Polyunsaturated Fatty Acids

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The ultraviolet absorption of the conjugated diene hydroperoxides arising from the lipoxidase catalysed oxidation of *cis*-polyunsaturated fatty acids has been used to measure the concentration of the enzyme in the presence of excess substrate[1]. The method described here[2] measures the substrate concentration by oxidation in the presence of excess enzyme. All polyunsaturated fatty acids, whose double bonds are separated by *cis*-methylene groups (linoleic, linolenic and arachidonic acids), react quantitatively. The extinction coefficients of the diene hydroperoxides at 234 mμ are the same for all the polyunsaturated fatty acids so far examined[2]. Irrespective of the number of double bonds, only one mole of diene hydroperoxide is formed from each mole of fatty acid, therefore the method cannot be used to differentiate the individual polyunsaturated fatty acids. This is accomplished spectrophotometrically after alkaline isomerization[3] or by gas chromatography[4,5].

The lipoxidase method allows the determination of as little as 5 μg. (0.018 μmoles) of linoleic acid. It has been applied to free fatty acids, esters, oils, fats, hydrogenated vegetable oils, blood plasma, plant seeds and micro-organisms. For the application of the method to the determination of unsaturated fatty acids in serum, see[6].

Principle

Lipoxidase catalyses the reaction between dissolved atmospheric oxygen and *cis*-polyunsaturated fatty acids to give the conjugated diene-hydroperoxides of the fatty acids:

(1) 2 Linoleate + 2 O_2 ⟶ 9-hydroperoxy-octadeca-$\Delta^{10,12}$-dienoate + 13-hydroperoxy-octadeca-$\Delta^{9,11}$-dienoate

The absorption of the conjugated diene-hydroperoxides at 234 mμ is a measure of the total fatty acids containing double bonds separated by *cis*-methylene groups. The absorption obeys *Beer*'s Law between 5 and 25 μg. substrate.

Reagents

1. Boric acid, H_3BO_3, A. R.
2. Potassium hydroxide, A. R.
3. Hydrochloric acid, A. R., conc.
4. Ethanol, benzene-free
5. Lipoxidase
   commercial preparation, see p. 987.

Purity of the enzyme preparation

In order to test the enzyme solution described under "Preparation of Solutions", a fatty acid solution is prepared from cottonseed, corn or soya-bean oil (see Section "Experimental

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1) *R. T. Holman* in *D. Glick:* Methods of Biochemical Analysis. Interscience Publ., New York 1955, Vol. II, p. 113.
2) *J. MacGee*, Analytical Chemistry *31*, 298 [1959].
3) American Oil Chemists' Soc., Tentative Method Cd-7-58, revised 1959, in: Official and Tentative Methods of the American Oil Chemists' Society. Chicago 1959.
4) *C. H. Orr* and *J. E. Callen*, Ann. N. Y. Acad. Sci. *72*, 649 [1959].
5) *B. M. Craig* and *N. L. Murty*, J. Amer. Oil Chemists' Soc. *36*, 549 [1959].
6) *H. Süllmann*, Klin. Wschr. *39*, 386 [1960].

material, fatty acid ester"). Pipette 3 ml. of this solution into two 1 cm. silica cuvettes. To the first cuvette add 0.1 ml. boiled, dilute enzyme (solution V), place the cuvette in the light path of a spectrophotometer and set the optical density at zero at 234 mμ. To the second cuvette add 0.1 ml. of the dilute enzyme (solution IV), mix and place this cuvette in the light path. Read the optical density at 1 minute intervals. It should reach a maximum in less than 5 minutes. If not, more concentrated solutions IV and V must be prepared or a more active lipoxidase preparation obtained.

Preparation of Solutions

I. Potassium borate buffer (1 M; pH 9.0):
Dissolve 61.9 g. boric acid and 25.0 g. potassium hydroxide in about 800 ml. distilled water by warming on a steam bath, with stirring. Cool the clear solution to room temperature, adjust pH to 9.0 with 1 N HCl or 1 N KOH and dilute with distilled water to 1000 ml.

II. Potassium borate buffer (0.2 M; pH 9.0):
Dilute 200 ml. solution I to 1000 ml. with distilled water.

III. Lipoxidase (stock solution, 1 mg. protein/ml.):
Dissolve 10 mg. lipoxidase in 10 ml. ice-cold buffer (solution II).

IV. Lipoxidase (dilute solution, 0.2 mg. protein/ml.):
Dilute 2.0 ml. solution III with 8.0 ml. ice-cold buffer (solution II).

V. Lipoxidase (boiled dilute solution, 0.2 mg. protein/ml.):
Pipette 5 ml. of solution IV into a test tube in such a way that the walls above the surface of the liquid remain dry. Place the test tube for 5 min. in a boiling water bath, so that up to about 1 cm. above the surface of the solution is heated. Under these conditions the enzyme is completely inactivated.

VI. Hydrochloric acid (0.5 N):
Dilute 50 ml. conc. HCl to 1000 ml. with distilled water.

VII. Potassium hydroxide (10 N):
Dissolve 56 g. KOH in about 80 ml. distilled water, cool and dilute to 100 ml.

VIII. Alcoholic potassium hydroxide (0.5 N):
Mix 0.5 ml. 10 N KOH (solution VII) with 9.5 ml. ethanol. Prepare freshly each day.

IX. Alcoholic potassium hydroxide (1.5 N):
Mix 1.5 ml. 10 N KOH (solution VII) with 8.5 ml. ethanol. Prepare freshly each day.

