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New Procedure To Readily Investigate Lactase Enzymatic Activity Using Fehling's Reagent

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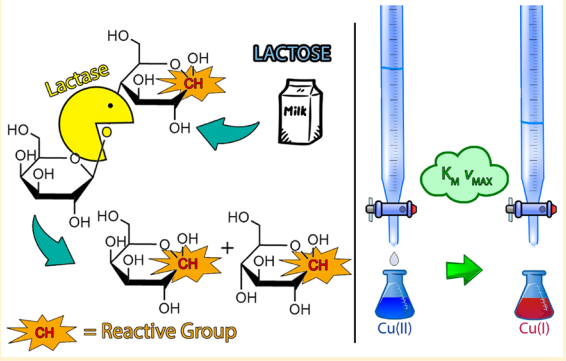
Supporting Information

ABSTRACT: Determination of enzymatic activity is a relevant experiment in teaching chemistry and biochemistry to high school and college students. A method for measuring the kinetic parameters of lactose cleavage by the enzyme lactase is described. This enzyme catalyzes the hydrolysis of lactose to galactose and glucose. The kinetic parameters can be determined easily and quickly by titration using Fehling's test for reducing sugars. In this way, it is possible to determine the rate of the reaction without using any instruments.



KEYWORDS: *Second-Year Undergraduate, Inquiry-Based/Discovery Learning, Biochemistry, Bioanalytical Chemistry, Bioorganic Chemistry, Enzymes, Kinetics, Titration/Volumetric Analysis*

INTRODUCTION

The determination of enzyme kinetic parameters is a relevant experiment in low-level chemistry and biochemistry courses. Not only can students become familiar with kinetic experiments and the Michaelis−Menten equation, but also they can experience different methods to collect and analyze experimental data. This experiment was conducted in the last year of an Italian high school that specializes in chemistry. Before that, these students had been studying chemistry for four years. This level is probably comparable to a two-year college in the United States that offers classes for first- and second-year undergraduate students. Lactase (EC 3.2.1.23) is a β-galactosidase enzyme that catalyzes the hydrolysis of lactose to galactose and glucose. The lack of or the shortage of this enzyme causes digestion problems and intolerance in a significant fraction of the population. Lactase deficiency affects about 20% of the population in Northern Europe and the United States, up to 95% of the population of Asia and about 75% worldwide.[1,2] Avoiding milk derivatives is the most common strategy to manage lactose intolerance. Still, problems may arise because lactose is present not only in dairy products, but also in many drugs and several types of food (e.g., meat, bread, frozen food). When avoidance of lactose-containing food is not possible or desired, enzymatic lactase supplements may be administered to patients. Such relevance in everyday life makes the study of lactase more interesting and stimulating for the students.

Methods for the determination of kinetic parameters of lactase and similar enzymes were already reported in this *Journal*.[3,4] The experimental procedures were based on the cleavage of *o*-nitrophenyl-β-D-galactopyranoside (Figure 1, structure **I**) to release *o*-nitrophenolate, a yellow compound, which is easily quantified by UV−vis spectroscopy.[3,4]

2- and 4-Nitrophenolesters are commonly used to study enzymes and to screen biomimetic catalysts (Figure 1).[5,6]

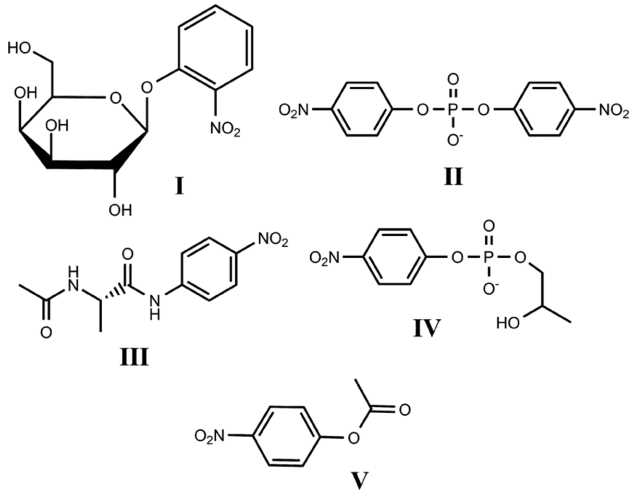


Figure 1. Examples of activated substrates used for the study of hydrolytic enzymes.

