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The Effect of Pectinase on the Bubble Fractionation of Invertase from α-Amylase

VEARA LOHA, ROBERT D. TANNER*, AND ALES PROKOP

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

ABSTRACT

Fermentation broth normally contains many extracellular enzymes of industrial interest. To separate such enzymes on-line could be useful in reducing the cost of recovery as well as in keeping their yield at a maximum level by minimizing enzyme degradation from broth proteases (either the desired enzymes or the proteases could be removed selectively or both removed together and then separated). Several large-scale separation methods are candidates for such on-line recovery such as ultrafiltration, precipitation, and two-phase partitioning. Another promising technique for on-line recovery is adsorptive bubble fractionation, the subject of this study. Bubble fractionation, like ultrafiltration, does not require contaminating additives and can complement ultrafiltration by preconcentrating the enzymes using the gases normally present in a fermentation process. A mixture of enzymes in an aqueous bubble solution can, in principle, be separated by adjusting the pH of that solution to the isoelectric point (pI) of each enzyme as long as the enzymes have different pIs. The model system investigated here is comprised of three enzyme separations and the problem is posed as the effect of pectinase (a charged enzyme) on the bubble fractionation of invertase (a relatively hydrophilic enzyme) from α-amylase (a relatively hydrophobic enzyme).

The primary environmental variable studied, therefore, is the pH in the batch bubble fractionation column. Air was used as the carrier gas. This prototype mixture exemplifies an aerobic fungal fermentation process for producing enzymes. The enzyme concentration here is measured as total protein concentration by the Coomassie Blue (Bradford) solution method *(1)*, both as a function of time and column position for each batch run. Since, from a previous study *(2)*, it was found that inver-

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

tase and α-amylase in a two-enzyme system can be partially separated in favor of one vs the other at two different pHs (pH 5.0 and 9.0) with significant separation ratios, emphasis is placed on the effect of pectinase at these pHs. In this study, the addition of pectinase reduced the total separation ratio of the α-amylase-invertase mixture at both pHs.

Index Entries: Pectinase; invertase; α-amylase; enzymes; bubble fractionation; protein separation.

INTRODUCTION

Robert Lemlich *(3)* coined the expression "adsorptive bubble fractionation" to describe the separation process by which surface active solutes are separated from dilute solution by bubbling an inert gas through a column containing the solute–solution mixture. Generally, surface active solutes, such as enzymes and other proteins, strongly adsorb at air–liquid interfaces. A typical protein structure is comprised of hydrophobic and hydrophilic parts that contribute to high surface forces. In bubble fractionation, gas bubbles are generated by forcing the gas through a sparger, which is typically a nozzle, a porous ceramic plate, or a sintered metal distributor. As bubbles rise through the column, proteins move from the dilute bulk solution to those bubbles, attaching to an adsorptive layer at the gas bubble–liquid interface. In that bubbling process, the bubbles burst at the liquid surface at the top of the column, releasing the proteins. The surface protein concentration thus increases as the bubbles rise in the column, and this increase continues until the surface concentration reaches a maximum. A steady-state protein gradient along the axis of the column is reached after a given time, typically 15–20 min. At steady-state, the amount of released proteins transferred downward by bulk diffusion from the protein rich surface is exactly countered by the proteins rising with the bubbles. Thus, if the protein is actively adsorbed by the bubbles at a particular processing condition, the highest concentration of that recovered protein is typically reached at the liquid surface of the column. When proteins are removed by surface skimming, a continuously changing protein gradient profile will describe the shift in concentration below the original steady-state profile.

The notion of bubble fractionation occurring within a fermentor came about as a result of the observation by Park and coworkers *(4)*, in which protein stratification was observed in the broth of a Baker's yeast fermentation process even though the broth was mixed vigorously. Effler et al. *(5)* replicated this phenomenon in a graduated cylinder, and Potter et al. *(6)* showed that this process could be modeled using the notion of bubble fractionation to affect protein stratification. The fermentation experiments *(5)* were performed at 32°C and pH 5.0 with and without sparging gases. These experiments, carried out within a graduated cylinder, were

sampled at three positions along the axis and at 4, 7, and 10 h. Air and carbon dioxide were alternately used as the sparging gas. It was shown that the separation occurred for both the sparged and nonsparged gas systems. The highest axial protein concentration gradients along the column occurred when carbon dioxide, at levels above those naturally present in the fermentation processes, was used as the sparging gas.

