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# The Effect of pH and Gas Composition on the Bubble Fractionation of Proteins

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## ABSTRACT

Studies were conducted to establish the effect of the variation of environmental factors on the separation occurring in protein systems, resulting from bubble fractionation in a bioreactor. The measure of separation was selected to be the separation ratio. This is defined to be the ratio of either the top or the middle position concentration in the vessel to the bottom concentration of the vessel. Invertase and α-amylase were the two "model" enzymes considered. It was observed that, under certain conditions, i.e., a combination of the nature of the sparging gas and the medium pH, varying degrees of protein separation were achieved. The pH of the system dramatically influenced the separation. It was found that the best separation occurred at a certain pH, assumed to be at or close to the p*I* of the protein in question. Furthermore, it was observed that systems sparged with $CO_2$ exhibited greater separation than systems sparged with air. In fact, in the case of invertase, almost threefold separation was observed at the top port when the solution was sparged with $CO_2$.

**Index Entries:** Bubble fractionation; proteins; protein separation; α-amylase; invertase.

## INTRODUCTION

Bubble fractionation is a nonfoaming adsorptive bubble separation technique. In this process, surface active materials, like proteins, are transferred to the upper portion of a column of liquid by adsorption on rising

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bubbles followed by release at the top of the column as the bubbles burst. If the bubbles arriving at the top of the column are insufficiently stable to form a foam, or if the foam is not removed and allowed to collapse back into the liquid, the surface active species are swept from the bottom to the top of the bulk solution. Species also move back down by axial dispersion, setting up a protein concentration gradient. In effect, the upper portion of the column is enriched, while the lower portion is stripped.

As long as the proteins are not inactivated, this process can be used as an initial step to isolate proteins. Isolation may be accomplished by adjusting the system environmental parameters, so that the protein of interest is carried over, and is removed from a point in the bioreactor, where its concentration is the highest (normally at the top).

The usual method of forming bubbles for small-scale experiments is to force gas into the liquid through sparger nozzles, or through perforated or sintered plate distributors under rotating stirring bars at the bottom of the vessel. The gas volume flow rate is one of the most significant operating parameters, and it determines principally the number of bubbles passing through the liquid in a given time. The purpose of the rotating impeller is to "chop off" the extruded bubble into smaller bubbles. Maintaining a small bubble diameter is important, since this increases the interfacial area presented by the gas to the solution. A small bubble diameter also reduces the rise velocity of the bubbles through the solution, thereby increasing the contact time of bubbles and solution.

The four process variables most significant in determining the degree of separation for most surface active materials have been found to be:

1. The concentration of surface-active material in the solution
2. The superficial gas velocity
3. The height of the column and
4. The gas composition.

In the separation of proteins, environmental factors that affect protein structure and thus its surface activity are also important. The most significant of these factors is the pH. Proteins are more likely to be separated near their isoelectric point, where they are least soluble in the solution and more likely to adhere to a rising gas bubble. Other factors that may affect protein separation are ionic strength and the presence of organic solvents. The solution temperature has not been found to have any significant effect (*1*).

Foam fractionation has been used in the past to separate various enzymes, e.g., separation of albumin from potato and beet juices, and the separation of pepsin from a mixture of pepsin and rennin (*2,3*). Bubble fractionation itself has been used to concentrate solutions of weakly surface active dyes. It has been observed that low gas bubbling rates gave better separation and that changes in the solution pH resulted in changes in the efficacy of separation.

One of the main objections to foam fractionation of proteins is the possibility of denaturation. However, it has been shown that many enzymes resist denaturation when subjected to foaming, e.g., phosphatase, catalase, $\beta$-amylase, alcohol dehydrogenase (*4*). What these enzymes have in common is that they are all extracellular enzymes, and are inherently more rugged and stable than enzymes retained within the cell wall. The possibility of denaturation of these extracellular proteins can be diminished by:

1. Using inert gases
2. Using low gas flow-rates
3. Avoiding mechanical agitation and
4. Using a once-through process.

Bubble fractionation is an inexpensive method for protein recovery. As a preconcentration step in purification, it obviates the necessity for processing large volumes of medium in order to recover the small amounts of protein present. It is of most relevance as a rapid technique for initial separation of proteins from aqueous solutions.

