Academy of Sciences—National Research Council for the Quartermaster Food and Container Institute for the Armed Forces, and Pioneering Research Division, Quartermaster Research and Engineering Center, May 1957, pp. 86–100.
(7) Kjaer, A., Rubenstein, K., *Acta Chem. Scand.* **7,** 528 (1953).
(8) Konigsbacher, K. S., Hewitt, E. J., Evans, R. L., *Food Technol.* **13,** 128 (1959).
(9) Mackay, D. A. M., Hewitt, E. J., *Food Res.* **24,** 253 (1959).
(10) Mackay, D. A. M., Hewitt, E. J., Bazinet, M. L., Bailey, S. D., Abstracts, page 35A, 134th Meeting, ACS, Chicago, September 1958.
(11) Reese, E. T., Clapp, R. C., Mandels, M., *Arch. Biochem. Biophys.* **75,** 228 (1958).
(12) Schwimmer, S., Carson, J. F., Makower, R. U., Mazelis, M., Wong, F. F., *Experientia* **16,** 449 (1960).
(13) Schwimmer, S., Guadagni, D. G. *J. Food Sci.* **27,** 94 (1962).
(14) Schwimmer, S., Weston, W. J., J. AGR. FOOD CHEM. **9,** 301 (1961).
(15) Virtanen, A. I., Matikkala, E. J., *Acta Chem. Scand.* **13,** 1898 (1959).
(16) Weurman, C., *Food Technol.* **15,** 531 (1961).

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LACTASE ACTIVITY MEASUREMENT

Evaluation of Lactase Preparations for Use in Breadmaking

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The lactase activity of preparations from yeast, fungal, and bacterial sources was determined by measuring manometrically the carbon dioxide formed by yeast fermentation of enzymatically hydrolyzed lactose. The effects of substrate concentration, temperature, and pH on the activity of lactases from fungal and bacterial sources were studied. The measured response was governed by amount and activity of tested lactases and was essentially linear for the enzyme preparations employed. The procedure was advantageously employed in an evaluation of lactases from various sources from the standpoint of their use in panary fermentation.

TWO PRINCIPAL METHODS are used for determining β-galactosidase (lactase) activity (*3*). In one method, *o*-nitrophenyl-β-galactoside (ONPGal) is used as substrate, and the freed *o*-nitrophenol is determined colorimetrically. This method has been extensively used to determine the β-galactosidase activity in bacterial sources. However, the affinities of enzymes from different sources for the substrate vary considerably (*18*). The affinity of β-galactosidase from *Escherichia coli* for *o*-nitrophenyl-β-galactoside is 10 times as large as for lactose (*6*); for other bacterial sources, it is three times as large (*11*). A commercial fungal lactase (*12*) shows hydrolysis rates of *o*-nitrophenol-galactoside higher than hydrolysis of lactose by a factor of about 100 times. Consequently, the results obtained by action of the enzyme on ONPGal cannot be transposed directly to the rate of hydrolysis of lactose. In the second assay method, lactose is used as substrate and the hydrolysis is measured by one of several techniques. Tauber and Kleiner (*16*) developed a colorimetric method for the determination of monoses in the presence of bioses. However, large amounts of lactose interfered with the determination of the hydrolytic products of lactose. Glucose formed from hydrolysis of lactose also can be determined by a gasometric method employing a Warburg apparatus (*3*).

During a survey of different sources of lactase and their application in bread baking (*10*), the authors deemed it desirable to evaluate the lactase activity of various preparations under conditions compatible with those found during panary fermentation. The method used is based on the fact that *Saccharomyces cerevisiae* neither assimilates nor ferments lactose (*4*). By employing yeast, a buffered nitrogen base medium, and the glucose which is split off from lactose by lactase as the only source of energy for yeast fermentation, the amount of carbon dioxide evolved is measured manometrically, using pressuremeters (*14*).

Experimental

Materials. The six lactases used in this study were: a crude bacterial extract containing 24 mg. protein per ml. (*1*), a lyophilized extract of *E. coli* containing about 5% lactase (*13*), a crude extract of lactase from *E. coli* (*2*), a commercial enzyme preparation from yeast (*9*), and two fungal, A and B, commercial enzyme preparations having optimum activities over pH ranges of 6.0 to 7.0 and 4.5 to 5.5, respectively.

