HYDROLYSIS OF LACTOSE IN ACID WHEY BY LACTASE
BOUND TO POROUS GLASS PARTICLES IN TUBULAR REACTORS

INTRODUCTION

IMMOBILIZATION of enzymes on particles, fibers, or membranes may allow multiple reuse of the enzyme, control of the selectivity between various reactions in multi-enzyme reactions, modification of the optimum pH of the enzyme, and may prevent contamination of the product by the enzyme (Katchalski, 1970; Jackson and Edwards, 1973; Wingard, 1972; Zaborisky, 1973).

The low tolerance for lactase of many humans beyond early childhood, the millions of pounds of lactose available annually as an edible by-product in whey, and the processing difficulties associated with its use as a food additive have led to research aimed at the production of new low-lactose foods. Lactose may be hydrolyzed to more digestible glucose and galactose using microbial lactases (β-galactosidase, EC 3.2.1.23) in solubility (Giacin et al., 1973; Hood, 1971; Kosi-kowski and Wierzbicki, 1971, 1973; Kosi-kowski et al., 1972; Olson and Stan-ley, 1973; Wendorff et al., 1971; Wierzbicki and Kosi-kowski, 1973) and immobilized forms (Dahlqvist et al., 1973; Esaunus et al., 1973, Giacin et al., 1973; Harper and Okos, 1973; Olson and Stanley, 1973; Stanley and Palmer, 1973; Wierzbicki et al., 1973a, b; c; Woychik and Wondolowski, 1972, 1973).

The well-characterized lactases from Escherichia coli have also been immobilized and tested with synthetic substrates for scientific purposes (Broun et al., 1970; Jackson and Edwards, 1973; Wingard, 1972; Zaborisky, 1973).

This paper summarizes research on the rate of hydrolysis of lactose in acid whey pumped continuously through cylindrical reactors packed with porous glass particles to which lactases from Aspergillus niger have been immobilized by diazotization of the glass surface. This work differs from previous work in the combination of the following features. First, the carrier, coupling method and enzyme were selected for optimum stability and activity in acid whey. Second, film diffusion effects on the reaction rate were evaluated. Third, both whole and deproteinized whey of various concentrations were tested as substrates. The results can be used in further study of reactor design and economic evaluation of a process using immobilized lactases to hydrolyze lactose in acid whey, acid whey concentrates, or deproteinized acid whey.

MATERIALS & METHODS

Preparation of glass-immobilized lactase (LBG)

Methods described earlier (Wierzbicki et al., 1973a, b) were used to prepare a diazonium salt derivative of porous 96% silica glass particles (86.5 nm mean pore diam, 75-125 μm particle diameter; Corning Glass Works, Corning, N.Y.) that had been silanized with 10% γ-aminopropyltrimethoxysilane in acetone by a modification of a previous procedure (Robinson et al., 1971). Lots numbers 2 and 3 of partially purified β-galactosidase (EC3.2.1.23) from A. niger (Baxter Laboratories, Chicago) were mixed and coupled to the diazotized glass in phosphate buffer of pH 6.8 containing 2% lactose to produce stable and active glass-bound lactases (LBG) that have been characterized in earlier reports (Wierzbicki et al., 1973a, b). The LBG was stored in acid whey at 4°C until use.

Substrate

In most experiments, lactose in freeze-dried acid whey powder from the Food Science Dept. was used directly or in deproteinized form. The concentration of the reconstituted whey most commonly used in these experiments contained about 4% lactose, 0.8% protein and 1% ash. The whey was deproteinized by boiling for 5 min and then decanting and filtering prior to use (Wierzbicki et al., 1973a, b). Some spray-dried whey was obtained from the Dairy Research and Development Corp., Vernon, N.Y. and reconstituted with distilled water when ready for use. Reported total solids concentrations (TS) of reconstituted whey may be in error by about 0.5% due to volumetric errors and to water uptake by whey powder before and during weighing as shown by measurements of the refractive index of the reconstituted whey.

Assay of enzymic activity

After contact with LBG, 5 ml samples of whole or deproteinized whey were heated to 80°C for 5 min to inactivate lactases, cooled and assayed for the extent of lactose hydrolysis by either one dimensional thin-layer chromatography (TLC) or by a glucose oxidase procedure (GS) which were both described earlier (Wierzbicki et al., 1973a, b).