The objective of this research was to establish the effect of the variation of environmental factors, namely, the nature of the sparging gas and variation in the pH of the medium, on the on-line separation occurring in enzyme-producing systems in the bioreactor. The separation ratio, defined to be the quotient of the top protein concentration to the bottom protein concentration, was chosen to be a measure of the fractionation occurring in the bioreactor. Invertase and $\alpha$-amylase were the two "model" enzymes considered in this microbe-free test system.

## MATERIALS AND METHODS

Four sets of experiments were conducted in a column bioreactor of height to diameter ratio of 5.65, with individual enzyme systems. An additional experiment with a mixed (binary) enzyme system was also conducted.

### Bioreactor

A schematic diagram of the bioreactor setup is depicted in Fig. 1 (*5*). The bioreactor consisted of a 35-cm (height)×6.2-cm (diameter) Nalgene polycarbonate 1-L graduated cylinder. The upper rim was modified to enable the column to be sealed with a rubber stopper. Sample ports fitted with rubber septa were inserted into the side of the column at the top position (1 cm below the 1000 mL mark), the middle position (500 mL mark), and the bottom position (1 cm above the bottom) of the column. The bottom of the reactor was modified to hold a 3.0-cm diameter flat disc

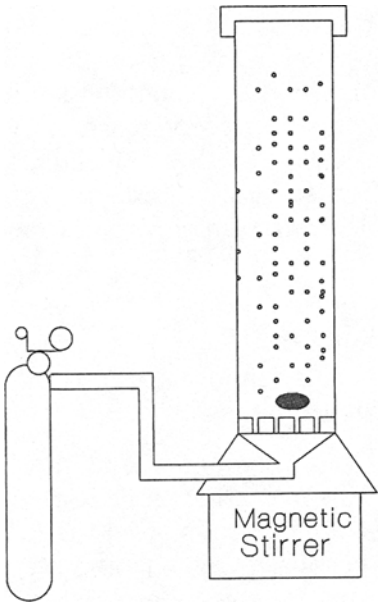


Fig. 1. Schematic of bioreactor setup.

sparger, made of fritted glass, fitted flush with and sealed to the bottom of the column. The cylinder was filled up to the 1000-mL mark with enzyme solution and rested on a magnetic stirring plate. A 2.54-cm stirring bar (ratio of bar length to bioreactor diameter = 0.4) rotated at 400 rpm at the bottom of the bioreactor, centered above the flat disc sparger.

Experiments were conducted at ambient temperature (23°C ± 1°C). The sparging gas, either air or $CO_2$, was introduced into the medium at ca. 0.14 vvm (volume of sparging gas per volume of aqueous medium per minute) through the bottom-fitted sparger in the cylinder. The gas flow was regulated by a valve attached to a flowmeter. The gas supply was filtered by an in-line Speedaire filter (Model No. 4Z034, Dayton Electric Manufacturing Co., Chicago, IL 60648) to remove any contaminants.

## Reagents

Coomassie Blue Protein Assay Dye Reagent Concentrate (No. 35370) was purchased from Bio-Rad Laboratories (Richmond, CA). The enzyme bacterial α-amylase (from *Bacillus subtilis;* also called *B. amyloliquefaciens*), in the powdered form, was purchased from Sigma Chemical Company of St. Louis, MO (1988 Catalog No. A-1278, Lot No. 0113F-0516). The yeast enzyme invertase (from *Saccharomyces cerevisiae*) was also purchased from Sigma Chemical Company (1988 Catalog No. I-9253, Lot No. 101F-0147). Certain values for the isoelectric points of these (or similar) enzymes are given in the Results and Discussion section.

### Sampling Procedure

Samples were taken at the "0 min" (actually ca. 30 s prior to commencing the gas flow) and 20-min time points. Using a 3-mL disposable syringe with an 18-gage needle, 2.4 mL samples were withdrawn from the top, middle, and bottom of the bioreactor by inserting the needle through the respective septum until it just entered the medium. The sample was drawn slowly (~60 s) to minimize protein shear and the disruption of the gas flow pattern. The syringe contents were dispensed into 10-mm × 65-mm testtubes for determination of total protein concentration by the Coomassie Blue (Bradford) method (*6*).

