Copyright © 2004 by Humana Press Inc.
All rights of any nature whatsoever reserved.
0273-2289/04/114/0619–0625/$25.00

Degeneration of β-Glucosidase Activity in a Foam Fractionation Process

Vorakan Burapatana, Ales Prokop, and Robert D. Tanner*

Department of Chemical Engineering,
Vanderbilt University,
Station B, PO Box 351604,
Nashville, TN 37235,
E-mail: rtanner@vuse.vanderbilt.edu

Abstract

Foam fractionation is a promising technique for concentrating proteins because of its simplicity and low operating cost. One such protein that can be foamed is the enzyme cellulase. The use of inexpensively purified cellulase may be a key step in the economical production of ethanol from biomass. We conducted foam fractionation experiments at total reflux using the cellulase component β-glucosidase to study how continuous shear affects β-glucosidase in a foam such as a fermentation or foam fractionation process. The experiments were conducted at pH 2.4, 5.4, and 11.6 and airflow rates of 3, 6, 15, 20, and 32 cc/min to determine how β-glucosidase activity changes in time at these different conditions. This is apparently a novel and simple way of testing for changes in enzyme activity within a protein foam. The activity did not degenerate during 5 min of reflux at pH 5.4 at an airflow rate of 10 cc/min. It was established that at 10 min of refluxing, the β-glucosidase denatured more as the flow rate increased. At pH 2.4 and a flow rate of 10 cc/min, the activity remained constant for at least 15 min.

Index Entries: β-Glucosidase; foam fractionation; cellulase; reflux time; airflow rate.

Introduction

Cellulase is a collection of enzymes that together can hydrolyze cellulose to glucose, a key step in the low-cost conversion of biomass to ethanol. Such a process would be useful for three reasons. First, in large scale it could contribute to the substitution of the renewable fuel ethanol for petroleum-based gasoline. Second, it is known that the addition of 5% ethanol to

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

present-day gasoline can reduce carbon monoxide, carbon dioxide, and hydrocarbon emissions from automobiles. Third, at the 5% addition level, no engine modification is needed. Unfortunately, the use of ethanol for a fuel addition from a waste crop source for biomass is not financially feasible at present *(1)*. Without a significant decrease in the production cost, the petroleum-sparing and environmental benefits of this "bioethanol" will likely not be achieved.

Foam fractionation can be used as a low-cost concentration and/or separation process for surface-active materials such as proteins *(2–5)*. It is possible to recover the protein cellulase via a foam fractionation process *(2)*, thereby possibly lowering the cost of ethanol derived from biomass. This lowering in cost follows because the production cost of bioethanol is heavily dependent on the cost of cellulase. However, foaming is thought to denature proteins such as enzymes and thus lower enzymatic activity. Surface denaturation, shear stress, and oxidation are three important factors known to denature proteins in a foam fractionation process *(6,7)*. A protein can denature at a gas-liquid interface owing to a change in folding of its structure. Generally, the hydrophobic parts of a protein will orient themselves toward the gas phase and the hydrophilic parts toward the liquid water phase. This orientation becomes important in an air-water system such as a fermentation process producing cellulase and in a foam recovery process of that fermentation broth since it can also change the shape of the cellulase molecule. The resulting degree of denaturation depends on the ability of a protein to refold back to its original structure following foaming. Shear stress caused by rising and bursting air bubbles can also damage proteins such as cellulase. In addition, it is suspected that an interaction between oxygen and enzymes may cause enzymes to denature. Changes in pH and temperature can denature proteins as well.

The loss of cellulase activity in a foam fractionation process has been observed previously *(2)*. Since cellulase is a collection of enzymes, and different proteins denature differently, it is not obvious which foam fractionation strategy (such as the best pH and temperature conditions to use) is best to invoke to minimize the loss in cellulase activity in each of the individual primary components of cellulase during a foam fractionation process. In particular, if recovery of the "combined" cellulase directly from a fungal fermentation process as it is being produced is desired, which single condition should be followed to maximize the activity of the cellulase grouping? Cellulase has three main components: endo-β-glucanase, exo-β-glucanase, and β-glucosidase.*(8)* In this article, we focus on the loss of activity of β-glucosidase caused by different pH values, airflow rates, and reflux times during a foam fractionation process. In previous experiments, it was shown that the relatively hydrophilic β-glucosidase can be recovered from a water solution via foam fractionation with a modest amount of foaming *(9)*. We studied β-glucosidase as a key component for characterizing activity degeneration in the "combined" cellulase because of the three cellulase components it is the one that is commercially avail-

