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# Effect of Invertase on the Batch Foam Fractionation of Bromelain

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**Abstract** Foam fractionation can be used to enrich a hydrophobic protein such as bromelain from an aerated dilute protein solution because the protein foams. On the other hand, a protein such as invertase, which is hydrophilic, is not likely to foam under similar aerated conditions. While a foam fractionation process may not be appropriate for recovering a hydrophilic protein alone, it is of interest to see how that non-foaming protein affects the foaming protein when the two are together in a mixture. The bromelain enrichment, activity and mass recovery were observed as a function of the solution pH in order to explore how invertase can affect the recovery of bromelain in a foam fractionation process.

*Keywords*: foam fractionation, bromelain, invertase , protein recovery process

## INTRODUCTION

Foam fractionation is a simple and inexpensive process used to concentrate a dilute solution of a hydrophobic protein such as bromelain. In this process, a stream of air bubbles flows through the protein solution from a sparger placed in the bottom of a glass column. The bromelain concentrates in the foam, which is then collected from the top of the column after the foam collapses to create a concentrated liquid foamate. The volume of the foamate is typically small relative to the initial volume of the protein solution [1].

Another method for concentrating bromelain is to adsorb the bromelain on polyphenols. In an earlier study, Liang *et al.* [2] used tea polyphenols obtained from Chinese green tea to complex bromelain molecules and then extract them from a solution of pineapple juice. While this method is effective for concentrating bromelain, the foam fractionation procedure may be less expensive. Moreover, foam fractionation does not require chemical additives like polyphenols: contaminants which must be removed after the bromelain is recovered [3].

Bromelain is a hydrophobic protein found localized in the core of pineapples. It is used in the pharmaceutical industry in various applications ranging from anti-inflammatory drugs to coping with certain *Escherichia coli* bacterial infections such as traveler's diarrhea [4]. The activity of the bromelain present in a solution can be determined using a simple and inexpensive activity test based on the well-known observation that bromelain can break down gelatin. In this test bromophenol blue, a potent blue dye, is added to a liquid test mixture of gelatin and ethyl cellulose, which is then solidified. When the dyed gelatin cubes are added to a bromelain solution the bromelain breaks down the gelatin, releasing the dye. The photometrically determined dye concentration can then be used as a measure of the activity of the bromelain. The activity of bromelain is then determined from a linear calibration curve relating the concentration of blue dye released to the bromelain activity.

Bromelain solutions were foamed at different pH's to determine the pH at which bromelain foams best. Invertase, a hydrophilic protein, was then added at one fourth the concentration of the bromelain solution to test the binary protein foaming procedure. The protein solution was then foamed to determine the effect of a hydrophilic protein on the foam fractionation of the dilute bromelain solution.

Invertase, a protein that breaks down sucrose into glucose and fructose, is produced by a myriad of microorganisms and is also, along with bromelain, produced in pineapples. Neutral invertase, acid invertase and cell wall invertase are types of invertase present in pineapples expressed at different concentrations throughout the fruit's development. For example, the concentration of invertase in pineapples decreases as the fruit ripens resulting in the presence of an increasing amount of sucrose [5]. This presence of invertase in pineapples has the potential to create a problem in the foam fractionation of bromelain from pineapples since it may interact with the bromelain. The invertase used in these experiments was derived from the yeast strain *Saccharomyces cerevisiae* since it was readily available and the pineapple invertase was not. It is presumed here that the yeast invertase has many of the

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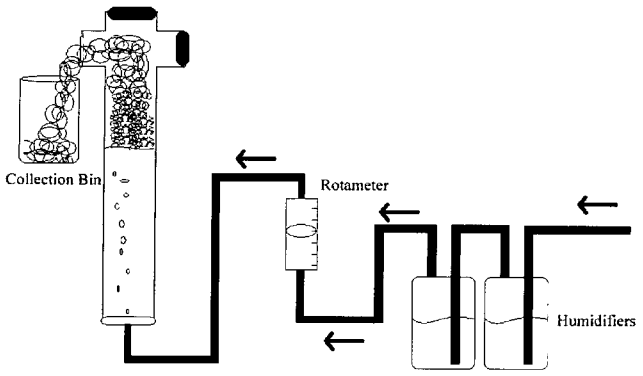


