Lipid-Lipase Interactions. 2. A New Method for the Assay of Lipase Activity

ABSTRACT

A simplified procedure for the measurement of activity was developed for lipases (EC 3.1.1.3) from *Chromobacterium viscosum, Candida rugosa, Aspergillus niger* and *Rhizopus arrhizus.* It differs from existing procedures in that olive oil, refined with a bleaching clay, was used as the substrate, periodic sonication was applied to promote efficient emulsification, the hydrolysis time was extended to 1 hr, and no additives such as protective colloids and surfactants were used. The activity was determined arbitrarily for that amount of lipase required to bring about 24% hydrolysis/hr at room temperature. Unlike currently used assay procedures, the present method gives results which are highly reproducible with a maximum standard deviation of 2.5% and, more commonly, less than 1.00%.

INTRODUCTION

In view of the increasing interest in biotechnology, the enzymatic hydrolysis or synthesis of lipids has become of more than academic interest. Several microbial lipases are now available commercially, and a reliable method of measurement of lipase activity is needed to evaluate the feasibility of enzymatic hydrolysis or synthesis. The presently available assay methods are tedious, often requiring operator skill, a number of reagents and/or expensive equipment, and give results that may vary by as much as ±20%. The assay values reported by the commercial enzyme producers bear little relation, for example, to industrial enzymatic total hydrolysis of a given fat or oil.

In a recent publication (1) on the total hydrolysis of various lipids with the lipase from *Candida rugosa* (formerly *C. cylindracea*), we pointed out that there is an approximately linear relationship between the extent of hydrolysis and the logarithm of the amount of enzyme present, and that an analogous relationship exists between the extent of hydrolysis and the logarithm of reaction time. It also was shown that additives such as calcium or sodium ions, albumin and surfactants which commonly are used in various lipase assays have no positive effect on the time required to attain essentially complete (96-98%) enzymatic hydrolysis (1).

The Worthington assay (2) probably is the most widely used test method. In this test, an emulsion of olive oil in water is produced in a Waring blender. A number of stabilizers are added to improve the rather poor emulsion stability. Reproducibility of this test depends largely on the efficiency of the blender and the operator's skill at concluding the test before the emulsion breaks. The literature contains a fairly large number of assay methods, which have been reviewed recently by Jensen (3). The cited methods involve fairly complex procedures, a number of reagents, and frequently the use of expensive and sophisticated equipment. A complex radioisotope assay was developed by Matlashewski et al. (4) for lipase activity in oat flour suspensions and soluble extracts.

In the course of our current research on lipid-lipase interactions, we were hampered greatly by the lack of a simple and reproducible assay method, and accordingly we set out to develop the methodology described below, which, however, may not be suitable for all types of lipid substrates and may not be applicable to all lipase preparations. It is well suited for olive oil lipolysis with lipases from *Candida rugosa, Rhizopus arrhizus, Aspergillus niger* and *Chromobacterium viscosum.*

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EXPERIMENTAL

Materials

Lipase from *C. viscosum* was purchased from Behring Diagnostics, San Diego, California. Enzeco lipase concentrate (30,000 U/gm, according to supplier) from *C. rugosa (cylindracea)* was supplied by Enzyme Development Corporation, New York, New York. Palatase lipase (2250 U/g) from *A. niger* was furnished by Novo Laboratories, Incorporated, Wilton, Connecticut, and Lipase S (46,000 U/g) from *R. arrhizus* was supplied by GB Fermentation Industries, Incorporated, Charlotte, North Carolina. All enzymes were kept in sealed containers at 3 C and checked periodically for lipase activity by the present method. Olive oil (free fatty acid 0.12%) imported from Filippo Berio, Lucca, Italy was purchased locally. Triolein, 99%, was obtained from Sigma Chemical Company, St. Louis, Missouri. The bleaching clay Filtrol Grade 4 was furnished by the Harshaw/Filtrol Partnership, Oakland, California.

Titrations were carried out in a radiometer (Copenhagen, Denmark) PHM 52 pH meter, with TTT-80 titrator and ABU-80 autoburette. The low heat emitting magnetic stirrers were supplied by Bellco Glass, Vineland, New Jersey, and ultrasonic agitation was generated by the Model W-185 Sonicator of Heat Systems-Ultrasonics, Incorporated, Farmingdale, New York.