To elucidate the protein separation phenomenon, DeSouza and coworkers (*2*) studied the effect of both pH and gas composition on the bubble fractionation of two enzymes in a nonfermentation system using the same graduated cylinder. Invertase (a highly glycosylated and relatively hydrophilic enzyme) and α-amylase (a relatively hydrophobic enzyme) were used individually and in combination. Air and carbon dioxide were used as the sparging gases. The experiments were conducted batchwise at ambient temperature. As expected, the highest separation ratios (defined as the ratio of the concentration of proteins at the top to the bulk protein concentration at the bottom of the column) reached locally maximum levels when the solution pH was positioned near the respective protein isoelectric point (pI). In the DeSouza et al. study (*2*), the apparent pIs were at pH 5.0 for invertase and pH 8.0 for α-amylase. Sparged carbon dioxide led to larger separation ratios than sparged air at these two pHs.

Mixtures of enzymes are normally found in living systems such as fermentation broths, plant, and animal cells. Fruits that contain the combination of α-amylase, invertase and pectinase, the mixture of enzymes used in this study, include apples, pears, and papayas. Such enzymes in fruits are usually found in dilute concentration and within a mixture of hundreds of enzymes. To even partially separate such enzymes from such fruits or a fermentation broth would be useful in reducing the separation cost of one or more of these enzymes. Generally, separation costs reach 80 to 90% of the total manufacturing cost of many enzymes. In a fermentation process, on-line separation of either proteases or other extracellular enzymes can contribute towards maximum yields of desired enzymes by lowering the contact time of enzyme degradation from broth proteases. One of the imposed constraints of this study is to not add any contaminating additives, such as surfactants or salts, because these additives usually must be removed in a subsequent time-consuming purification step. Enzymes such as α-amylase and invertase may be sufficiently surface-active to be bubble fractionated without the use of such additives. Bubble fractionation processes also have the advantage of requiring little additional energy above that normally needed by the fermentation process itself, when the fractionation is performed on-line. Bubble fractionation itself does not typically lead to highly purified protein products, but its simplicity in achieving a crude separation indicates that it may be a promising technique for the initial isolation and purification step of enzymes.

In this preliminary study of a three enzyme mixture, air alone was used as the carrier gas. Pectinase (a positively charged enzyme, since its

pectin substrate is negatively charged), invertase, α-amylase, and various combinations of these three different type of proteins were selected for these experiments. The objective was to determine the effect of pectinase on the bubble fractionation of invertase from α-amylase by varying the pH and the concentration of pectinase in the bubble fractionation column.

MATERIALS AND METHODS

A schematic diagram of the bubble fractionation column previously developed *(2)* is depicted in Fig. 1. The bubble column is a Nalgene polycarbonate 1 L graduated cylinder of 6.2 cm diameter and 35 cm height. The column was modified to have three sample ports perpendicular to the column as shown: top, middle, and bottom positions. The bottom of the column contained a fritted glass sparger that was tightly fitted and glued to the inner diameter of the column. Compressed air, introduced below the column sparger, was prehumidified by bubbling it through water in order to minimize both column water loss and protein contamination from the influent air.

The bacterial (*B. subtilis*) crude α-amylase (Lot No. 113F-0516) and Baker's yeast crude invertase (Lot No. 101F-0147) used in the experiments were purchased in powder form from Sigma Chemical Company (St. Louis, MO). Solid powder pectinase produced by L.D. Carlson Company (Kent, OH) packaged as pectic enzyme, was purchased from a local winery store.

The Coomassie Blue (Bradford) method *(1)* was used to determine the total protein content in the solution samples. The Coomassie Blue binding reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 (purchased from Bio-Rad Laboratories, Richmond, CA) in 50 mL of 95% ethanol (produced by Midwest Grain Products, Atchison, KS) and 100 mL of 85% phosphoric acid (purchased from Fisher Scientific, Fairlawn, NJ), then adding deionized water bringing the solution up to 1 L. The solution was filtered to remove any undissolved dye. Excess reagent was stored in the refrigerator at 4°C and used within 2 wk.

In all of these experiments, 2 mL of sample and 3 mL of Coomassie Blue binding agent were used for the total protein content analysis. Following complete mixing of the sample and the reagent, the absorbance of that mixture was read at 5 min (at a wavelength of 595 nm in a Bausch and Lomb Spectronic 20 spectophotometrer).

