Journal of Chromatography, 202 (1980) 313–316
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,140

Note

Efficient lipoxygenase assay by gas–liquid chromatography

EMMANUEL A. C. NWANZE
Department of Biochemistry, University of Benin, P.M.B. 1154, Benin City (Nigeria)
(First received September 11th, 1979; revised manuscript received July 10th, 1980)

Lipoxygenase (E.C. 1.13.1.13) catalyzes the aerobic oxidation of unsaturated fatty acids with a *cis*-1-*cis*-4-pentadiene system to optically active conjugated *cis,trans*-diene hydroperoxides[1,2]. The enzyme has been isolated from a variety of plant sources[3–5] and has been implicated in rancidity and general food decay[6].

Many assay techniques have been adapted to the study of this enzyme, including manometry and iodiometry[7], Cupplate diffusion[8], polarography[9] and spectrophotometry[10]. The last two methods are more widely used and have been the subject of a recent assessment[11]. The authors concluded that, although the polarographic method is to be preferred, it is not sufficient to fully characterize the reaction mechanism.

The present report shows that gas–liquid chromatography (GLC) can be adapted to measure the enzyme activity. It is much more sensitive and devoid of many of the limitations of the other techniques. It may also be comparable in rapidity to the spectrophotometric method.

MATERIALS AND METHODS

Materials

The boron trifluoride–methanol reagent was supplied by Applied Science Labs. (State College, PA, U.S.A.). Linoleic acid (*cis*-9-*cis*-12-octadecadienoic acid) and soy bean lipoxygenase Type II were obtained from Sigma (St. Louis, MO, U.S.A.).

The substrate was 3.5 mg/ml sodium linoleate in a 0.5 *M* NH_4OH–NH_4Cl buffer, pH 7.0, plus 0.5% (w/v) sodium cholate as emulsifier.

Enzyme assay

A preparation containing 0.1 mg protein per ml in 50 m*M* phosphate buffer, pH 7.0 was used as the enzyme solution. A 7-ml volume of the substrate solution was placed into each of two 25-ml volumetric flasks. These were then placed in a water-bath at 30°C and saturated with clinical grade oxygen, bubbled in for 5 min. A 0.2-ml volume of the enzyme solution was then added to flask 1 and to the second flask (blank) were added 0.2 ml of the phosphate buffer. The time was noted and, at 15-sec intervals, 1 ml was withdrawn from each flask and placed into separate round-bottomed flasks containing 5 ml of chloroform–methanol (1:1, v/v) acidified with

0021-9673/80/0000–0000/$02.25

0.01 ml of 10% H_2SO_4. The chloroform layer was separated and reduced to dryness in a rotary evaporator.

Methylation and GLC

Fatty acid methyl esters (FAME) were prepared by a slight modification of the method of Metcalfe *et al.*[12] using a boron trifluoride–methanol mixture. Typically, 0.5 ml of 0.5 *N* methanolic sodium hydroxide was added to the dried extract and warmed gently on a steam-bath for 1 min. A 1-ml volume of the boron trifluoride–methanol mixture was then added and the solution boiled for 2 min. One millilitre of a saturated solution of sodium chloride was added and 2 ml of diethyl ether were then used to extract the FAME. After successive washings with water, the extracts were dried with Na_2SO_4 and used directly for GLC.

A 0.5-μl volume was injected into a Model GCD Pye Unicam gas chromatograph, equipped with a glass column (1.5 m × 4 mm I.D.) packed with 8% HI-EFF 1,4-butanediol succinate polyester (Applied Science Labs.) on Chromosorb W (80–100 mesh) and with dual hydrogen flame ionization detectors. The carrier gas (nitrogen) flow-rate was maintained at 40 ml/min. The detector and column temperatures were kept at 200°C and 190°C respectively.

RESULTS AND DISCUSSION

Fig. 1 shows the GLC profiles obtained from the sample as a function of time. A pilot spectrophotometric assay (inset of Fig. 1) showed that, although linearity exists in the initial 3 min of reaction, the reliability of the curve is doubtful owing to the high noise level. At 234 nm, which is the $\lambda_{max.}$ of the hydroperoxides present in the spectrophotometric assay, there are "ill-defined" chromophore interferences. This together with the lipophilic nature of the substrate (solubilizing agents required) can lead to considerable spectral fluctuations. Furthermore, the hydroperoxides have been shown to be immediately converted into secondary oxidation products[13], complicating their use as a basis of the enzyme assay. Because of the high

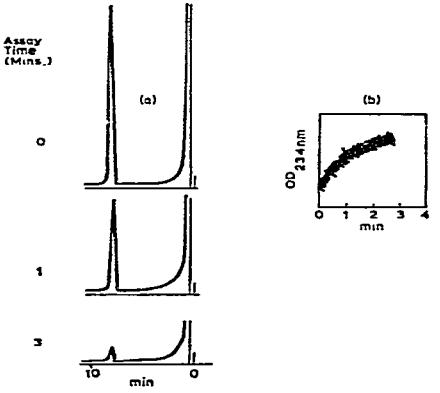

Fig. 1. GLC profiles (scan rate = 60 sec/cm) of linoleate as a function of time: (a) enzyme assay, conditions are described in Materials and methods; (b) activity profile of the enzyme assay at $\lambda_{max.}$ 234 nm.