Lipolysis

The olive oil was freed of lipase inhibitors by treatment with Filtrol Grade 4 as described in a previous publication (1), and 3.01 g (3.33 millimoles or 10 milliequivalents) of the purified oil was placed in a 25-ml Pyrex glass-stoppered Erlenmeyer flask supplied with a 1-in. Teflon™ coated magnetic stir bar. A previously weighed sample of enzyme was dissolved in 3.5 ml distilled water or in 3.5 ml 0.1 M pH 5.5 sodium phosphate buffer solution, and the solution was added to the Erlenmeyer flask. The Palatase *A. niger* lipase solution was weighed accurately, and sufficient distilled water added to bring the aqueous phase to 3.5 g. The reaction mixture was stirred at room temperature at a rate of 40-60 rpm, and the ultrasound probe was inserted close to the bottom of the flask without touching the glass. Contact between probe and glass may shatter the flask. Sonication was applied for 30-90 sec with a sonicator setting of 6 corresponding to 25 watts output. After sonication, the reaction flask was placed on a Bellco stir plate and the stirring rate increased to 90-120 rpm. Sonication was repeated after exactly 15 min reaction time and optionally also after 30 and 45 min. After 60 min reaction time, the product was transferred to the titration vessel and the reaction flask was rinsed with a total of 30 ml ethanol adjusted to pH 9.5. The ethanol addition inactivated the enzyme. After the rinsings had been added to the titration vessel, the sample was titrated to pH 9.5. Blanks were obtained by adding the substrate and lipase solution, which had been deactivated by prior addition of 30 ml of alcohol

into the titration vessel and titration into the automatic apparatus. Five replicate runs were carried out on most determinations, and standard deviations were calculated. Because of the small standard deviations obtained, the number of replicates eventually was reduced to three. Mean values of replicate data are recorded in the tables below or plotted in the figure.

RESULTS AND DISCUSSION

A major difficulty encountered in enzymatic lipolysis is the lack of emulsification of the lipid substrate in water. Triglycerides are notoriously difficult to emulsify without addition of surfactants. Yet, in order to obtain meaningful and repeatable enzymatic hydrolysis data, it is mandatory to obtain a good initial emulsion. Once hydrolysis has begun, the mono- and diglycerides formed act as internal emulsifying agents. Since enzymatic lipolysis takes place at the water-oil interface only, good initial emulsification would give rise to a large interfacial area and result in less scatter of data. Unless ultrasonic agitation or possibly homogenization are used at the initial reaction period, a lag period may persist during which virtually no reaction takes place. The lag time phenomenon has been observed frequently and probably is the major cause of scatter of data. Unfortunately, most currently used assay tests are based on initial reaction time, and Verger et al. (5) stated correctly that initial velocity measurements have no meaning. The commonly used practice of addition of surfactants, protective colloids and various cations stabilizes the emulsion so that a more or less linear relationship may be obtained; however, the foreign additives create an artificial situation, and data thus obtained yield little practical information on complete lipid hydrolysis. It has been shown, in fact, that nonionic surfactants and calcium ion may be detrimental to enzymatic lipolysis (1).

In the present study, it was observed that initial ultrasonic agitation and periodically repeated sonication produced immediate emulsification, and repeatable analytical data could be obtained without the addition of extraneous material. Another departure from traditional assay methodology was that the extent of hydrolysis was determined after 1 hr reaction time, which would effectively wipe out small differences in initial emulsification. All tests were carried out at room temperature. Precise temperature control was not required, because it already had been determined (1) that temperature deviations do not greatly affect the hydrolysis rate.

The effects of wattage applied and number and length of sonications were studied in detail. It was found that with the instrument used, an input of less than 25 W did not produce emulsions; hence, all data reported were obtained with 25 W or higher settings. An input of 25 W, as shown i Table I, produced fairly repeatable results, and an increase to 30 W did not reduce the standard deviation. Hence, we set a power input of 25 W. The higher hydrolysis obtained with 3 mg of lipase under conditions of two 30-sec sonications at 25 W appears anomalous. We have observed that higher wattage and prolonged sonication tend to reduce enzyme activity. Table I shows the effects of number and length of sonication periods on olive oil hydrolysis by various amounts of *C. rugosa* lipase. Two 30-sec or 60-sec sonications at 0 and 15-min reaction time appear adequate to result in low standard deviations, as shown in Table I. Additional sonications after 30- and 45-min reaction time have little effect on the extent of hydrolysis or standard deviation. The length of the sonication period has a more profound effect, insofar as hydrolysis is increased while the scatter of data is reduced up to an optimum point. Thus, lower standard deviations were obtained for 60-sec sonication periods, whereas 90 sec increased data scatter. Therefore, a 60-sec sonication period was adopted as standard.