Calibration curves for α-amylase, invertase, and pectinase were developed by taking absorbance readings over a range of enzyme concentrations by using the Bradford procedure *(1)*. For all three enzymes, the linear relationship, $A_i = K_i C_i$, i = 1, 2, 3, described the resulting calibration curves in the selected concentration ranges. At pH 6.0 these were: $K_1 = K_{amylase} = 1.95 \times 10^{-3}$ L/mg; $K_2 = K_{invertase} = 5.36 \times 10^{-4}$ L/mg; and $K_3 = K_{pectinase} = 6.99 \times 10^{-5}$ L/mg. Different calibration curves are

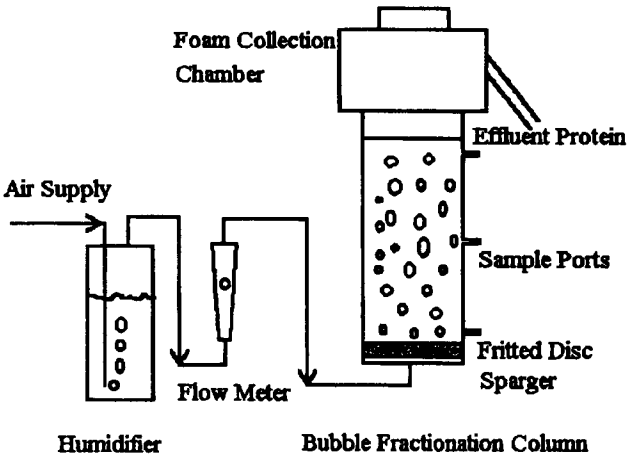

Fig. 1. Schematic diagram of the semibatch bubble fractionation process.

needed in general for each protein since each contains different amounts of arginine, the amino acid that is complexed with Coomassie Blue.

The impure invertase powder used dissolved only partially in water at room temperature. Invertase solution, therefore, was prepared by first dissolving invertase powder in deionized water, followed by filtering through Whatman No. 4 filter paper to remove the undissolved solids. In general, the concentration of this filtrate was then determined from its absorbance reading using $A_i = K_i C_i$. The K_i value in the $A_i = K_i C_i$ calibration curve was determined from a single concentration/absorbance (C_i, A_i) point where the absolute concentration was determined by taking the solution to dryness and then weighing it.

Both pectinase and α-amylase were weighed and mixed with deionized water before being used in the bubble fractionation experiments. Fresh mixtures used for the experiments were prepared in 1250 mL of deionized water without filtration and the pH was adjusted by using 0.1 N NaOH or HCl. No buffer was added. The pH of these three enzyme solutions was adjusted between 4.0 and 10.0 in the experiments. The air flow rate was held constant at 47 cm^3/min for all of the runs (ca. 0.05 vvm, where vvm = vol of air per vol of liquid solution, per minute). Bubble fractionation experiments were performed for one enzyme, two enzyme and then, the complete three enzyme system. The protein concentration conditions used in these experiments are listed in Table 1. The protein concentrations were selected so that the different enzyme components would each contribute about the same toward the solution absorbance reading. These concentrations differ from the previous experiment *(2)* where a 50–50% (100 mg/L each) mixture was used for the α-amylase-invertase system and may account for why the results for this mixture were somewhat different.

Samples for all of the enzyme cases listed in Table 1 were taken every 15 min up to 1 h at all three sampling ports. Since local maximum separa-

Table 1
Bubble Fractionation Conditions for the Experiments using α-Amylase, Invertase, and Pectinase.

System	Protein solution	Protein concentration (mg/L)	pH range
Single enzyme	α-amylase	200	4-10
	invertase	600	4-10
	pectinase	2,000	4-10
Two enzymes	α-amylase and invertase	100 (α-amylase) and 300 (invertase)	4-10
	α-amylase and pectinase	100 (α-amylase) and 1,000 (pectinase)	4-10
	invertase and pectinase	300 (invertase) and 1,000 (pectinase)	4-10
Three enzymes	α-amylase, invertase and pectinase	100 (α-amylase), 300 (invertase) and 1,000 (pectinase)	4-10
	α-amylase, invertase and pectinase	100 (α-amylase), 300 (invertase) and 0-1,250 (pectinase)	5 and 9

tion ratios were obtained for α-amylase and invertase at pH 5.0 and 9.0, studies of the effect of pectinase on these other two enzymes were focused at these two pHs. The concentrations of pectinase were varied up to 2000 mg/L. The concentrations of α-amylase and invertase were kept constant at 100 mg/L and 300 mg/L, respectively, in the two and three enzyme experiments and at 200 and 600 mg/L in the single enzyme cases.

