0273-2289/91/2829—0647$02.00

Determination of the Partition Coefficient for Yeast Invertase Between Microwater Droplets in Air in Contact with Liquid Water

PRASHANT B. KOKITKAR AND ROBERT D. TANNER*

Department of Chemical Engineering, Vanderbilt University, Nashville, TN 37235

Index Entries: Equilibrium; enzyme separation; invertase; protein partitioning between air and water phases.

INTRODUCTION

Previously, Baker's yeast was grown on a semisolid substrate (homogenized whole potatoes) in an air-fluidized bioreactor. During the batch bioprocess, certain proteins were trapped by sparging the effluent air stream into a water chamber. Surprisingly, the proteins carried over were specific ones and not the most abundant ones available in the process mixture (*1*).

Simple experiments were performed to determine equilibrium data and to estimate the equilibrium constant K_{eq}, for a single enzyme between a condensed phase and a gas phase containing entrained liquid droplets as is observed in the air-fluidized-bed-bioreactor system that has been previously described (*1*). The purpose of this experimental study is to test whether the estimated value for K_{eq} derived from a postulated model (*2*), is, in fact, a directly measurable quantity. If the measured value of K_{eq} is close to that inferred for the postulated model, then the validity of the model for describing the fluidized bed protein recovery process would be greatly strengthened.

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

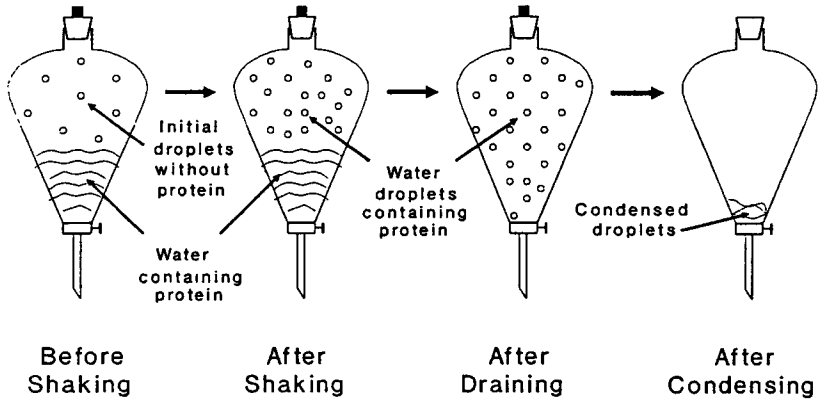

Fig. 1. Schematic diagram of the experimental steps performed in a separatory funnel in order to evaluate the equilibrium constant (partition coefficient) for invertase.

EXPERIMENTAL

The experiments were performed in a separatory funnel as shown in the schematic in Fig. 1. Like the previously used air-fluidized bed apparatus, the separatory funnel was made of glass. The enzyme selected was invertase (No. I-9253, Sigma Chemical Company), which is one of the enzymes secreted by yeast, and is presumably present in the proposed bioprocessing system (3). A known concentration (100 mg L^{-1}) of invertase was prepared in deionized water and 100 mL of this feed solution was added to the separatory funnel (600 mL in volume; Fig. 1). The solution was mixed with unfiltered air by gentle inversion for about 10 min and then the liquid was drained, leaving behind humidified air and some entrained water droplets. Since the deionized water used in the invertase solution samples contained some amounts of proteins, it was used as a blank for the Spectrophotometer (Bausch & Lomb, Spectronic 20) in order to remove their effects on the invertase absorption readings in the Coomassie blue protein assay. The invertase, as well as some of the water proteins, was transferred (by shaking the separatory funnel) from the water phase to the microwater droplets in the air phase. The proteins (invertase + water proteins) trapped on (and possibly in) the water droplets in the air phase were recovered by condensing the droplets. This condensation was achieved by cooling the separatory funnel below the freezing point of water, after the liquid had been drained from it (*see* Fig. 1). The cooling was performed by immersing the funnel in an acetone-dry ice bath for about 25–30 min. A control run of deionized water plus unfiltered air without invertase revealed that dust contributed proteins as well. This artifact was taken into consideration by subtracting off the control run proteins (called base proteins) from the total protein concentration in the invertase run.

