

# QUANTITATIVE DETERMINATION OF LACTASE

LENORE JASEWICZ AND AARON E. WASSERMAN

Eastern Regional Research Laboratory, USDA, Philadelphia 18, Pennsylvania

## SUMMARY

A simple and quick assay is described for the determination of the lactase activity of crude enzyme preparations. Results are available in 2 hr. from as little as 2 mg. of material. Fifteen minutes' incubation at 37° C. is used for the enzymatic reaction on a pure lactose substrate. The lactase activity is related to the amount of glucose released by the hydrolysis, as determined by an enzymatic micro-method. The effects of substrate concentration, pH, temperature, and related factors are investigated. The assay procedure was advantageously employed in a preliminary quantitative screening of lactase-producing organisms which could utilize whey in their growth.

Lactase, the enzyme that hydrolyzes lactose to glucose and galactose, is of potentially great importance to the dairy industry. In the course of establishing a screening program to find the best microbial sources of the enzyme, it became apparent that a rapid, but sensitive, lactase assay was necessary.

The few methods cited in the literature for testing commercial lactase are quite lengthy and complicated. In each, a 4-hr. hydrolysis step is followed by chemical determination of the hydrolysis products. The lactase acts upon a substrate of 30% condensed skimmilk in two of the methods (5, 9) and upon a 10% lactose solution in the other (7). The resulting mixture of reducing sugars is extremely difficult to analyze, particularly in the presence of condensed skimmilk components. Potter and Webb (5) chose to determine the intensity of the molybdenum blue color caused by the glucose-galactose mixture and reported the degree of lactose hydrolysis.

This paper describes some preliminary work on the production of crude lactase and presents a procedure by which small amounts of the crude enzyme preparations can be accurately analyzed in less than 2 hr. The enzyme reacts upon pure lactose for 15 min., then the hydrolysis products are analyzed for glucose by the specific enzymatic technique developed by Salomon and Johnson (6).

## MATERIALS AND METHODS

*Propagation of organisms.* Lactase is generally found in microorganisms growing in milk or other products containing lactose. Many organisms, therefore, were isolated from such sources. These were propagated on a supplemented whey medium prepared as follows:

Cheese whey was autoclaved 15 min. at 15-lb. pressure and the precipitated proteins removed by filtration through cheese cloth. The clear solution was adjusted to pH 7.0 with concentrated KOH and again autoclaved. The precipitated residue was removed by filtration through coarse paper. The following materials were added to the clear solution:

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	—	1.0% (w/v)
K <sub>2</sub> HPO <sub>4</sub>	—	0.5% (w/v)
Dry yeast extract	—	0.1% (w/v)

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The medium was adjusted to the desired pH and autoclaved in Fernbach flasks in 500-ml. volumes. These were inoculated with suspensions of cells washed from nutrient agar slants containing 0.5% lactose. The cultures were agitated 18 hr. on a reciprocating shaker at 28° C. The heavy growths were centrifuged, the cellular residue washed with distilled water and recentrifuged.

TABLE 1  
Optimum temperature for lactase activity

° C.	$\mu M$ glucose liberated
20	13.9
25	24.4
30	33.4
37	46.7
45	41.2

*Preparation of crude lactase.* Lactase is an endoenzyme; therefore, whole cells exhibit very little lactase activity. It is necessary to treat the cells to release the enzyme. By destruction of the cell permeability with acetone, the total lactase activity is quickly made available for assay purposes. The centrifuged cells were reconstituted in 200 ml. distilled water and slowly added to 400 ml. cold acetone with constant vigorous mixing. After 15 min. in an ice bath, with occasional stirring, the treated cells were filtered through paper, washed first with 200 ml. cold acetone and then with 50 ml. ethyl ether. The crude material was air-dried on the filter paper and refrigerated in a desiccator until assayed.

Yields of the crude enzyme preparations varied considerably. Many of the organisms were well adapted to growth in the whey medium and yielded many cells in 18 hr. A few did not. However, since only 10-50 mg. of a preparation was needed for the lactase assay, sufficient quantities were obtained.