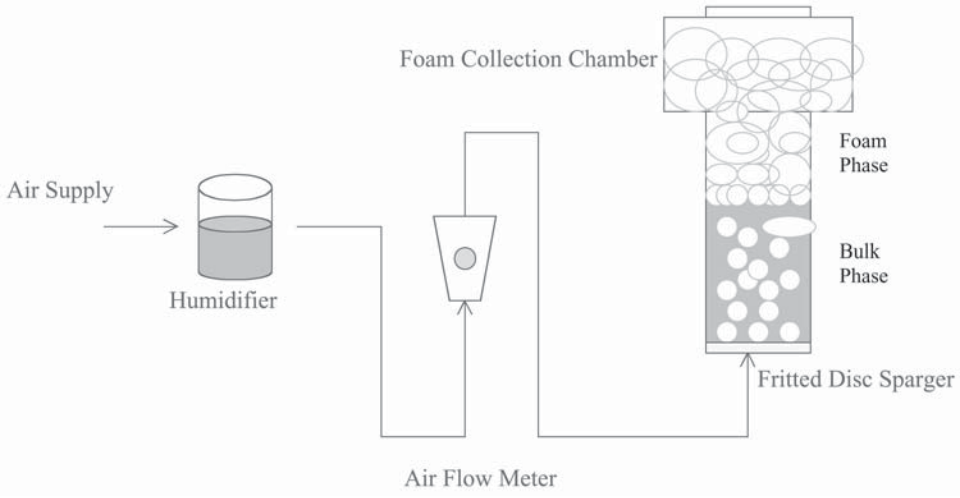

Fig. 1. Schematic of apparatus for foam fractionation of β-glucosidase at total reflux.

able. Herein, we define the amount of activity recovered as the relative activity (RA):

$$RA = \frac{\text{Activity}}{\text{Activity}_{\text{base case (pH 5.4)}}} \quad (1)$$

The base case is set at pH 5.4, the initial activity. RA is the ratio of the activity after the foam experiment to the activity before the foam experiment (referred to as time zero).

Materials and Methods

Effect of Airflow Rate

Twenty milligrams of glucosidase (containing both α and β glucosidase) derived from *Aspergillus niger* purchased from Fluka (catalog no. 49291) was dissolved and mixed well in 200-mL of deionized water to make a 100 mg/L solution. Ten milliliters of this glucosidase solution was poured into the foam fractionation column depicted in Fig. 1. This small column of 2-cm diameter and 9-cm length was used because the high cost of purified glucosidase prohibited multiple runs in a larger column. The semibatch (continuous in air) column was operated at total reflux at different air velocities for 10 min for each run. One- milliliter samples were taken from the liquid phase at airflow rates of 3, 6, 15, 20, and 32 cc/min. The concentration of β-glucosidase remained relatively constant throughout the experiment because we did not collect the foamate.

Effect of Reflux Time

A 100-mg/L glucosidase solution was used throughout this part of the experiment. Ten milliliters of this solution was poured into the foam fractionation column. The air velocity was then set at 10 cc/min and the pH at 5.4 (the unadjusted solution pH). A 1-mL sample was taken from the liquid phase at several different times: 0, 1, 1.5, 2, 3, 4, 5, 7, and 10 min. The experiment was repeated with 100-mg/L of glucosidase solution for more acidic and basic conditions, at pH 2.4 and 11.6, respectively.

Activity Assay

One hundred microliters of each sample from the foam fractionation column was mixed with 100 µL of 15 m*M* glucosidase substrate solution in a 1.5-mL microcentrifuge tube. The samples were incubated at 50°C for 30 min. After incubation, the samples in the microcentrifuge tubes were put in boiling water for 5 min. Then, the tubes were cooled down in an ice bath for 10 min. After cooling, 10 µL of solution from each tube was added, along with 90 µL of deionized water, into a selected well in a 96-well microplate. Next, 100 µL of glucose oxidase reagent (catalog no. G3660; Sigma Aldrich; the assay is included as part of the assay kit) was added to each filled well. After 15 min, 100 µL of 12 N H_2SO_4 was added to each filled well to stop the glucose reaction. The microplate was scanned at 540 nm using a µQuant Plate Reader from Biotek.

Results and Discussion

Bubbling air into a foam fractionation column containing a glucosidase water solution can cause damage to the glucosidase molecules. Figure 2 shows that at pH 5.4, as the airflow rate increased, the glucosidase activity decreased in a decaying, exponential manner. It is seen that the change in activity was rapid for airflow rates in the range of 0–6 cc/min. Then, the rate of change in relative activity decreased as the airflow rate increased. Even at the relatively slow flow rate of 3 cc/min (held for 10 min), the activity dropped to 93% of the original β-glucosidase activity. The degradation of activity owing to air shear can be crudely estimated as $0.92e^{-kF}$, in which $k = 0.0086$ min/cc and F = airflow rate (cc/min). Here, the R^2 of the fit is 0.72.