Solution samples (5 mL) for all cases were taken by syringe. The samples that were assigned time 0 were actually taken 2 min after pouring the solution into the bubble fractionation column. Sampling was taken every 15 min up to 60 min, from rubber septum sampling ports, located at the

top, middle, and bottom of the column. For each pH profile case for a given enzyme system, 15 samples were analyzed for total protein content by the Coomassie Blue method *(1)*.

RESULTS AND DISCUSSION

Air bubbles can cause proteins to rise in a dilute solution to the upper surface by adsorbing on the bubble-solution interface. Proteins concentrating on the bubbles lead to a protein concentration gradient from the top to the bottom of column as the bubbles rise up through the column. After the bubbles burst at the liquid-air surface of the bubble fractionation column, the released proteins build up to their highest concentration at that position.

Proteins tend to be hydrophobic and at their isoelectric point (where there is no charge in a water solution) they move toward the bubble—solution interface, thereby leaving the water solution by attaching to the gas bubbles. Once they latch onto a gas bubble, they are carried up to the top of the bulk solution, and thus lower the residual protein concentration of the remaining bulk solution. Since the protein-carrying bubbles collapse at the top surface, the protein concentration at that position reaches its highest level. The proteins, therefore, transfer downward and reflux by bulk diffusion. At steady state, the flux of proteins upward is equal to the flux of proteins downward. For the case of a mixed proteins, each of the enzyme molecules competes with each other to attach to adsorption sites on the bubbles. The more successful the protein is in competing for these interfacial sites, the better its separation within the bubble column. The separation ratio, defined as the ratio of a particular protein concentration at the top of the column to the bottom position, is used here to quantify the separation of a particular protein. In this study, the individual protein concentrations were not determined, in order to simplify the procedure. Here, the lumped protein optical absorbance ratio is used as a rough indicator of the partitioning. A mixed separation ratio, expressed as the ratio of optical absorbance at the top of column to the optical absorbance at the bottom of column, is used to infer the extent of the overall separation for the mixture of enzymes. Since certain proteins tend to favor particular pHs (at their isoelectric points) to achieve maximum separation as determined by their individual separation ratios, determining the optical absorbance ratio as a function of pH can provide clues for determining the best conditions to achieve maximum separations of components within a mixture.

Based on the experience gained in this study, steady state generally is achieved by 15–20 min. To ensure the samples were taken at steady-state, measurements were all taken at 60 min in the batch bubble fractionation process. Figure 2 shows the individual enzyme separation ratio vs pH trajectory for α-amylase, invertase, and pectinase solutions bubbled with air. α-amylase reaches a local maximum separation ratio at pH 8.0–9.0 and a bounded maximum at pH 4.0. Previous work *(2)* reported that a local max-

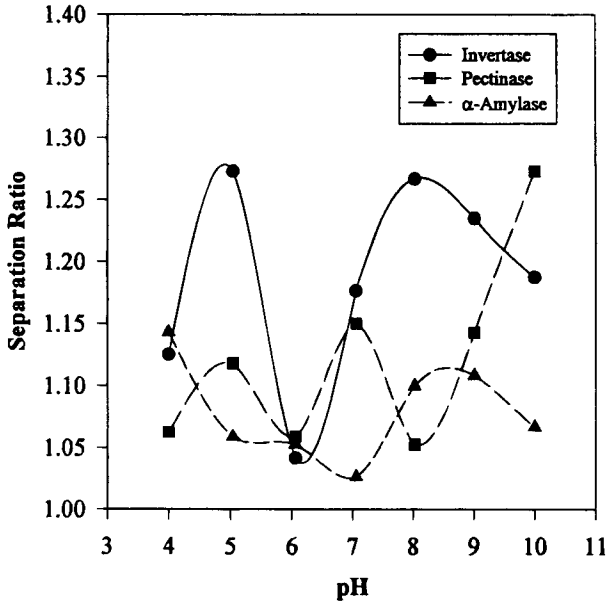

Fig. 2. Bubble fractionation of each of the three enzymes.