**Fig. 1.** Schematic of the foam fractionation column used to foam bromelain solutions. The arrows indicate the direction of airflow through the water traps and into the column. The bubbles in the column depict how the air flows through the water phase in the bottom of the column.

same foaming and bromelain interference characteristics as the pineapple invertase. Since invertase is widely used in the baking and confectionary industries, it's potential recovery from pineapples could not only improve bromelain recovery, but also contribute economically to the bromelain enzyme recovery process. In this paper we investigate the recovery of active bromelain in the foam fractionation process using the bromelain blue dye assay. The experiments focused on the invertase-free foam fractionation case to provide the setting for the problem. Then the foam fractionation results for one concentration of invertase will be presented to show how invertase can affect the resulting bromelain activity.

## MATERIALS AND METHODS

### Chemicals

Yeast invertase (lot no. I-9253), bromophenol blue (lot no. 83H3638), bromelain (lot no. B-2252), gelatin (58H0150), sodium citrate (lot no. 66F0345), and sodium hydroxide (lot no. 873487) were purchased from Sigma (St. Louis, MO, USA). Coomassie brilliant blue G-250 (lot no. 23242) was purchased from Bio-Rad (Richmond, CA, USA). Sodium sulfite (lot no. 785778) and citric acid (lot no. 795790) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

### Foam Fractionation

A 100-mg/L stock solution of bromelain was used for the bromelain solution foaming experiments. A fresh solution was prepared for each set of experiments performed on a given day. The solution was prepared by dissolving powdered bromelain into deionized water. The pH was then set at values of 2, 3, 4, 5, 6, 7, 8 and 9 by the addition of 1 N hydrochloric acid or 1 N sodium hydroxide. A volume of 100 mL bromelain solution of the

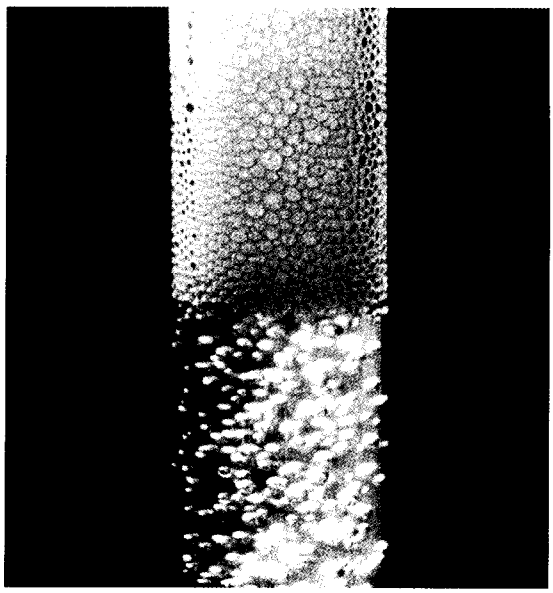

**Fig. 2.** A typical dispersion picture of foam (top) and bubbles (bottom) in a foam fractionation column. Photograph taken by Hytham A. Al-Saati.

desired pH was then charged to the foam fractionation column, *ca.* 3.9 cm in diameter and then foamed with air as shown Fig. 1. An in-line rotameter was used to determine the constant flow rate set at 21 $cm^3$/sec. The supplied air was purified by bubbling through two water baths in separate beakers in series (to remove most of the proteins that are inherently present in the laboratory compressed air). The air entered the porous sparger at the bottom of the column, creating bubbles, *ca.* 0.5 to 1.5 cm in diameter, in the protein solution as shown in Fig. 2. The bubbles flowed through the solution from the bottom of the fractionation column to the top where they produced foam. The solution was allowed to foam until the foam could no longer leave the top of the column. The foamate was collected from the top part of the column through a side port (see Fig. 1) into the collection bin. The liquid that remained in the column was collected as the residue. The foamate volume was then measured using a pipette. The concentration and activity of bromelain were determined using 3 mL samples of the following solutions: foamate, initial solution, and residue. If there was less than 3 mL of foamate, the foamate sample was diluted two fold to provide the minimum volume needed to test for bromelain activity. The dilution was later accounted for by doubling the activity value. A typical pattern of foam and gas bubbles near the gas–liquid interface inside of the foam fractionation column is pictured in Fig. 2.