### Assay of Protein Concentration

The Coomassie Blue (Bradford) method (*6*) was used to determine the protein concentrations in the samples withdrawn from the bioreactor. This method was chosen because of its simplicity and the speed at which it could be used to determine protein concentration. The optical densities were read in a Bausch and Lomb Spectronic 20 Spectrophotometer at 595-nm wavelength, at 2 min following complete mixing of sample and reagent. The blank used was either Coomassie Blue + deionized water or just deionized water alone. Both are generally equivalent, except that since Coomassie Blue "reacts" with the water proteins the Coomassie Blue blank has a slow drift. Also, for many of the readings, the difference between the Coomassie Blue sample and the Coomassie blank readings is so close that the errors tend to get magnified. Such errors tend to get damped out with a water blank. α-amylase (from *Bacillus subtilis*) was chosen as the calibration standard.

## EXPERIMENTAL CONDITIONS

Five sets of experiments were conducted. In all cases, enzyme solutions were stirred and sparged with either air or $CO_2$ in the bioreactor described in the previous section. Each of the first four experiments was conducted over a pH range varying from 4–10 (in pH increments of one) using standardized 0.1*N* solutions of HCl and NaOH, respectively. The aim of the study was to investigate the effect of pH and the nature of the sparging gas on the separation of enzymes in solution. These experiments are described in Table 1.

In an additional experiment, a binary mixture containing 100 mg/L of α-amylase and 100 mg/L of partially purified invertase was sparged with $CO_2$. The experiment was repeated twice, once each at each of the approximate isoelectric points of both enzymes, as determined in Experiments 3

Table 1
List of Experiments

| Experiment Number | Protein Soln. (100 mg/l conc) | Sparging gas | pH range |
|---|---|---|---|
| 1 | α-amylase | Air | 4-10 |
| 2 | α-amylase | $CO_2$ | 4-10 |
| 3 | Invertase | Air | 4-10 |
| 4 | Invertase | $CO_2$ | 4-10 |
| 5 | 100 mg/l α-amylase and 100 mg/l invertase | $CO_2$ | 5 and 8 (each replicated) |

and 4. The aim of the experiment was to see if a separation between an enzyme mixture could be effected by varying environmental factors.

The system pH was left uncontrolled in all of the above experiments. In each of these experiments, the pH measured at the end of the run was practically the same as the starting pH ($\pm 0.1$), hence no buffer was needed. Furthermore, the addition of buffering salts would tend to charge the system, perhaps adding a "salting out" effect to the proteins.

## RESULTS AND DISCUSSION

It was observed that, under certain conditions, i.e., a combination of the nature of the sparging gas and the medium pH, varying degrees of protein separation were achieved. The measure of separation was selected to be the separation ratio. This is defined to be the ratio of either the top or middle position concentration in the vessel to the bottom concentration of the vessel. Hence, the separation ratio is a function of the vertical height.

If the system pH is near the isoelectric point of one of the dissolved proteins, that protein will be less soluble in water and will tend to come out of solution (in our case by adhering to the rising bubbles) to a greater extent than one whose isoelectric point differs from the pH of the system (*7,8*). Since different proteins have different isoelectric points, we expect various enzyme systems to exhibit different degrees of separation at a given pH.

Figure 2 represents the Separation Ratio vs pH profiles for an α-amylase solution sparged with air. Maximal separation is seen to occur at pH = 8. The SR value (top) value is about double the SR (middle) value at pH = 8. The SR value at pH = 6 ($< 1$) may indicate that insoluble α-amylase and/or its impurities are settling out of solution at the top and accumulating at the bottom and the middle of the column at this pH.