Table 1
Sample Experimental Data Set and Calculations

Enzyme : Invertase ; Initial pH = 5.25

	Feed (Initial liq)	Liquid (Final)	Droplets (in air)
Temp (°C)	22.5	25.5	Drained at 13.2 Measured at 22.7
pH	5.25	6.13	8.13
Concentration:			
a. O.D.	0.16	0.11	15.3
b. mg/L	100.00	68.75	9562.5
Liq. Vol (L)	0.1	0.0989	0.001
Air Vol (L)			0.5
mass of enzyme and/or Proteins (mg)	10.00	6.799	3.201 (By diff.)
			9.563 (From O.D.-Uncorrected)
			3.263 (From O.D. - Corrected)
Mass of base proteins in condensed water droplets at pH = 5.81 for above correction (mg)			6.30
Equilibrium Constant K_{eq} (mg L^{-1})	By diff.		93.109
	From O.D. (Corrected)		94.909

In principle, the experimental procedure results in data similar to that for a binary distillation or extraction system, except that, here, the two components are the invertase protein and water. Equilibrium is assumed to be reached in 10 min between the liquid phase (representing the semi-solid broth in the fluidized bed) and the microwater droplets in the air phase, all within the separatory funnel. It is expected that since there is no solid adsorbed in the liquid phase, the directly measured K_{eq} value would be slightly higher than the K_{eq} indirectly obtained for the semisolid adsorbed case (air-fluidized bed).

Two methods were used to determine the invertase equilibrium constant from Eq. (1), given in the Results Section and in Table 1. In the first method, the amount of protein in the condensed water droplets was determined directly from the spectrophotometric absorbance measurements (at 595 nm), after appropriate dilution, using a calibration curve with deionized water as the blank. In the second method, the mass of

protein in the condensed water droplets was determined as the difference between the amount of protein in the original feed liquid and that in the residual liquid, which was drained from the separatory funnel, both of which were determined spectrophotometrically.

RESULTS

The equilibrium data were used to calculate the equilibrium constant for invertase, partitioned between the liquid water phase and the microwater droplets in the air phase. In the model developed previously for the fluidized bed (*2*), the partition or equilibrium constant was defined as

$$K_{eq} = \frac{\left[\frac{\text{mg of Protein}}{\text{L of Air}}\right]}{\left[\frac{\text{mg of Protein}}{\text{g of Semi-solid broth}}\right]} \simeq \frac{\left[\frac{\text{mg of Protein}}{\text{L of Air}}\right]}{\left[\frac{\text{mg of Protein}}{\text{g of Water}}\right]} \quad (1)$$

Here, the studied protein is invertase. Implicit in Eq. (1) is the assumption that the semisolid broth is 100% water. This assumption was made, initially, in order to compare the direct measurement of K_{eq} in the single glass flask with that inferred from the previous experiments in the air fluidized bed. When corrected for the actual water content of 80% (assuming no solids) in the semisolid broth, it gives what appears to be an upper bound for K_{eq}. The 20% solid material may alter the equilibrium constant value by providing both an adsorbent as well as additional volume.