### Bromelain Activity

Dyed solidified gelatin cubes for bromelain activity analysis were prepared prior to each foam fractionation [6]. The enriched gelatin solution was prepared as fol-

lows: 5.00 mg of bromophenol blue dye (this step requires some precision because the spectrophotometer absorbency reading is very sensitive to minute variations in the amount of dye) was added to 100 mL of boiling deionized water. The solution was mixed using a magnetic stirring bar. As the solution was being stirred, 13.3 g of gelatin were blended in slowly. Once the gelatin was fully dissolved, 1.0 g of ethyl cellulose was added to the solution to harden the gelatin once cooled so it wouldn't liquefy at room temperature (unless bromelain was added). When the solution was thoroughly mixed, it was poured into a small dish and allowed to solidify when refrigerated overnight.

After foam fractionation was complete, 3 mL samples of solution were taken from each of the initial, residue and foamate phases for each pH and placed in separate small tubes. The pH of each 3 mL solution was then set to 4.28 by adding 1 mL of 0.05 M citrate buffer. This was done to ensure that differences in gelatin breakdown were not due to pH differences. The tubes containing the bromelain solutions were then placed in heating wells, maintained at 37°C, for 15 min. Portions of the dyed solidified gelatin weighing in the range of 0.50 ± 0.02 g, were diced into cubes of side length *ca.* 3 mm, were prepared for each experiment. Each diced portion of 0.50 g was placed in a tube containing bromelain solution. All of the tubes were then placed on a vortex mixer for 5 min (± 10 sec) to mix the resulting reacting solution of bromelain and dyed gelatin. The tubes were then immediately placed in an ice bath for an additional 5 min dropping the solution temperature to 0°C causing the bromelain-gelatin reaction to slow to a negligible rate, effectively stopping the reaction. The tubes were then allowed to reach room temperature. The solution was decanted into a spectrophotometer tube, leaving the undigested gelatin in the reaction tube. The spectrophotometer (Hitachi Model 100-10, Hitachi Ltd., Tokyo, Japan) absorbance was initially set to zero using a solution of deionized water (0 mg/L protein concentration) plus the components of the bromelain activity test (buffer and gelatin). The absorbance of the initial, foamate, and residue bromelain solutions were then measured using the spectrophotometer at a wavelength of 590 nm. The bromelain activity was determined by the absorbance corresponding to the amount of bromophenol blue dye released from the gelatin using a previously developed calibration curve [6] and a linear equation relating absorbance to the activity of bromelain. The concentration of the blue dye is determined from the absorbance, measured by the spectrophotometer, using the following equation:

$$\text{Concentration of bromophenol blue dye (g/L)} = 0.01434 \times (\text{absorbance @ 590 nm})$$

This concentration of bromophenol blue dye is then converted to the defined bromelain hydrolysis activity unit, where 1 unit corresponds to the hydrolysis of 5.2 mg of gelatin in 1 min at 37°C and pH 4.7 as:

$$1\ \text{Unit} = 4433.3 \times \text{Concentration of bromophenol blue dye (g/L)}$$