imum separation ratio of α-amylase, using either air or carbon dioxide as a carrier gas, was reached at pH 8.0. Local maximum separation ratios for invertase were obtained in these studies at pH 5.0 (close to the pH value used for invertase activity measurements by Vitolo and Borzani *[7]*) and pH 8.0. In previous experiments *(2)*, with both air and carbon dioxide as the carrier gas, only one local maximum was reported at pH 5.0. The addition of a second peak may perhaps indicate that, under the conditions of this study, subunits of invertase (with pIs of 8) were released to the solution. The difference in air flow rate between previous (140 cm^3/min) experiments and these (47 cm^3/min) might cause the different degree of denaturation of proteins. Typically, the major cause of damage to proteins is from bursting bubbles *(8)*. The longer sampling time for these experiments (60 min) may have led to more denaturation of enzymes than in previous experiments (20 min), but this longer time is countered by shorter residence times of oxygen contacting the recovered enzyme. The structure of denatured protein generally differs from the original protein. It is possible then that the denatured protein has a new isoelectric point from the undenatured protein that leads to a previously unobserved second peak. For invertase in these experiments, the second local maximum separation ratio appeared at pH 8.0. It is also noted that in these experiments the separation ratio trajectory decreases for α-amylase in the low pH range.

Pectinase seems to have the local maximum separation ratio at the pH bound of 10.0. Normally, pectinase is a mixture of three enzymes *(9)*: pectinesterase, polygalacturonase, and pectate lyase. Multiple peaks of the

pectinase separation ratio vs pH trajectory may indicate that three pectinase enzymes have separate and distinct isoelectric points. It is observed that the local maximum protein separations all occur in the pH range of 4.0 to 10.0: between pH 5.0 and 8.0 for invertase, between pH 5.0 and 7.0 for pectinase and between pH 8.0 and 9.0 for α-amylase.

The three combinations of two enzyme mixtures are comprised of the binary components of α-amylase-invertase, invertase-pectinase, and α-amylase-pectinase as listed in Table 1. The optical absorbances of the isolated α-amylase and invertase components in these binary combinations (prior to being combined) were chosen to be about 0.15, which corresponds to a concentration of 100 mg/L for α-amylase and 300 mg/L for invertase. Since pectinase is such a poor absorber of light in the Bradford procedure, the concentration of pectinase used in the binary enzyme combinations was raised to 1000 mg/L, which corresponds to an optical absorbance of 0.07. The optical absorbance of the α-amylase-invertase mixture is about 0.3, which is about twice that of the original optical absorbance of α-amylase and invertase, as expected, indicating that Beer's law holds in this concentration range. The optical absorbance of single enzyme systems (α-amylase and invertase) was selected to be the same as the α-amylase-invertase mixture at about 0.3 for α-amylase (at a doubled concentration of 200 mg/L) and for invertase (at a doubled concentration of 600 mg/L). Unfortunately, the optical absorbance of pectinase solution could only be raised to 0.14, even for a concentration of 2000 mg/L, the upper concentration limit explored in this study. Optical absorbances of 0.3, therefore, were not reached with pectinase mixtures.

Both the α-amylase-pectinase mixture and the invertase-pectinase mixture have optical absorbances around 0.22, inferring that Beer's law holds for these cases, as well as the α-amylase-invertase case. Like the α-amylase-invertase mixture, these experiments are designed to explore the behavior of two enzyme systems at an optical absorbance value in the range of 0.2 and 0.3. Figure 3 illustrates the mixed separation ratios of α-amylase-invertase, α-amylase-pectinase, and invertase-pectinase mixtures as a function of pH. The mixed separation ratio is the ratio of optical absorbances, A_{Top}/A_{Bottom}, for these systems. The absorbance, A, in dilute solutions can usually be represented by Beer's Law as $K_1C_1 + K_2C_2$. A can be used in place of C in this preliminary study in which only optical absorbance is measured. Since only one wavelength, λ, is appropriate for the Coomassie Blue method, the concentrations cannot be estimated from the linear relationship $A_{mixture} = \sum_{i}^{n} K_iC_i$, where $A_{mixture}$ and K_i are functions of the given λ. An additional procedure such as UV absorption needs to be used to provide extra information in order to estimate both C_1 and C_2 in this mixed enzyme system. It is noted that the mixed separation ratio, A_{Top}/A_{Bottom}, reduces to the regular separation ratio, C_{Top}/C_{Bottom}, for a single enzyme system, since A becomes KC for dilute systems and the K cancels out.