Apparatus. Pressuremeters (*14*) were employed to determine lactase activity. The manometers were filled with ethyl lactate (1.031 grams per ml. at 20° C.) colored with crystal violet (*8, 17*) to provide high sensitivity.

Reagents. Reagent grade lactose, galactose, and glucose (chromatographically pure).

Nitrogen base medium for carbon assimilation tests with yeast prepared in the laboratory according to the formula in the Difco Manual (*5*).

Yeast suspension, 7.5 grams in 95 ml. of water, prepared from Fleischmann's wet cake baker's yeast.

Analytical procedure. Crystalline lactose was weighed in one side of the aluminum cup of the pressuremeter. At 1 minute intervals, 10 ml. of nitrogen base medium, 10 ml. of yeast suspension, 10 ml. of 0.1*M* phosphate buffer solution, aliquots of enzyme suspension, and water to give a total volume of 40 ml. of liquid were added. The contents were mixed well prior to tightening the lids, and the pressuremeters placed in a water bath at 30° C. (except for the series of studies on temperature effect on lactase activity). After 5 minutes, the pressure was released; and after equilibration for 15 minutes, four pressure readings were taken at 30-minute inter-

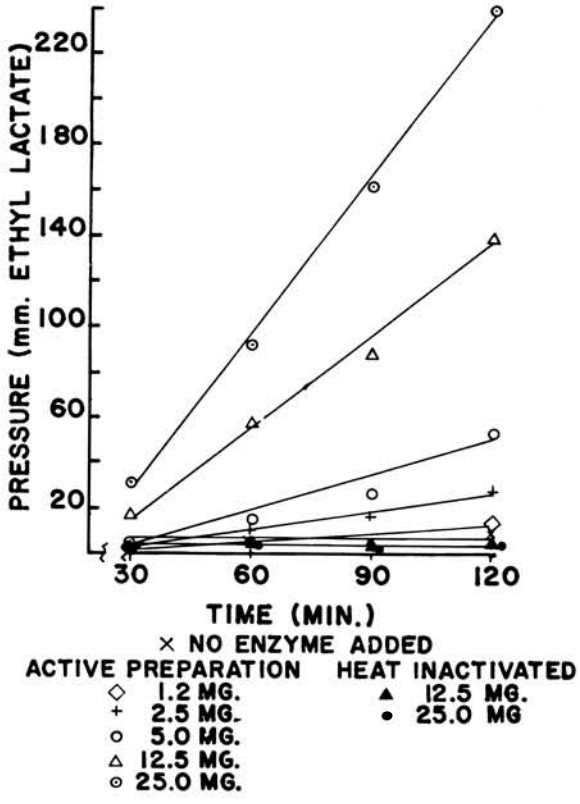

Figure 1. Fungal lactase activity as influenced by digestion time and enzyme concentration