*Enzyme assay.* The procedure consists of two steps: (a) hydrolysis of pure lactose by the test preparation and (b) the micro-determination of glucose in the hydrolysis products.

1. Lactose hydrolysis. A tentative lactose hydrolysis method was followed before the standard assay conditions could be established. The enzyme reaction is performed in test tubes in a water bath at 37° C. One milliliter of a 5% (0.139 *M*) lactose solution in 0.1 *M* potassium or sodium phosphate buffer of pH 6.3 and 0.8 ml. of the buffer solution are mixed and equilibrated in the bath. Two-tenths milliliter of a distilled water suspension of the dried lactase (10 mg. per milliliter) is added, bringing the total test volume to 2.0 ml. After a 15-min. incubation period, the activity is stopped by heating the tubes in a boiling water bath for 5 min. The cellular residue is removed by centrifugation, and the supernatant is analyzed for glucose.

2. Glucose determination. The composite reagent of Salomon and Johnson (6) is used for the determination of glucose. To 700 mg. *o*-tolidine dihydrochloride in a few milliliters of water add 150 ml. 2 *M* acetate buffer of pH 4.1-4.2. Dissolve 200 mg. glucose oxidase and 15 mg. horseradish peroxidase in a few

milliliters of buffer (both obtainable from Sigma Chemical Company, St. Louis, Missouri.)<sup>1</sup> Add to the *o*-toluidine solution and make up to 300 ml. with the buffer. Filter to remove turbidity and store at 5° C.

In the presence of glucose, the following reactions occur:

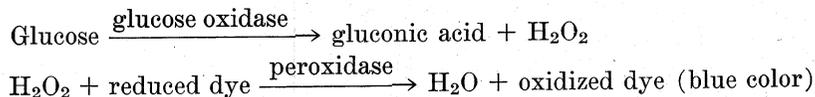


TABLE 2  
Lactase activity of various organisms

Organisms	Lactase units/mg dry weight
Bacterium A	7.56
Bacterium B	13.4
Yeast A	14.25
Yeast B	19.8
<i>Aerobacter cloacae</i>	2.89
<i>Escherichia coli</i>	5.0
<i>Saccharomyces fragilis</i>	2.2

To determine the concentration of glucose in the lactase-hydrolyzed lactose solutions, 2 ml. of 1:20 and 1:40 dilutions of the supernatants are placed in test tubes. At the same time, a standard curve is prepared by diluting a glucose stock solution (1 mg/ml) to cover a range of 10-100  $\gamma$  glucose per 2 ml. One and a half milliliters of composite reagent is added to all the tubes, which are then incubated at room temperature (25  $\pm$  2° C.). Color development in the samples is visually compared to the standards at intervals. If the blue color developed is considerably deeper than the most concentrated standard, higher dilutions of the hydrolyzed supernatant are necessary. After 1 hr. the solutions are transferred to cuvettes and read in a spectrophotometer at 630-635 m $\mu$ . The amount of glucose present is determined from the standard curve.

The Salomon and Johnson composite reagent gave consistent color development at 25° C., but incomplete color formation has been noticed at 30° C., especially in the absence of light. A new standard curve should be prepared for each new batch of composite reagent. Several reference standards should be included in each assay.

#### RESULTS AND DISCUSSION

Several factors of importance in the study of enzyme reactions were investigated in establishing conditions for the lactase assay procedure. The standard lactase preparation used was an unidentified bacterium isolated from a milk agar plate exposed to air. The organism grew well on whey medium and produced lactase of high activity.

*Effect of substrate concentration.* The rate of the lactase reaction was studied as a function of the lactose concentration. The reaction mixture con-

<sup>1</sup>It is not implied the USDA recommends the above company or its product to the possible exclusion of others in the same business.

sisted of 5 mg. crude enzyme in several concentrations of lactose in 0.1 *M* sodium phosphate buffer, pH 7.0. The results plotted in Figure 1 are for micromoles glucose liberated by 5 mg. lactase in 30 min.