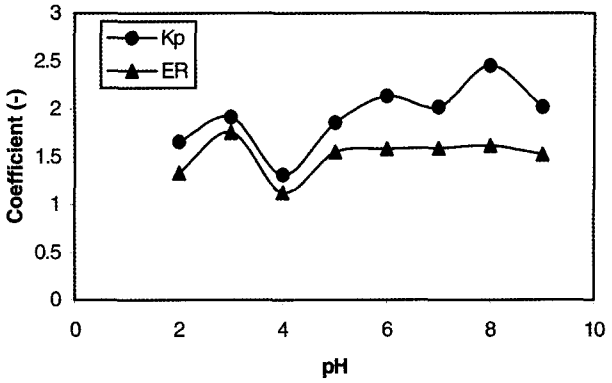


**Fig. 3.** Partition coefficient ($K_p$) and enrichment ratio (ER) for a bromelain solution with an initial concentration of 100 mg/L.

These two relationships can then be combined to give a bromelain hydrolysis unit in terms of absorbance [6]:

$$1\ \text{Bromelain hydrolysis unit (BHU)} = 63.574 \times (\text{absorbance @ 590 nm})$$

### Bromelain Activity of Solutions Containing Invertase

Samples of dilute invertase solutions were prepared by centrifuging an impure invertase-debris mixture for ten minutes at 6,000 rpm in order to remove the larger particulates. Portions of bromelain were weighed out to add to the particulate-free invertase solution in order to create two different solutions, each with 100 mg/L of bromelain. Mixtures of 0 mg/L of invertase and 100 mg/L bromelain (baseline point) and 25 mg/L of invertase and 100 mg/L of bromelain were prepared. The pHs of these solutions were then set to the pH range where the bromelain activity is maximized, by the addition of hydrochloric acid or sodium hydroxide. Separately, 100 mL of each of these solutions were then foamed in the fractionation column. Samples of the foam and residue for each experiment were collected and labeled. Volumes of 3 mL of each the initial, foam, and residue solutions were placed in small test tubes in order to carry out the bromelain activity test described above.

## RESULTS AND DISCUSSION

The partition coefficient (the concentration of protein in the foamate divided by the concentration in the residue: $K_p$) varied with pH, with a low at pH 4 and a high at pH 8, as shown in Fig. 3. Similarly, the enrichment ratio (the concentration of the protein in the foamate divided by the initial concentration: ER) also varied with pH in a similar manner. The partition coefficient and enrichment ratio should have a similar graphical pattern if the initial concentration and the residue concentration don't vary significantly (as when only a small amount [mass] of the foaming protein is removed in the foamate). It is observed that at pH 8 the partition coefficient devi-

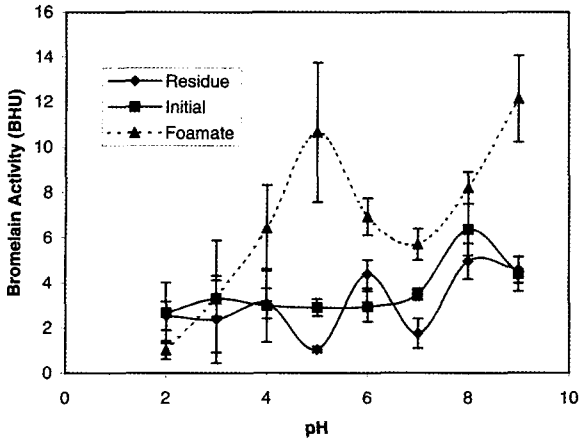


**Fig. 4.** Bromelain activity in residue, initial and foamate solutions. Each pH experiment started with a 100 mg/L bromelain solution before foaming.

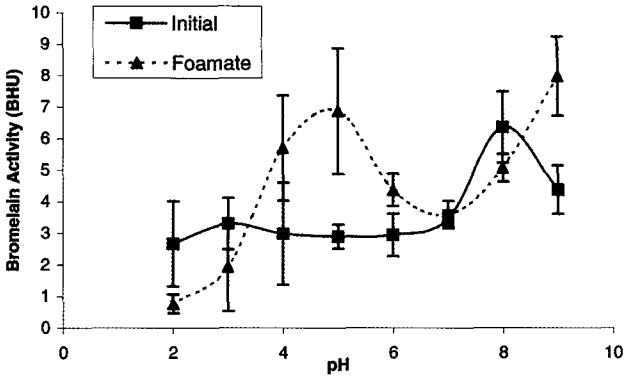


**Fig. 5.** Comparison between the normalized foamate bromelain activity (Bromelain Foamate Activity / Enrichment Ratio) and the initial bromelain activity as a function of pH. This shows the relative possible denaturation of protein resulting from the foam fractionation. The points on the curves represent the average value of two data points. Each pH experiment started with a 100 mg/L bromelain solution.