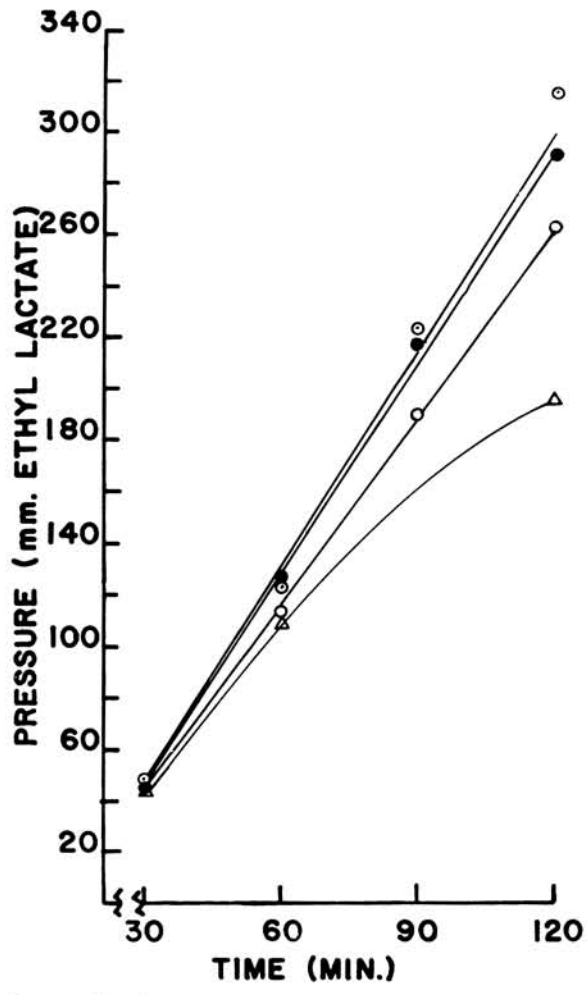

Figure 2. Fungal lactase activity as influenced by digestion time and substrate concentration

(substrate added: △ 1 gram, ○ 2 grams, ● 5 grams, ⊙ 10 grams)

vals. The pressure was recorded as the increase from that measured at the end of the 15-minute equilibration period. This time between initial release of pressure and the 15-minute reading permitted the ethyl lactate to drain to a stable level in the capillary tube.

Results and Discussion

Digestion time and enzyme concentration. Figure 1 shows the effects of various levels of active and heat-inactivated (boiled 15 minutes on a water bath) lactase preparations per 40 ml. of total reaction volume on the availability of glucose for yeast fermentation. There was no release of glucose by heat-inactivated preparations, and an essentially linear response from active lactase. These data are in agreement with previous findings (*15*) which show that lactases from various sources obey the monomolecular law at least during the first part of the reaction.

Since the net effect of two separate systems, hydrolysis of lactose by lactase and fermentation of the free hexose by yeast, is measured in the proposed procedure, the rate of each of these two major reactions must be considered. Instead of using lactose, a mixture of 1 gram of glucose and 1 gram of galactose was used as substrate for yeast fermentation. Use of this mixture of monosaccharides, in the absence of lactose and lactase, resulted in a pressure of 7211 mm. ethyl lactate, which necessitated use of mercury filled manometers to take the readings. This constitutes a 30-fold increase over the pressure of gas evolved by use of 25 mg. of the potent fungal lactase which was used in obtaining the data given in Figure 1. The rapid utilization of glucose by yeast in the presence of galactose shows that the rate of lactose hydrolysis is limiting and that as soon as glucose is available, it is rapidly fermented by yeast. Thus, the method as used measures essentially the rate of lactose hydrolysis.

Substrate Concentration. The effect of substrate concentration on lactase activity is given in Figure 2. The results show that increasing the amount of substrate resulted in increased carbon dioxide which reached a maximum at about 5 grams of lactose per total reaction volume of 40 ml. This amount was used in all subsequent experiments.

Digestion Temperature. The literature indicates that maximum lactase activity is attained at 49° to 53° C. and that at 58° C. inactivation of yeast lactase takes place (*9*). Similar data for fungal and bacterial lactases are not available. Such high temperatures, however, likely affect adversely some of the moieties involved in yeast fermentation. Data given in Figure 3 show the composite effect of temperature on fungal (Rohm & Haas Co., preparation A) lactase activity and yeast fermentation. Because of technical difficulties, only the temperature range between 5° to 44° C. was covered. The results (Figure 3) confirm the advantage of employing higher temperatures for increased hydrolysis of lactose by the enzyme.

Influence of pH. To follow the effect of pH on optimal lactase activity, a series of buffered solutions of lactase preparations from various sources was examined. Changes in pH took place as a result of fermentation. The data presented in Figure 4 refer to pH measurements taken at the end of the 2-hour fermentation period. The optimal pH determined is not that for lactase action alone, but for the whole system involving the hydrolysis of lactose by lactase and subsequent fermentation of glucose by yeast.

The enzyme preparation from various sources varied widely in their activity.