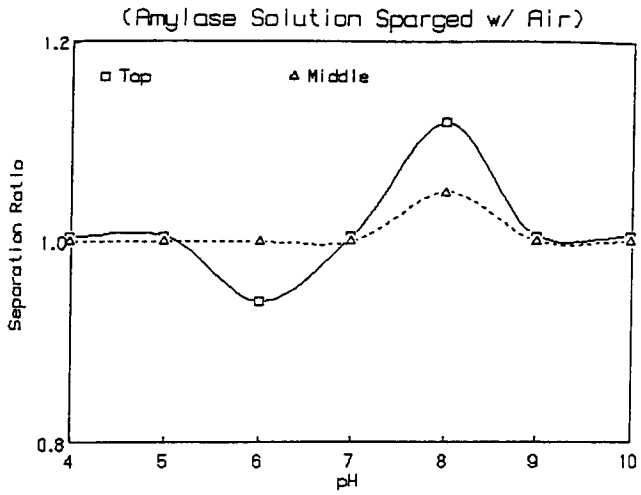


Fig. 2. Variation of separation ratio with pH for α-amylase solution sparged with air.

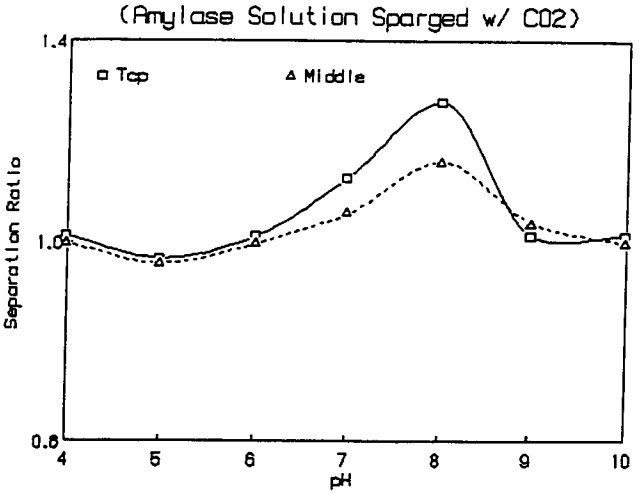


Fig. 3. Variation of separation ratio with pH for α-amylase solution sparged with $CO_2$.

Figure 3 represents the Separation Ratio vs pH profiles for an α-amylase solution sparged with $CO_2$. Again, maximal separation is seen to occur at both top and middle ports at pH = 8.

Figure 4 represents the Separation Ratio vs pH profiles for an invertase solution sparged with air. Maximal separation is seen to occur at pH = 5. The SR (middle) value is only about 70% that of the SR (top) value at pH = 5.

Figure 5 represents the Separation Ratio vs pH profiles for an invertase solution sparged with $CO_2$. Again, maximal separation is seen to

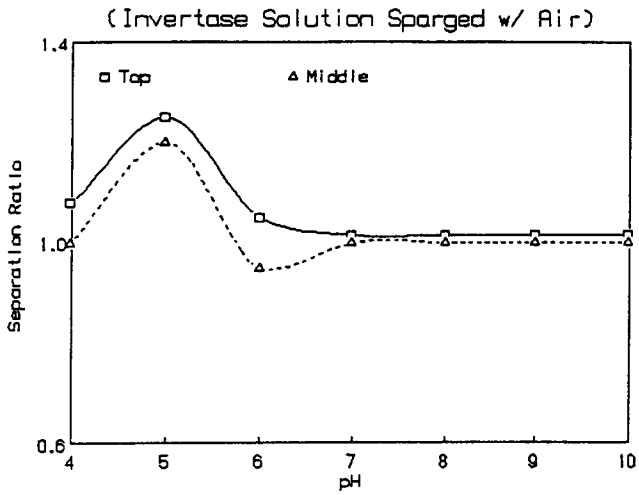


Fig. 4. Variation of separation ratio with pH for invertase solution sparged with air.