The addition of 0.1 M pH 5.5 sodium phosphate buffer further reduced standard deviation, and this addition was made a standard condition, unless the enzyme preparation already was buffered, as is the case with the commercial *A. niger* solution. Finally, we examined the effect of the substitution of the substrate by triolein. As the table shows, the differences are not large enough to warrant the use of the high-priced triolein.

The preferred conditions for the assay methods are: purified olive oil substrate, room temperature, 60-sec sonication periods at zero, and 15-min reaction times with sonication input power of 25 W, and addition of 0.1 M pH 5.5 sodium phosphate buffer unless the enzyme already has been buffered by the supplier as in the case of the *A. niger* lipase. Under these conditions replicate assay data with a standard deviation of less than 1% usually can be attained, and in only one instance did we obtain a deviation of 1.2% for *C. rugosa* lipase.

Standardization curves were prepared from mean values of three to five replicate runs for the commercial lipase preparations from *C. viscosum, C. rugosa, R. arrhizus* and *A. niger.* Hydrolyses were carried out at room temperature under conditions described above with and without buffer. *A. niger* lipase was run unbuffered only because it was

TABLE I

Enzymatic Hydrolysis of Olive Oil in Presence of *C. rugosa* Lipase

Sonication conditions			Millimoles of fatty acid titrated in presence of				
Number of sonications	Sonication period sec	Power input W	1 mg	3 mg	5 mg	10 mg lipase	Special conditions
4	30	25	0.690 (0.66)	1.744 (1.60)	2.964 (1.17)	5.198 (3.31)	
3	30	25	0.575 (0.42)	1.710 (1.70)	2.878 (0.53)	5.229 (2.04)	
2	15	25	0.610 (0.73)	1.463 (1.88)	2.756 (2.39)	4.493 (3.40)	
2	30	25	0.574 (0.82)	2.868 (0.33)	3.089 (1.50)	5.195 (0.14)	
2	60	25	0.872 (0.64)	2.274 (0.51)	3.566 (0.44)	5.246 (0.08)	
2	60	25	1.300 (0.47)	2.806 (0.01)	3.940 (0.03)	5.436 (0.76)	pH 5.5 buffer added
2	90	25	0.775 (0.99)	2.245 (0.39)	3.467 (1.33)	5.538 (0.95)	
2	30	30			3.329 (1.01)		
2	60	30			3.496 (2.00)		
2	90	30			3.353 (1.56)		
2	30	25		2.340 (1.13)	3.355 (0.66)		Triolein substrate

One-hour reaction time data at room temperature for 3.33 milliequivalents of olive oil or triolein and various conditions of sonication and enzyme level. Percent standard deviations of 3-5 replicate runs in parentheses.

