Instrumental Assay of Microbial Lipase at Constant $pH^1$

CHARLES L. SAN CLEMENTE AND DHARAM V. VADEHRA

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan

Received for publication 15 August 1966

ABSTRACT

A rapid, accurate method with high sensitivity and reproducibility, and having the advantage of a short incubation period under constant $pH$, has been developed for routine measurement of microbial lipase. Assembled from readily available and economical instrumental components, the apparatus includes a $pH$ meter, a thermoelectric heating and stirring device, a motor-driven buret, and an automatic recorder. The reaction mixture, consisting of 5 ml of a 10% olive oil-gum arabic emulsion, 2 ml of 3 M NaCl, 2 ml of sodium taurocholate (15 mg/ml) of 0.075 M CaCl$_2$, 5 ml of water, and 1 ml of enzyme solution, was adjusted to $pH$ 8.0 and 37 C. The $pH$ was maintained at a constant value by automatic addition of 0.01 N NaOH during the incubation period, which usually lasted 5 min. A lipase unit, derived from the use of this technique, may be defined as the number of microequivalents of acid liberated per minute under the specified conditions. The method was sensitive to 0.01 units. Various organisms tested produced 0.17 to 1.32 units per ml of the cell filtrate. An Arrhenius plot for staphylococcal lipase yielded 14,500 cal for function A (energy of activation).

Several methods for the assay of microbial lipase have been proposed, but comparative values of lipolytic activity are difficult to evaluate because of the variation in parameters (3) employed, such as the assay conditions: preparation and stabilization of the emulsion, time, $pH$, the degree of fat dispersion, and temperature of incubation. Among the several methods currently in common use, one of the following basic techniques was employed: (i) chromogenic substrates, Foster et al. (4); (ii) manometric procedures, Willart and Sjostrom (8); (iii) surface tension reduction, Dunkley and Smith (2); and (iv) spectrophotometric determination, Börgström (1).

The object of this report is to present a rapid instrumental method especially developed to permit meaningful comparisons of lipase determinations from several laboratories.

MATERIALS AND METHODS

Cultures. Various organisms (Bacillus cereus, Achromobacter butyri, Brevibacterium linens, Pseudomonas fragi, P. fluorescens, and Staphylococcus aureus) were cultivated in Trypticase Soy Broth (BBL) for 48 hr at 37 C. Cell-free supernatant fluid was used for assay of lipase.

Enzyme extraction. Partially purified staphylococcal lipase (6) was prepared from cell-free supernatant fluids by alcohol precipitation, redissolution in water, and sequential fractionation at $pH$ 8.6 and 4.3; column chromatography with Sephadex G-200 was used in the final step.

Substrate. The lipid emulsion was prepared by blending 20 ml of quality olive oil (Fisher Scientific Co., Pittsburgh, Pa.), 165 ml of 10% gum arabic, and 15 g of ice.

Reaction mixture. The reaction mixture consisted of 5 ml of the lipid emulsion, 2 ml of sodium taurocholate (15 mg/ml), 2 ml of 3 M NaCl, 1 ml of 0.075 M CaCl$_2$, and 5 ml of deionized water. Finally, 1 ml of the lipase solution was added at the appropriate time.

Apparatus. The instrumental assembly (E. H. Sargent and Co., Detroit, Mich.) was composed of a thermoelectric temperature-regulating magnetic stirrer, a motor-driven buret, an automatic recorder (model SR), and a $pH$ meter (Zeromatic Beckman, model 96; Beckman Instruments, Inc., Fullerton, Calif.). The electrical impulses from the $pH$ meter were transferred to the recorder input, which in turn activated the motor-driven buret through a microswitch to maintain a constant $pH$ of the assay mixture.

Assay procedure. By means of the $pH$ meter, the recorder was set at the predetermined optimal value of the particular lipase ($pH$ 8.0 in this case). This setting required a minor adjustment of the microswitch. A 1-ml amount of the lipase preparation was added to the reaction mixture (15 ml) previously

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1 Published with the approval of the Director of the Michigan Agricultural Station as Journal Article 3907.
conditioned to the desired temperature (37°C). A 5-min period was usually allowed for the determination; volume of the 0.01 N NaOH was recorded from the digital readout. A lipase unit is defined as the number of microequivalents of acid produced per minute under the conditions specified.

Effect of pH. Lipolytic activity of staphylococcal lipase was measured at various pH values (6.68, 7.48, 8.02, 9.11, and 10.05) maintained by automatic addition of standard alkali for 5 min at 37°C. Relative lipase activity was calculated for each pH condition with reference to pH 8.02, which was arbitrarily assigned a value of 100%.

Effect of unbuffered reaction mixture. A 1-ml amount of purified staphylococcal lipase was added to the reaction mixture predosed to pH 8.0 and 37°C. The change in pH by the action of lipase was recorded continuously for 1 hr.

