Lipase

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Titrimetric methods and other non-colorimetric[1–4] methods available for the assay of serum lipase differ in their respective substrates: tributyrin[1], Tween[2], and olive oil[3]. Assay of lipase in the presence of large amounts of other esterases requires a relatively specific system and activation of lipase by bile salts is especially important in making the distinction. Tributyrin and Tween, and even the so-called natural substrate, olive oil, are relatively unspecific and are therefore unsuitable as substrates. Of the phenol and naphthol esters, 2-naphthyl nonanoate is the most sensitive[5]. However, because of its high esterase sensitivity as well, use of this compound has not resulted in a useful serum method. It was recently noted that taurocholate was not a reliable activator of pancreatic lipase in human serum[6]. Substitution of esterase-resistant 2-naphthyl myristate[7] for 2-naphthyl laurate and sodium cholate for sodium taurocholate resulted in a serum method useful in the diagnosis of human pancreatitis[6].

Principle

Lipase and esterase hydrolyse 2-naphthyl myristate to 2-naphthol and myristic acid. In the absence of sodium cholate the slight hydrolysis that occurs is due to esterase while in the presence of cholate the hydrolysis is almost entirely due to lipase and very little to esterase. The difference corresponds to the lipase activity. Two molecules of 2-naphthol are coupled with tetrazotized o-dianisidine to give a purple azo dye which is determined colorimetrically. Two incubation times are used for serum assay, one hour and 5 hours. For diagnosis of pancreatitis the 5 hour test is more reliable. However a positive 1 hour test consistently indicates a positive 5 hour test.

Reagents

1. Tris-hydroxymethyl-aminomethane
2. Hydrochloric acid, A. R., 1 N
3. Cholic acid *)
4. Sodium hydroxide, A. R., 1 N
5. 2-Naphthyl myristate, naphthol-free[7] **)
6. Ethyleneglycol monoethyl ether, purified +)
7. Tetrazotized o-dianisidine (Fast Blue B) ++)
8. 2-Naphthol
9. Glacial acetic acid

Purity of the reagents

The 2-naphthyl myristate should not contain free naphthol, otherwise too high a blank will be obtained. To check the purity of the substrate, dissolve it in acetone-water and add Fast Blue B in tris buffer. The substrate can be purified by recrystallization from alcohol.

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*) Obtainable from Mann Research Laboratories Inc., New York 6, N. Y.

**) Obtainable from Cyclo Chemical Corporation, 1922 E. 64th Street, Los Angeles 1, California.

+) Obtainable from Fisher Scientific Company, Silver Spring, Maryland.

++) Obtainable from General Aniline and Film Corporation, 435 Hudson Street, New York 14, N. Y.

1) *N. P. Goldstein, J. H. Epstein* and *J. H. Roe*, J. Lab. clin. Med. *33*, 1047 [1948].

2) *R. M. Archibald*, J. biol. Chemistry *165*, 443 [1946].

3) *I. S. Cherry* and *L. A. Crandall jr.*, Amer. J. Physiol. *100*, 266 [1931].

4) *R. Ammon* and *M. Jaarma* in *J. B. Sumner* and *K. Myrbäck*: The Enzymes, Academic Press, New York 1950, Vol. I, Part 1, p. 390.

5) *S. P. Kramer, L. D. Aronson, M. G. Rosenfeld, M. D. Sulkin, A. Chang* and *A. M. Seligman*, Arch. Biochem. Biophysics *102*, 1 [1963].

6) *S. P. Kramer, M. Bartalos, J. N. Karpa, J. S. Mindel, A. Chang* and *A. M. Seligman*, J. Surgical Research *4*, 23 [1964].

7) *H. A. Ravin* and *A. M. Seligman*, Arch. Biochem. Biophysics *42*, 337 [1953].

Preparation of Solutions

I. Tris buffer (0.4 M; pH 7.4):
Dissolve 48.44 g. tris-hydroxymethyl-aminomethane in distilled water. Add 33.2 ml. 1.0 N HCl and make up to 1000 ml.

II. Sodium cholate (0.232 M):
Suspend 94.9 g. cholic acid in distilled water. Titrate slowly with vigorous stirring to pH 7.4 with 1 N NaOH (approximately 232 ml. required). Make up to 1000 ml. Only a negligible quantity of material remains undissolved. Filter.

III. Substrate solution (1.4×10^{-2} M 2-naphthyl myristate):
Dissolve 125 mg. of 2-naphthyl myristate in ethyleneglycol monoethyl ether and make up to 25 ml.

IV. Tetrazotized o-dianisidine (4 mg./ml.):
Just before use dissolve 40 mg. tetrazotized o-dianisidine in 10 ml. of cold distilled water.

V. 2-Naphthol standard solution (7×10^{-4} M 2-naphthol):
Dissolve 10 mg. 2-naphthol in 20 ml. of ethyleneglycol monoethyl ether and make up to 100 ml. with distilled water.

VI. Ethyleneglycol monoethyl ether solution (0.2 ml./ml.):
Dissolve 20 ml. of ethyleneglycol monoethyl ether in distilled water and make up to 100 ml. with distilled water.