Continuous flow reactor

Various amounts of LBG were packed between two adjustable piston-like distributors in a jacketed, isothermal chromatographic column with an internal diam of 1.6 cm (Pharmacia Fine Chemicals, Piscataway, N.J.). Whey from a flask in a constant temperature water bath (55°C unless otherwise noted) was pumped peristaltically at various constant rates downward through the column. A second pump circulated water from the constant temperature bath through the column jacket.

During assays of column activity, a third pump supplied fresh whey from a 20-liter reservoir to the flask in the water bath and the reaction effluent was collected for analysis and discarded. Between assays, the reactor effluent was returned to the supply flask in the bath and continuously recirculated through the reactor. When operating in this mode, the spent whey in the recirculating system (about 800 ml) was replaced daily with fresh whey. Periodic backwashing of the column of LBG with 2 liters of distilled water containing 5 ml toluene per liter further served to disinfect the LBG and to remove colloidal matter, including microorganisms, which accumulated during normal operation and led to a high pressure drop across the column after extended periods of operation (several weeks). The hydrolytic activity of LBG showed no apparent effects of toluene treatment.

After continuous flow studies of diffusional effects in columns packed with 1, 5 and 10.5 cm of LBG, most experiments were done with the longest column to minimize the possibility of film diffusion effects. The actual length of the 10.5 cm column decreased from an initial value of 10.8 cm to 9 cm after 3 months of use due mainly to losses of LBG during frequent backwashing. All calculations were based on the actual column length during any given experiment.

RESULTS

Consistency of assays for lactose hydrolysis

Usually both TLC and GS methods were used to determine the extent of reaction, but only GS data are reported in Figures 2, 3 and 4. Figure 1 includes all data in Figures 2, 3 and 4 and shows that TLC indicated consistently higher lactose conversions than the GS assay. Deviations were greatest at Intermediate conversions and least at high conversions. Samples at lower total solids (TS) concentrations...
showed smaller systematic differences despite dilution of all samples to similar concentrations before assay. The two methods differed by 10% or less in 20 of the 27 samples of whole whey containing 4-6% TS. Nine data for deproteinized whey showed much better agreement between the TLC and GS methods. The two methods agree very well considering

![Fig. 1 - Comparison of lactose hydrolysis determined on identical samples by both thin-layer chromatography (TLC) and a glucose oxidase method (GS); acid whey: (*) 4-6% TS; (+) 15% TS; (--) 25% TS; deproteinized acid whey: (**) 4-6% TS.](image1)

![Fig. 2 - Glucose production at various fluid residence times in column 4 (1.6 cm diam x 10.5 cm, LBG) from lactose in acid whey, pH = 4.5, T = 55°C. (---) 4% TS; (---) 5% TS; (---) 6% TS; (---) composite curve for 5.5% TS.](image2)

![Fig. 3 - Glucose production at various residence times in column 4 from lactose in deproteinized acid whey, pH = 4.5, T = 55°C, 5.5% TS. Experiment number: (*) 1; (+) 2; (---) 3, backwashed and repacked before experiment; (**) 4, approximately 2 wk of semicontinuous operation after repacking of LBG column; (-----) composite curve for 5.5% TS.](image3)

![Fig. 4 - Glucose production at various residence times in column 4 from lactose in concentrated acid whey, pH = 4.5, T = 55°C. (---) 25% TS; (---) 15% TS; (---) composite at 5.5% TS from Fig. 2; (-----) 100% hydrolysis of lactose at 5.5% TS; (------) 100% hydrolysis of lactose at 15% TS.](image4)
that the TLC method depended upon visual estimation of percent hydrolysis.

Reproducibility of continuous flow reactions
Variations in conversion at constant flow rate arise from experimental errors, concentration of lactose in the acid whey fed to the column, use of whole or deproteinized whey and channeling due to imperfect packing in the LBG column.