*Effect of enzyme concentration.* In enzyme assay procedures it is desirable that the relationship between the quantity of enzyme used and the amount of product released also be linear. Figure 2 shows this. Distilled water suspensions of the acetone-dried cells were used to hydrolyze solutions of 25 mg. pure lactose per milliliter under the conditions described. Saturation of the enzyme within this range of substrate did occur. From these data the choice of 1 mg. enzyme per milliliter of assay solution was justified.

*Effect of time.* A straight-line relationship between the time allowed for lactase activity and the amount of lactose hydrolyzed was also desired. To ascertain whether this was achieved under standard assay conditions, a time

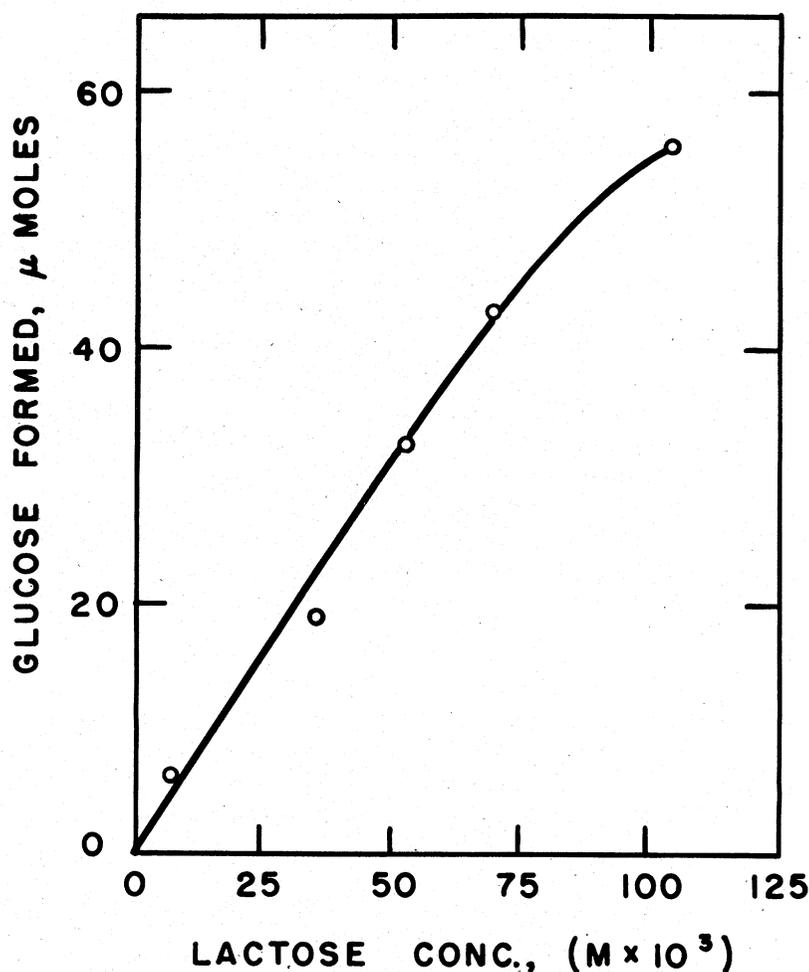


FIG. 1. Lactase activity as a function of substrate concentration.

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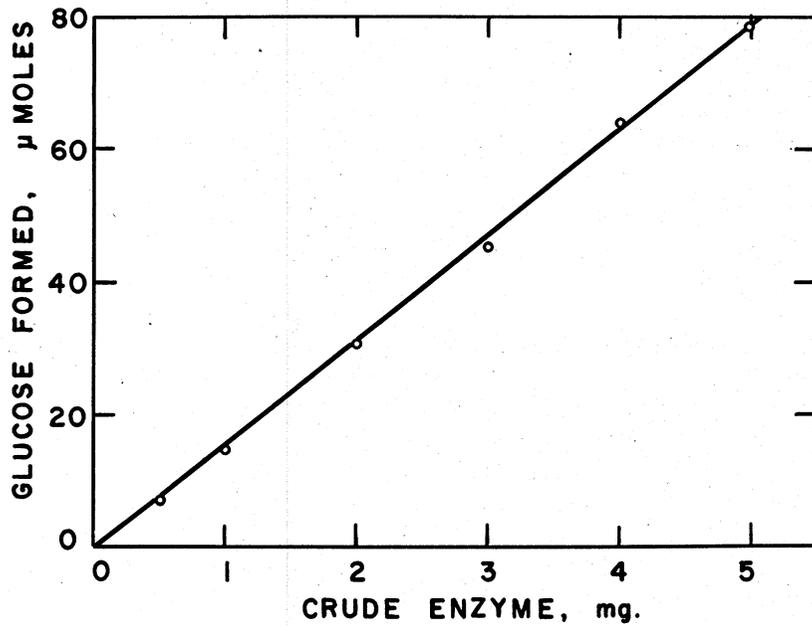