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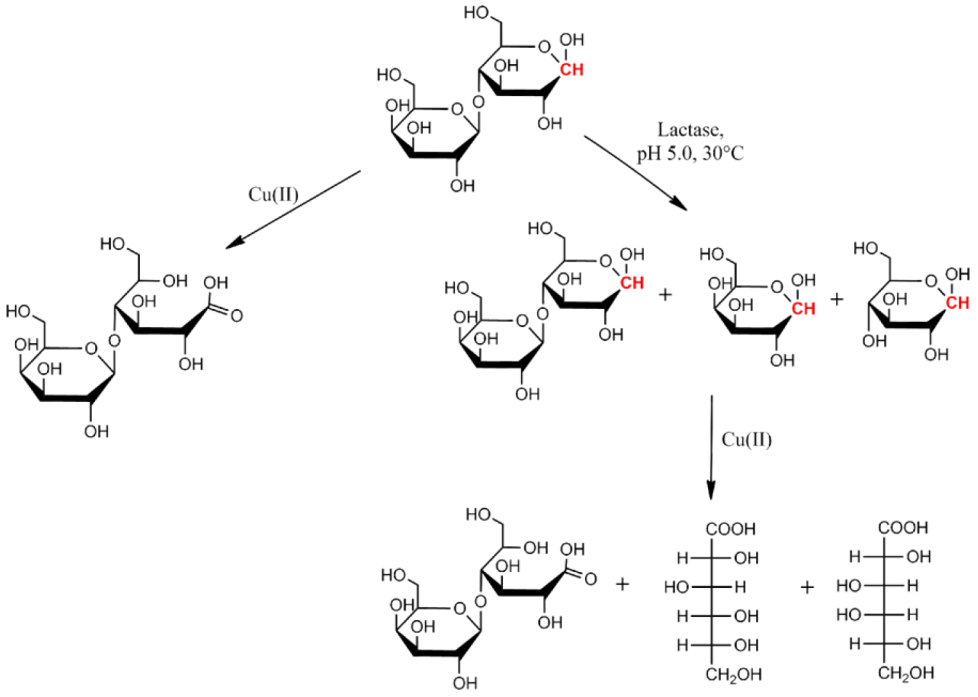
Scheme 1. Oxidation of Sugars in the Absence (left) and in the Presence (right) of Lactase[a]



[a]Reducing groups are written in red.

These compounds allow the rapid investigation of hydrolytic catalysis to obtain the relative kinetic parameters. However, they have a much better leaving group with respect to the natural substrate. This may change the reaction mechanism and consequently affect the determined kinetic parameters. Indeed, results obtained were quite different from those reported with the natural substrate.[7−9] As an example, the K_M obtained using **I** was 1.7 mM,[4] a value that is quite different from the one reported for lactose (18 mM).[10]

Later, the use of a blood glucometer was proposed to measure the quantity of glucose produced by the hydrolysis.[11,12] The data obtained in this way needed a postmeasurement correction because glucometers are calibrated to work in the blood matrix, and they are not accurate in aqueous buffered solution.[11]

Herein, a quick and easy method is described to determine the amount of hydrolysis products, and consequently the kinetic parameters of lactase, by titration. As long as the hydrolytic reaction takes place, the number of reducing groups present in the sample increases progressively (Scheme 1). Lactose has only one reducing group, because only the glucose unit is in equilibrium with the aldehydic open-chain form. Upon the hydrolysis, both the glucose and galactose products are reducing. The overall concentration of lactose, glucose, and galactose is related to the extent of cleavage and can be determined by titration with a basic solution of Cu(II)−tartrate complex (Fehling's method).[13−16] The reducing sugars present are oxidized by Cu(II) forming corresponding gluconic acid and copper(I) oxide.

The titration with Fehling's reagent is quite accurate and precise. It is commonly used in analytical laboratories for determining reducing sugars contained in fruits and derivatives (wines, juices, worts, etc.).[17] Measuring the total saccharide concentrations after a fixed time allows for the collection of reaction rates at different initial lactose concentrations. Data can then be fitted according to the Michaelis−Menten model. The experiment is addressed to students of biochemistry or analytical chemistry courses in a high school. Students can take advantage of their knowledge of analytical chemistry, organic chemistry, and biochemistry in a single experiment.

STUDENT LEARNING OBJECTIVES

The key learning objectives are to

- Familiarize students with the Michaelis−Menten equation
- Carry out a redox titration in a precise and accurate way
- Interpret and analyze experimental data
- Work in teams/groups in order to reach a common outcome

The improvement of the students was evaluated through an observation of them during the activity, through students' surveys, and a summative test that concentrates on enzymatic kinetics (see the Instructor Information in the Supporting Information).