ates a little from this pattern. It is interesting to note (jumping ahead to Fig. 6) that the mass recovery in the foamate is less than 10% for pH's between 3 and 9, as expected, and that the local foamate mass recovery is maximized at pH 8 where there is the largest deviation on Fig. 3 The bromelain concentration values used to determine the partition coefficients and enrichment ratios in the invertase free solutions were obtained using the Bradford (Coomassie Blue) Total Protein Method [7]. The partition and enrichment coefficients, $K_p$ and ER varied from *ca.* 1.3 to 2.5.

Fig. 4 shows the bromelain activity of each solution, initially, and in the foam and residue after foaming is performed. The points on Fig. 4 represent the average activity values of two sets of data, where the error bars correspond to the standard deviation between the data. This representation applies to Figs. 5 and 6 as well. The foamate activities are observed in Fig. 4 to generally increase with an increase in pH. There is shown to be a local maximum in the foamate activity at a pH of 5 as well as a local minimum at pH 7. The bromelain activity increased about three-fold in the foamate of a pH 5 solution (not taking into consideration the effect of concentration) when compared to the other pHs. The only foamate solution with a bromelain activity comparable to the pH 5 case is at pH 9. The bromelain activities in the initial samples are nearly equal, up to pH 7, which is due to the fact that they are measures of essentially identical solutions that underwent a pH change and were returned to the pH 4.28 test condition (100 mg/L solutions of bromelain buffered to a pH of 4.28 before the bromelain activity test). Even when the determined activity appears to have increased, as for pH 8 case, the pH 8 standard deviation seems to indicate that it may just be an artifact (compare the standard deviations for the pH 3 and pH 8 cases which may overlap). It is expected that the activity in the residue decreases as the activity in the foamate increases just from the loss of bromelain to the foamate phase. This apparent loss in activity appears to be clear for the pH 5, 7 and 8 cases.

The bromelain activity can be corrected to "remove" the effect of bromelain concentration in the foamate by dividing the foamed protein activity by the enrichment factor, ER, defined as the concentration of foamate over the concentration of the initial bromelain solution. When the foamate activity was corrected for this concentration effect, the bromelain activities for the foamate and initial samples became virtually the same within experimental error(with the possible exception of the pH 2 point), as shown in Fig. 5 (corrected foamate activities greater than initial activities are considered here to be the same within experimental error). This indicates that the bromelain activity appears to be preserved during the foam fractionation process, except perhaps for pH 2. With negligible loss of activity due to foaming in the very low pH region, the unnormalized bromelain activity values can be used to infer the partition coefficient ($K_p$) of bromelain merely by dividing the unnormalized activity values by the initial activity values. This information could be especially important in mixtures of enzymes where we are unable to measure the mass of bromelain in the foamate directly.

The bromelain mass recovery rose slightly between pH 4 and pH 8, excluding the very acidic pH 2 and 3 points, as shown in Fig. 6. There was a sharp dropoff in the mass recovery of greater than 30% at pH 2 to a level less than 10% at pH 3, where it begins to level out. The protein may denature at pH 2 since it is observed that the mass recovery is more than three times that of the other pH cases and high mass recoveries are often associated with denatured proteins. This possible denaturation is also seen at the pH 2 case of normalized activity in Fig. 5.