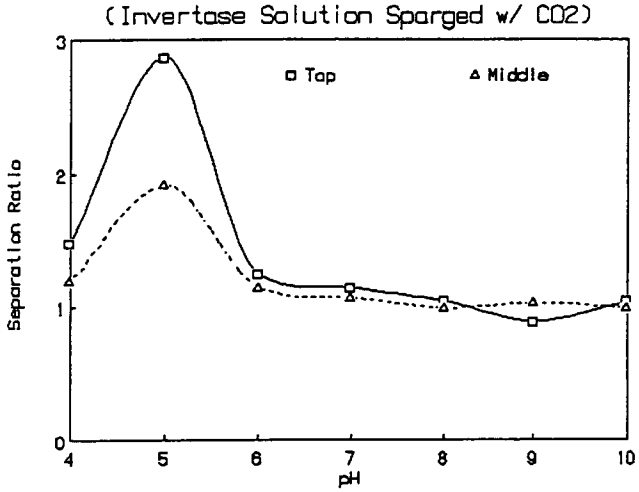


Fig. 5. Variation of separation ratio with pH for invertase solution sparged with $CO_2$.

occur in both cases at pH = 5. However, the SR (top) value is nearly double the SR (middle) value for this case, indicating that lengthening the column can lead to significantly enhanced separation of invertase by $CO_2$.

Consistent with our belief that different enzyme systems would exhibit different degrees of separation at a given pH, $\alpha$-amylase exhibited its greatest separation at pH = 8, whereas invertase exhibited its highest separation at pH = 5. Thus, the pH of our system dramatically influenced the separation. The pH at which maximal separation occurred is assumed to be close to the isoelectric point (p*I*) of the protein under consideration,

since it caused the greatest separation and presumably the most insolubility of the enzyme in the aqueous solution. Surprisingly, numerical values for the p*I*s of these two well-studied enzymes were not available in the literature (e.g., reference [*9*]). However, a historical reference (*10*) does report a p*I*=5 for yeast invertase. Further, indirect evidence for a similar mol wt of 50,000 fungal (but not bacterial) $\alpha$-amylase of ca. 8 for the p*I* is inferred by its industrial recovery at a pH of around 8 (*11*).

It was observed that systems sparged with $CO_2$ exhibited higher separation than systems sparged with air. In fact, in the case of invertase, almost threefold greater separation was observed at the top port when the solution was sparged with $CO_2$. A similar experiment conducted by Effler et al. (*5*), in which a solution of partially purified invertase dissolved in a fermentation medium was sparged with $CO_2$, yielded a fivefold greater separation at the top port. The presence of salts in the fermentation medium renders the ionic strength of the fermentation medium different from that of an aqueous solution. The difference in ionic strengths is possibly responsible for the enhanced separation achieved when the invertase solution dissolved in fermentation medium is sparged with $CO_2$.

Possible reasons why higher degrees of separation were achieved when $CO_2$ was the sparging gas as opposed to air are: (1) Since the protein has no net effective charge and $CO_2$ is less polar than water, the protein would be attracted to the $CO_2$ bubble and repelled by the highly polar water. (2) The $CO_2$ sparged environment might make the protein molecules more hydrophobic, since the $CO_2$ in water in the $HCO_3^-$ form may pull the water away from the protein, leaving less water for the protein to solubilize. Therefore, more protein could come out of solution and attach to the rising $CO_2$ bubbles. This, however, is expected to be a relatively small effect.

Basically, the original, thermodynamically stable one-phase system has been rendered appreciably unstable with respect to precipitation when sparged with $CO_2$, compared to when the system was sparged with air. However, it is to be noted that the system pH did not change when the solution was sparged with $CO_2$.

What is interesting, however, is that, in the case of the $\alpha$-amylase separation, the separation ratio at the top position was not significantly greater than that at the middle position for either sparging gas. It reached its greatest separation by the time it reached H/2, where H was the height of the liquid in the separating column.

The above limitation also appears to hold when an invertase solution is sparged with air. In contrast, however, when an invertase solution is sparged with $CO_2$, the separation achieved at the top port is significantly higher than that obtained at the middle port. For $CO_2$, then, increasing the height of the column (stages) does enhance the separation.

Interestingly, with reference to Figs. 2, 4, and 5, it can be observed that the separation ratio is less than one at either pH=6 or pH=9. This

phenomenon is ascribed to the settling out of impurities ("junk proteins") from the aqueous partially purified α-amylase and invertase solutions. These impurities, in all probability, are not surface active and hence settle down to the bottom, since they are not adsorbed on the rising bubbles.

