Monitoring In Vitro Enzymatic Digestion of Lactose in Milk Using Microdialysis with Pulsed Amperometric Detection

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In vitro microdialysis is used for monitoring the hydrolysis of lactose to glucose and galactose in milk. The reaction is catalyzed by Lactaid® drops, which contain β-galactosidase. A microdialysis cell designed for in vitro use is described. Use of a low molecular weight cut-off membrane as well as high perfusion rates permits direct injection and analysis of the dialysate without additional sample clean-up or dilution. Separation and detection of lactose is accomplished by high performance anion exchange chromatography (HPAEC) followed by pulsed amperometric detection (PAD). Observed rate constants for this reaction at 5° C are determined in skim (fat free) as well as whole milk. This technique is generally applicable to the study of exo- and endoglycosidases and other enzyme reactions with carbohydrate-based substrates.

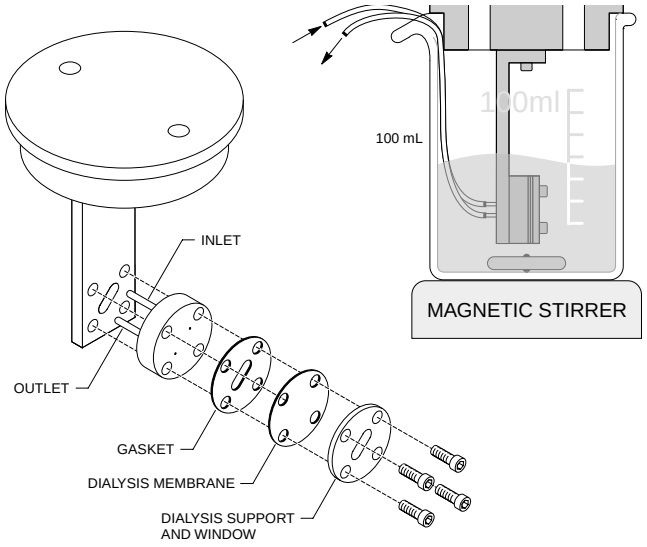
Between 30 and 50 million Americans are lactose intolerant (1). Lactose intolerance is the inability to digest significant amounts of lactose, the predominant sugar in milk. The intolerance, which originates from a diminished production of lactase by the cells of the small intestine, may manifest itself in the form of nausea, cramps, bloating, gas, and diarrhea. These symptoms are a consequence of undigested lactose, which is fermented in the colon by bacteria to metabolic gases, lactic acid, and short-chain fatty acids (1). No treatment exists to improve the body's ability to produce lactase, except through modification of one's diet. The approach is to consume foods and drinks that naturally have little or no lactose or that are enzymatically processed to reduce lactose levels (2). Hence, a better understanding of the lactase enzyme is essential to harnessing its power for health and commercial reasons.

The study of enzymes (e.g., exo- and endoglycosidases) which utilize carbohydrate substrates has been hindered by the poor optical detection properties of their substrates and/or products. Kinetic analysis has often relied upon derivatized substrates or the monitoring of side products (e.g., peroxide released from glucose oxidase reaction). These approaches can give incongruent results (different rates/kinetics for a chemically modified vs. unmodified substrate) and can be less specific (side products may be from multiple sources), respectively. Many glycosidase reactions do not even produce an easily measurable product. In addition, substrate modification and indirect methods may not be applicable to on-line process monitoring of real systems (e.g., enzymatic hydrolysis of lactose in milk).

Microdialysis has been developed and used mostly for in vivo applications, particularly in the neurosciences (3-6) and pharmacokinetic studies (5,7,8). Other than characterization studies (e.g., calibrations, use of alternative membranes) of in vivo probes external to the biosystem of study, only a limited number of in vitro applications (all using in vivo design probes) have been published (9-15). These studies have focused on microdialysis as a one-step clean up approach (typically based on size), an alternate technique to equilibrium dialysis for drug bind-

F1

Expanded view of in vitro *microdialysis cell.*



ing studies, and a sampling of carbohydrates post-hydrolysis of large substrate (i.e., mannan) enzyme systems. In each of these applications, the microdialysis probes developed for in vivo applications have been used to sample from the fruit or reactors.

A powerful feature of microdialysis is that it provides a means of continuous sampling, which makes it amenable to monitoring reactions in progress and to determining kinetics of the reaction. In addition, removing the analyte from the matrix also removes it from the reaction, in effect quenching the reaction at the moment of sampling, which is ideal for studying enzyme systems. We first utilized a prototype in vitro microdialysis cell, designed to accept a wide range of commercially available flat membranes, to study and determine kinetic parameters for the glucose oxidase reaction (16).