The effect of reflux time at pH 5.4 (holding the airflow rate at 10 cc/min) is shown in Fig. 3. It is observed that the activity held at about 95% of the initial activity until about 6 min of refluxing, at which time the activity dropped to about 80% of the initial activity. This means that with comparable aeration shear, experienced in a fermentor or a foam fractionation column, the activity of this cellulase component remains relatively intact for up to 6 min. At pH 3.5 and 4.0, when the activities were measured, following foaming for 6–8 min, the activities decreased only slightly (to about 95%). This low airflow rate usually led to foamate (recovered solution at the top of the foam) that was highly enriched.

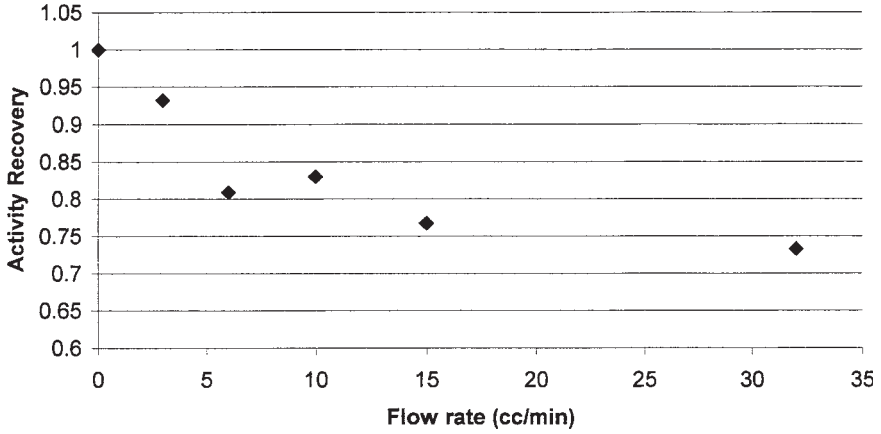

Fig. 2. Shearing effect of air on β-glucosidase activity at pH 5.4 and 10 min of refluxing.

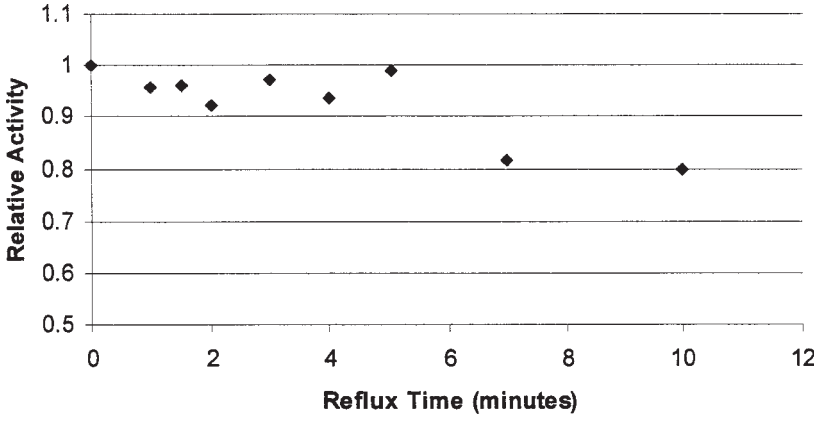

Fig. 3. Effect of reflux time on β-glucosidase activity at pH 5.4. The airflow rate was set at 10 cc/min.

The effect of aeration on glucosidase activity can be dependent on the reflux time, as seen in Fig. 3. A short time period of foaming apparently does minor (about 5% activity loss) damage to glucosidase. Figure 3 shows that the relative activity of glucosidase fluctuated between 0.9 and 1 for the first 5 min of foaming. Then the activity dropped to 80% by about 7 min of foaming and remained at that level for the next 3 min. The data indicate that glucosidase molecules can withstand the air bubble shear quite well for up to about 6 min of foaming at pH 5.4 and an airflow rate of 10 cc/min in the small reflux foam fractionation column.

