Lipase Assays

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) have broad applications in the food, oleochemical, pharmaceutical, and detergent industries, as well as in diagnostic settings (Hafkenscheid et al., 1983; Schmid and Verger, 1998). Over 100 lipases have been characterized to some extent, and more than 30 of these are commercially available. The identification of novel sources of lipases with unique patterns of reaction selectivity remains a strategic objective of lipase studies. Various levels of sophistication are required in the assessment of lipase activities, and they increase as one attains the differential goals of (1) screening for activity (qualitative and semi-quantitative); (2) quantifying activity; and (3) characterizing kinetic patterns of selectivity. The focus of this unit will be quantification of lipase activity, since methods developed for this purpose can often be adapted for the other two purposes as well.

Lipases are unusual hydrolytic enzymes because they act on substrates providing an interface (with few exceptions). This feature has been historically used to distinguish lipases from esterases, the latter of which act on substrates in true solution (Jensen, 1983). The distinction of lipases as interfacial catalysts can make kinetic characterization a challenge, because relevant substrate concentrations are expressed in terms of area and not concentration.

Even though esterases and lipases differ in their ability to act at interfaces, some assays can be used to measure both types of enzyme activities, since they share the general ability to hydrolyze carboxyl esters of various alcohols. In general, the use of water-soluble substrates (generally, shorter acyl chain length derivatives) is considered diagnostic for esterases, and the use of water-insoluble substrates (longer acyl chain length derivatives) is considered diagnostic for lipases. Of the three most commonly used assays, two exploit the ability to measure the free carboxylic acid or fatty acid residues liberated during lipase hydrolysis of native substrates, whether it be by titration of the released acid (Basic Protocol 1), or detection of the fatty acids by complexation with a colorimetric reagent of cupric acetate (Basic Protocol 2). Alternatively, the use of *p*-nitrophenyl acyl esters as chromogenic substrate analogs provides for a continuous, spectrophotometric assay (Basic Protocol 3). Throughout this unit, the term "fatty acid" will be used instead of the popular term "free fatty acid" since the former is preferred by IUPAC-IUB (1977), according to rules of nomenclature.

TITRIMETRIC DETERMINATION OF LIPASE ACTIVITY

BASIC PROTOCOL 1

In this procedure, native substrates (triacylglycerols) are hydrolyzed to yield fatty acids. Subsamples are withdrawn from reactive mixtures at predetermined intervals, and reactivity is quenched by the addition of ethanol. The amount of fatty acids released during the reaction is determined by direct titration with NaOH to a thymolphthalein end point.

Materials

95% (v/v) ethanol
1% (w/v) thymolphthalein indicator
Olive oil/gum arabic emulsion substrate (see recipe)
Enzyme
0.05 N NaOH
50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*)
Burette

Contributed by Praphan Pinsirodom and Kirk L. Parkin

1. Into each of six 25-ml Erlenmeyer flasks, place 10 ml of 95% (v/v) ethanol and 2 to 3 drops of 1% (w/v) thymolphthalein indicator.

   This titration cocktail is used to quench the reactivity of subsamples of the reaction mixture. Six flasks are used for five time points plus a reagent blank.

2. Into a 50-ml Erlenmeyer flask with stopper, place 50 ml of 5% (w/v) olive oil/gum arabic emulsion substrate and preincubate 15 min in a 37°C water bath with magnetic stirring.

3. Add an appropriate amount of enzyme to initiate lipolysis on the emulsion substrate, start timer, and continue stirring.

4. At five suitable reaction intervals (e.g., 5, 10, 15, 20, and 25 min), remove 5 ml reaction mixture and transfer each subsample to a separate flask containing titration cocktail prepared in step 1. Swirl contents immediately to stop the reaction.

   The quenched subsamples may be turbid. Samples may be put aside (up to 2 to 3 hr at 20° to 22°C) for later titrimetric analysis.

5. Titrate the contents of each flask with 0.05 N NaOH using a burette until a light blue color appears.

   The pH indicator range of thymolphthalein is 9.3 to 10.5.

6. Into the last 25-ml Erlenmeyer flask containing titration cocktail, add 5 ml phosphate-buffered olive oil/gum arabic emulsion substrate and mix well. Using a burette, titrate the contents of this flask with 0.05 N NaOH to serve as a reagent blank.

