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Sonic Wave Separation of Invertase from a Dilute Solution to Generated Droplets

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Abstract

It has previously been shown that a droplet fractionation process, simulated by shaking a separatory funnel containing a dilute protein solution, can generate droplets richer in protein than present in the original dilute solution. In this article, we describe an alternative method that can increase the amount of protein transferred to the droplets. The new method uses ultrasonic waves, enhanced by a bubble gas stream to create the droplets. The amount of protein in these droplets increases by about 50%. In this method, the top layer of the dilute protein solution (of the solution-air interface) becomes enriched in protein when air is bubbled into the solution. This concentrating procedure is called bubble fractionation. Once the protein has passed through the initial buildup, this enriched protein layer is transferred into droplets with the aid of a vacuum above the solution at the same time that ultrasonic waves are introduced. The droplets are then carried over to a condenser and coalesced. We found that this new method provides an easier way to remove the protein-enriched top layer of the dilute solution and generates more droplets within a shorter period than the separatory funnel droplet generation method. The added air creates the bubbles and carries the droplets, and the vacuum helps remove the effluent airstream from the condenser. The maximum partition coefficient, the ratio of the protein concentration in the droplets to that in the residual solution (approx 8.5), occurred at pH 5.0.

Index Entries: Sonic waves; droplet fractionation; invertase; hydrophobicity; enzymatic activity.

Introduction

Bubble fractionation, a nonfoaming adsorptive bubble separation process, is well known as a simple and inexpensive method for concentrating

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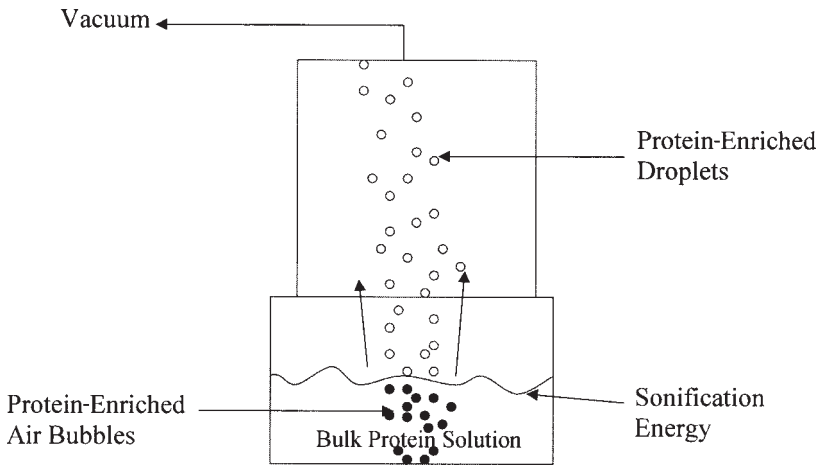

Fig. 1. Schematic of the protein-enriched droplet separation (fractionation) method.

proteins in a dilute nonfoaming protein solution. In this process, air or other gaseous bubbles travel through an elongated column of a bulk aqueous phase, transporting surface-active material such as proteins to the top of the column. The enriched surfactant/protein surface layer is removed periodically in a batch process or continuously in a steady state by collecting this upper layer of the liquid *(1)*. However, not all of the enriched protein is collected in the top enriched section because typically the concentration at the top can reach only about double that in the bulk liquid (e.g., from initially 100 to 200 ppm at the top); thus, by itself, it cannot concentrate a dilute protein solution enough to make this an industrially usable process.

Droplet fractionation has been suggested previously as an enhancement to the bubble fractionation process concentrating dilute protein bulk solution up to three to five times that of the residual solution *(2,3)*. The past studies indicate that certain nonfoaming proteins such as invertase can be partitioned by generating and recovering the now concentrated proteins in droplets of the dilute protein solution. In this present study, we show that the previous droplet separation equipment can be redesigned by incorporating a sonic wave generator to the bubble fractionation scheme to create more droplets in a shorter period of time.