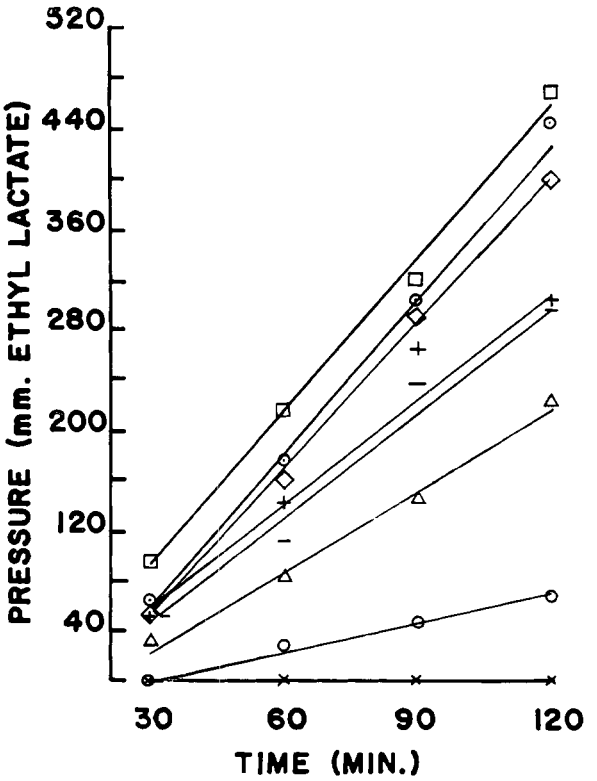

Figure 3. Fungal lactase activity as influenced by digestion time and temperature

(× 5° C., ○ 17° C., △ 23° C., — 30° C., + 34° C., ◇ 37° C., ⊙ 40° C., □ 44° C.)

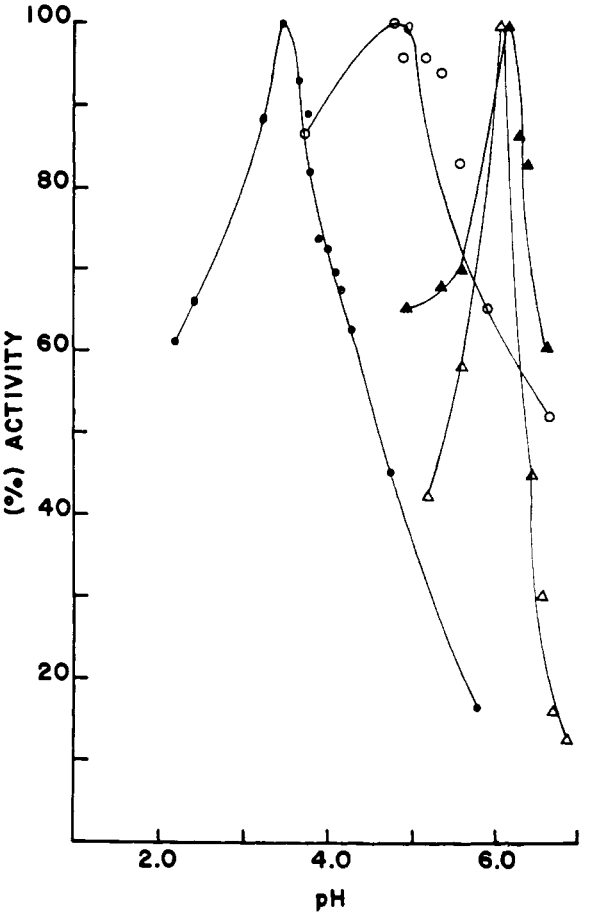

Figure 4. Lactase activity as influenced by pH

(·——· fungal lactase A; O——O fungal lactase B; ▲——▲ bacterial lactase A; △——△ bacterial lactase B)

The data given in Figure 4, therefore, refer to percentage activity, assuming the highest activity for each preparation at optimal pH to be 100%. The results of testing only four of the six tested preparations are given. One of the preparations was inactive under conditions of the experiments. The sixth preparation, lactase of bacterial origin, showed essentially the same results as the other two bacterial preparations.

Determination of β-galactosidase activity from bacterial sources with *o*-nitrophenyl-β-D-galactoside as substrate and in a solution buffered at pH 7.0 (*7*) or 7.25 (*3*) has met with wide acceptance. The enzyme isolated from yeast also has its pH optimum at 7.0, but animal lactase has its maximum activity at pH 5.4 to 6.0 (*14*) and almond β-galactosidase acts best at pH 4.2 (*15*).