Further, since the protein molecules have no net charge at the p*I* and some local charge at different pHs, electrostatic repulsive forces between protein molecules are minimized at the p*I*. This, coupled with the fact that the protein solubility exhibits a minimum at the p*I*, suggests a fractional precipitation procedure for separating proteins with different isoelectric points. At a given pH, the proteins with the nearest p*I* will tend to precipitate. By varying the system pH, fractions containing different proteins could then be separated. However, care must be taken while using this procedure, since imposing wide pH variations on a protein solution runs the risk of denaturing the "fragile" protein components (*9*).

In order to test the hypothesis that a variation in the system pH, in a sparged system, could be used as a first step in isolating a particular protein from a mixture, two experiments were conducted. These consisted of sparging a solution mixture consisting of 100 mg/L each of α-amylase and invertase with $CO_2$, at pH=5 and pH=8, respectively. These particular combinations of sparging gas and pH were chosen to maximize the separation of the individual proteins used, as indicated by the prior single protein experiments.

As can be seen from Table 2 (and Fig. 6, for the top separation ratio alone), the separation ratios at both the top and middle positions in the bioreactor, when the mixture was sparged with $CO_2$ at pH=5, were quite close to the separation ratios obtained when invertase was the only component in the system under these conditions. This is consistent with our conjecture that the protein carried over for this binary case will consist mostly of invertase. At the apparent p*I* of 5, the invertase apparently has minimal interaction with the other protein α-amylase whose apparent p*I* is 8. The slight deviation (within 10%) could be ascribed to an interaction between the components in the mixture or to experimental error. A similar conclusion could be drawn from the data for the case when the above experiment was repeated at pH=8. As was expected, the separation ratios were within 10% when α-amylase was the only component in the system.

## CONCLUSIONS

Thus, as seen above, combination of the isoelectric pH and a sparging gas that gives the maximum separation could be used for "isoelectric focusing" of a particular component within an enzyme mixture. This may enable us to achieve a rapid, cost-effective initial separation (a preconcentration step) of a protein from a mixture.

Table 2
Results of the "Isoelectric Focusing" Experiments

A. pH = 5, Sparging Gas = CO2

| Column Position | Mixture Separation Ratio | Invertase Separation Ratio |
|---|---|---|
| Top | 2.7 | 2.88 |
| Middle | 1.76 | 1.92 |

B. pH = 8, Sparging Gas = CO2

| Column Position | Mixture Separation Ratio | Amylase Separation Ratio |
|---|---|---|
| Top | 1.2 | 1.28 |
| Middle | 1.1 | 1.16 |

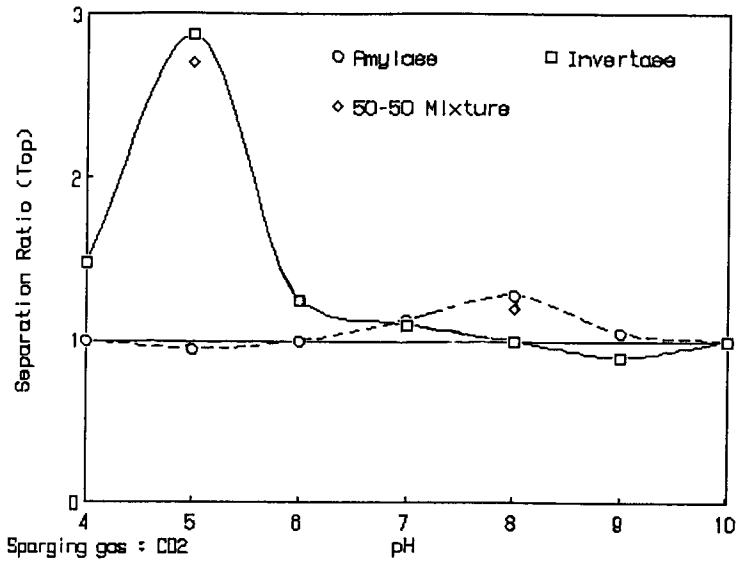


Fig. 6. Summary graph for the "isoelectric focusing" experiments.

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