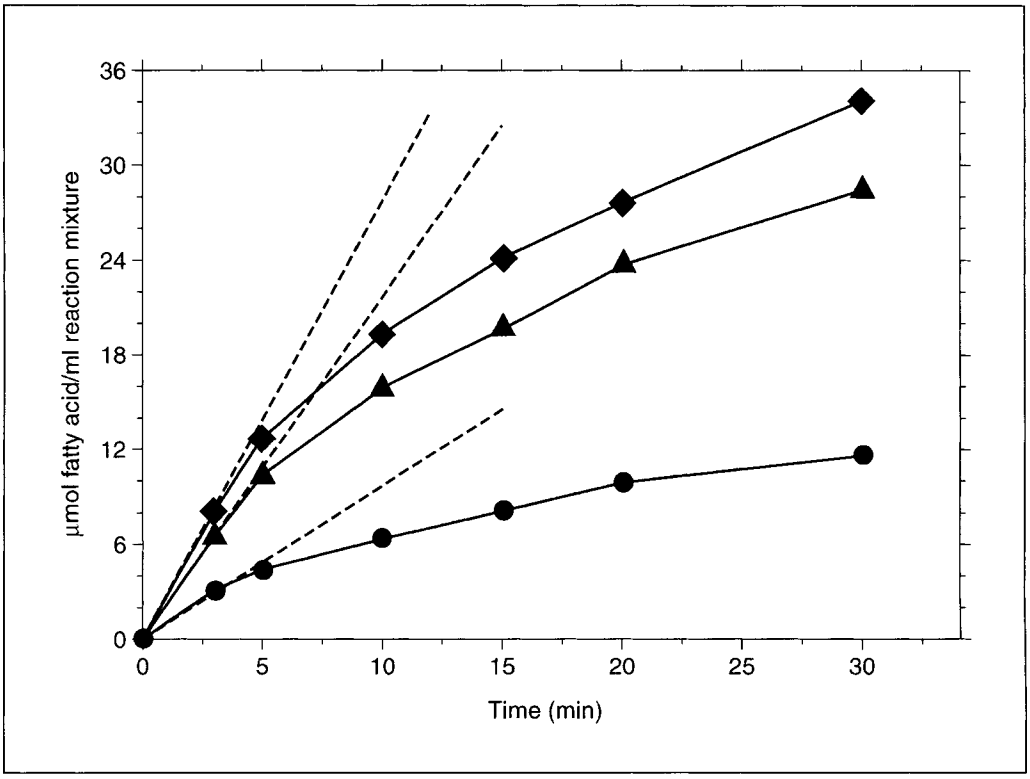

Figure C3.1.1 Comparison of lipase activities using the titrimetric assay. The lipases used in the reaction mixture, all at 0.1 mg/ml, were from *B. cepacia* (diamonds), *C. rugosa* (triangles), and porcine pancreas (circles). Broken lines represent estimation of initial reaction rates.

7. Calculate the quantity of fatty acids liberated in each subsample based on the equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following equation:

   μmol fatty acid/ml subsample =

   [(ml NaOH for sample − ml NaOH for blank) × N × 1000]/5 ml

   where N is the normality of the NaOH titrant used (0.05 in this case).

8. Create a reaction progress curve by plotting the quantity of fatty acid liberated over the time of reaction (Fig. C3.1.1). Determine the activity (initial velocity, v_0) of the lipase from the slope of the linear portion (see Critical Parameters) using the following equation:

   $$v_0 = \text{slope} = (y_2 - y_1)/(x_2 - x_1)$$

   where units are μmol/(ml × min), equivalent to mM/min.

9. Determine the specific activity (sp. act.) of the lipase preparation, if the protein content of the added enzyme preparation is known, using the following equation:

   sp. act. = $v_0 \div$ [a mg protein/(50 + b) ml reaction volume]

   where a is mg protein added in b volume to the reaction mixture, and units are μmol/(min × mg protein preparation).

   If a liquid form of the enzyme is added, such as lipases in broth samples from microbial cultures, calculate specific activity on a normalized basis by substituting a ml enzyme sample added (instead of mg protein) using the equation above.

10. Determine the number of units (U) of lipase activity, which is defined as the amount that produces 1 μmol of fatty acid per minute under the specified assay conditions.

    Depending on the nature of the original enzyme sample, specific activity can be expressed as U/mg protein or as U/ml for liquid forms of an enzyme of unknown concentration.

COLORIMETRIC ASSAY OF LIPASE ACTIVITY USING THE COPPER SOAP METHOD

BASIC PROTOCOL 2

Fatty acids liberated during hydrolysis of an olive oil substrate by lipase can be determined colorimetrically using a cupric acetate/pyridine reagent. Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range (λ_{max} 715 nm), yielding a blue color. Quantification of fatty acid released by lipase is determined by reference to a standard curve prepared using oleic acid.

Materials

25 mM oleic acid standard solution
Benzene
Cupric acetate/pyridine reagent (see recipe)
Olive oil/Triton X-100 emulsion substrate (see recipe)
50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*)
Enzyme

15- to 20-ml screw-cap test tubes
Spectrophotometer (visible lamp)
Glass cuvettes

Prepare standard curve

1. Into ten individual 15- to 20-ml screw cap test tubes, place 0.1 to 1.0 ml of 25 mM oleic acid standard solution and dilute each to 5 ml with benzene for the standard curve (final 2.5 to 25 μmol oleic acid).

   CAUTION: *Benzene is toxic to humans, and is listed as a carcinogen by the United States Environmental Protection Agency. Handle with care, wear gloves, and work in a well-ventilated fume hood.*

2. Add 1 ml cupric acetate/pyridine reagent to each tube and vortex 2 min. Centrifuge 5 min at 1000 × *g*, room temperature.

3. Transfer a sufficient volume of the upper, clear benzene phase into a glass cuvette.

4. Measure the A_{715} of the benzene layer against a benzene blank and compose a standard curve by plotting A_{715} versus the amount of oleic acid in 5 ml benzene.

Perform lipase activity assay

5. Into each of seven 15- to 20-ml screw-cap test tubes, place 5 ml benzene and 1 ml cupric acetate/pyridine reagent.

   These reagents quench reactivity in subsamples of the reaction mixture. Seven tubes are used for six time points plus a reagent blank.

6. Prepare a reagent blank by adding 0.3 ml olive oil/Triton X-100 emulsion substrate to one of the tubes, vortex 2 min, and centrifuge 5 min at 1000 × *g*, room temperature.

7. Into a 50-ml Erlenmeyer flask with a stopper, place 25 ml olive oil/Triton X-100 emulsion substrate. Preincubate 15 min with magnetic stirring in a water bath set at 37°C.

8. Add a sufficient amount of enzyme (limit to 0.5 ml in solution form) to initiate lipolysis on the emulsion substrate, start timer, and continue stirring.

9. Remove duplicate 0.3-ml subsamples of the reaction mixture at predetermined time intervals (e.g., 4, 8, 12, 16, 20, and 30 min) and place in individual assay tubes prepared in step 5. Immediately vortex 2 min to stop the reaction and form the colored fatty acid cupric soaps. Centrifuge 5 min at 1000 × *g*, room temperature, to obtain the clear benzene upper phase.

   Samples are stable at this point for up to 3 days (Lowry and Tinsley, 1976) and can be set aside for later analysis.

10. Zero the spectrophotometer at 715 nm with the benzene layer obtained from the reagent blank. Measure the A_{715} for the benzene layer of each sample using glass cuvettes.