In this paper, in vitro microdialysis mated with high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is used for monitoring the hydrolysis of lactose to glucose and galactose in milk. HPAEC-PAD is routinely used for the separation and direct detection of carbohydrates (17-19). The reaction is catalyzed by Lactaid® drops, which contain a β-galactosidase/lactase and are used commercially to remove lactose from milk. A microdialysis cell designed for in vitro use is described. Using commonly available cellulose-based membranes with a low molecular weight cut-off (MWCO) and high perfusion rates, the loss of lactose is monitored directly. Observed rate constants for this reaction at 5° C are determined in skim (fat free) and whole milk.

Experimental Procedures

Reagents

All solutions were prepared from reagent-grade chemicals. Sodium hydroxide solutions were diluted from 50% (w/w) stock solution (J.T. Baker Inc., Phillipsburg, NJ). All mobile phases were filtered with 0.2 µm Nylon-66 filters (Rainin Corp., Woburn, MA) and a solvent filtration apparatus (Microfiltration Systems, Rainin). All mobile phases were deaerated with dispersed N_2. Water was purified using a reverse osmosis system coupled with multitank/ultraviolet ultrafiltration stations (US Filter/IONPURE, Lowell, MA). Lactaid® drops (McNeil-PPC, Inc., Fort Washington, PA) and milk samples were purchased locally and used without modification.

Voltammetry

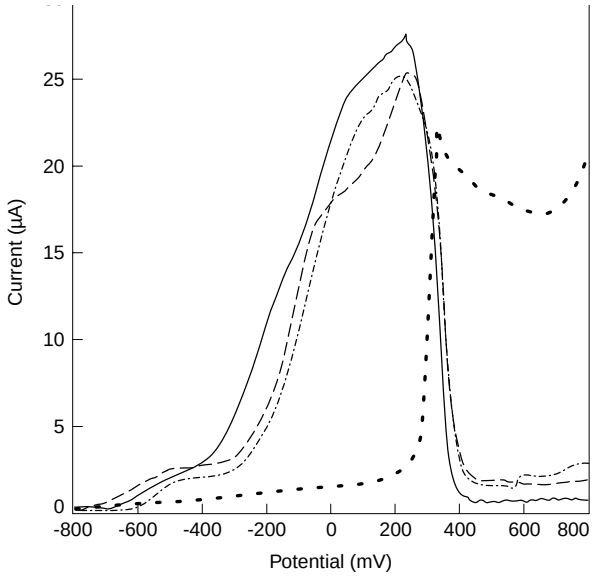
Pulsed voltammetric data were obtained at a gold rotating disk electrode (RDE) using a Model AFMSRX rotator and a Model AFRDE4 potentiostat (Pine Instrument Co., Grove City, PA). Potentiostat control and data acquisition were accomplished with a 286/16 MHz IBM™ compatible computer interfaced using a DAS-20 AD/DA expansion board (Keithley Data Acquisition, Taunton, MA). Pulsed voltammetric waveforms were generated using ASYST scientific software (Asyst Software Technologies, Inc., Rochester, NY). Pulsed voltammetry (PV) for the study of carbohydrates and optimization of PAD waveforms has been described (20). The Au RDE used was 3 mm in diameter (Pine), and the auxiliary electrode was Pt wire. All electrode potentials are reported versus an Ag/AgCl reference electrode (Model 13-620-45, Fisher Scientific, Pittsburgh, PA). The electrochemical cell (ca. 125 mL) was constructed of Pyrex glass with two side arms separated from the cell body with fine glass frits. The side arms housed the auxiliary and reference electrodes.

Microdialysis

The microdialysis system used included a cell constructed at Bioanalytical Systems (BAS, West Lafayette, IN) (***F1***). A top faceplate with the active dialysis window and a matching gasket sandwich the membrane, which is held against the platform with four bolts. The membranes used were 33 mm cellulose ester membrane discs with a molecular weight cut-off of 1000 daltons (Spectrum, Houston, TX). Inlet and outlet tubing are attached to the platform. FEP tubing (0.12 mm ID) connects a Baby Bee™ syringe pump (BAS) to the inlet port of the cell and the outlet port of the microdialysis cell to an adaptor (Upchurch Scientific, Inc., Oak Harbor, WA), then to 1/16" PEEK tubing. The PEEK tubing is connected directly to the chromatography injection valve. The entire cell is fitted into a 100-mL jacketed flask. Temperature is maintained using a circulating water bath (VWR, South Plainfield, NJ) with a portable immersion cooler. In addition, the top of the cell holder had

F2

Pulsed voltammetric response as a function of Edet for (_____) lactose, (———) galactose, and (-.-.-.-.-) glucose at an Au RDE in 100 mM NaOH. Residual response (• • • •) shown for reference. Background substracted responses shown for 0.1 mM solutions at a rotation speed of 900 rpm.



two holes—one to accept a thermometer and the other to allow for the introduction of liquids (e.g., enzyme solution) by micropipet.