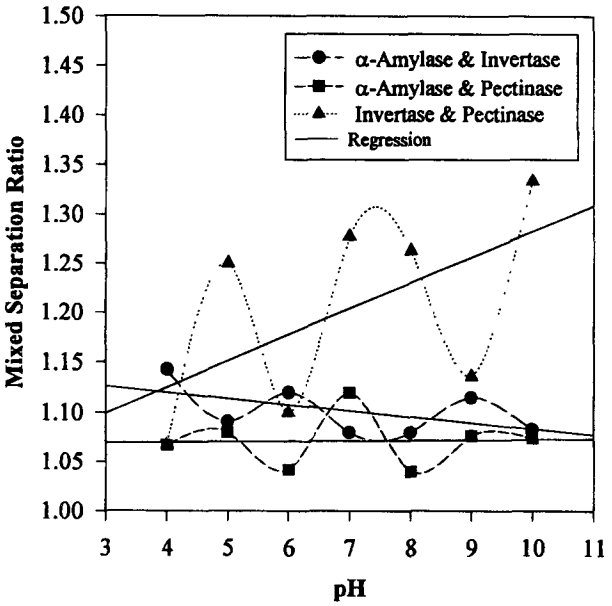

Fig. 3. Air bubble separation of two enzyme mixtures.

The mixed separation ratio in a multi-enzyme system is the ratio of the absorbance at the top position to the absorbance at the bottom position of the column. For dilute solutions this ratio may infer the weighted ratio of the total protein concentration at the top position to that at the bottom position since $A_{mixture} = \sum_{i=1}^{n} K_i C_i$, where $n = 3$. Here, the weighting factors are the absorbance coefficients, K_i. A value for total protein can be estimated if the values of $K_1C_1 = K_2C_2 = K_3C_3 = K_iC_i = A_i$ are about equal. The mixed separation ratio using the absorbance ratio can be implied from the concentration ratio as follows:

$$A_{top} = \sum_{i=1}^{3} A_{i,Top}$$

where $A_{i,top} = [K_iC_i]_{Top}$ so that:

$$A_{top} = 3A_{i,Top}$$

Similarly,

$$A_{bottom} = 3A_{i,Bottom}$$

The mixed separation ratio (S.R.) becomes:

$$S.R. = \frac{A_{Top}}{A_{Bottom}} = \frac{A_{i,Top}}{A_{i,Bottom}} = \frac{[K_iC_i]_{Top}}{[K_iC_i]_{Bottom}}$$

If the additional approximation of $K_{i,Top} = K_{i,Bottom}$ is made for this "mean" value of K_i, then:

$$S.R. = = \frac{C_{i,Top}}{C_{i,Bottom}}$$

To qualitatively infer the separation tendency of these mixtures undergoing bubble fractionation, a linear regression of the mixed separation ratio, A_{Top}/A_{Bottom}, as a function of pH, was developed (in Fig. 3). It is observed that the mean value of the mixed separation ratio of the α-amylase-invertase mixture decreased slightly and the α-amylase-pectinase held constant as the pH of the mixture increased. It is also observed that the mean value of the mixed separation ratio of invertase-pectinase may increase when the pH of solution increases, albeit with significant oscillations. This seems to indicate that the standard deviation for the mixed separation ratio of invertase–pectinase is particularly large (more than double that observed in the other two binary systems). Comparing Fig. 2 and 3 it appears that pectinase does not seem to impair the separation of invertase (or at least the total protein) under the conditions studied, even though the pectinase concentration was more than three times that of the invertase concentration: enzyme activities were not measured in this study and should be checked in subsequent investigations to observe how they vary in response to the changing conditions. In contrast, the mixture of α-amylase-pectinase seems to track the pectinase pH profile more closely than the α-amylase profile, which is not surprising since the pectinase concentration is 10 times that of the α-amylase concentration. These experiments seem to indicate that pectinase can either cause the separation to remain unchanged (invertase) or enhance the separation (α-amylase). The effect of pectinase in the presence of another protein in bubble fractionation, therefore, depends on both the bubble adsorptive force of the protein being separated and the competition for the given bubble adsorption sites by the pectinase. The competition for adsorption sites depends on the protein structure of each enzyme, and their polarity, hydrophobicity, and concentration.