FIG. 2. The effect of enzyme concentration on the rate of lactose hydrolysis.

curve was run using 1 mg. enzyme per milliliter of assay solution. Samples were incubated, quickly inactivated, and measured for glucose formation at frequent intervals during a 2-hr. period. Figure 3 is a plot of these data. Although the rate of hydrolysis was linear during a 1.5-hr. period, it was decided to determine the activity of the enzyme during the initial 15-min. period, when the rate

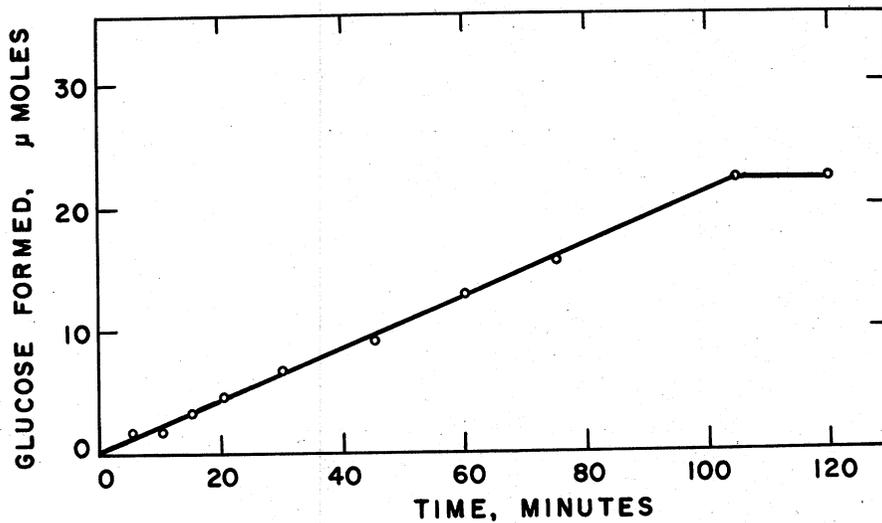


FIG. 3. Time course for hydrolysis of lactose by crude lactase preparation.

more closely represented the true reaction velocity. This would be of importance with enzyme preparations of greater activity.

*Effect of temperature.* The effect of temperature on the rate of lactase activity was investigated. Temperatures studied were those easily available in the laboratory. The lactose and buffer components of the reaction mixtures and the cell extracts were equilibrated separately at the selected temperatures for 30 min. before mixing and timing the assay. Results given in Table 1 point to the selection of 37° C. for the standard assay conditions. The decline in enzymatic activity at 45° C. may possibly indicate heat inactivation of the enzyme.

*pH and buffer.* The optimum pH for lactase varies with the enzyme source. Cajori (1) reported an optimum of 5.4 to 6.0 for the intestinal lactase of dog. Cohn and Monod (2) selected a pH of 7.0 for *E. coli* lactase ( $\beta$ -galactosidase) activity, while Wallenfels *et al.* (8) observed maximum at pH 7.3.

The optimum pH for the enzyme preparation used in these experiments was ascertained by using 0.1 M potassium and sodium phosphate buffers of various pH levels in the assay procedure. The 5% (0.139 M) lactose substrate and the lactase suspension were prepared in distilled water for these tests. Figure 4 shows that maximum hydrolysis occurred with a pH range of 6.3 to 6.6. Since activity was sharply inhibited on either side of pH 6-7, a buffer of pH 6.5 was selected for the standard assay.