EXPERIMENTAL PROCEDURES

The experiment was included in the biochemical chemistry course of a chemistry-focused high school in 2016, 2017, and 2018. In total, 54 students from three classes participated to the laboratory activity. Students of each class were divided into nine groups. The experiment was divided into two parts, each one lasting 2 h. In the first, each group prepared a solution with a different lactose concentration, incubated it at pH 5.0 (acetate buffer) and 30 °C for 15 min, added Na_2CO_3 to raise the pH (and quench the reaction when lactase is present), and measured the concentration of lactose contained by titrating a known amount of Fehling reagent. Methylene blue was employed as indicator (added just before the end point), and the titration was considered complete when the color of the

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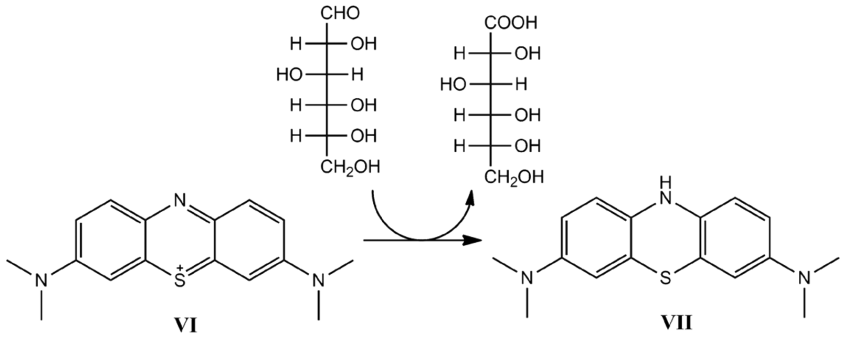
Scheme 2. Reduction of Methylene Blue: Oxidized Form (VI) and Reduced Form (VII)



solution turns from blue to red. The end point of the titration is signaled because all copper(II)-ions are consumed and the sugars reduce the indicator, giving leucomethylene blue (Scheme 2, structure **VII**, colorless), so copper(I) oxide (red) will be visible. To consider the titration a reliable source of information, it had to occur in less than 3 min. These expedients are necessary to limit oxygen interference and to minimize evaporation of Fehling's solution.[16]

It is relevant to stress that in the Fehling's procedure the analyte solution is added to the titrant solution. Such a procedure is used to make it easier to determine the end point, but the counterintuitive outcome is that more concentrated saccharide solutions require smaller added volumes. In the second part of the experiment, a solution of commercial lactase was added to the lactose-buffered solution. The mixture was treated as the first and then used to titrate the Fehling's reagent.

Lactase hydrolyzes lactose to glucose and galactose, both containing a reducing group; consequently, the second titration measured a greater concentration of reducing groups than the first titration. The difference between the reducing units' concentrations before and after the reaction corresponds to the amount of cleaved substrate (see the student handout in the Supporting Information). In this way, the initial rates for the reaction at different initial concentrations of the substrate can be measured. Every titration was carried out at least three times, and the end point volumes of reducing sugars solutions used were averaged. The dispersion of the end point volumes was in any case within 0.1 mL.

HAZARDS

The experiment uses dilute solutions, which can be prepared in advance by laboratory assistants to minimize the risk of pure compounds and concentrated solutions to students' health. Labeled containers for wastes must be available. Gloves, lab coats, and safety glasses must be worn throughout the experiment. All laboratory activities must be carried out under the supervision of trained and qualified personnel. Copper(II)-ion solution (Fehling A) may cause eye and skin irritation; it may be harmful if it is absorbed through the skin, swallowed, or inhaled. Sodium hydroxide (Fehling B) is corrosive: it may cause eye and skin burns as well as severe damage to the digestive tract if ingested. Methylene blue hydrate is toxic if it is swallowed. The diluted acetate buffer and potassium sodium tartrate solutions used for the preparation of the reactive Fehling B have no known hazards. The heating plates (set at the temperature of 180 °C) may cause burns if accidentally touched. In order to move the Erlenmeyer flasks necessary for the titration, it is necessary to use a pair of metal pliers. The solutions containing lactose, glucose, and galactose could be dangerous only if ingested in high quantity.