Stability of the solutions

The buffer solution (I) and the sodium cholate solution (II) are stable for up to a month at 4°C. The substrate solution (III) can be kept for at least 1 week. Solution IV must be used immediately and solution V and VI are prepared fresh when needed.

Procedure

Assay

This method is valid for serum. — Wavelength: 540 mμ (520—580 mμ); light path: 1 cm.; final volume: 8.2 ml.; temperature: 37.5°C (constant temperature water bath); measure against water. — Each determination requires 4 test tubes: 2 samples with cholate and 2 samples without cholate.

Pipette successively into test tubes:	with cholate	without
buffer (I)	2.0 ml.	2.0 ml.
cholate solution (II)	0.5 ml.	—
distilled water	1.3 ml.	1.8 ml.
sample (serum)	0.2 ml.	0.2 ml.
Incubate at 37.5°C for 10 min. Add with an automatic pipette substrate solution (III)	0.2 ml.	0.2 ml.
Mix well. Incubate for 1 or 5 hr. at 37.5°C. Add with an automatic pipette tetrazotized o-dianisidine solution (IV).	1.0 ml.	1.0 ml.
After 3 min. add with an automatic pipette glacial acetic acid	3.0 ml.	3.0 ml.

and mix. Read optical densities against water. All tubes reading greater than 1.000 dilute

with glacial acetic acid and read again; multiply by the dilution factor (final volume over initial volume) to give the reading for the undiluted assay mixture.

E_1 = mean of optical densities of the solutions with cholate

E_2 = mean of optical densities of the solutions without cholate

2-Naphthol standard curve

Since cholate increases the colour intensity it is necessary to prepare two calibration curves, one with and one without cholate. For serum, however, the ratio of the optical densities obtained for absence/presence of cholate is well established and found to be 0.79. In this case only one standard curve without cholate but with serum need be prepared.

Pipette into test tubes 0 to 1.0 ml. of 2-naphthol standard solution (V) and make up to 1.0 ml. with glycol ether solution (VI). The solutions contain 0 to 0.7 μmoles 2-naphthol/ml. Add to each test tube 2.0 ml. buffer (I), 0.2 ml. serum and 1.0 ml. dist. water. Mix and add 1.0 ml. tetrazotized o-dianisidine solution (IV) to each test tube. Continue as described under "Assay". Plot the optical density (ordinate) against μmoles 2-naphthol (abscissa). To confirm the value 0.79 a second standard curve with cholate has to be prepared in which 0.5 ml. water are substituted by 0.5 ml. cholate solution (II).

Calculations

1 unit is that amount which splits 1 μmole substrate per minute. 1/1000 unit is one milli-unit.

In spite of the use of a non-natural substrate, an extremely long incubation time and a higher temperature (37.5°C instead of 25°C according to the international definition) it is possible to calculate the lipase unit from the one or five hour values.

Read the μmoles 2-naphthol formed (which are equal to the amount of substrate used) from the standard curve(s). c_1 and c_2 correspond to the optical densities E_1 and E_2. For experiments with serum where only one standard curve is prepared (see above) E_1 is to be multiplied by 0.79 before entering the standard curve.

$\Delta c = c_1 - c_2$ is the amount of naphthol myristate split in 1 or 5 hours by 0.2 ml. serum.

For one hour incubation: $\Delta c \times 5 \times 1/60 \times 1000 = \Delta c \times 83$ = milli-units/ml. serum.

For five hour incubation: $\Delta c \times 16.7$ = milli-units/ml. serum.

Normal serum ranges from 0.04 to 2.5 milli-units/ml. (average of 1.4 milli-units/ml.).

Details for Measurements in Tissues [6]

Weigh fresh tissue and homogenize in distilled water using a mechanically driven ground-glass homogenizer. Freeze and thaw 4 times and then use uncentrifuged or centrifuge for 10 min. at $1200 \times g$. Assay the supernatant as described for serum. Use two standard curves, one with and one without cholate. Calculate lipase activity from $\Delta c = c_1 - c_2$. For five hour incubation it is $\Delta c \times 3.3 \times 1/g.$ = milli-units/g. (g. = fresh or dry weight tissue.) Larger amounts and higher activities of the sample allow the incubation time to be reduced to 1 hour or less. The lipase activity is proportional to the amount of 2-naphthol liberated over the range of 0.0002 to 0.005 mg. fresh pancreas per tube.

Sources of Error

The substrate conversion is linear with respect to time. The maximal deviation does not exceed ±20%. Not more than 80% of the substrate should be hydrolysed, otherwise the hydrolysis rate is no longer proportional to the activity of the enzyme. Minimally lipaemic, haemolyzed, or icteric sera do not affect the results, but moderately haemolyzed or lipaemic sera do. Highly icteric sera interfere greatly with colorimetry. A precipitate (substrate) in the assay mixture may be observed which is finely dispersed and does not interfere too much with the colorimetric measurements. Injection of heparin has been reported to change the value of serum lipase obtained by this method [8].

[8] *M. Bartalos* and *F. Györky*, Clin. Chim. Acta *9*, 273 [1964].