When fermentation of lactose by yeast in the absence of lactase was studied, there was hydrolytic breakdown within the range of pH 2.1 to 3.3, whereas in the range of pH 3.3 to 6.1, the buffer had no effect on lactose. Beyond pH 6.1 (up to pH 7.2) there developed a negative pressure, probably due to the higher solubility of carbon dioxide in the solution buffered at the high pH values. These effects were compensated for in plotting the data in Figure 4. The results represent the net effect of pH during a 2-hour fermentation period on enzymatic hydrolysis of lactose by lactase and fermentation of the glucose by yeast. The results show that, whereas in the presence of bacterial lactases an optimal fermentation was achieved at pH 6.1 to 6.2, the use of fungal lactases permits maximum carbon dioxide evolution at lower pH levels which are more compatible with those present during panary fermentation.

One unit of lactase activity is defined as the amount of enzyme preparation in mg. dry weight required to increase the pressure 1 mm. under the established conditions of the test. A comparison of lactase activity of the various preparations gave the results summarized in Table I.

Figure 5 shows lactase activity of graded increments of three of the lactase preparations. Limited amounts of experimental material prevented including comparative results for the other two active bacterial preparations.

The results show that the method as established from these experiments permits one to distinguish between the lactase activities of various preparations. The response was essentially linear for the less active preparations and curvilinear for the more potent lactase. In the latter case, the response over the lower range of concentrations was essentially linear. The results obtained by this method permitted the use of enzyme preparations in baking with equivalent results when adjusted to a common lactase activity. The advantage in using this method is its simplicity and use of conditions which approximate those encountered during breadbaking. The manometers used are those which are usually available in cereal and baking laboratories.

Table I. Lactase Activity of Various Preparations

Source	*Lactase Units per Mg. Dry Weight*
Bacterial extract from *E. coli*	1.4
Crude bacterial preparation	2.7
Lyophilized bacterial preparation from *E. coli*	14.0
Fungal lactase A	10.5
Fungal lactase B	2.7
Yeast lactase	Below 1

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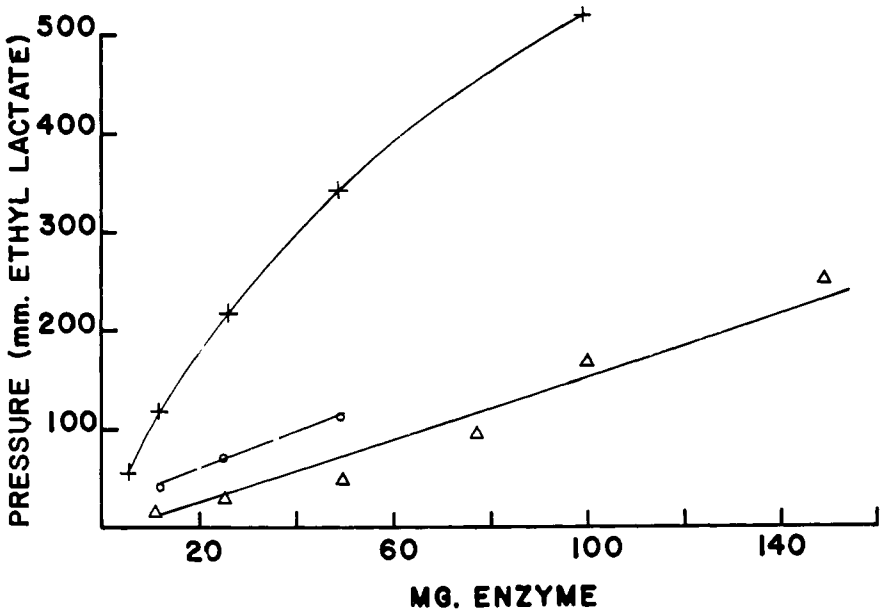

Figure 5. Fungal and bacterial lactase activity as influenced by enzyme concentration

(× fungal lactase A; ○ fungal lactase B; △ bacterial lactase)

Rickenberg, University of Indiana, Bloomington; B. Rotman, Veterans Administration Hospital, Albany, N. Y.; National Dairy Products Corp., Glenview, Ill.; and Rohm & Haas Co., Philadelphia, Pa. They furnished the bacterial extract from *E. coli*, the crude bacterial extract, the lyophilized extract of *E. coli*, the preparation from yeast, and the two fungal preparations, respectively.

