Effect of pH on Successive Foam and Sonic Droplet Fractionation of a Bromelain-invertase Mixture

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Abstract A droplet fractionation method was previously developed to concentrate a dilute non-foaming protein solution. In that earlier study with invertase, it was demonstrated that droplets created by ultrasonic energy waves could be enriched up to 8 times that of the initial dilute invertase solution. In this study, a mixture of bromelain (a foaming protein) and invertase (a non-foaming protein) is investigated as a preliminary step to determine if droplet fractionation can also be used to separate a non-foaming protein from foaming proteins. The foaming mixture containing bromelain is first removed by bubbling the binary mixture with air. After the foam is removed, the protein rich air-water interfacial layer is skimmed off (prior to droplet fractionation) so as not to interfere with the subsequent droplet production from the remaining bulk liquid, rich in non-foaming protein. Finally, sonic energy waves are then applied to this residual bulk liquid to recover droplets containing the non-foaming protein, presumed to be invertase. The primary control variable used in this droplet fractionation process is the pH, which ranged for separate experiments between 2 and 9. It was observed that the maximum overall protein partition coefficients of 5 and 4 were achieved at pH 2 and 4, respectively, for the initial foaming experiment followed by the post foaming droplet fractionation experiment.

Keywords: bromelain, invertase, foam fractionation, droplet fractionation, sonic waves

INTRODUCTION

Foam fractionation, a foaming protein adsorptive separation process, has been studied for decades to recover proteins from dilute water solutions [1]. This process is not applicable, however, if the protein does not foam. Droplet fractionation, a non-foaming adsorptive droplet separation process, on the other hand, previously introduced to enhance the bubble fractionation process as a simple and inexpensive method for concentrating proteins [2], may complement the foaming process by providing a means of recovering proteins from a dilute non-foaming solution. In the droplet fractionation process, air or other gaseous bubbles travel through an elongated column of a bulk aqueous solution and transport surface-active materials such as proteins to the top of the column via bubble fractionation (as is also the case in foam fractionation). The enriched surfactant/protein droplets are then generated from this protein-enriched bulk liquid-air interface. These droplets can be easily formed at the interface by shaking the bulk solution, or by introducing ultrasonic waves (focused acoustic beams) to the bulk solution. Ultrasonic waves can produce a high volume of droplets in a short period of time. In order to minimally denature the concentrated protein in the droplets, the system is carefully controlled so as not to significantly heat up the bulk solution.

In this study, a mixture of bromelain (a foaming protein) and invertase (a non-foaming protein) was prepared in order to test the premise that foaming proteins can be first removed from a bulk fluid followed by the removal of the residual non-foaming proteins using droplet fractionation. The total protein separation ratios are determined here to establish whether successive foam and droplet fractionation processes can separate and concentrate the foaming and non-foaming proteins. Determination of the activity of the bromelain in the foamate is used to verify that the foamate is enriched in bromelain.

The foaming protein, bromelain, used in this study, is a proteolytic enzyme obtained from a pineapple. It is currently used as a digestive and anti-inflammatory products for the pharmaceutical industry [3]. It also has been reported that bromelain may have value in suppressing tumor growth [3].

MATERIALS AND METHODS

Chemicals

Yeast invertase (lot no. 101F-0147), bromelain (lot no. 104F-0043), gelatin (58H0150), and sodium hydroxide (lot no. 873487) were purchased from Sigma (St. Louis, MO, USA). Coomassie brilliant blue G-250 (lot no.

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23242) was purchased from Bio-Rad (Richmond, CA, USA). 100% ethyl alcohol was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY, USA). Dinitrosalicylic acid (DNS) (lot no. 09026LW) was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Sodium sulfite (lot no. 785778), citric acid (lot no. 795790), glucose (lot no. 793019), sucrose (lot no. 771722) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Experimental Procedure