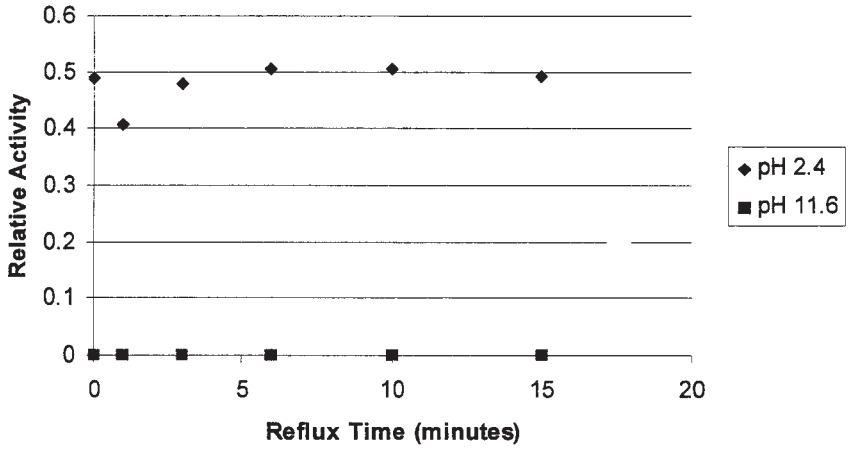

Fig. 4. Effect of reflux time (at two extremes pH values) on β-glucosidase activity. The airflow rate was set at 10 cc/min.

The glucosidase molecules appear to behave like an elastic spring. Like a spring losing some of its elasticity after being stretched for a significant time period, glucosidase loses some of its activity after being sheared by air bubbles over a given time. At an air-liquid interface (such as at the surface of an air bubble), a protein molecule such as glucosidase can be oriented so that the hydrophilic part stays in the liquid while the hydrophobic part tends to point toward the air (within the gas bubble). This orientation is thought to cause enzymes (such as glucosidase) to denature at air-water interfaces like those found in bubbles of a foam fractionation column. Such enzymes can also refold back to their original structure following temporary denaturation in an air shear environment like refluxing in a foam fractionation column. The ability to refold might be lost, however, because the molecules remain under shear at the interface over long time periods. In most foam fractionation experiments, the entire separation of concentrated foam in the foamate phase is completed well before the 3–7 min refluxing times noted here. The data presented here indicate that foam fractionation over shorter periods of time (less than 3–7 min) does not significantly denature glucosidase.

Foaming experiments were also conducted at pH 2.4 and 11.6 to determine how glucosidase is affected at very acidic and very basic environments; the findings are shown in Fig. 4. At pH 2.4, the activity dropped to 47% of that present initially (essentially at zero time) but then remained constant at this level throughout the subsequent 15 min of foaming. At pH 11.6, the activity was essentially all lost on the addition of base and did not recover on refluxing.

Conclusion

Glucosidase activity is best retained in a moderately acidic environment during refluxing in a foam fractionation column. Activity retention is also dependent on the airflow rate. The greater the airflow rate (the greater the bubble shear stress on glucosidase), the greater the reduction in glucosidase activity. Foaming time is another important factor: keeping the foaming time below a certain time threshold (i.e., 6 min) at pH 5.4 and an airflow rate of 10 cc/min minimizes loss of glucosidase activity. This means that glucosidase in a foam fractionation column experiences minimal loss of activity with a typical foaming time (residence time) of less than 1 min.

Acknowledgments

We are pleased to thank Prof. R. Robert Balcarcel for allowing us to use the μQuant Plate Reader (Biotek). We also greatly appreciate financial support from the US Department of Agriculture (grant no. 2001-52104-11476).

References

1 Sheehan, J. and Himmel, M. (1999), *Biotechnol. Prog.* **75,** 817–827.
2 Loha, V., Prokop, A., and Tanner, R. D. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 701–712.
3 Varley, J. and Ball, S. (1994), *Separat. Biotechnol.* **158,** 525–531.
4 Uraizee, F. and Narsimhan, G. (1990), *Microbiol. Technol.* **12(4),** 315–316.
5 Lockwood, C., Bummer P., and Jay, M. (1997), *Pharma. Res.* **14(11),** 1511–1515.
6 Clarkson, J. R., Cui, Z. F., and Darton, R. C. (1999), *J. Colloid Interface Sci.* **215,** 323–332.
7 Clarkson, J. R., Cui, Z. F., and Darton, R. C. (1999), *J. Colloid Interface Sci.* **215,** 333–338
8. Bhat, M. K. and Bhat, S. (1997), *Biotechnol. Adv.* **15,** 583–620.
9 Lambert, W. D., Du, L., Ma, Y., Loha., V., Burapatana, V., Prokop, A., Tanner, R. D., and Pamment, N. B. (2003), *Bioresour. Technol.* **87,** 247–253.