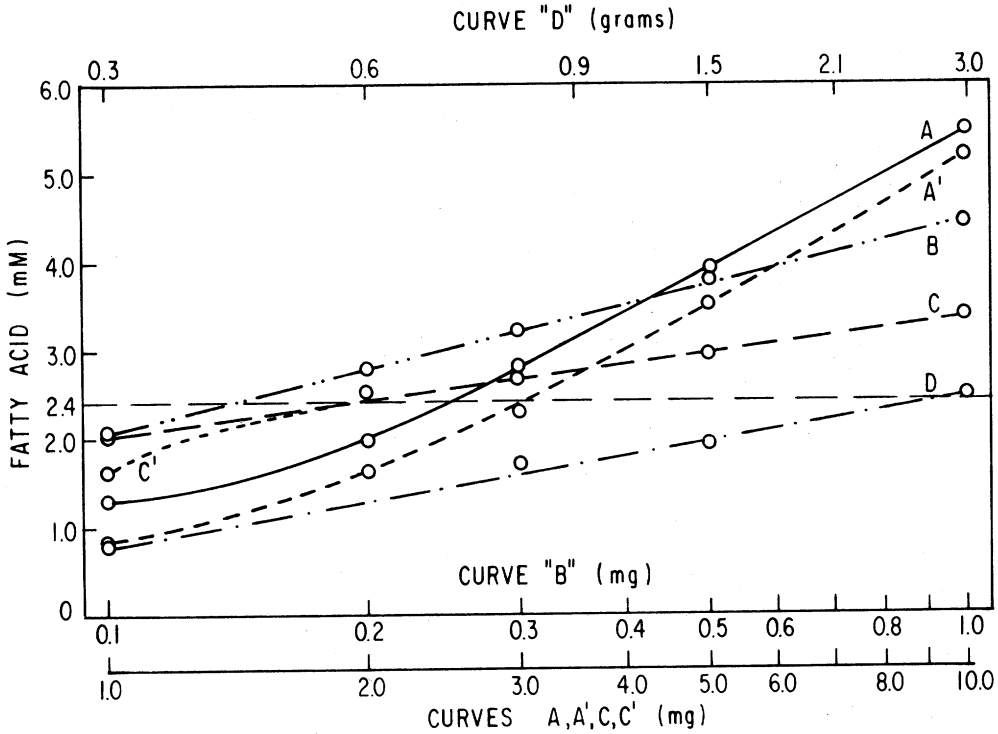

FIG. 1. Calibration curves for four lipase preparations from: *C. rugosa* buffered (A); *C. rugosa* unbuffered (A'); *C. viscosum* buffered (B); *R. arrhizus* buffered (C); *R. arrhizus* unbuffered (C'); *A. niger* no buffer added because the product contains manufacturer's buffer (D).

buffered by the supplier. The standard deviation for lipase from *C. viscosum* was in the range of 0.03-1.2%, that for the *A. niger* lipase was in the range of 0.38-1.38%, that for buffered *R. arrhizus* lipase 0.13-2.52%, and the deviations for *C. rugosa* are shown in Table I. It should be pointed out that the bulk of the assay showed a standard deviation of less than 1.00%. The curves are shown in Figure 1, where millimoles of fatty acid titrated are plotted on a semi-logarithmic scale against weight of enzyme used. As mentioned above, the logarithmic relationship is reasonably linear for buffered lipase from *C. viscosum,* for *A. niger* and buffered *R. arrhizus* lipases and in the range of 2 to 10 mg for *C. rugosa* lipase. It should be noted that the abscissa of Figure 1 is calibrated from 0.3 to 3 g for the rather dilute solution of *A. niger* lipase, from 0.1 to 1 mg for the highly concentrated, crystalline *C. viscosum* lipase, and from 1 to 10 mg for powdered *C. rugosa* and *R. arrhizus* lipases. Buffering affects the *R. arrhizus* lipase only in the 1 mg to 2 mg range, where buffering gives rise to linearity. Buffering has a more dramatic effect on *C. rugosa* lipase insofar as the extent of hydrolysis is greater in the presence of buffer and the range of linearity is extended down to 2 mg lipase.

The four lipases differ from each other in their mode of action, the *C. viscosum* and *C. rugosa* enzymes being non-specific whereas the other two are specific for the α-positions of the lipid substrate. It was pointed out, however, that the commercial *A. niger* lipase appears to contain non-specific components, because complete lipid hydrolysis was achieved with this enzyme (6).

In view of the logarithmic relationship between the extent of hydrolysis and the amount of enzyme present, an arbitrary point on the standardization curve (Fig. 1) had to be chosen for the assay of lipolytic activity. We selected 2.4 millimoles of fatty acid titrated (24% hydrolysis) because all four plots were linear from this point up. The interpolated values from Figure 1 for the four lipases were 0.14 mg for *C. viscosum,* 270 g for *A. niger,* 1.90 mg for *R. arrhizus* and 2.50 mg for *C. rugosa.* Activity is calculated as 2,400/(60 × g of lipase), corresponding to micromoles of fatty acid/min/g. The assays for these commercial lipases are 285,714 U for *C. viscosum,* 14.8 U for *A. niger,* 21,053 U for *R. arrhizus* and 16,000 U for *C. rugosa.*

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