A mixture of dilute invertase solution and bromelain solution (approx. 100 mg/L each) was prepared by dissolving invertase granules and bromelain powder into deionized water. The dilute mixture was adjusted initially to the desired pH (between 2 to 8) by adding 1 N hydrochloric acid or 1 N sodium hydroxide. The initial volume of the bromelain/invertase mixture used for the batch foam fractionation experiment was 400 mL. The foam fractionation apparatus set up is shown in Fig. 1. Since the foam fractionation column can only hold 100 mL volume of the initial solution at a time, the batch experiment was repeated 3 times to complete 400 mL of the initial volume. Each of the 4 successive batch experiments was terminated after negligible foaming occurred at the air/liquid interface, and the remaining foam was skimmed off. The residual non-foaming residues for all four batch experiments were combined and then placed in a container containing the ultrasound generator (Serial No. 11496, Laboratory Supplies Co.) for the droplet fractionation experiment. The droplet fractionation apparatus was set up as shown in Fig. 2. The condenser was immersed in a dry ice bath for 10 min prior to starting the sonified droplet experiment. A porous ceramic disc sparger (porous size 40-60 μm) was placed snuggly at the bottom of the droplet experiment cylinder to act as a bubble generator. The air inlet tube was connected to the air supply copper tube by aluminum flanges. An air flow meter (rotameter), located prior to the flanges was used to aid in calibrating a constant airflow rate at 12 cm^3/s. The supplied air (purified with cotton to remove protein-containing dust) was humidified by bubbling it through water in a separate container prior to the rotameter (humidification chamber). This humidification step helped prevent further protein contamination in the influent air by scrubbing much of the influent air residual protein in the humidification chamber. Air entered the porous sparger at the bottom of the liquid containing cylinder. The bubbles entered the bulk protein solution, rose up to the liquid surface where the ultrasonic wave-assisted droplets were generated. Droplets were continuously carried up to the top of the column into the open (air) space above the liquid, assisted by a continuously drawn vacuum, and then coalesced in the subsequent dry ice cooled condenser. The droplet fractionation experiments were terminated after generating droplets for 15 min since a previous experiment showed that almost 10% of the residual enzymatic activity was lost by 15 min [2]. Pre-
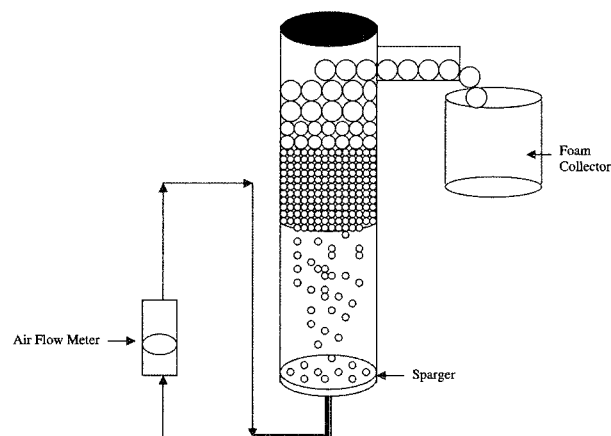

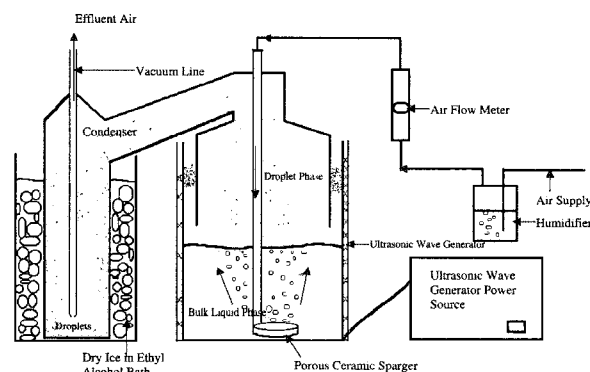**Fig. 1.** Schematic of the foam fractionation process.

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

sumably, cavitation generated shear and localized heating led to the loss of that enzymatic activity in the previous study. Both the droplet and the residual volumes were measured after each 15 min run. Since these volumes were typically less than 3 mL, the collected droplet volumes were each diluted to 3 mL for the respective total protein assay (Bradford method) in order to achieve the minimum cuvette volume needed by the spectrophotometer [4]. The initial, residual, and collected droplet solutions were all assayed for their total protein content.

Total Protein Assay for Both the Foamate and Droplets

The total protein content in the invertase solution was determined by the Coomassie blue (Bradford method) using a Bausch and Lomb Spectronic 20 spectrophotometer set at 595 nm [4]. In all of 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 relationship developed previously for the total protein content, was used for this invertase assay [2]:

Invertase (protein) concentration (mg/L)
= 1429 × (absorbance at 595 nm)

Bromelain Activity Assay for the Foamate

Preparation of Solidified Gelatin

A solidified form of dyed gelatin was prepared prior to the bromelain activity test. First, 100 mL of deionized water was heated until it boiled. 5 mg of bromophenol blue dye (a dye which does not complex with proteins) was dissolved into the boiling water while stirring with a magnetic stirring bar. 13.3 g of gelatin and 1 g of ethyl cellulose were placed in the solution and well mixed until the gelatin was completely dissolved. Ethyl cellulose was used to harden the gel upon solidification, reducing the liquefaction of the gel while heating during the activity test. It, thus, maximized the liquefaction occurring by enzymatic hydrolysis of gelatin with the bromelain by minimizing pre-enzymatic losses. This pre-made gelatin was solidified by cooling at 4°C in a refrigerator for 1 h.