Figure 2 shows three separate experiments with reconstituted freeze-dried whey containing 4, 5 and 5.5% TS. Glucose concentrations were measured by the GS method. The absissa is the apparent or superficial residence time, which is the ratio of the column volume (V) divided by the flow rate (F). Except for the gradual loss of LBG during backwashing, the column volume was constant and reproducible on a single repacking within the limits of accuracy of our detection method (ruler).

The data show little scatter and trend towards the final glucose concentration expected if all lactose were hydrolyzed in whey solutions containing 4, 5 and 6% TS of which 80% by weight of the original solids was anhydrous lactose. Points for each experiment could be drawn at the origin to symbolize the absence of detectable glucose in untreated controls and the equivalent reactor conditions of infinitesimal contact times between whey and enzyme. The dashed line in Figure 2 is a composite curve of the data of the two experiments at 5 and 6% TS (average = 5.5% TS) and is thus extrapolated to the origin. At a product concentration of 10g glucose/liter, the specific activity from the composite curve for 5.5% TS is 98 μmoles/min/g dry LBG, based on 0.85g dry LBG/ml of packed column.

Figure 3 compares data of four subsequent experiments with the same column using four batches of reconstituted, deproteinated whey which originally contained 5.5% TS. The first two experiments were conducted on successive days. The lower conversion in experiment 2 may be due to a lower initial TS concentration. TS concentrations are accurate to about ±0.5%. Experiments 3 and 4 are in good agreement with the data of the earlier experiments, despite backwashing and repacking of the column, followed by about 2 wk of semi-continuous operation prior to experiment 4 and variability between batches of whey. Because no simple kinetic model fits the data, dashed lines were drawn to enclose most of the data and then a solid line was drawn by eye to maximize representation of all data in Figure 3 and to extrapolate to the origin. At a glucose concentration of 10g/liter, the specific activity from the composite curve in

Figure 3 is 78 μmoles/min/g dry LBG, based on a packing density of 0.85g dry LBG per ml of column.

Effects of total solids concentration
At equal fluid residence times in the LBG column, increased lactose concentrations increase rates of lactose hydrolysis (Fig. 2 and 4), showing the hydrolysis reaction is not zero order in whey solids concentration. Glucose concentrations determined by the GS method are reported in Figure 4, although Figure 1 shows that at the higher TS values, the TLC data indicate about 10–20% higher lactose conversions. As lactose concentration is increased, the fractional hydrolysis of lactose to glucose and galactose is less at any given residence time (Fig. 4), showing that the reaction is also not first or higher order in whey solids concentration. The same conclusions apply when conversions measured by TLC are plotted instead of the GS data.

The GS data appear to extrapolate to a common slope at low conversions and holding times, where diffusional effects and product inhibition would be least important and where lactose would also have least effect in the absence of substrate inhibition or activation. However, more experiments at high whey solids concentrations and low conversions would probably show that the reaction is not zero order in whey solids concentration (Fig. 2 and 3).

Subsequent experiments with deproteinized whey also showed higher rates of lactose hydrolysis at higher TS concentrations. The results will not be presented here because they are similar to those in Figure 4.

During these first four groups of experiments, which lasted about 2 months, the LBG column was continuously maintained at 55°C and the length decreased from 10.8 to 10.3 cm. Although a nominal value of 10.5 cm is used throughout the discussion, the actual column length was used to compute the superficial holding times reported in Figures 2–5.

Diffusional effects
Figure 5 compares glucose production in whey at equal fluid residence times for LBG columns of different lengths (Columns 1 and 2, 1 cm; column 3, 5 cm; column 4, 10.5 cm; numbers indicate the time sequence of column preparation and experimental usage). The column and experimental conditions were identical except for the use of differing amounts and batches of LBG and the use of pasteurized whey in column 1. If catalyst and reactant properties are held constant, such a comparison can be used to detect influences on the reaction rate of diffusional resistances in the fluid film surrounding the catalyst particles of LBG.
(Levenspiel, 1962). Examination of the curves and data for whole whey shows that conversions were the same for the 5-cm (triangles) and 10.5-cm (dotted line) columns within the limits of experimental error, but that lower conversions were obtained with the 1-cm column (inverted triangles) at all holding times. Confidence limits are shown as bars for one point of the six points shown for the 1-cm column, because that point was based on seven replicate measurements and the student's "t" distribution could be applied to compute the 95% confidence limits on the sample mean (Hoel, 1954). Because conversions were lower at all holding times, it is likely that the intrinsic lactase activity per unit volume was less in the 1-cm column, which was packed with an earlier preparation of LBG that was a 50/50 mix of toluene- and acetone-silanized LBG (Wierzbicki et al., 1973b). Also, TLC instead of GS assays were used to measure percent hydrolysis in column 2, so the differences between the results for columns 2 and 4 are even more significant (Fig. 1).