Analyze data

11. Convert A_{715} values to μmol oleic acid/ml subsample to yield mM concentration as follows:

    μmol fatty (oleic) acid/ml subsample =

    (A_{715} − *y* intercept)/(slope × 0.3 ml subsample)

    In a typical assay, respective values of 0.0407 and 0.0433 are recorded for the y intercept and slope from the standard curve (see Anticipated Results).

12. Construct a reaction progress curve by plotting the concentration of oleic acid versus reaction time. Draw a tangent to the initial portion of the progress curve to obtain initial reaction rates (v_0 in mM/min) as follows:

$$v_0 = \text{slope} = (y_2 - y_1)/(x_2 - x_1)$$

13. Determine specific activity (sp. act.) of the lipase preparation by accounting for the level of protein (mg) in the added enzyme:

$$\text{sp. act.} = v_0 \div [a \text{ mg protein}/(25 + b) \text{ ml reaction volume}]$$

where a is mg protein added in b volume to the reaction mixture, and units are μmol/(min × mg protein preparation).

Specific activity can be expressed in U (μmol/min) per mg protein, or as U/ml if the enzyme is in a liquid form and the concentration of protein is unknown. All calculations in this protocol are similar to those used in Basic Protocol 1. However, differences occur in the use of the standard curve, magnitude of volumes and quantities of subsamples, the reaction mixture, and level of enzyme added.

SPECTROPHOTOMETRIC DETERMINATION OF LIPASE ACTIVITY USING *p*-NITROPHENYL LAURATE AS SUBSTRATE

BASIC PROTOCOL 3

This method quantifies the level of *p*-nitrophenol (λ_{max} 400 to 410 nm) released following the hydrolysis of *p*-nitrophenyl laurate substrate by lipase. Activity of lipase can be calculated by comparing sample A_{410} values to those of a standard curve prepared with *p*-nitrophenol. *p*-Nitrophenyl laurate (and other carboxylic acid esters) are model or "synthetic" substrates. As with other model substrates, the ease of use of *p*-nitrophenyl acyl esters is balanced by providing only a presumptive test for lipase activity. However, this assay is often the method of choice for screening purposes or to provide an initial assessment of suitable assay conditions. Lipase is classified by the IUB Enzyme Commission as triacylglycerol acylhydrolase (EC 3.1.1.3) and, by definition, the native substrate is a triacylglycerol. Other lipolytic enzymes, and some proteolytic enzymes, may hydrolyze *p*-nitrophenyl acyl esters but be inactive toward triacylglycerols. Use of a shorter chain length *p*-nitrophenyl acyl ester, especially the water-soluble acetate derivative, would provide a preliminary indication of esterase-type activity of an unknown preparation.

Materials

0.5 mM *p*-nitrophenol standard solution (see recipe)
0.1 M Tris·Cl, pH 8.2 (*APPENDIX 2A*)
420 μM *p*-nitrophenyl laurate substrate solution (see recipe)
Lipase solution

15- to 20-ml test tubes
Spectrophotometer
Cuvettes

1. Place 0.05 to 0.50 ml of 0.5 mM *p*-nitrophenol standard solution into ten individual 15- to 20-ml test tubes and dilute each to 5 ml with 0.1 M Tris·Cl buffer, pH 8.2.

   This yields a standard curve of 0.005 to 0.05 μmol p-nitrophenol/ml.

2. Measure A_{410} using 0.1 M Tris·Cl buffer, pH 8.2, as a blank, and make a standard curve by plotting A_{410} versus the *p*-nitrophenol concentration in each tube.

3. For each lipase activity assay, place 2.5 ml of 0.1 M Tris·Cl buffer, pH 8.2, and 2.5 ml of 420 μM *p*-nitrophenyl laurate substrate solution into a 15- to 20-ml test tube. Prepare one extra tube for a reagent blank.

4. Add 1 ml water to the reagent blank.

5. Add 1 ml lipase solution to the next substrate-containing tube to initiate the reaction. Start the timer, vortex briefly, and immediately transfer the reaction mixture into a cuvette. Record A_{410} every minute (or every 30 sec) for up to 15 min.

   For some spectrophotometers, continuous recording is available, and preferable. Some degree of automation may also be used if available.

6. Use the *p*-nitrophenol standard curve to convert absorbences to mM substrate hydrolyzed (see Anticipated Results) as follows:

   μmol *p*-nitrophenol/ml reaction mixture =

   (A_{410} – *y* intercept)/(slope × 6 ml reaction mixture)

   In a typical assay, respective values of 0.002 and 17.2 are recorded for the y intercept and slope from the standard curve (see Anticipated Results).

7. Determine lipase activity by constructing a reaction progress curve of concentration of *p*-nitrophenol (mM) released versus reaction time. Draw a tangent to the initial portion of the progress curve to obtain initial reaction rates (v_0 in mM/min) as follows:

   v_0 = slope = $(y_2 - y_1)/(x_2 - x_1)$

8. Determine specific activity (sp. act.) of the lipase preparation by accounting for the level of protein (mg) in the added enzyme:

   sp. act. = $v_0 \div$ [*a* mg protein/6 ml reaction volume]

   where *a* is mg protein added to 6 ml reaction mixture, and units are μmol/(min × mg protein), which is equivalent to U/mg protein.