The proteins present in the unfiltered air in the glass separatory funnel experiments (an unexpected artifact) also concentrated in the microwater droplets in the gas phase. Therefore, this quantity of protein determined by spectrophotometric absorbance measurements had to be subtracted from the quantity of total protein measured, in order to obtain correct values for invertase. Using the same calibration curve for determining both invertase and trapped dust proteins allowed us to determine the K_{eq} independent of the slope of the calibration curve. This independence follows from Eq. (1), since the slopes of the calibration curves cancel out. The equilibrium constant seems to be invariant over most of the pH range, but decreases at a pH of less than 2.0 or greater than 9.0, as shown in Fig. 2. At a pH of about 5.7 (which is close to the isoelectric point of invertase), the equilibrium constant seems to fluctuate, possibly indicating an instability in either the calculation method or the experimental procedure. With both methods of determination, K_{eq} for invertase has a constant value of 93 ± 2 g L^{-1} for feed pH values varying between 2 and 9 (the feed pH is the pH of the "feed" solution before shaking the separatory funnel). Modifying this K_{eq} value for the 20% nonwater part in the semisolid broth present in the air-fluidized bed (*see* the assumption made in Eq. (1)) would increase the value of the equilibrium constant by 25% to 116 g broth L^{-1}. These K_{eq} values compare favorably to those estimated from

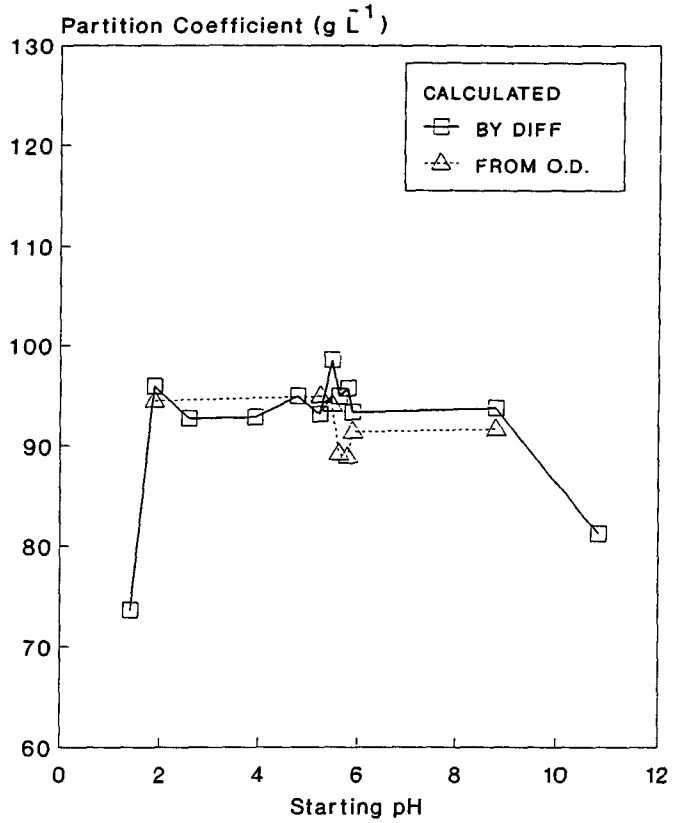

Fig. 2. Plot of the equilibrium constant for invertase against the initial pH of the feed solution.

fluidized bed data for *S. cerevisiae* produced extracellular protein of ca. 270 kDa (*1*); $K_{eq}=63$ g L^{-1} for a bed efficiency (relative to equilibrium) of 10% and $K_{eq}=88.3$ g L^{-1} for a 7% bed efficiency (*2*). It is interesting to note that when the partition coefficient is expressed in terms of mg protein/L liquid in the droplets per mg protein/L liquid in the residual bulk water phase, using the data from Table 1, the partition coefficient becomes 47.5. In other words, there is a 47.5-fold concentration of invertase in the vapor droplets (over the residual liquid solution) for the case in which the initial pH is 5.25. This equilibrium can also be expressed as a 32.6-fold concentration over the original feed concentration, with about one-third of the protein recovered in the droplets (a single equilibrium separation stage process).