If film diffusion was entirely responsible for the differences in the reaction rates, the curves for columns 2 and 4 should approach the same limiting slope at sufficiently low conversions and holding times, diverging at higher residence times until very high conversions force convergence again. Because the two assay methods differ most at intermediate concentrations (Fig. 1), film diffusion is thus even less likely to be dominant. However, fluid velocities in columns 1 and 2 are 10 times less than in column 4 at any given apparent residence time (V/F), so diffusion may decrease the reaction rate in the shorter columns for the entire range of data shown in Figure 5. Alternatively, channeling might account for erroneous low conversions in short columns (Li et al., 1972). Intraparticle diffusion effects were not evaluated, but could substantially decrease the apparent activity of LBG, especially in the presence of product inhibition (Jackson and Edwards, 1973). Product inhibition has been demonstrated for A. niger lactase in soluble and immobilized form by Woychik and Wondolowski (1972).

Temperature and pH optima of lactase-BG

After more than 100 days of operation of column 4 in a stability experiment (Wierzbicki et al., 1973c), the optimum pH and temperature of the LBG column was determined in continuous flow assays for comparison with batch shake flask results, in which diffusional limitations had been shown to exist (Wierzbicki et al., 1973b). Table 1 summarizes the results of experiments conducted with deproteinized whey containing 5% TS in column 4, which by then was 9 cm long.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Glucose conc (GS OD at 405 nm)</th>
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<tbody>
<tr>
<td>29</td>
<td>0.12</td>
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<tr>
<td>41</td>
<td>0.26</td>
</tr>
<tr>
<td>55</td>
<td>0.32</td>
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<tr>
<td>59</td>
<td>0.35</td>
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The glucose concentration in the product stream was only 6.8g/liter or about 17% conversion at the highest temperature of 59°C, due to severe loss in activity during one period of microbial contamination during the stability experiment which followed the experiments already described. Kinetics of lactose hydrolysis were proportional to fluid residence times at lower conversions at all temperatures tested. Although the observed reaction rates do not necessarily obey the Arrhenius equation because no kinetic model could be found that would fit all the data presented here, an activation energy of 6.2 kcal/g mole was calculated from points at 30 and 55°C on a smoothed curve drawn through the data. Operation at 59°C was brief to avoid possible further inactivation of the LBG.

Experiments were next conducted at 55°C and V/F = 7 to 8 min with deproteinized whey containing 5.5% TS and adjusted to pH values between 3.5 (the optimum observed in batch shake flask experiments conducted between pH values of 1.0 and 9.9; Wierzbicki et al., 1973b) and 6.5, a value closer to that most useful in hydrolysis of lactose in sweet whey, skim milk, or whole milk. The highest conversions were at pH values of 3.5, with a 25% rate reduction at pH 4.5, a fourfold reduction at pH 5.5 and a tenfold reduction at pH 6.5, in good agreement with the results of batch assays (Wierzbicki et al., 1973b). Results at lower flow rates led to similar conclusions.

**DISCUSSION**

**FIGURE 5** compares composite curves obtained with column 4 for both whole (Fig. 2, dotted line) and deproteinized (Fig. 3, dashed line) whey with results obtained earlier with the same column packed with smaller amounts of LBG, including a correlation published elsewhere (Wierzbicki et al., 1973b) and data of Woychik and Wondolowski (1973). Their data was obtained with the glutaraldehyde derivative of acetone-silanized porous glass and the same commercial lactase.