Combining an airstream with a sparger improved the circulation of the sonified dilute protein solution, relative to no air sparging, enriching the top section of the short, new, circular cylindrical chamber (*see* Fig. 1) with the protein. The aerated/sonified system then created protein-enriched droplets (above the liquid surface) from that top layer. The generated droplets were then carried over to a condenser by employing a vacuum. These droplets were then coalesced in the condenser, which was cooled with a dry ice bath, as shown in Fig. 2.

In the present study, the partition coefficient, K_p, the ratio of the protein concentration in the droplet phase to that in the residual bulk phase,

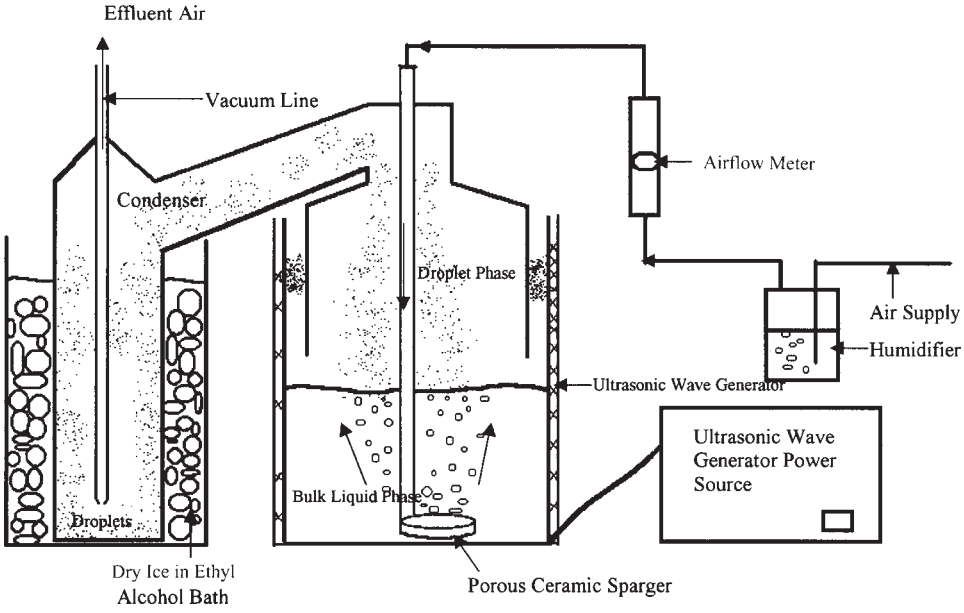

Fig. 2. Schematic of the ultrasonic wave droplet fractionation process.

was determined as a function of pH, and the resulting relationship was compared with the results of the previous (separatory funnel shaking) droplet separation technique *(2)*.

Materials and Methods

Chemicals

Yeast invertase (lot no. I-9253) and sodium hydroxide (lot no. 873487), were purchased from Sigma (St. Louis, MO). Coomassie brilliant blue G-250 (lot no. 23242) was purchased from Bio-Rad (Richmond, CA). Ethyl alcohol (100%) was purchased from Aaper Alcohol and Chemical (Shelbyville, KY). Dinitrosalicylic acid (DNS) (lot no. 09026LW) was purchased from Aldrich (Milwaukee, WI). Sodium Sulfite (lot no. 785778), citric acid (lot no. 795790), glucose (lot no. 793019), and sucrose (lot no. 771722) were purchased from Fisher Scientific (Fair Lawn, NJ).

Experimental Procedure

Dilute invertase solution (approx 100 mg/L) was prepared by dissolving invertase granules into deionized water followed by centrifugation (Marathon 21K; Fisher Scientific) at 3785*g* for 10 min. The supernatant was collected, kept in a refrigerator at 8°C, and used within 1 wk. The dilute invertase solution was adjusted initially to the desired pH (between 2.0 to 8.0) by adding hydrochloric acid or sodium hydroxide. The initial volume of the invertase solution used for the batch droplet fractionation experiment was 400 mL. The apparatus was set up as depicted in Fig. 2. The

