carlson, A. (1988), *Sep. Sci. Technol.* **23,** 765.
18. Eiteman, M. A. and Gainer, J. L. (1991), *Bioseparation* **2,** 31–41.
19. Dove, G. B. and Mitra, G. (1986), in *Separation, Recovery and Purification in Biotechnology,* Asenjo, J. A. and Hong, J., eds., ACS Symposium, Series 314, Washington, p. 93.