Chromatography

HPAEC was performed on an advanced gradient chromatography system (Dionex Corp., Sunnyvale, CA). Separations were done with a CarboPac-PA1 anion-exchange analytical column (Dionex), preceded by a CarboPac-PA1 guard column at a flow rate of 1.00 mL/min. A second injection valve was placed in-line before the microdialysis injection valve for manual injection of standards. PAD was accomplished using an LC-4C Amperometric Detector (BAS). Output of waveform and data collection were controlled by ChromGraph® software (BAS) interfaced using a DA-5 ChromGraph Interface (BAS) to a 486 IBM™ compatible computer. The detection cell was comprised of a 3 mm Au working electrode, an Ag/AgCl reference electrode (MF-2021, BAS) and a stainless steel auxiliary electrode. The cell was housed in a grounded CC-5 liquid chromatography column and cell compartment (BAS). All injection volumes were 10 μL unless otherwise noted.

Procedure

Before use, each membrane was rinsed with de-ionized water, then mounted carefully in the microdialysis cell, and the cell was placed in a stirred beaker of deionized water. The cell was perfused with water, and injections of dialysate were made to insure no contamination in the cell. The cell was then placed in the jacketed flask, filled with 80 mL of milk or other standard solution. Standard solutions were prepared in phosphate or citrate buffers. The cell was perfused with deionized water and injections of dialysate were made until the peak heights were constant. The peak heights were quantitated against external standards, and the percent recovery for each compound determined. Performance of a membrane was described as % recovery of a particular analyte, where

$$\% \text{ recovery} = \frac{\text{concentration of dialysate}}{\text{concentration of sample}} \times 100$$

After establishing the % recovery of the membrane for each analyte, enzyme was added and injections were made every ten minutes beginning at time zero, unless otherwise noted. An internal standard, deoxyglucose (d-glc), was added to all samples. This compound is not a substrate for β-galactosidase, and elutes prior to the substrate and products. The % recovery for the internal standard was determined along with the analytes of interest, and this value is used to correct for variations in % recovery over time.

Results and Discussion

Pulsed Voltammetry

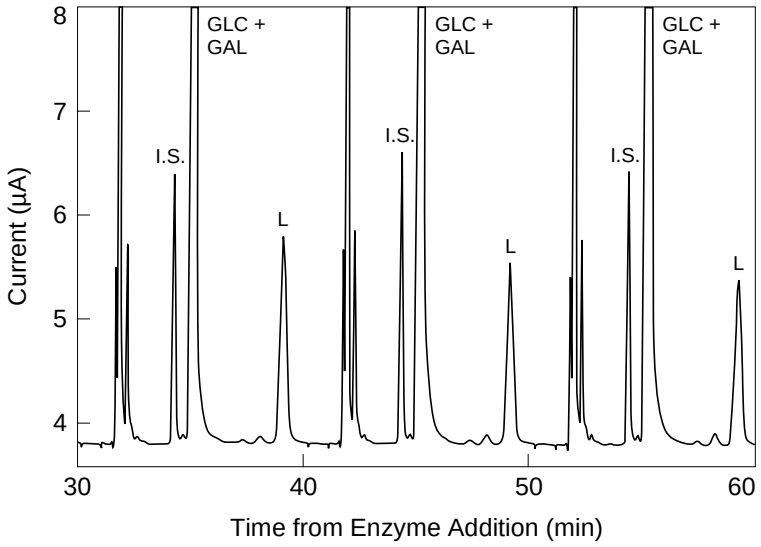
Pulsed voltammetry (PV) is used to determine detection parameters for PAD (20), changing one PAD parameter over a range of conditions while holding others constant. ***F2*** shows the PV response of lactose (_ _ _ _ _) at an Au RDE in degassed 100 mM NaOH. This plot is background corrected, and the background is shown (. . . . .) for clarity. The anodic current commencing at ca. -600 mV corresponds to oxidation of the aldehyde group on the reducing end of lactose. The increased current beginning at ca. -400 mV and continuing through ca. + 400 mV corresponds to oxidation of hydroxyl groups. Attenuation of lactose oxidation current is due to onset of gold oxide formation. Galactose (———) and glucose (_ . _ . _ . _) behave similarly to lactose, so only one waveform is needed for optimal detection of virtually all carbohydrates. The waveform used in this paper is comprised of a detection step (+150 mV) held for 420 ms, during the last 200 ms of which current is collected, an oxidation step (+800 mV) held for 180 ms, and a reduction step (-800 mV) held for 340 ms.