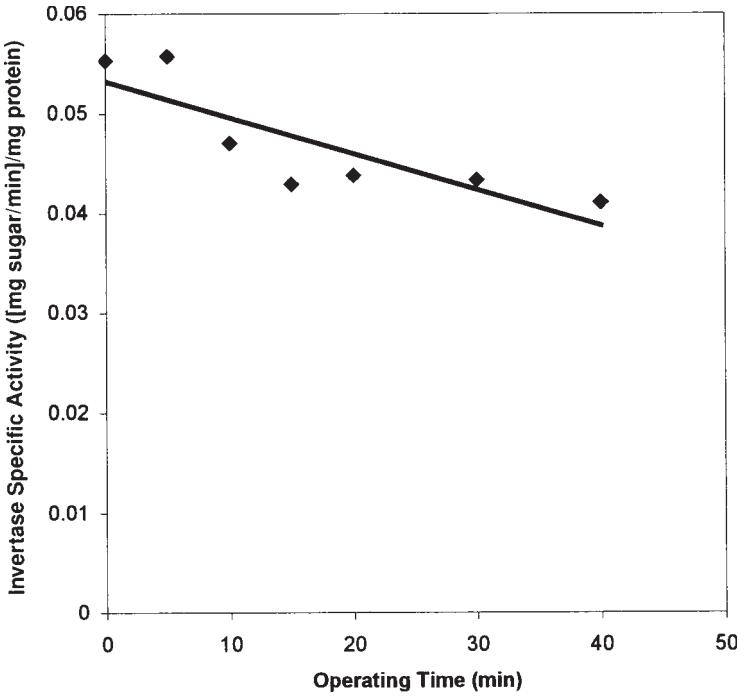

Fig. 3. Effect of sonification operating time on the determination of the invertase specific activity. The initial protein concentration was 100 mg/L.

condenser was immersed in a dry ice bath for 10 min prior to starting the sonified droplet experiment in the column. Initially, 400 mL of invertase solution was placed in the column containing the ultrasound generator (serial no. 11496; Laboratory Supplies, Hicksville, NY). A porous ceramic disc sparger was attached to the effluent gas line and placed at the bottom of the column as a bubble generator. It was connected to the air supply copper tube by aluminum flanges. An airflow meter (rotameter) was used to ensure a constant airflow at 12 cm^3/s. The supplied air (purified with cotton to remove protein containing dust) was humidified by bubbling it through water. This humidification step prevented further protein contamination in the influent air by scrubbing any remaining protein in the humidification chamber. The air was introduced to the porous sparger at the bottom of the column. The bubbles entered the bulk protein solution and rose up to the liquid surface and then the ultrasonic wave–assisted droplets were generated above the liquid surface. Droplets were continuously carried up to the top of the air column in the open space above the liquid, assisted by the vacuum, and then condensed and coalesced in the dry ice condenser.

The droplet fractionation experiments were terminated after generating droplets for 15 min. The experiments were allowed to run for only fifteen minutes because almost 10% of the enzymatic activity was lost by

then, as seen in Fig. 3. Presumably, the cavitation generated shear and localized heating, which led to the loss of enzymatic activity. Both the droplet and the residual volumes were measured after 15 min. The collected volume of droplets was diluted to 3 mL for the total protein assay (to achieve the minimum cuvet volume used for the spectrophotometer). The initial, residual, and collected droplet solutions were all assayed for their total protein content.

The effect of pH on the droplet fractionation of invertase was compared with the previous study that used a separatory funnel *(2)*. The correlation between the partition coefficient and the surface tension of the invertase solution, determined in the previous study, was also compared with the correlation obtained for these sonic-generated droplets.

Total Protein Assay

The total protein content in the invertase solution was determined by the Coomassie blue (Bradford *[4]* method) using a Bausch and Lomb Spectronic 20 spectrophotometer (Rochester, NY) set at 595 nm. In all the assays, 2 mL of Coomassie blue reagent was added to 3 mL of each sample solution. The optical absorbance was read at 5 min after adding the reagent. The following calibration curve, developed previously for the total protein content, was used for this invertase assay *(2)*:

$$\text{Invertase (protein) concentration (mg/L)} = 1429 \times (\text{absorbance @ 595 nm})$$