As the pH increases from 2 to 9, the objective function, defined as $\phi$ = (mass recovery × partition coefficient × normalized bromelain activity divided by the initial activity) [7], shown in Fig. 7, increases from a base of 0.9 at pH 3 up to a local maximum at pH 5, followed by a de-

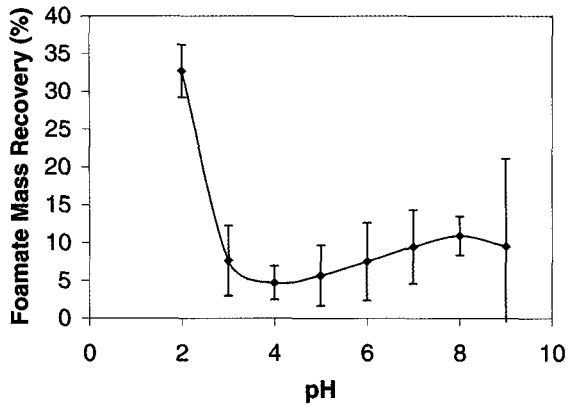


**Fig. 6.** Bromelain protein mass recovery in foamate. Each experiment was started with a 100 mg/L bromelain solution before foaming. The points on the curve represent the average values of two sets of data.

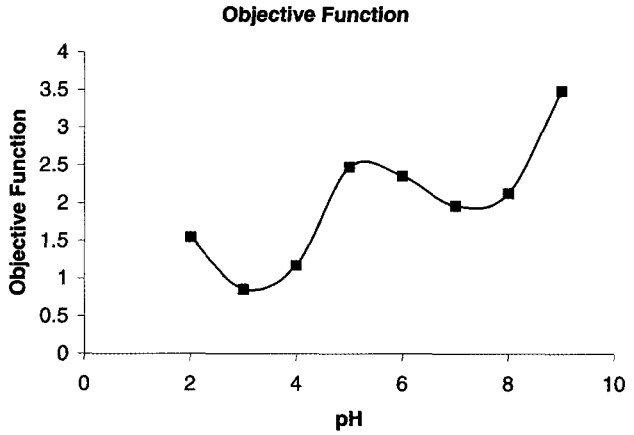


**Fig. 7.** The effect of pH on the overall unweighted objective function, defined as: Mass Recovery * Partition Coefficient * Normalized Bromelain Activity / Initial Bromelain Activity.

crease after pH 5 until pH 8 where an increase occurs up to a global maximum value of 3.5 at pH 9. An objective function determines how good a solution in reaching the objective of simultaneously maimizing the concentrating effect of the foam fractionation while maximizing both the mass recovery and the retained enzyme activity [8]. In this case it is used to help define the optimal pH for extracting bromelain from solution using foam fractionation while considering the tradeoffs among possibly conflicting goals. Fig. 7 shows that the optimal pH for the foam fractionation of bromelain from a water solution, where denaturation does not occur, is about pH 4 to 5 and pH 9. The easiest recovery between these two choices is at pH 4-5, the nominal starting point of the experiments and also the native range of a pineapple, where bromelain and invertase coexist [9].

The addition of invertase, the nominal subject of this paper, causes the apparent (measured) bromelain activity to drop, as shown in Fig. 8. The addition of 25 mg/L of

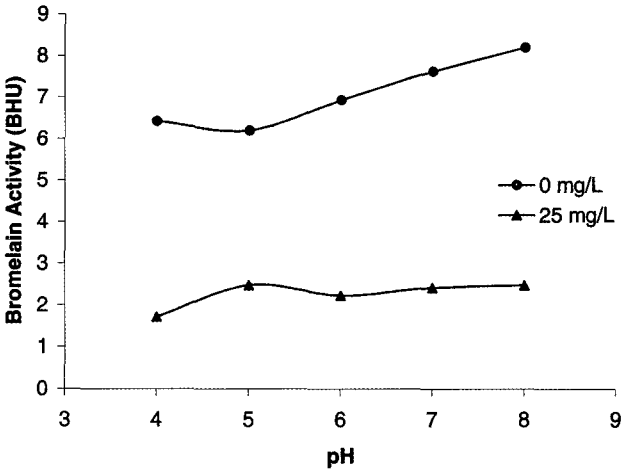


**Fig. 8.** Bromelain activity of two 100 mg/L bromelain foamate solutions (containing 0 mg/L and 25 mg/L invertase , respectively) foamed at various pH's over a pH range of pH 4-8. The activity levels are considerably lower for the solutions containing invertase. BHU is a bromelain hydrolysis unit.