DISCUSSION

This technique represents an interesting new approach to protein separation and purification. For example, such a technique could be used

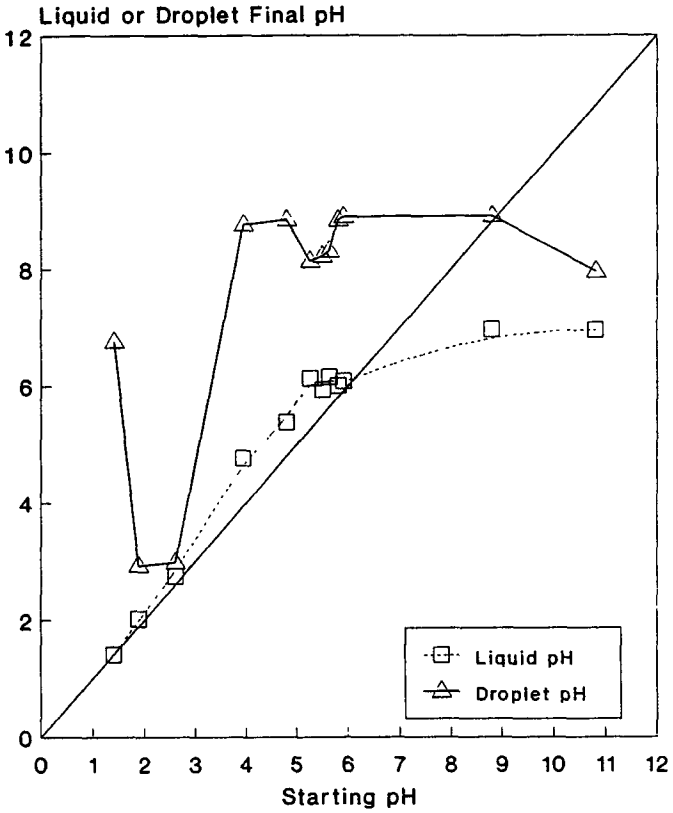

Fig. 3. Plot of the initial feed pH against the pHs of the residual liquid and the condensed droplets for invertase.

to purify surface active extracellular products from a fermentation broth (such as antibiotics, as well as proteins) by transferring the desired products into the condensed droplets. The major cost in such a concentration process is that of condensing the resulting microwater droplets. Other protein systems could also be considered. Further work needs to be done to help elucidate the mechanisms underlying the process. This would be particularly appropriate in dealing with the air-fluidized-bed system in which about 50% of the total protein recovered in the overhead collector was perhaps comprised of one polypeptide, a 33 kDa protein (*1*).

It is interesting to note that the carried-over-proteins are positively charged molecules (*1*), indicating that the protein charge affects the selectivity of the proteins carried out of the fluidized bed by the water droplets in the effluent air stream. In the experiments with the use of a separatory funnel, it was found that both the liquid and condensed droplet pHs tended to increase, except at high starting pH values. These pH changes are seen in Fig. 3 for invertase and in Fig. 4 for the control runs without invertase addition. The control runs (with a feed solution of relatively protein-free deionized water) have pH profiles similar to the invertase pH profiles. These observations may lead to a better understanding of the