RESULTS AND DISCUSSION

A spreadsheet (see the .xslx file in the Supporting Information) was provided to students to determine the kinetic parameters. Each group shared the obtained titration values. Initial rate values (r), at a different initial lactose concentration, were obtained by calculating the amount (concentration) of substrate cleaved and dividing it by the reaction time. The initial rate values versus initial substrate concentrations data were reported in the spreadsheet and used to plot the Michealis–Menten profile. Data were then linearized to obtain the Lineweaver–Burk plot (see the student results in the Supporting Information). Linear regression fitting of the data provided the Michealis–Menten parameters V_{MAX} and K_M. A detailed discussion of the Michaelis–Menten models has already been reported[11] and is provided in the Supporting Information. The quality of the fit was very good, as demonstrated by the R^2 value of 0.990, 0.993, and 0.994 (see Figures II3b, II6b, and II7b in the Supporting Information) obtained, respectively, by the first, second, and third classes. The kinetic parameters obtained were, respectively, $K_M = 25 \pm 3$ mM and $V_{MAX} = 0.50 \pm 0.05$ mM min^{-1} by the first class, $K_M = 35 \pm 3$ mM and $V_{MAX} = 1.6 \pm 0.2$ mM min^{-1} by the second class, and $K_M = 46 \pm 3$ mM and $V_{MAX} = 1.6 \pm 0.2$ mM min^{-1} by the third class (the last two data points were obtained with triple the quantity of enzyme, see the student results in the Supporting Information). K_M values are comparable to each other and with the values found in the literature under different pH, buffer, and temperature conditions.[10,18,19]

Only one group of students ($N = 18$) was not able to obtain a correct plot because of an erroneous use of the spreadsheet. After the correct procedure was re-explained, this group also obtained the correct plot.

The improvement of the students was evaluated through an observation of them during the activity (see the evaluation grid in the Instructor Information in the Supporting Information) and through a conclusive text that concentrates on enzymatic kinetics. After the second titration, it was noticed that students' skills were improved, mostly in the use of pipettes and burets. The marks of the laboratory part rose from 6.2 to 7.3 in a 1–10 range from the first to the second titration. Indeed, students carried out the subsequent titrations in a more precise way because it was observed that the dispersion between the measures had decreased by about 20% just during the second experiment. The final test results (questions 1–4 of the Supporting Information) were compared with the results of other two classes that did not carry out the experiment: the

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J. Chem. Educ. 2018, 95, 1238–1242

average of the marks rose from 6.1 to 6.7 in a 1−10 range (see Instructor Information in the Supporting Information).

Reagents used in the experiment are quite inexpensive. The average cost for each student was about 4 € (~$5). The total cost of this experiment (equipment excluded) was about 75 € (~$93), and only basic equipment and glassware for analytical chemistry were used. The total cost of the experiment is considerably lower when compared to the literature experiment.[3,4] The first titration on the prepared lactose solution is useful to help students become familiar with the method and end point (change of color) detection. Performing this experiment, students could verify Michaelis−Menten kinetics and reinforce their skills in titration methods and data analysis. The Michaelis−Menten law's trend was clearly visualized by students through the rate versus substrate concentration graph (see the student results in the Supporting Information). In fact, at the experimental lactose concentrations, students could observe the curvature of the kinetics rate. Moreover, it could be noticed that students, thanks to this experiment, improved their ability to work in a team. Indeed, the experience greatly increased the positive interdependence within the class because each student clearly perceived how the work was relevant, not only for the results, but also for the realization of the common outcome. Every group obtained a single point of the Lineweaver−Burk plot in order to achieve the final graph. Advice and know-how were exchanged between the groups during the experiment.

CONCLUSION

During this experience, students learned that it is possible to follow a chemical reaction by measuring the concentration variation of selected functional groups. They also learned basic principles of kinetic investigations. This experiment used a standard and well-known analytical technique to study the reactivity of an enzyme of high social interest. Indeed, students already knew about lactase from everyday experience, such as use by lactose-intolerant people in restaurants, and news items in newspapers and on television. As a didactic item, students had the opportunity to combine tools from different fields of chemistry such as analytical chemistry, biochemistry, and organic chemistry. The experiment received positive feedback from the students, with comments such as these (translated by the authors):

"I enjoy that we used a drug we had bought in a pharmacy."
"In the restaurant menu there are some foods for people who suffer from lactose intolerance: now I understand how important lactase is!"
"In my family, there are people who take this enzyme before meals".
The fact that the procedure has been demonstrated to be reliable enough to provide kinetic values in good agreement with the literature was also a point of satisfaction for the students.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.7b00637.

Data analysis, concentration data and students' results, evaluation rubric, and results of students' assessments (PDF, DOCX)

Detailed procedure of the experiment for the students, including a list of chemicals and materials for the experiment and hazards (PDF, DOCX)

Spreadsheet for the students (XLSX)

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Notes

The authors declare no competing financial interest.

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