Activity Test

3 mL of room temperature bromelain-invertase mixture was placed in a small (10 mL) test tube and incubated at 35°C for 15 min. 0.500 g (± 0.001) of the pre-made solidified dyed gelatin was added to the incubated solution. The test tube containing the gelatin solution was well mixed with a vortex mixer at room temperature for 1 min. The test tube was then immediately placed in an ice water bath to terminate the enzymatic reaction between bromelain and gelatin. 1 mL of 0.05 M sodium acetate solution (buffer solution, pH 4.7) was then added to the solution, and the temperature of the resulting mixture was allowed to reach room temperature (in *ca.* 5 min). The optical absorbance of the spilled dye from the bromelain-gelatin reaction at room temperature was determined at 590 nm wavelength spectrophotometrically. A linear equation correlating the absorbance measurements for the spilled bromophenol blue dye concentration was determined using a previously determined gelatin concentration vs. absorbance calibration in which small cubes of solidified gelatin containing bromophenol blue dye were completely reacted with bromelain:

Concentration of bromophenol blue dye (g/L) = 0.01434 × (absorbance at 590 nm)
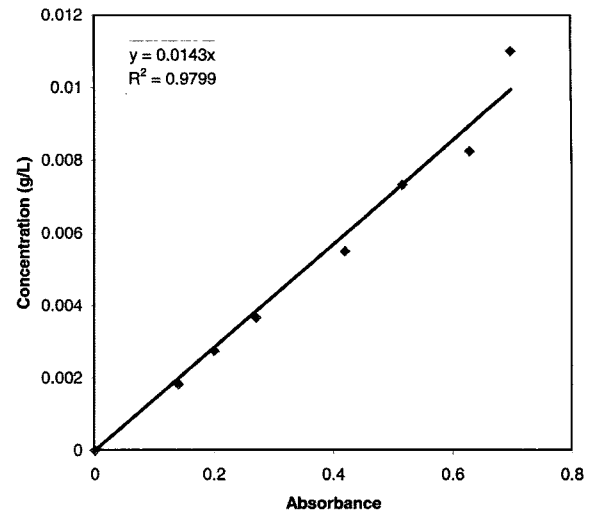
This concentration of bromophenol blue dye was again converted to the bromelain activity unit, where 1 unit hydrolyzes 5.2 mg of gelatin in 1 min at 37°C and pH 4.7.

1 unit = 4433.3 × Concentration of bromophenol
blue dye (g/L)

These two relationships can then be combined to give:
1 unit = 63.574 × (absorbance at 590 nm)

Invertase Activity Assay for the Droplets

100 mg/L sucrose solution was used to determine the invertase enzyme activity. A 0.05 M sodium acetate

Fig. 3. A linear correlation between concentration of bromophenol blue (g/L) and absorbance at 590 nm.
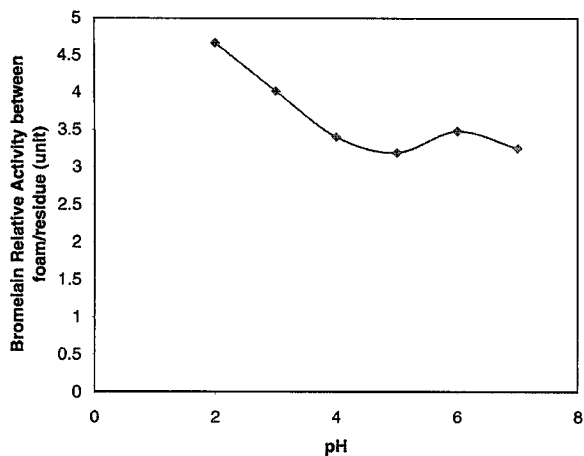
solution was used as the buffer solution (pH 4.7). 1.5 mL of the enzyme solution (from the droplets), 0.5 mL sugar solution and 1.0 mL of the buffer solution were added in sequence to the 30-mL test tube. After incubation (at 37°C for 15 min), the enzymatic reaction of invertase was stopped by quenching the test tube in an ice bath. 3 mL of DNS (dinitrosalisylic acid) reagent was pipetted into each 30 mL test tube containing 3 mL of the enzyme sugar and buffer mixture [5]. This test tube was then placed in a boiling water bath for 5 min (±5 sec.). The test tube was then removed and cooled in cold (tap) running water (at *ca.* 10-15°C) for 2 min to reduce the solution temperature to room temperature. The optical absorbance of the room temperature solution was determined at 575 nm wavelength using a Bausch and Lomb Spectronic 20 spectrometer. The linear equation correlating the absorbance measurements for invertase activity was previously developed [2]:

Reducing sugar concentration (mg/L)
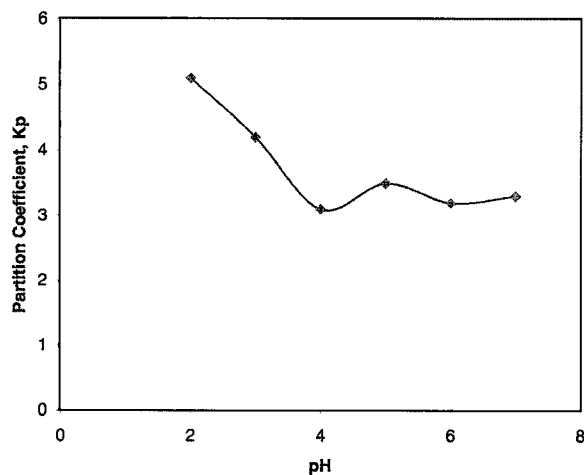= 136.99 × (absorbance at 575 nm).

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

RESULTS AND DISCUSSION

In the first foam fractionation step, presumed bromelain enriched bromelain-invertase mixture was collected in the foamate. This was followed by a foam skimming step to collect the residual foam in the column. Since bromelain is a foaming protein, and negligible foaming was observed in the residue, the majority of recovered protein in this foamate was assumed to be bromelain and removed from the mixture, leaving an invertase rich bulk solution in the residue. Preliminary experiments for the actual separation between bromelain and
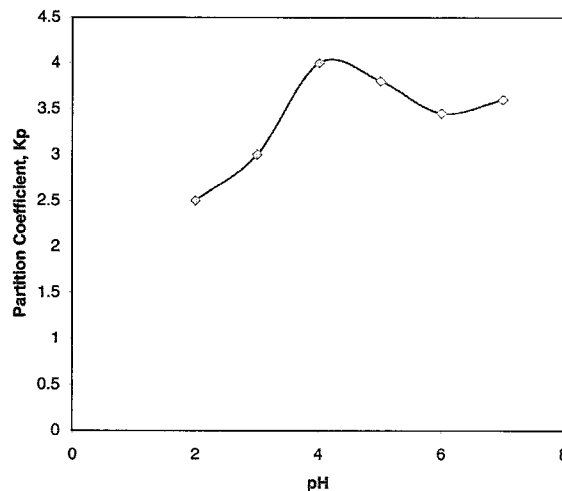
Fig. 4. Relative bromelain activity between foamate and residue bromelain activities for the foam fractionation experiments and ranging between pH 2 and 7 (Relative bromelain activity = foamate bromelain activity/residual bromelain activity).

Fig. 6. Partition coefficient of post foaming protein mass from the droplet fractionation experiment conducted in the pH range of 2 to 7.

covered in the foamate (seen in Fig. 5), most of the enrichment appears to be foamate. The shapes of the two curves are even qualitatively similar. The maximum total foam partition coefficient, K_p of 5, was found at pH 2 as shown in Fig. 5. Allowing for a *ca.* 80% (based on previous experiments with other enzymes) partial denaturation of bromelain at pH 2, the activity and mass recovery partition coefficient, are in agreement at pH 2.

The droplet partition coefficient, K_p, is defined as the ratio of the protein concentration in the droplet (*Cdroplet*) to the remaining protein concentration in the residual solution (*Cresidue*) [2]:

$$K_p = (Cdroplet)/(Cresidue)$$

Here *C* is determined in units of mg protein/liter. The volume of the foam generated from the bromelain-invertase mixture was about 30% less than the volume of the foam generated from a solution containing only bromelain (both experiments at 100 mg/L bromelain concentration) at pH 2, 4, and 6. In the binary enzyme mixture experiments, invertase, a non-foaming protein may have acted as an anti-foaming agent, suppressing some of the bromelain from foaming the bulk solution.