The mixture used in the three enzyme bubble fractionation experiment is comprised of 100 mg/L α-amylase, 300 mg/L invertase, and 1000 mg/L pectinase. As previously noted, these concentrations were selected to enable the optical absorbance of α-amylase and invertase components to each contribute about equally to the optical absorbance, whereas pectinase contributes to the optical absorbance only about one half of the other two enzymes. The resulting mixed separation ratio for the three enzyme system is shown as a function of pH in Fig. 4. The local maxima of this mixed separation ratio of the ternary mixture appear at pH 5.0, 7.0, and between 9.0 and 9.5. Unlike the single and binary cases shown in Figs. 2 and 3, the highest mixed separation ratio was reached at 1.10 (vs ca. 1.25 in the previous

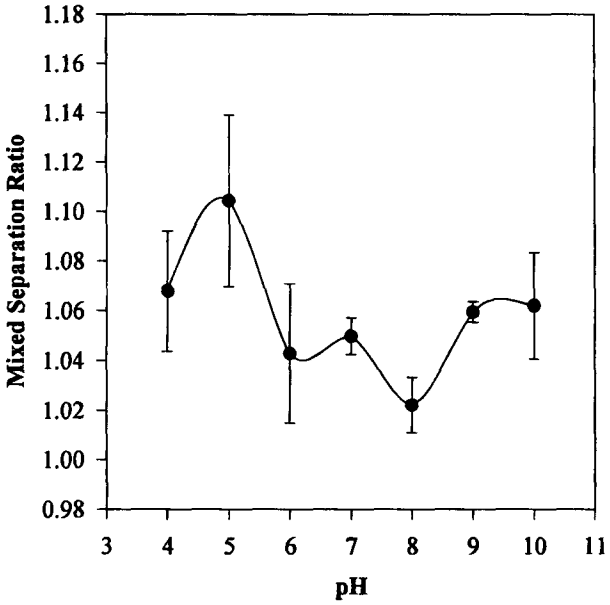

Fig. 4. Air bubble fractionation of the three enzyme mixture.

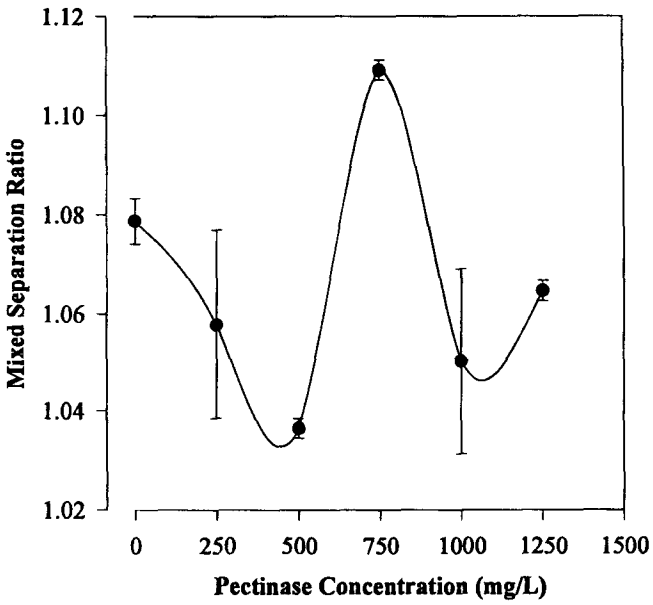

Fig. 5. The effect of pectinase on the bubble fractionation of α-amylase and invertase at pH 5.

cases). The largest two of the three local maxima located at pH 5.0 and 9.0 were thus selected (to effect the greatest resolution) for subsequent study of the effect of pectinase on the air bubble fractionation of α-amylase from invertase. These pHs also turn out to be close to the isoelectric values (pIs) of invertase and α-amylase, respectively (2).

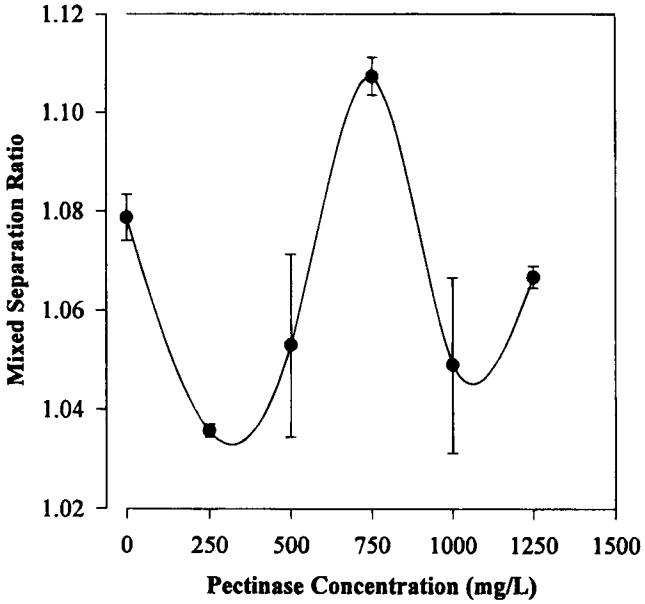

Fig. 6. The effect of pectinase on the bubble fractionation of α-amylase and invertase at pH 9.