   If the enzyme is in a liquid form and the concentration of protein is unknown, then U (μmol/min) can be expressed on the basis of ml instead of mg. All calculations in this protocol are similar to those used in Basic Protocol 2.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cupric acetate/pyridine reagent

Place 5 g cupric acetate into a 100-ml volumetric flask and bring to volume with water. Swirl to dissolve, then filter through Whatman no. 1 filter paper. Adjust pH of the solution to 6.0 to 6.2 using pyridine. Store at room temperature (stable for a year or more).

p-Nitrophenyl laurate substrate solution, 420 μM

Place 0.0135 g *p*-nitrophenyl laurate (mol. wt. 321.4), 0.017 g sodium dodecyl sulfate (SDS), and 1.00 g Triton X-100 into a 100-ml volumetric flask and bring to volume with water. Heat the mixture in a water bath at 65°C for 15 min, mix well, and let the solution cool to ambient temperature prior to use. Store up to 3 days at 4°C. Reheat if the solution becomes turbid.

p-Nitrophenol standard solution, 0.5 mM

Place 0.0869 g *p*-nitrophenol (mol. wt. 139.1) in a 25-ml volumetric flask and bring to volume with 0.1 M Tris·Cl, pH 8.2 (*APPENDIX 2A*). Store up to one month in a tightly sealed vessel at room temperature. Dilute 1 vol with 49 vol of 0.1 M Tris·Cl buffer for a final 0.5 mM *p*-nitrophenol standard solution.

Olive oil/gum arabic emulsion substrate

Combine 10 g each olive oil and gum arabic in a 400-ml beaker. Bring volume to 200 ml with 50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*), and homogenize 5 min using a domestic blender or sonic-probe homogenizer at a setting that does not cause excessive foaming. Prepare fresh daily and rehomogenize periodically, or continuously subject the emulsion to low-speed magnetic stirring during an entire day of use.

Olive oil/Triton X-100 emulsion substrate

Weigh 5 g each olive oil and Triton X-100 in a 100-ml volumetric flask, and dissolve the mixture by adding 5 ml chloroform. Evaporate the chloroform under a stream of nitrogen gas at 60°C. This takes ~15 min, and the mixture becomes turbid. Slowly add warm (60°C) 50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*), while swirling the flask and bring to 100 ml. Transfer the olive oil emulsion to a 250-ml beaker and homogenize 5 min using a homogenizer at a setting that does not cause excessive foaming. Let the emulsion stand at room temperature until most of the foam subsides. Adjust the final pH to 8.0 using 2 N NaOH. Prepare fresh daily and rehomogenize periodically, or continuously subject the emulsion to low-speed magnetic stirring during an entire day of use.

CAUTION: *Avoid exposure to chloroform. Use in a well-ventilated fume hood.*

COMMENTARY

Background Information

There are many procedures available for assaying or characterizing hydrolytic activity of lipases (Jensen, 1983; Vorderwülbecke et al., 1992; Schmid and Verger, 1998; Thomson et al., 1999). Owing to the nondistinct spectral nature of the products of triacylglycerol hydrolysis (fatty acids and partial glycerides), direct spectrophotometric analysis of reaction mixtures is not suitable for assessing lipase activity on native substrates. Consequently, most lipase assays have been developed on the basis of measuring liberated fatty acids either specifically or nonspecifically. Alternatively, the use of chromogenic or fluorogenic model substrates (analogs) affords the option to use spectrophotometry to directly and continuously follow the course of lipase (esterolytic) reactions. Other approaches are less commonly used and are based on sophisticated instrumentation and/or exploit the ability to measure physicochemical changes brought about by lipase action on native substrates and interfaces.

Titrimetric method

The most common method of nonspecific measurement of fatty acids is the titrimetric method (Basic Protocol 1). This is perhaps the simplest method, from both conceptual and procedural viewpoints (Benzonana and Desnuelle, 1968). Titrimetry can also be considered the benchmark method, since other assay methods, especially during the phases of development or modification, are routinely tested for how well they correlate to titrimetric assays. Fatty acids are weakly acidic, with pK_a values on the order of 4.7 to 4.9 for saturated 4- to 14-acyl carbon species, to 7.7 to 8.9 for oleic acid in the presence of 0 to 0.1 M Na^+ (linoleic acid has a pK_a of 7.9). The fatty acids liberated during the course of reaction can be determined quantitatively by titration using a standard alkali titrant and an appropriate end point.

Reactions are quenched by the addition of ethanol, which not only attenuates enzyme action but also facilitates solubilization of the fatty acids during subsequent titration to yield sodium salts (the presence of Ca^{2+} or Mg^{2+} can interfere in the assay by forming fatty acid soaps recalcitrant to titration). One will note a clearing of the titration vessel as titration progresses, even though the ethanol-quenched subsample may initially be turbid because of insoluble fatty acids. The choice of a thymolphthalein end-point (pH 9.3 to 10.5) indicator is essential to ensure the complete titration of all fatty acids, in view of the relative high pK_a values for oleic acid noted earlier.

The choice of the 50 mM sodium phosphate buffer at pH 8.0 is consistent with the optimal pH of lipases often being in the range of 7 to 9, although plant seed lipases are notable exceptions (Jensen, 1983). Phosphate provides buffering capacity over the pH range of 6.3 to 8.1

($pK_{a2} = 7.2$) and helps maintain reaction pH as product (fatty acid) is formed. This buffer system contributes little to titratable acidity of quenched reaction mixtures ($pK_{a2/3} = 7.2/12.3$), and the low background levels of titratable phosphate are easily accounted for in appropriate reagent blanks. This feature renders the titration step primarily responsive to newly evolved acidic species (i.e., fatty acids) in the reaction mixtures, conferring a large signal-to-noise ratio to this procedure. The Na^+ reduces the pK_a of oleic acid to a range that ensures complete titration. Thus, although the titration step itself is not specific to fatty acids, the manner in which the procedure is carried out affords the specificity necessary for the procedure to be accurate. Precision is afforded by careful preparation and assaying of reagent blanks. The coefficient of variation in analyses of lipase activity using titrimetry is on the order of 5% to 10% (Benzonana and Desnuelle, 1968; Hafkenscheid et al., 1983).