HPAEC-PAD

Separation conditions were chosen in order to have resolution of d-glc (internal standard) and lactose from peaks corresponding to Lactaid® components and products of the reaction. Under these conditions, lactose elutes in 8.93 min (k'= 5.29). Deoxyglucose, the internal standard, elutes in 4.15 min (k'=1.92). The responses due to d-glc and lactose are linear from 1 mM to their LODs (over three orders of magnitude). Concentrations greater than 1 mM were not tested. Limits of detection were 1 ng (0.6 μM) and 3 ng (0.8 μM) for d-glc and lactose, respectively. Correlation coefficients (r^2) over this range are greater than

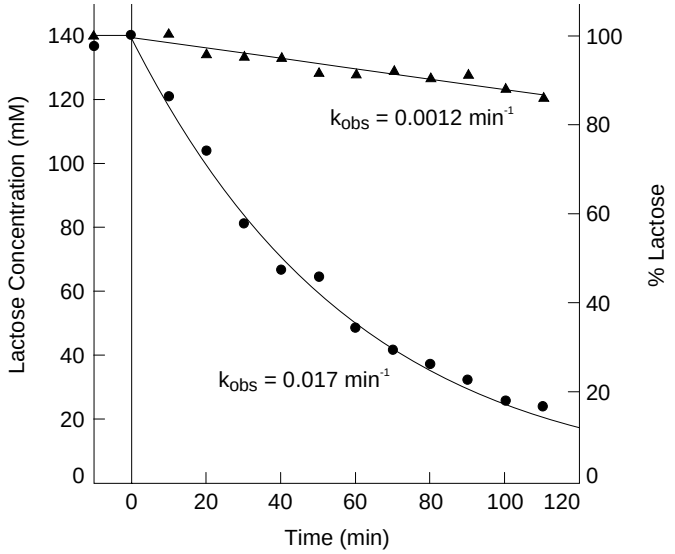
F3

Sequential chromatograms of lactose (L), unresolved glucose (GLC) and galactose (GAL), and internal standard (I.S.) from lactase reaction (enzyme added at t=0, 0.5 mL Lactaid®) in skim milk.



F4

Plot of extent of lactase reaction showing decrease in concentration of lactose at (●) 0.5 and (▲) 0.05 mL Lactaid® in skim milk.



T1

Summary of recovery data.

conditions	deoxyglucose (n=6)		lactose (n=6)	
	% recovery	% rsd	% recovery	% rsd
standard[a]	1.51	4.0	0.30	13.5
standard[a]	1.32	3.0	0.22	4.7
standard[b]	1.52	1.7	0.24	1.7
average	1.45	2.9	0.25	6.6
skim milk	2.72	5.4	0.44	4.3
skim milk	2.62	4.2	0.43	4.2
whole milk	2.59	5.4	0.40	4.5
average	2.64	5.0	0.42	4.4

[a]100 mM phosphate buffer, pH 6.62.
[b]100 mM citrate buffer, pH 6.62, 23 mM Ca^{2+}.

0.99994 for both compounds. Repeatability for each compound is below 1% relative standard deviation. These statistics are consistent with published values (16,21).

F3 shows a chromatogram of sequential injections of dialysate for lactose hydrolysis in skim milk. Note that the lactose peak is diminishing with time. Separation of glucose from galactose was not attempted in order to minimize the time between injections. Quantitation of the coeluting compounds would be possible since the ratio of glucose and galactose from the reaction is constant. However in this case, the "coeluted" peak was not quantitated due to its response being out of the linear range of the detector.