Figs. 5 and 6 qualitatively depict the effect of various levels of pectinase on the separation of α-amylase from invertase at pH 5.0 and 9.0, respectively. Both graphs show similar curve patterns. The oscillation of these curves may be from the ionic charge on pectinase driving the separation ratio of the system to neutrality and polarity. For low pectinase concentration, $0 \leq C_{pectinase} \leq 500$ mg/L, the mixed separation ratio decreases as the pectinase concentration increases. Perhaps the ionic charges on pectinase neutralize the opposite charges on α-amylase and invertase to drive the system to neutrality. When the charges are balanced, the proteins generally tend to move freely and disperse evenly in the bubble column. This charge leads to a minimum in mixed separation ratio (at about 1.03) of this three enzyme mixture for a pectinase concentration of 500 mg/L for pH 5.0 and 300 mg/L for pH 9.0. When more pectinase is added beyond the neutral balance point, the ionic charges build up in the solution. An additional adsorptive effect of pectinase itself develops at the air-liquid interface leading to an enhanced pectinase layer that is brought up to the top by the air bubbles. The two pH systems reach a maximum of protein separation when the mixed separation ratio is about 1.11, at a pectinase concentration of 750 mg/L for both the pH 5.0 and 9.0 cases. Adding more pectinase beyond this 750 mg/L level, leads to excess ionic charges in the solution, and resulting repulsive forces. Therefore, it is difficult for proteins to form a layer on the bubbles and the resulting fractionation is degraded.

Since all of the mixed separation ratios determined in these experiments are greater than 1.0, this seems to indicate that some protein sepa-

ration occurs over the entire pH range ($C_{i,Top} > C_{i,Bottom}$ for all i). Unfortunately, for the mixed enzyme mixture cases, the individual protein concentrations were not measured since the expected separation (which matched the individual protein cases at their isoelectric points) previously found by DeSouza et al. *(2)* for a 50–50% mixture of α-amylase and invertase, was not replicated here for any binary combination of enzymes. The total protein technique (Coomassie Blue method) used here only determines the concentration of individual enzymes in single protein systems. Polyacrylamine gel electrophoresis, HPLC, or a direct enzyme activity method must be employed in future work to sharpen these results. It seems clear, however, that a change in pectinase level has a strong effect on the apparent separation of the total protein mixture even when the pectinase concentration is less than 500 mg/L at pH 5.0 and less than 250 mg/L at pH 9.0.

CONCLUSIONS

In these preliminary experiments, pectinase does not seem to enhance the separation of an invertase-pectinase mixture (relative to the invertase system alone) and even seems to inhibit the bubble separation of α-amylase in an α-amylase-pectinase mixture. When pectinase was mixed with invertase and α-amylase to form a three enzyme system, the local maxima mixed separation ratios were reached at pH 5.0 and 9.0. The addition of pectinase to the mixture of α-amylase and invertase seemed to reduce the separation of these two enzymes by bubble fractionation at both pH 5.0 and 9.0.

REFERENCES

1. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
2. DeSouza, H. G. A., Tanner, R. D., and Effler, W. T. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 655–666.
3. Lemlich, R. (1972), in *Adsorptive Bubble Separation Techniques*, Academic Press, New York, NY, pp. 133–143.
4. Park, D. H., Baker D. S., Brown K. G., Tanner R. D., and Malaney G. W. (1985), *J. Biotechnol.* **2,** 337–346.
5. Effler, W. T., Tanner, R. D., and Malaney, G. W. (1989), in *Bioproducts and Bioprocesses*, Fiechter, A., Okada, H., and Tanner, R. D., eds., Springer-Verlag, New York, NY, pp. 235–256.
6. Potter, F. J., DeSouza, H. G. A., Tanner, R. D., and Wilson, D. J. (1990), *Separation Sci. Technol.* **25,** p. 673–687.
7. Vitolo, M. and Borzani, W. (1983), *Analyt. Biochem.* **130,** 469, 470.
8. Wright, K. I. T. and Cui, Z. F. (1994), in *Separations for Biotechnology-3*, The Royal Society of Chemistry, London, pp. 577–585.
9. Whitaker, J. M. (1994), in *Principles of Enzymology for the Food Sciences*, Marcel Dekker, New York, NY, pp. 425–435.