The titrimetric method also allows for virtually any triacylglycerol to be used as a substrate, as long as the substrate can be dispersed in liquid form; high-melting glycerides that partially solidify may cause anomalous results (Jensen, 1983). Because of this feature, titrimetry is a popular choice among these three methods for surveying or characterizing substrate selectivity by making use of pure or natural sources (e.g., vegetable oils) of glyceride substrates of different acyl chain compositions. In fact, assessment of lipolytic action on separate emulsions of monoacid triacylglcyerols (of 4 to 18 saturated acyl carbon lengths) and olive oil (or triolein) are often used to provide an initial indication of chain length selectivity for the enzyme (lipase) preparation of interest. Including an assessment of activity on triacetin (a water-soluble substrate) would expand the analysis to include an estimate of the balance of esterase and lipase activities in the preparation. The substrate tributyrin would lead to ambiguous results in this regard, because it has limited solubility and would exist as both soluble and interfacial forms in an assay system when included at levels above ~1% (solubility is also dependent on medium composition).

A pH-stat or automatic titration instrument greatly simplifies titrimetric analyses in that no buffer is required and the time course of tritrant addition may be available as a continuous recording (facilitating estimates of initial rates; see Critical Parameters). However, one limitation of the automatic titrator compared to the manual titration protocol is that not all fatty acids may be titrated if the pH-stat is set to maintain pH 7 to 8, making correction factors necessary for substrates evolving oleic or linoleic acids (Lowe, 1999).

Colorimetric method

Conceptually, assays for lipase activity using the colorimetric method (copper-soap procedure; Basic Protocol 2) are similar to titrimetry in that liberated fatty acids are being measured; however, the colorimetric method is more specific for fatty acids (Lowry and Tinsley, 1976). Quenched subsamples of emulsified acylglycerol/lipase reaction mixtures are combined with the biphasic mixture of cupric acetate/pyridine and benzene. Cupric salts of the fatty acids are formed (molar stoichiometry of fatty acid to Cu^{2+} of 4:2) and these soaps, which are blue in color, are partitioned into benzene to allow for quantification by measuring absorbance of the clear benzene phase at 715 nm.

The presence of pyridine is critical on two accounts. It appears to prevent cupric fatty acid salts from forming micellar aggregates of 40 or more fatty acids, which is important because the formation of these aggregates limits partitioning of cupric fatty acid salts into benzene. Consequently, sensitivity of the assay is increased by the inclusion of pyridine in the assay cocktail. Pyridine is also suspected of displacing water of hydration of the cupric ions of the soap, thereby enhancing copper soap solubility in the apolar phase (benzene). This not only increases sensitivity, but also normalizes the partitioning/solubility parameters of copper soaps of fatty acids of saturated 12- to 20-acyl carbons. This gives rise to a single response factor for these fatty acid species, although oleic acid gives a greater response because of its greater solubility as a cupric soap in organic media. Copper soaps of fatty acids less than 12 acyl carbons are progressively less soluble in benzene as acyl chain length is reduced, making sensitivity of the assay to these species variable, and less than is seen for longer chain length fatty acids. This requires correction factors to be obtained by the user in the event that 6- to 10-acyl chain length substrates are the subject of evaluation.

When identical lipase reaction subsamples are measured for fatty acid levels by both titrimetry (Basic Protocol 1) and colorimetry (Basic Protocol 2), estimates by the colorimetric procedure are only 60% of those obtained by titrimetry. However, the nature of this rela-

tionship may also be embedded in subtle differences between assay systems. When isooctane is used to replace benzene in Basic Protocol 2 to obviate the suspected toxicity of benzene, estimates are only 20% of that obtained by titrimetry (Kwon and Rhee, 1986). This indicates a semi-empirical nature to the copper soap procedure in that only a proportion of the cupric fatty acid soaps are partitioned into the organic layer. This partitioning is solvent dependent and compromises some degree of sensitivity of the assay. However, the results from the colorimetric and titrimetric procedures correlate highly (Hafkenscheid et al., 1983). The estimated coefficient of variation for results from the copper soap procedure is ~5% (Lowry and Tinsley, 1976). Another reagent that forms complexes with fatty acids is rhodamine B. However, this reagent is most often used to detect fatty acids on thin-layer plates or lipolytic activities on agar plates based on fluorescence of the fatty acid-dye complex (Kouker and Jaeger, 1987).

Spectrophotometric method

The most common spectrophotometric procedure used for lipase assays, and the one described here, is based on using the substrate analogs *p*-nitrophenyl acyl esters (Basic Protocol 3). The basis of this procedure is that lipases possess general esterolytic activity toward a variety of native and non-native carboxyl ester substrates. The *p*-nitrophenyl acyl substrate analogs are hydrolyzed to yield the fatty acid and *p*-nitrophenol, which is a chromophore (λ_{max} 400 to 410 nm) in slightly alkaline media (pK_a = 7.2) with a large extinction coefficient (ε_{mM} 18.3 at 400 nm and pH 10.2). Since *p*-nitrophenyl acyl esters may be hydrolyzed by nonspecific esterases in a given enzyme preparation, results with this type of substrate are only presumptive for lipase activity, and this would have to be confirmed by other assays using native substrates (such as Basic Protocol 1 or 2). Other less commonly used substrate analogs include β-naphthyl- (chromogenic) or umbelliferyl- (fluorogenic) acyl esters, or resorufin-linked acylglycerols (fluorogenic); assays based on their use are similarly presumptive for lipase activity (Jensen, 1983).

Despite the aforementioned limitation, the use of *p*-nitrophenyl acyl esters is the most sensitive of the three protocols described, and has an estimated coefficient of variation in the procedure of ~5%. It is essential for the user to prepare a standard curve for *p*-nitrophenol, because the pK_a and user-selected pH of the assay system may not afford complete spectrophotometric detection (i.e., ionization) of the released *p*-nitrophenol to *p*-nitrophenoxide.