Effect of time. The relationship between time and lipolysis was determined by continuously recording the acid produced over a 15-min period. The experimental conditions were the same as described for the assay procedure.

Effect of temperature. With all other conditions kept constant as described, the effect of temperature upon lipolysis was measured at 5° intervals from 25 to 50°C.

Effect of lipase concentration. By use of the assay procedure previously outlined, a series of measurements was made with different amounts of lipase preparation to determine the nature of the correspondence between lipase concentration and microequivalents of acid produced.

Lipase activities of various microorganisms. A 1-ml amount of cell-free supernatant fluid from cultures of various organisms was assayed for lipase activity at 37°C and pH 8.0 for a period of 5 min.

RESULTS

Effect of pH on lipase. The pH of the reaction profoundly affected the lipase activity (Table 1). A change of 1 unit from the apparent optimal value caused a 75% reduction in activity.

Effect of unbuffered reaction mixture. The maximal change in pH occurred during the first 10 min (Fig. 1). However, acid production con-
temperature (Fig. 3A) under the conditions specified was about 45 C. However, when the data were plotted according to Arrhenius (Fig. 3B), the optimal temperature became closer to 40 C. Surprisingly, a straight line was not obtained. The graph expressed a discontinuity of the slope which approximates two straight lines, meeting at a point from which the optimal temperature was determined. The line with the negative slope permitted a calculation of 14,500 cal. per mole for the Arrhenius function A (energy of activation).

Table 2. Lipolytic activity of cell-free supernatant fluids obtained from 48-hr-old cultures grown in Trypticase Soy Broth at 37 C

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lipase (units per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>0.27</td>
</tr>
<tr>
<td>Achromobacter butyri</td>
<td>0.60</td>
</tr>
<tr>
<td>Brevibacterium linens</td>
<td>0.17</td>
</tr>
<tr>
<td>Pseudomonas fragi</td>
<td>1.32</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>0.48</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Effect of lipase concentration. The amount of lipase solution used in the reaction mixture was varied from 0.1 to 1.0 ml, and the data obtained from such an experiment are shown in Fig. 4, for which the microequivalents of acid produced were calculated from the digital units on the automatic titrator. A straight-line relationship existed between the two parameters.

Lipase activity of various microorganisms. The lipolytic capacity per milliliter of the cell-free supernatant culture fluid from several microorganisms was determined (Table 2). In the group

![Fig. 3(A). Effect of temperature (25 to 50 C) upon lipolysis (microequivalents μEq) for 5 min during constant maintenance of pH 8.0 by automatic addition of standard alkali. (B) Arrhenius plot of the same data used in Fig. 3A. A line representing the slope (negative) corresponds to 14,500 cal.](image)

![Fig. 4. Relationship (linear) between lipase concentration and total microequivalents (μEq) of acid produced during lipolysis at 37 C and constant pH of 8.0 maintained by automatic addition of standard base.](image)
selected, the lipase activity varied from 0.17 to 1.32 units per ml.

**DISCUSSION**

We have presented a standardized method for the determination of lipase activity. This method, which specifies the use of instrumental components to maintain pH stasis, has the advantage of being accurate, reproducible, rapid, and sensitive. It is well known that the pH has a profound effect on enzyme activity, and the current methods incorporating buffers may not assure the pH stasis necessary for accuracy. This report on lipase confirms the inhibitory effect of adverse pH condition on the enzyme activity and the ease of overcoming this deficiency with an automatic titrator. Additionally, our study on rate of enzyme activity points out the fallacy of the unjustified long periods (30 to 60 min) of incubation for most enzyme determinations. For lipase, we found that the linear relationship for reaction rate actually ceased after 6 to 8 min. Current methods previously mentioned, therefore, are not only unduly prolonged, but actually give values that err on the low side. Moreover, pH stasis guarantees a straight-line relationship between lipase concentration and acid production over a wide range. This fact permits a definition of an accurate and reproducible lipase unit. Significant comparisons of lipase activity from various sources can be readily achieved. The apparatus described in this paper is composed of commonly available components which are reasonably priced and easily assembled.

The A value of 14,500 cal for our purified microbial lipase does not agree with the value of 2,600 cal for pancreatin reported by Sizer and Josephson (5). Besides the difference of purity and source of lipase, the chief reason for disagreement may be the substrate; we used emulsified olive oil rather than nonemulsified tributyrin. A major discordance, however, is the nature of the slope itself. Over a corresponding temperature range, Sizer and Josephson (5) obtained the linear relationship of a typical Arrhenius plot. The discontinuity of slope that we found is explained by Walter (7) in terms of: (i) phase change of solvent, (ii) two parallel reactions by different active centers with different temperature coefficients, (iii) two successive reactions with different temperature coefficients, or (iv) two different forms of the active enzyme with two different activation energies.

**ACKNOWLEDGMENT**

This investigation was supported by Public Health Service research grant AI-05926-02 from the National Institute of Allergy and Infectious Diseases.

**LITERATURE CITED**