invertase to a 100 mg/L bromelain solution causes the bromelain activity to drop by 2/3 of its original activity, compared to the same foaming case except for the addition of invertase. Further studies need to be conducted in order to better understand this observation. It is not clear as to whether the drop was caused by the interference of the invertase in the bromelain activity test or it was due to the interference of the hydrophilic invertase in the foaming experiment, or both. If the activity drop was due to an artifact in testing and truly remained constant, then the concentration of bromelain in the foamate would have dropped to one third of its original value (the ER dropped one third to a value less than one due to the presence of invertase). To support the argument of a change in the bromelain recovery conditions, it was observed that the foaming time decreased from *ca.* 5 to 2 min and the diameter of the bubbles decreased from *ca.* 1.0 to 0.5 cm when invertase at a concentration of 25 mg/L was added to the 100 mg/L bromelain solution. On the other hand, if the bromelain activity dropped because of the presence of invertase in the gelatin test, then invertase bound to the gelatin or complexed with the bromelain kept the bromelain from decomposing the gelatin at it's invertase-free rate. Further studies need to be conducted to elaborate on the effect of invertase on the gelatin test and the foam fractionation process. Particular attention should be paid to the effect of invertase on the foaming time and on the resulting foam bubble size of this binary hydrophilic-hydrophobic protein mixture.

## CONCLUSION

The addition of 25 mg/L invertase to a 100 mg/L bromelain solution causes the bromelain activity to drop

significantly. It is not clear whether this drop is due to the presence of invertase reducing the ability of the foam fractionation process to concentrate bromelain in the foamate or just as an artifact in the bromelain activity assay. The reductions in foam recovery time and foam diameter when invertase is added seem to lend support to an inherent change in foaming conditions which may cause a reduction in recovered bromelain. Bromelain appears to become denatured at pH's below 3. The optimal pH for the extraction of bromelain from a solution using foam fractionation appears to be around pH 4 and 5, the native pH of pineapple, and pH 9.

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## REFERENCES

[1] Santana, C. C., L. Du, and R. D. Tanner (2002) Downstream Processing of Proteins Using Foam Fractionation, Entry No. E6-58-02-06 in Encyclopedia of Life Support Systems, K. Matsura, Eolss Publishers Co., Ltd., Oxford, UK.

[2] Liang, H. H., H. H. Huang, and K. C. Kwok (1999) Properties of tea-polyphenol-complexed bromelain. *Food Research International.* 32: 545-551.

[3] http://www.invertase.net

[4] http://www.wholehealthmd.com. (2001) *WholeHealthMD LLC,* 11150 Sunset Hills Road, Suite 210, Reston, VA 20190, USA.

[5] http://www.actahort.org/books.htm.Chen, C. and R. E. Paull (2001). Changes in sugar contents and activities of sugar metabolizing enzymes in pineapple fruit flesh during development. *III International Pineapple Symposium.*

[6] Ko, S., A. Prokop, and R. D. Tanner (2002) Effect of pH on successive foam and sonic droplet fractionation of a bromelain-invertase mixture. *Biotechnol. Bioprocess Eng.* 7: 26-30.

[7] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

[8] Cherry, J., S. Ko, R. Grainger, A. Prokop, and R. D. Tanner (2000) Developing an objective function to characterize the tradeoffs in salting out and the foam and droplet fractionation processes. *Brazilian J. Chem. Eng.* 17: 233-238.

[9] http://vm.cfsan.fda.gov/~comm/lacf-phs.html. U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition. June 2000. Approximate pH of Foods and Food Products.

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