This protocol is also suitable for assessing chain length selectivity of lipolytic-active fractions, as *p*-nitrophenyl acyl esters are readily available for acyl chain lengths of 2 to 18 (in fact, *p*-nitrophenyl laurate is not always the best analog for assaying all lipases). Activity toward the shorter chain length derivatives in this series of substrates, especially *p*-nitrophenyl acetate, is diagnostic for esterase activity. Both the spectrophotometric and titrimetric methods are advantageous over the colorimetric method in terms of assessing chain length selectivity of lipolytic enzymes. This is because the latter method requires complicated correction factors for measuring fatty acids released from monoacid triacylglycerols of 6 to 10 acyl carbon chain lengths, and lacks sensitivity in measuring lipase activity on monoacid substrates of shorter acyl chain lengths (acetic and butyric).

The greatest advantage of the spectrophotometric method is that it is direct and rapid, requires no sample workup, and allows for continuous assays of lipase activity compared to the multiple fixed-time-point analyses incumbent within Basic Protocols 1 and 2. The spectrophotometric method can also be done using very small volumes (as small as 1 ml) and is suitable for following the course of purification (such as in chromatographic fractions) or adaptable to 96-well plates (and subject to automation, if available). Thus, it is the method of choice for screening several samples or preparations for lipase (esterase) activity.

Choosing an assay method

In addition to assay features already mentioned, other factors may influence the choice of assay by the user. In terms of sensitivity of the assay, the threshold of detection of lipase activity, using the procedures as described in this unit, is on the order of 10^{-2} U for titrimetry, 10^{-1} U for colorimetry, and 10^{-4} U for spectrophotometry (where U is the amount of enzyme required to yield 1 μmol product per minute). The smallest amounts (volumes) of materials, including enzyme, are required for the spectrophotometric method, and progressively more material is required for the colorimetric and titrimetric methods. Unless a flow cell adapter is available, the spectrophotometric method is not suitable for analysis of particulate (immobilized) enzyme preparations, whereas the other assay procedures are.

Critical Parameters

Enzymes and assays

It is recommended that all enzyme assays be replicated three times. Powdered enzymes can be dissolved in assay buffer fresh daily and kept on ice prior to assay.

Substrate emulsion

All of the described procedures use emulsified substrate. Although the *p*-nitrophenyl laurate assay cocktail is stable for 3 days at 4°C, the emulsified olive oil substrates (or other triacylglycerol-based substrate systems) should be made fresh daily and rehomogenized periodically and when separation is visually evident. Use of day-old emulsion substrate will yield increased blank values for titratable acidity, and this effectively compromises the limit of detection of activity. Emulsified substrates should be in liquid form at common assay conditions (20° to 50°C), and partially solidified substrates (those rich in long-chain saturated fatty acids) will cause interfacial irregularities and confound the assessment of lipases in ways that cannot be accounted for.

Once a method for emulsifying the substrate is defined, it should be reproduced as carefully as possible from one day to the next, as this will help maintain a constant surface area-to-concentration ratio for the substrate preparation. The substrate concentrations listed for these protocols provide for saturating conditions in "fine" emulsions. In addition, the surfactants used in each case help maintain a constant surface area by promoting the formation of emulsion droplets of fairly discrete sizes. Each surfactant has a characteristic critical micelle concentration and aggregation state(s), and these properties will largely determine interfacial area for an emulsified substrate (even though it may not be known to, or easily determined by, the user). However, over time, coalescence of emulsion particles will occur, giving rise to reduced surface area to the point where reaction rates may become limited in part by substrate. In addition, the resulting heterogeneity caused by "creaming" of the emulsion may compromise the ability to procure representative subsamples for conducting the lipase assay. Replicate analysis of a given enzyme preparation should take place over the course of different days (different substrate emulsion

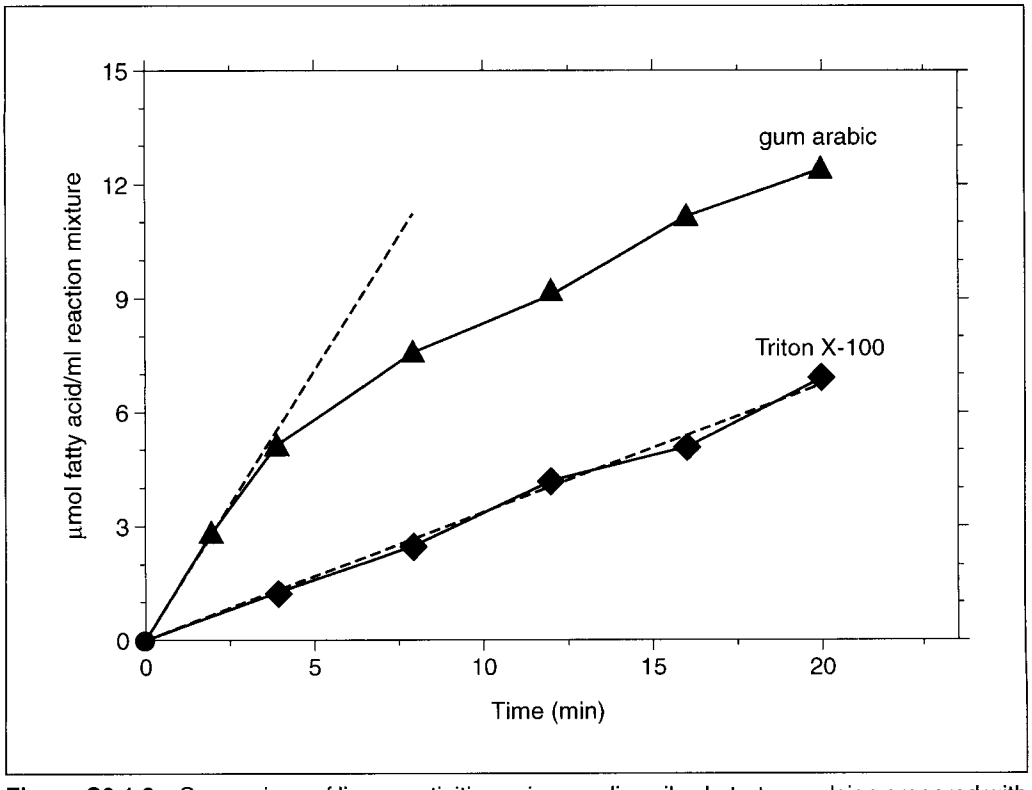

Figure C3.1.2 Comparison of lipase activities using an olive oil substrate emulsion prepared with different emulsifying agents. The lipase used in the reaction mixture was from *C. rugosa* (at 0.06 mg/ml) in the presence of 5% (w/v) each of gum arabic (triangles) or Triton X-100 (diamonds). Reaction progress analysis was obtained using titrimetry. Broken lines represent estimation of initial reaction rates.