Microdialysis System

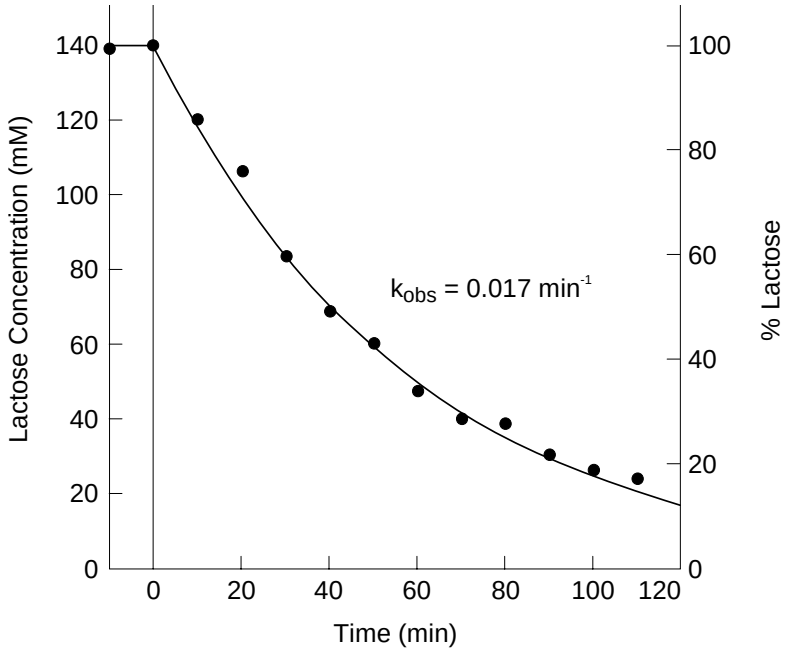
The amount of lactose in milk is on the order of 4.6%, or 140 mM. In order to use the sample "as is," the approach is to minimize the % recovery by using a low MWCO membrane (i.e., 1000 daltons) and a high perfusion flow rate (10 μL min^{-1}). Membrane selection was also based on previous work with oligosaccharides (16). ***T1*** shows that the recoveries for d-glc and lactose in milk were 2.64 and 0.42%, respectively. Even at these low % recoveries, the RSDs are relatively low (less than 5% on average) and acceptable for the analysis. Interestingly, the % recoveries of d-glc and lactose standards in buffer are on the order of 50% less than in the presence of milk (see ***T1***). At this perfusion rate (10 μL min^{-1}), the 10 μL sample loop of the injection valve filled in only 1 min.

Enzyme Reactions

The prescribed use of Lactaid® drops is 5-15 drops per quart of milk, depending on personal lactose sensitivity, with incubation in the refrigerator (ca. 5° C) for 24 hours. ***F4*** plots (▲) the first two hours of data for lactose sampled from skim milk under these conditions (e.g., 15 drops/quart, 5° C). The corresponding concentration-time curve was fitted to a first-order exponential decay curve

F5

Plot of extent of lactase reaction showing decrease in concentration of lactose at 0.5 mL Lactaid® in whole milk.



(i.e., $y = a \cdot e^{-bx}$); where a is the initial concentration and b is the observed rate constant. If desired, the first 10% of this curve can be used to determine the initial velocity of the reaction. Under these conditions, the observed rate constant is 0.0012 min^{-1}, and the total hydrolysis of lactose would require at least 24 hrs. This agrees well with the instructions on the Lactaid® bottle.

The rate of hydrolysis can be accelerated by increasing the amount of enzyme. ***F4*** also shows the reaction plot (●) for a ten-fold greater amount of Lactaid,® or 150 drops. As expected, the observed rate constant was determined to be 0.017 min^{-1}. The curve denotes that after only two hours, more than 84% of the lactose had been hydrolyzed. A similar experiment which used whole milk had an observed rate constant of 0.017 min^{-1} (***F5***). Hence, the presence of milk fats has little effect on the enzyme reaction.

An attempt was made to study lactase in the absence of the milk matrix. Using conditions optimal for lactase (i.e., 100 mM phosphate buffer, pH = 6.62) (22), the rate in the absence of the milk matrix was an order of magnitude less. Similar results were obtained using a 100 mM citrate buffer, pH = 6.62 which contained Ca^{2+}—a necessary cofactor required for enzyme activity. This reduced observed rate constant effect is attributable to stabilization of the enzyme by milk components (23).

Conclusions

The in vitro microdialysis cell described here is an alternative to commercially available probes designed for in vivo use. The microdialysis cell allows control over membrane composition, temperature, hydrodynamics, and active dialysis area. It is more rugged than the commercially available probes and can be made from materials tolerant of harsh environments. Microdialysis coupled to HPAEC-PAD has been used to monitor lactose during its enzymatic digestion in milk by lactase. By following the time dependence of the concentration of substrate of the reaction instead of the product, false kinetics due to interfering substrates producing the same products are more easily avoided. This technique is easily adaptable to monitoring online processes.

Acknowledgments

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