Invertase Activity Assay

To determine the invertase enzyme activity, 100 mg/L sucrose solution was used. A 0.05 M sodium acetate solution was used as a buffer solution (pH 4.7). To each test tube were added 1.5 mL of the enzyme solution, 0.5 mL of sugar solution, and 1.0 mL of the buffer solution After incubation (at 37°C for 15 min), the enzymatic reaction of invertase was stopped by cooling the test tubes in an ice bath. Three milliliters of DNS reagent was pipeted and added to a 30-mL test tube containing 3 mL of the enzyme solution *(5)*. This test tube was then placed in a boiling water bath for exactly 5 min. The test tube was then removed and cooled in running water for 2 min to reduce the solution temperature to room temperature. The optical absorbance of the room temperature solution was determined at a wavelength of 575 nm using a Bausch and Lomb Spectronic 20 spectrometer. The linear equation correlating the absorbance measurements for invertase activity was predetermined using a previously determined reducing sugar concentration vs absorbance calibration:

$$\text{Reducing sugar concentration (mg/L)} = 136.99 \times (\text{absorbance @ 575 nm})$$

The invertase activity was then determined in terms of the amount of reducing sugar (glucose) released per mass of invertase per minute.

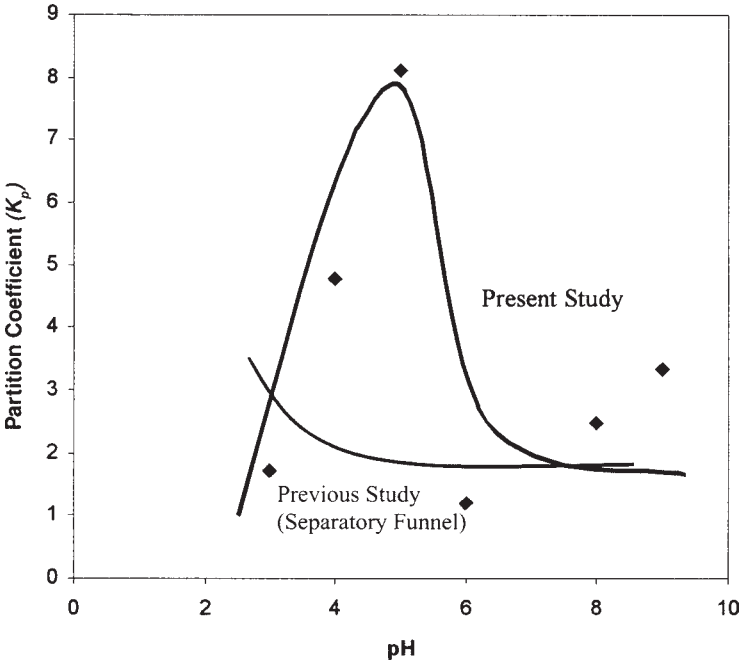

Fig. 4. Effect of pH on the partition coefficient of invertase solution at the initial protein concentration of 100 mg/L. The data points for the present study indicate the average of two sets of experiments. The *Kp*'s for both experimental sets were within 10% at a given pH.

Results and Discussion

Loss of invertase activity with respect to time is shown in Fig. 3. These results are from an experiment in which droplets were continuously generated in the column. Samples were taken every 5 min for the first 20 min and then at 10-min intervals. Much of the energy used to generate the droplets was converted to localized heating, increasing the average temperature of the bulk solution by about 5°C. The increase in temperature along with the shear resulting from the localized cavitation contributed to the slow degradation of the enzymatic activity of the bulk solution. Thus, experiments on generation of the ultrasonic wave–assisted droplets were terminated after 15 min in order to retain 90% of the initial enzymatic activity.

K_p is defined as the ratio of the protein concentration in the droplets ($C_{Droplet}$) to the protein concentration in the remaining residual solution ($C_{Residue}$) *(1)*:

$$K_p = (C_{Droplet})/(C_{Residue})$$

Here, *C* is determined in units of milligrams of protein/liter. As seen in Fig. 4, the maximum K_p value doubled from the previous study from about 4 to 8 in the pH 2.0–8.0 range. The K_p values ranged from 1.0 to 8.1