preparations), as the quality and reproducibility of the substrate emulsion is arguably the most critical assay feature impacting on experimental variance (Lowe, 1999).

Measurement of initial reaction rates

Perhaps the most critical parameter the user should be concerned with, regardless of the protocol selected, is to ensure the measurement of initial reaction rates. To use someone else's words, "It must be stressed...that the activity of an enzyme is related to the initial rate of the reaction...rather than to the amount of product released...after an arbitrarily selected period of time" (Benzonana and Desnuelle, 1968). Too often in the scientific literature, activity measurements are made using a discontinuous, fixed-time-point approach without regard for verifying that true initial rates are being measured. The initial, linear portion can be rather easily identified using a continuous assay (such as in Basic Protocol 3), but requires more careful deliberation when using discontinuous or multiple-sampling-point procedures (such as in Basic Protocols 1 and 2). In either case, a tangent must be drawn to the initial portion of the progress curve that approximates (by linearization) the reaction rate initially observed (Figs. C3.1.1 and C3.1.2). Generally, greater enzyme activities lead to earlier departure from linearity of initial reaction rates and, in this case, improved results will likely occur if the enzyme is assayed again at a more dilute level, as illustrated in Figure C3.1.3. It is also important to avoid any lag (pre-steady-state) period in estimating initial reaction velocities, as this phenomenon is sometimes encountered. Curvilinear progress curves result from the tendency of liberated fatty acids to partition at the interface and block (inhibit) further access of enzyme to substrate, or possibly to block enzyme interfacial inactivation during reaction. Increasing the rigor of agitation of the reaction mixture may help prolong linear rates by mitigating product inhibition.

Anticipated Results

Analysis of three lipase reactions using the titrimetric method illustrates typical reaction progress curves and how, as well as the need, to estimate initial rates by tangential analysis (Fig. C3.1.1). The corresponding initial reaction velocities were 27.5 U/mg for *Burkholderia cepacia* (formerly, *Pseudomonas cepacia*) li-

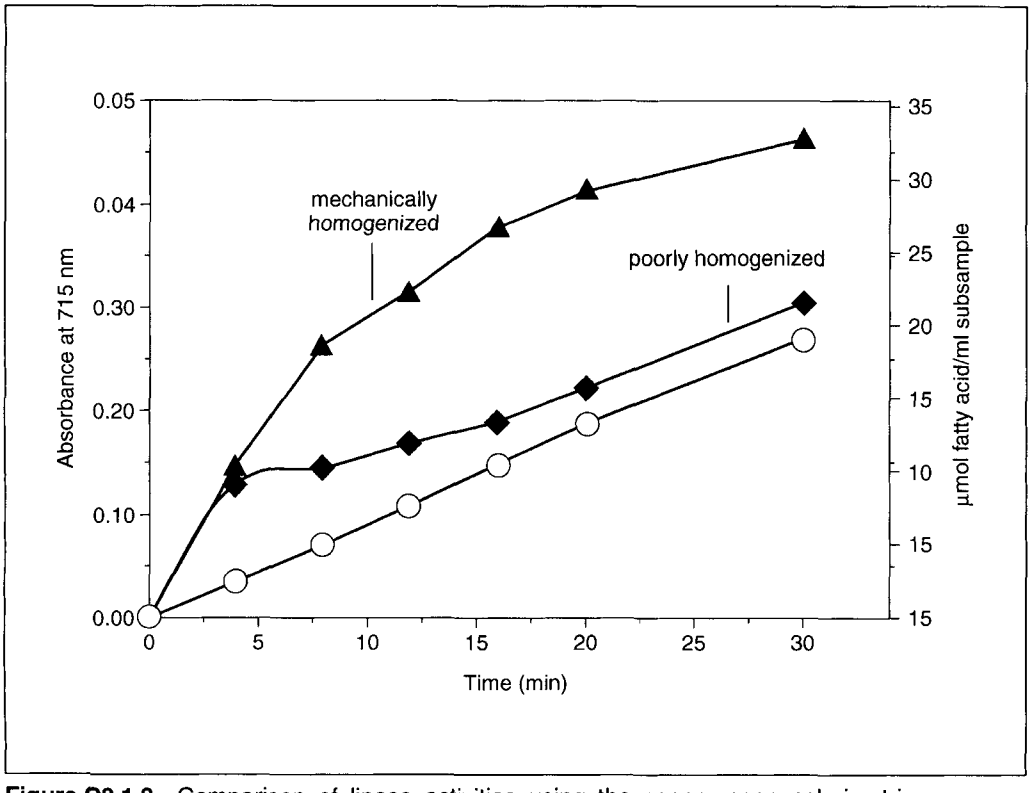

Figure C3.1.3 Comparison of lipase activities using the copper soap colorimetric assay as affected by the degree of homogenization of olive oil substrate. The lipase used in the reaction mixture was from *C. rugosa* (at 0.40 mg/ml) in the presence mechanically homogenized (triangles) or poorly homogenized (shaken by hand; diamonds) substrate emulsion. For comparison of linearity of reaction progress curves, the time course of *C. rugosa* lipase (at 0.20 mg/ml; circles) is provided.

pase, 21.8 U/mg for *Candida rugosa* lipase, and 9.70 U/mg for porcine pancreatic lipase. When Triton X-100 replaced gum arabic (gum acacia) in the emulsion system, the corresponding initial reaction rates for these same enzymes were 14.6 U/mg, 5.20 U/mg, and not detectable, respectively (data shown only for *C. rugosa* lipase in Fig. C3.1.2). These results indicate that the choice of surfactant influences lipase action either by modulating the nature or interfacial area of the dispersed phase and/or by direct influence on the lipase. Gum arabic also offers greater stabilization of the emulsion than does Triton X-100, and has evolved as the surfactant of choice when using titrimetric assays for lipase activity.