Stability of the solutions

The enzyme solutions can be stored frozen for several months without loss of activity. They may be thawed by placing in water at room temperature, but after thawing completely, they should be mixed well and placed immediately in an ice-water bath. Repeated freezing and thawing does not affect the enzyme activity.

All solutions containing polyunsaturated fatty acids must be protected from atomspheric oxygen, except during the enzymatic reaction where oxygen is necessary. Storage in oxygen-free conditions is accomplished by displacing the air above the solutions with nitrogen and then stoppering the vessels.

Procedure

Experimental material

Prepare a solution containing 5—25 μg. free, polyunsaturated fatty acid in 3 ml. 0.2 M borate buffer (solution II). Free fatty acids are mixed with 0.6 ml. 1 M borate buffer (solution I) and diluted with water to 3 ml.

Fatty acid esters must be saponified: mix the sample of fatty acid ester, oil, fat or hydrogenated vegetable oil, corresponding to about 0.5 mg. polyunsaturated fatty acid, in a 100 ml. volumetric flask with 1 ml. 0.5 N alcoholic KOH (solution VIII) and allow to stand at least 4 hours in the dark. Then add 20 ml. 1 M buffer (solution I) and 1 ml. 0.5 N HCl (solution V), and dilute with distilled water to 100 ml.

Blood plasma: Saponify 0.1 ml. as described above, but use a 25 ml. volumetric flask. Add 5 ml. 1 M buffer (solution I), 1 ml. 0.5 N HCl (solution V) and dilute with distilled water to 25 ml.
Because of the high absorption of the control cuvette at 234 mμ determine the optical density at 245 mμ and use the factor 5460 instead of 3964 for the calculations. (Other methods see under "Plant seeds.")

Micro-organisms: (Example: *Penicillium*). Allow 100 mg. of dried mycelium to stand 24 hours at room temperature with 2 ml. 1.5 N alcoholic KOH (solution IX) in a 100 ml. volumetric flask. Then add 20 ml. 0.1 M buffer (solution I) and 6 ml. 0.5 N HCl (solution VI), and dilute with distilled water to 100 ml. Filter through a dry filter paper and dilute a portion of the filtrate ten-fold with 0.2 M buffer (solution II).

Plant seeds: (Example: wheat kernels). Weigh five soft wheat kernels and slice each into five transverse sections. Saponify in a 200 ml. volumetric flask with 4 ml. 1.5 N alcoholic KOH (solution IX) by heating gently on a steam bath for 2 hours, then allow to stand in the dark at room temperature for 24 hours. Add 40 ml. 1.0 M buffer (solution I) and 6 ml. of 0.5 N HCl (solution VI), dilute with distilled water to 200 ml. and filter through a dry filter paper.

The optical density of this solution was also too high to permit accurate determination of the absorption at 234 mμ with the Beckman DU spectrophotometer. Therefore the optical density was read in a Cary recording spectrophotometer at 234 mμ against a control cuvette (see "Spectrophotometric measurements", control). To calculate the polyunsaturated fatty acid content of the sample the corresponding conversion factor of 3964 is used.

Spectrophotometric measurements

Wavelength: 234 mμ; light path: 1 cm.; final volume 3.1 ml.

Pipette 3.0 ml. of sample into two test tubes. To the first tube (*control*) add

0.10 ml. boiled dilute enzyme solution (V)

and mix. To the second tube (*experimental*) add

0.10 ml. dilute enzyme solution (IV)

and mix. Allow both to stand 30 minutes at room temperature with access to the air, then transfer to 1 cm. silica cuvettes. Read the optical density E of the experimental cuvette at 234 mμ against the control cuvette.

Calculations

From the optical density E calculate the percentage polyunsaturated fatty acids in the sample according to the formula

$$\% \text{ polyunsaturated fatty acids} = \frac{E \times 3964}{W}$$

W = μg. sample in 3.0 ml. solution.

The factor 3964 is obtained as follows:

where $$3964 = \frac{3.1}{3.0} \times \frac{1000}{78.2} \times 100 \times 3$$

$\frac{3.1}{3.0}$ = dilution factor (3 ml. of sample + 0.1 ml. enzyme solution)

78.2 = extinction coefficient of 1 g. reacted polyunsaturated fatty acid/litre with a 1 cm. light path at 234 mμ.

1000 = conversion factor from grams/litre to μg./ml.

100 = conversion to %

3 = volume of sample [ml.]

Sources of Error

1. Contamination of the control or boiled, dilute enzyme solution (V) with active enzyme (solution III or IV) results in oxidation of polyunsaturated fatty acids in the control with a corresponding increase in optical density.

2. Extremes of pH are to be avoided. Excess of acid or base in the sample solutions must be neutralized before the final dilution.

3. Organic solvents used in the preparation of the samples must be removed by gently heating in a stream of nitrogen. Ethanol up to about 3% does not interfere. An ethanol concentration of 5% causes a 50% inhibition of the enzyme, thus prolonging the time taken for completion of the reaction.

Specificity

Only those polyunsaturated fatty acids having double bonds separated by *cis*-methylene groups are oxidized and isomerized by lipoxidase. The biologically important linoleic, linolenic and arachidonic acids are substrates for the enzyme. Esterified polyunsaturated fatty acids are also attacked, but the rate is extremely slow compared to oxidation of the potassium salts of the free fatty acids.