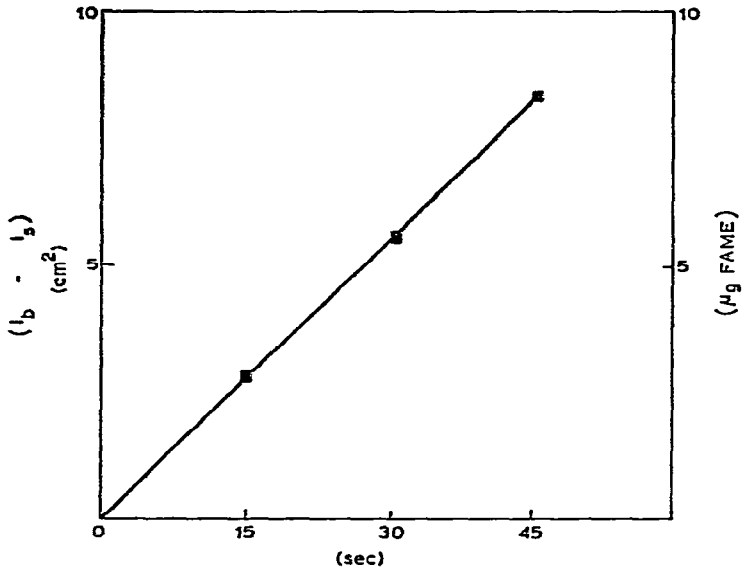

Fig. 2. Amount of linolcate consumed as a function of assay time. $I_b - I_s$ = Intensity of blank minus intensity of sample.

sensitivity of gas chromatography, even microgram quantities may be employed in the assay, thus obviating the problems posed by the poor solubility in water of the substrates.

The peak intensities were estimated by the triangulation method, and a plot of the amount of fatty acid consumed against time is linear over the period studied (Fig. 2). This method suffers from the disadvantage that comparatively longer times are required to conclude the assay. If, however, appropriate conditions are selected, a considerable time reduction can be achieved. For instance, a reduction of the percentage of stationary phase coating and an increase in operating temperature will presumably further shorten the retention times (8.6 min) observed here.

The enzyme has also been known to act directly on triglycerides, especially in the absence of lipolytic enzymes[14]. If it is desired to monitor such activity, it may therefore be necessary to employ a transesterification procedure in the preparation of the FAME. In such cases, longer reflux times with the methylating agent, such as in the method of Stoffel *et al.*[15] may be required. We have observed that there is no apparent decomposition of the substrate, especially when nitrogen is continually bubbled through the refluxing mixture. We have also observed that sodium cholate is preferred to many other dispersing agents, although at concentrations higher than 0.5% (w/v) in the assay medium the enzyme is inhibited[16].

ACKNOWLEDGEMENT

A research grant from the University of Benin is gratefully acknowledged.

REFERENCES

1 R. T. Holman, *Progr. Chem. Fats Other Lipids*, 2 (1954) 51–66.
2 O. S. Privett, C. Nickeli, W. O. Lundberg and P. D. Boyer, *J. Amer. Oil Chem. Soc.*, 32 (1955) 505–512.
3 C. E. Eriksson and S. G. Svensson, *Biochim. Biophys. Acta*, 198 (1970) 449–459.
4 M. Holden, *Phytochemistry*, 9 (1970) 507–512.
5 T. Galliard, *Biochem. J.*, 121 (1971) 379–390.
6 T. Galliard and J. A. Matthew, *J. Sci. Food Agr.*, 24 (1973) 623–632.
7 A. L. Tappel, P. D. Boyer and W. O. Lundberg, *Arch. Biochem. Biophys.*, 42 (1953) 293–298.
8 J. A. Blain, J. D. E. Patterson and M. Paerce, *J. Sci. Food Agr.*, 19 (1968) 713–719.
9 H. Mitsuda, K. Yasumoto, A. Yamamoto and T. Kusamo, *Agr. Chem.*, 31 (1967) 115–121.
10 K. Surrey, *Plant Physiol.*, 39 (1964) 65–71.
11 H. O. Berkeley and T. Galliard, *Phytochemistry*, 15 (1976) 1475–1484.
12 L. D. Metcalfe, A. A. Schmitz and J. R. Pelka, *Anal. Chem.*, 38 (1966) 514–518.
13 A. Graveland, *Lipids*, 8 (1973) 599–605.
14 H. Berkeley and T. Galliard, *Phytochemistry*, 15 (1976) 1481–1484.
15 W. Stoffel, F. Chu and E. H. Ahrens, Jr., *Anal. Chem.*, 31 (1959) 307–308.
16 E. A. C. Nwanze and J. Okoh, unpublished results, 1979.