**Table 1—Glucose production from deproteinized acid whey containing 5% TS by column 4 (after more than 100 days of operation) at V/F = 7.2 min and various temperatures**

**Effects of deproteinization**

Comparison of the composite curves for whole and deproteinized whey in Figure 5 shows a correlation obtained in the first series of experiments with LBG in toluene-silanized glass and the TLC assay (Wierzbicki et al., 1973b). These results (column 1, pasteurized whey, dashed line) are probably lower because of a lower specific activity of the enzyme preparation.

The data of Woychik and Wondolowski (1973) are also shown in Figure 5, showing about half the specific activity of our preparations. They used the same enzyme and porous glass of the same particle size. Differences that could explain the differences in specific activity include the use of 37°C instead of 55°C and glutaraldehyde coupling to the glass, which reacts primarily with epsilon amino groups (lysine residues) of the protein and secondarily with tyrosine, histidine and perhaps sulfhydryl residues (Tomiatsu et al., 1971). The diazotized glass used here reacts primarily with the tyrosine and other aromatic residues and secondarily with lysine residues (Jackson and Edwards, 1973). The optimum temperature and pH are very similar for both lactase derivatives. Activation energies were also similar; 6.2 kcal/g mole in this work and 4.7 kcal/g mole in theirs. Low activation energies usually imply processes controlled by diffusion or physical adsorption of the reactant and/or products, rather than the reaction itself (Satterfield, 1970). In this case, it is unlikely that the reaction is zero order (Fig. 2–5; Woychik and Wondolowski, 1972), so the low activation energies are probably due to lower reaction rates at higher conversions achieved at higher temperatures. The mixed order kinetics may be due in part to the product inhibition by D-galactose reported by Woychik and Wondolowski (1972) in 0.1M sodium acetate buffer at lactose concentrations of the same size as used here (up to 100
mM). Calculations by conventional methods (Satterfield, 1970) show that at V/F = 1 min, an average concentration difference of 10 mM lactose between the bulk solution and the LBG particles would be required to sustain a reaction rate of 80 mmole/g glass, min, which is the maximum rate observed. Columns lower than the maximum rates observed with columns 2, 3 and 4 (Fig. 5). Use of smaller particles or less active preparations of LBG would increase the effectiveness of the immobilized enzyme at the expense of higher pressure drops and possibly shorter catalyst lifetimes in the first case and larger reactors in the second.

Olson and Stanley (1973) have immobilized A. niger lactase to a phenol-formaldehyde resin by absorption, followed by cross-linking with glutaraldehyde. They obtained activities of 200 umoles glucose produced/(min, g drained enzyme resin) at pH 4.0 and 45°C using substantially larger particles (10-40 mesh). They found a pH optimum of 4.0, an increasing lactase activity up to 60°C, but 15% loss in activity of the immobilized lactase at 55°C in 16 hr contact with 0.4M lactose in pH 4.0 sodium acetate buffer. Other results were qualitatively similar to ours, and the results of tests with their immobilized lactase with acid whey will be very interesting.

Other considerations involved in practical uses

Toluene obviously could not be used as a disinfectant in food processing and was not completely effective during the long term stability test with column 4 (Wierzbicki et al., 1973c). The column was contaminated once by a mold and once by a mesophilic yeast during failure of a temperature control system while using pasteurized, toluene-free whey. The second contamination resulted in severe activity loss, but the original LBG still retained significant lactase hydrolyzing activity after 8 months of frequent use. Other solutions to the problems of sanitation are described elsewhere (Wierzbicki et al., 1973c).

The data presented here are consistent with previous and subsequent work and provide a basis for a preliminary design and economic analysis of processes using immobilized lactases to produce low lactose acid whey products. The effects of protein recovery by ultrafiltration are unknown, but prehydrolysis with immobilized lactases would decrease the difficulty of the ultrafiltration process by decreasing the molecular weight of the permeating solutes, although desalting for recovery of the more digestible sugars would be more difficult. Processes using the soluble enzyme are clearly uneconomical at present because of the enzyme cost, but the good stability of LBG suggests that the immobilized enzyme process may succeed economically as well as technologically, apart from the advantages of easier quality control and no enzymic contamination of the product. If carrier costs dominate, silica and organic carriers may offer less expensive alternatives.

REFERENCES


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