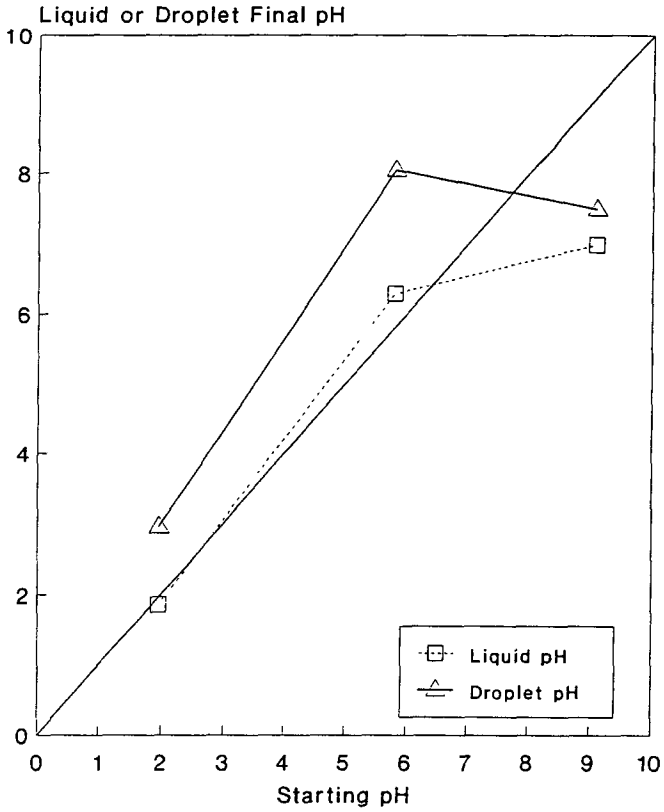

Fig. 4. Plot of the initial feed pH against the pHs of the residual liquid and the condensed droplets for plain deionized water without added invertase.

mechanism of protein separation. One possible explanation of this pH behavior may be that the inside glass wall of the funnel may be initially at a pH of about 7. This glass wall would then be relatively basic at low starting feed pH values. In that case, the positively charged end of a volatile water molecule could then attach itself to the wall, leaving the negatively charged end free. This negatively charged end could then remove H^+ ions from the feed solution, making the residual liquid and the condensed droplets more basic ($\Delta pH > 0$, where $\Delta pH = pH$ of residual liquid or droplet $- pH$ of feed). Since the droplets have a much larger holding capacity (for volatile molecules) in which to contact the glass wall compared to the liquid inside the glass funnel, the pH change could be expected to be more dramatic for the condensed droplets. Similarly, at high starting feed pH values, the glass wall could become "acidic" relative to the feed solution ($\Delta pH < 0$) and, following the same logic, the residual liquid and the condensed droplets could have a lower pH than that of the feed, as seen in Figs. 3 and 4 for high initial pH values. It would be interesting to repeat the experiments in a nonglass vessel to check if that substitution would change the results obtained from the fluidized bed and the glass separatory funnel.

It is surprising that the role of the protein charge does not appear to be associated with the isoelectric points of the separated proteins (*see* Fig. 2), since the isoelectric point plays a major role in the bubble fractionation (separation) of effluent proteins in a water solution (4). Perhaps, what is important is that once a protein reaches the interface between the bulk water phase and the water droplet phase, the protein's hydrophobicity determines how it is partitioned at the interface. If this were indeed the case, the so-called hydrocarbon tail (hydrophobic end) of the protein would stick out into the air, whereas the hydrophilic end would attach to the water surface of the microwater droplets. This surface adsorption is presumably superimposed on top of the protein absorption in the droplets themselves. Interestingly, the presence of the invertase on the microwater droplets reduces the amount of dust protein particles that can adhere to those droplets.

SUMMARY

The experiments carried out in the separatory funnel provide a simple technique for concentrating proteins up to 30 times. From a feed concentration of 0.1 g L^{-}, it is possible to obtain a condensed droplet solution with a concentration as high as 3 g L^{-1}. The directly determined equilibrium constant (from the separatory funnel experiments) matched well with that estimated from the air-fluidized bed data (using an equilibrium model corrected with an efficiency term).

ACKNOWLEDGMENT

The authors thank Alvin O. Converse of Dartmouth College for his insightful suggestions regarding the role of hydrophobicity in the mechanism of protein partitioning between the liquid water and the microwater droplet (in air) phases. Thomas E. Ward of Idaho National Engineering Laboratory kindly critiqued the Coomassie blue determination of proteins in water for the control run. His comments helped sharpen our interpretation of the reported data.

REFERENCES

1. Hong, K., Tanner, R. D., Malaney, G. W., and Danzo, B. J. (1989), *Bioprocess Eng.* **4,** 209–215.
2. Kokitkar, P. B., Hong, K., and Tanner, R. D. (1990), *Journal of Biotechnology,* **15,** 305–322.
3. Reed, G. and Peppler, H. J. (1973), *Yeast Technology,* The AVI Publishing Company, Inc., Westport, CT, pp. 358–361.
4. DeSouza, A. H. G., Tanner, R. D., and Effler, W. T. Jr. (1991), *Appl. Biochem. Biotechnol.* **28/29.**