Literature Cited

(1) Anderson, J. M., Rickenberg, H. V., *J. Bacteriol.* **80,** 297 (1960).
(2) Cohn, M., Horibata, K., *Ibid.*, **78,** 601 (1959).
(3) Colowick, S. P., Kaplan, N. O., "Methods in Enzymology," Vol. **1,** p. 241, Academic Press, New York, 1955.
(4) de Becze, G. I., *Wallerstein Lab. Commun.* **23,** 99 (1960).
(5) Difco Manual, 9th ed., p. 251, Difco Laboratories, Inc., Detroit, Mich.
(6) Dixon, M., Webb, E. C., "Enzymes," p. 508, Academic Press, New York, 1958.
(7) Hu, A. S. L., Wolfe, R. G., Reithel, F. J., *Arch. Biochem. Biophys.* **81,** 500 (1959).
(8) Linko, P., J. AGR. FOOD CHEM. **9,** 310 (1961).
(9) National Dairy Products Corp., Glenview, Ill., private communication, December 12, 1960.
(10) Pomeranz, Y., Miller, B. S., Miller, D., Johnson, J. A., *Cereal Chem.* In press.
(11) Rickenberg, H. V., University of Indiana, Bloomington, private communication, November 28, 1960.
(12) Rohm & Haas Co., Philadelphia, Pa., private communication, January 16, 1961.
(13) Rotman, B., *J. Bacteriol.* **76,** 1 (1958).
(14) Sandstedt, R. M., Blish, M. J., *Cereal Chem.* **11,** 368 (1934).
(15) Sumner, J. B., Somers, G. F., "Chemistry and Methods of Enzymes," 3rd ed., p. 111, Academic Press, New York, 1953.
(16) Tauber, H., Kleiner, I. S., *J. Biol. Chem.* **99,** 249 (1932).
(17) Umbreit, W. W., Burris, R. H., Stauffer, J. F., "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1957.
(18) Wallenfels, K., Malhotra, O. P., in "Advances in Carbohydrate Chemistry," Vol. **16,** p. 259, Academic Press, New York, 1961.

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ASCORBIC ACID MEASUREMENT

Polarographic Determination of Total Ascorbic Acid in Foods

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NUMEROUS METHODS for the estimation of ascorbic acid have been described. In general, these are based on decolorization by ascorbic acid of certain oxidation-reduction indicators. Of these, the one most widely used is the 2,6 - dichlorophenolindophenol (2,6-DCIP) titration. Unfortunately, this reagent is not specific for ascorbic acid as it is also reduced by sulfhydryl compounds, reductones, and various inorganic cations.

Roe and coworkers (*15, 16*) have described a colorimetric method based on condensation of oxidized ascorbic acid (dehydroascorbic acid) with 2,4-dinitrophenylhydrazine (2,4-DNPH) to produce a red color in sulfuric acid. This method also lacks the desired specificity and is somewhat tedious.

In recent years, the development of the polarographic method (*3, 6, 11–14*) has provided another approach to the problem of ascorbic acid analyses. The principle advantages associated with this method are its specificity, comparative rapidity, and applicability to highly colored solutions which otherwise are not easily analyzed by titrimetric or colorimetric procedures.

Unlike ascorbic acid, dehydroascorbic acid is not oxidized by the dropping mercury electrode, and hence cannot be determined polarographically. Since both the dehydro and reduced forms of ascorbic acid are physiologically active, a limitation of the polarographic method is its inability to determine total ascorbic acid.

A method is described for the polarographic determination of total ascorbic acid. It is based on reduction of dehydroascorbic acid to ascorbic acid with homocysteine, treatment with *N*-ethylmaleimide to remove the interference of homocysteine, and subsequent polarographic determination of the total amount of ascorbic acid present. This method has been applied to a variety of food products, and the results have been compared with total ascorbic