It has been observed previously that the presence of the potassium ion strongly activated *E. coli* lactase (2, 8). This was also found to be true with

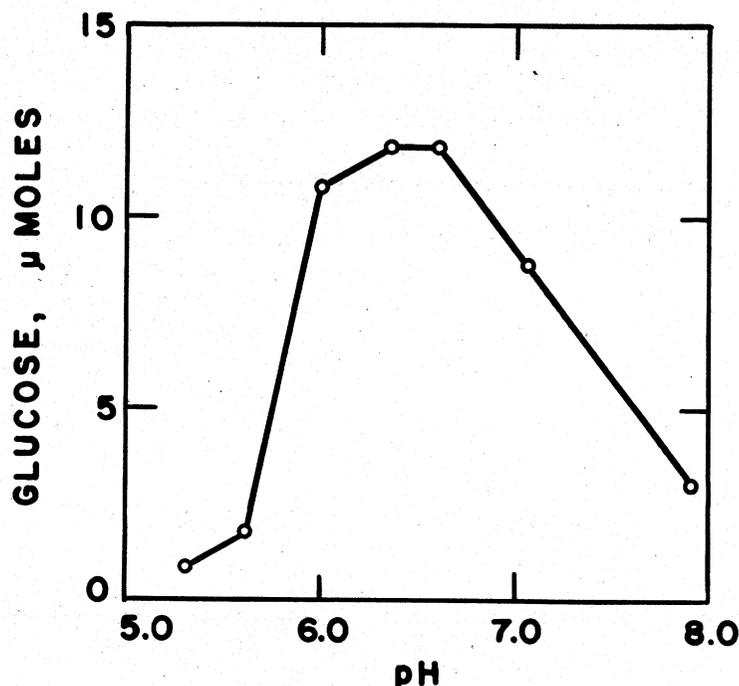


FIG. 4. Effect of pH on lactose hydrolysis by crude lactase.

the enzyme preparations tested in this study. When lactase preparations from two yeast and two bacteria were compared in 0.1 *M* sodium phosphate and 0.1 *M* potassium phosphate buffers, the activity in potassium phosphate was almost three times as great.

As a result of the above studies the hydrolysis conditions for the standard assay of lactase were selected. One milliliter of a 5% (0.139 *M*) lactose solution and 0.8 ml. 0.1 *M* potassium phosphate buffer of pH 6.5 are mixed and equilibrated in a 37° C. water bath. Two-tenths milliliter of lactase suspension is added. After 15 min. at 37° C. the enzyme action is stopped by heating for 5 min. in a boiling water bath. The centrifuged supernatant is analyzed for glucose.

A unit of lactase is defined as the amount of enzyme which will liberate 1  $\mu$ *M* of glucose from lactose in 15 min. at 37° C.

Pazur (4) reported that lactose hydrolysis by lactase results in the formation of varying quantities of oligosaccharides in addition to glucose and galactose, depending on the conditions of the hydrolysis. The effect of oligosaccharide formation in this determination was not investigated. It was assumed for the purposes of achieving a simple and rapid assay that a measure of the amount of glucose formed was also a measure of the degree of lactose hydrolysis.

*Use of standard assay in screening lactase producing organisms.* A routine quantitative screening procedure utilizing the assay was initiated to detect lactase producing organisms that could grow on the modified whey substrate. Both known and unidentified lactose-utilizing bacteria were grown on the supplemented whey medium under the conditions described. These were not the best growth conditions for each strain, so yields often were quite small. However, even as little as 2 mg. of the acetone-dried lactase could be assayed. Yield of cells, therefore, was of no consideration in prejudging enzyme activity. After a potent lactase producer is revealed, its optimum growth conditions can be determined.

Approximately 60 organisms were investigated in this preliminary study. Of these, 30 were able to grow on a whey medium and produced enough cell material to be assayed at 1 mg. per milliliter concentration. During the assay test all preparations that gave only a faint blue color in the 20-fold dilution were eliminated from further consideration. The enzyme activity of these organisms was less than one unit per milligram dry weight.

Several organisms not yet completely identified give promise of being excellent producers of lactase from whey. These are compared with lactase similarly prepared from known organisms in Table 2.

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