In the second droplet fractionation step, the maximum partition coefficient (of 4) occurred near pH 4 as shown in Fig. 6. Since the droplet fractionation and the foam fractionation steps are independent of each other, they can each be conducted at different pH's for better control. The droplet step experiments demonstrated that a significant amount of residual total protein can be transferred to the droplets, which are generated by sonic energy waves entering the bulk solution. The recovered protein in the droplets is presumed to be predominantly invertase for the following three reasons: 1. The solution rarely foams when bubbles are added to

Fig. 5. Total protein partition coefficient, Kp, for the foam fractionation process conducted between pH 2 and 7. The initial concentration of the bromelain-invertase mixed solution was 200 mg/L total protein (100 mg/L bromelain and 100 mg/L invertase). This experiment was conducted prior to the droplet separation experiement at a given pH.

invertase is explored in this paper using activity measurements of the bromelain to characterize the foamate and invertase to characterize the droplets. The relative bromelain activity between foam and residual solution shows that the bromelain activity in the foamate is up to 4.7 times higher than the bromelain activity in the residue, as shown in Fig. 4. With the assumption that 90% of the activity is retained in the foamate from the initial solution (based on our initial observation for bromelain foamed by itself), the relative bromelain activity indicates that the foamate phase is much more bromelain-enriched than the bromelain-invertase residue. In fact, it appears that based on the total mass re-

the bottom of the solution, and invertase is a non-foaming protein, 2. Much of the foaming mixture (mainly bromelain) appears to have been removed by foaming and by skimming off the residual foams in the foaming step. Unfortunately, using sonification under the present conditions the invertase activity in the droplets is nearly negligible. When this experiment was conducted using only invertase [4], it was clear that invertase was recovered in the droplets and was, in fact, denatured. Here, no such conclusion can be drawn but only inferred. Gel electrophoresis should be used in future work to provide additional information on how much protein separation has indeed occurred by comparing the strength of the color of the droplet phase band for bromelain and invertase to that of the respective bands for the foam fractionation experiment and the initial solution (in order to obtain bromelain and invertase mass balances for both the foam and the droplets). The volume of the collected droplets decreased by 20% with respect to the previous dilute invertase experiments for the same initial concentration solutions, indicating that the residual bromelain may have acted as an anti-droplet forming protein. Although most of the foam appears to have been removed in the initial skimming step, modest foam generation continued in the droplet forming experiment. Foam accumulated at the air-bulk solution interface and seemed to inhibit the formation of droplets by the sonic energy waves.

CONCLUSION

The feasibility of the separation of a mixture of a foaming protein mixture from a non-foaming protein mixture using successive steps of foam and droplet fractionation has tentatively been demonstrated. The maximum total foam partition coefficient, K_p of 5,

relative to the residual solution was found at pH 2 while the maximum partition coefficient of 4 occurred near pH 4 for the droplet fractionation. The maximum bromelain activity of 4.5 units appeared at pH 2.

Further analysis (*e.g.* by gel electrophoresis) should be performed to quantify the actual separation between bromelain (the original foaming protein) and invertase (the original non-foaming protein) in this combined foam and droplet protein separation scheme.

REFERENCES

[1] Karger, B. L., L. R. Snyder, and C. Horvath (1973) *An Introduction to Separation Science*, pp. 424-432, John Wiley & Sons, New York, NY, USA.

[2] Ko, S., V. Loha, L. Du, A. Prokop, and R. D. Tanner (1999) Partitioning invertase between a dilute water solution and generated droplets. *Appl. Biochem. Biotechnol.* 77: 501-510.

[3] Macrae, R., R. K. Robinson, and M. J. Sadler (1999) *Encyclopedia of Food Science Food Technology and Nutrition*, Vol. 6: p. 3603 Academic Press, New York, NY, USA.

[4] Bradford, M. M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye. *Anal. Biochem.* 72: 248-264.

[5] Miller, G. L. (1959) Using dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.

[6] Kokitkar, P. B. and R. D. Tanner (1991) Determination of the partition of the partition-coefficient for yeast invertase between microwater droplets in air in contact with liquid water. *Appl. Biochem. Biotechnol.* 28: 647-654.