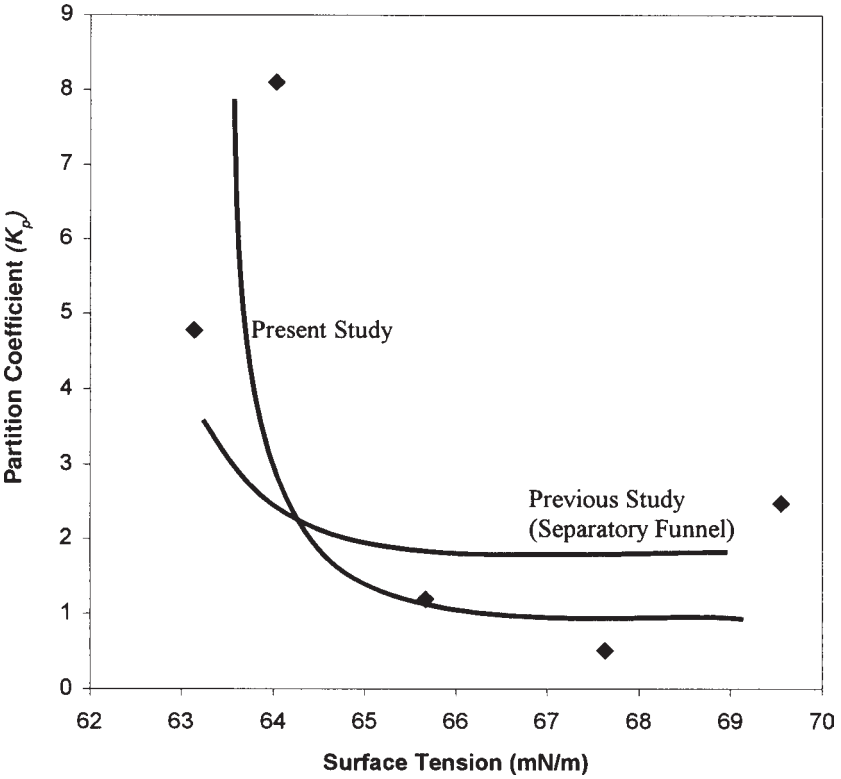

Fig. 5. Effect of surface tension on the partition coefficient of invertase solution at the initial protein concentration of 100 mg/L.

with a standard deviation of about 1. In this study, the K_p values increased almost linearly from pH 2.0 to 5.0 and decreased dramatically as the pH rises further. Note that the maximum K_p was close to the isoelectric point (p*I*) value of 4 *(6)*. These results indicate that invertase, a nonfoaming protein, behaves like most foaming proteins such as sporamin, which tend to be highly concentrated at their p*I*s *(7)*.

The volume of the collected droplets doubled with respect to the previous separatory funnel experiments as well. This larger droplet volume tended to increase the accuracy of the total protein assay since less water needed to be added to give the minimum volume needed for the assays. Not adding water in this step reduced the errors associated with this dilution step.

Figure 5 shows the correlation between *Kp* and the surface tension: K_p decreases as the surface tension increases. The maximum measured K_p occurred at a surface tension of 64 mN/m, far from the surface tension of water (72.6 mN/m). This implies that invertase, a hydrophilic protein, has hydrophobic properties as well. A similar trend, that the K_p decreases as the surface tension increases (and the hydrophobicity decreases), was observed in a previous study *(2)*.

Conclusion

The correlation of bubble and droplet fractionation using sonic waves in a bubbled gas environment increased the partition coefficient by ≈ 100% relative to the previous shake flask droplet fractionation. The maximum protein concentration (relative to the residual solution) into droplets increased from 4.8 to 8.1 times. The partition coefficient reached a minimum at pH 7.0, where the surface tension was at approx 67.8 mN/m, a binding similar to that obtained in the shake flask studies. Because this minimum K_p was <1, it may be possible to use droplet generation to largely remove waste and concentrate proteins in the residual bulk liquid. The use of ultrasonic waves for 15 min degraded the activity of enzyme by 10% owing to cavitation, the resulting generation of heat, and shear experienced by the proteins.

In one experiment, it was noted that dry ice (CO_2) could also be used to generate bubbles in the bulk solution in place of air. The use of dry ice potentially could reduce the degradation of the enzymatic activity owing to heat generation by internally cooling the protein bulk solution.

Acknowledgment

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