A direct comparison of the titrimetric and copper soap methods using olive oil emulsion and Triton X-100 as surfactant for *B. cepacia* lipase gave results of 14.6 U/mg and 5.32 U/mg, respectively, and 5.20 U/mg and 2.67 U/mg for *C. rugosa* lipase, respectively (Figs. C3.1.2 and C3.1.3). This illustrates the lower estimate obtained with the copper soap method compared to the titrimetric method. Triton X-100 is used as surfactant with the copper soap method because gum arabic forms a viscous emulsion in the presence of benzene during the sample workup steps. By the same token, if the user selects to substitute isooctane for benzene in this method (to avoid the toxicity of benzene), gum arabic is preferred over Triton X-100 as the surfactant, because Triton X-100 forms a viscous emulsion during sample workup in the presence of isooctane. A typical standard curve for oleic acid when using the copper soap method was: μmol oleic acid = 0.0433 A_{715} + 0.0407 (r^2 = 0.996).

The importance of the physical nature (often termed interfacial quality) of the substrate on the progress of assays for lipase activity is illustrated in Figure C3.1.3. Formation of fine emulsions by systematic, mechanical processes yields more typical progress curves (which are easier to analyze for initial reaction rates) than when using nonmechanically homogenized (coarse) emulsion, using *B. cepacia* lipase as an example. Even though initial reaction rates with the two substrate preparations may be similar, the reaction progress with the less-well-dispersed substrate can compromise or obscure the ability to make accurate estimates of initial reaction rates. The results with the "poorly homogenized" substrate emulsion are qualitatively similar to what would be observed if the emulsion was used throughout the day with evidence of visible phase separation and without periodic rehomogenization.

In the event that lipase preparations are too active to allow for facile estimation of initial rates, the enzyme can be diluted and assayed again. This is illustrated using the copper soap method where the reduced level of *C. rugosa* lipase addition afforded a longer period of linearity to the reaction progress curve than did the more active *B. cepacia* lipase (Fig. C3.1.3).

Assays using the spectrophotometric method (*p*-nitrophenyl acyl esters) are typically linear for 5 to 15 min depending on the levels of lipase activity. A typical standard curve for *p*-nitrophenol under the assay conditions described in this unit (pH 8.2) is: mM *p*-nitrophenol = 17.2 A_{410} + 0.002 (r^2 = 0.999). Results can range widely for different lipase preparations (Vorderwülbecke et al., 1992). For example *C. rugosa* and porcine pancreatic lipase preparations exhibited initial reaction rates of 1.39 U/mg and 0.0007 U/mg, respectively, using this assay method. Although these initial reaction rates are less than those observed using the assay systems designed for titrimetric and colorimetric analysis, the fact that such low activities can be measured underscores the sensitive nature and utility of the *p*-nitrophenyl acyl ester assay system, especially for screening dilute or crude preparations containing lipase.

Time Considerations

Aside from the time required to prepare reagents, the least amount of time is required per lipase assay by the spectrophotometric method, and the greatest amount of time is required per assay for the titrimetric method. Although all assays are described as requiring up to 30 min for the reaction mixture to be subsampled, time savings can be realized by subsampling more frequently over a shorter period of time, as long as one obtains valid initial rate data. Thus, for all assays, the time involved to run the lipase reaction can be normalized to be the same at ~10 to 15 min. The difference in time requirements for the protocols becomes embedded in sample workup procedures.

For the spectrophotometric method, there is no sample workup, allowing one to run ~4 assays/hr. This can be increased to ~16 to 100 or more samples/hr depending on equipment features and automation, such as multiple cuvette holders/changers and 96-well microplate readers. For the colorimetric procedure, sample workup requires ~10 min/subsample, but several samples can be "batch processed" simulta-

neously. Thus, one can comfortably complete 2 to 4 lipase activity determinations/hr, depending on how efficiently the user coordinates subsampling and sample workup. The titrimetric assay poses the greatest demands on time, as each subsample requires detailed attention during the titration step. One can reach the point of completing at most 2 activity determinations/hr, although this can be almost doubled using an automatic titrator or pH-stat instrument. The manual titration method is tedious and much more subject to user fatigue than the other protocols described in this unit.

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Key References

Benzonana and Desnuelle, 1968. See above.

Detailed account of kinetics and inhibition of pancreatic lipase action on emulsified triacylglycerol, including special treatment of initial rate measurements and pK_a of liberated fatty acids.

Jensen, 1983. See above.

Contrasts several popular and less common methods for detecting and assaying lipase activity. Discusses advantages, disadvantages, and the unique requirements of each method for obtaining accurate estimations of lipase activity.

Lowry and Tinsley, 1976. See above.

Describes a modified copper soap that is still in widespread use. Focus is placed on the development of solvent systems that simplified the procedure and enhanced sensitivity, and on issues relating to reproducibility.

Vorderwülbecke et al., 1992. See above.

An exhaustive account of 73 commercially available lipase preparations, comparing activities using six hydrolytic and five esterification assay substrate systems. Illustrates the wide range of activities between lipases and for a given lipase assayed by different protocols.

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