Biotechnology Techniques, Vol 12, No 8, August 1998, pp. 627–628

Simple gas chromatography method for lipase assay

Neelima Kulkarni and R.V. Gadre*

National Chemical Laboratory, Pune – 411 008, India
Fax 091 0212 333941; E Mail nene@ems.ncl.res.in

A gas chromatography method for lipase assay using tributyrin as substrate is presented. Tributyrin is hydrolyzed by lipase to produce free butyric acid that is directly quantified by gas chromatography. The estimation of lipase activity takes only 6 min after enzyme reaction. The technique needs a small enzyme sample and is useful for analysis of large number of lipase samples.

Introduction

Lipases (EC 3.1.1.3) are usually estimated using triolein or olive oil as substrate and fatty acids released from triacylglycerols are titrimetrically quantified. The spectroscopic lipase assay methods presently practised use either natural substrates or chromogenic synthetic substrates. The fatty acids released from natural substrates react with compounds like Rhodamine 6G or copper salts to form coloured products (Jaeger *et al*., 1994). The chromogenic synthetic substrates like *p*-nitrophenyl esters (Winkler and Stuckmann, 1979) and β-naphthyl caprylate (McKellar, 1986) allow direct spectrophotometric estimation of the enzyme activity. Omar *et al*. (1987) have described estimation of hydrolysis products of triolein by gas chromatography after converting them to trimethylsilyl derivatives.

We present here an assay method for lipase activity using gas chromatography. The synthetic substrate, tributyrin, is hydrolyzed by lipase to produce butyric acid that is directly estimated by gas chromatography.

Materials and methods

Chemicals

AmanoPS (*Pseudomonas fluorescens*) lipase was a kind gift from Amano Pharmaceutical Co. (Nagoya, Japan). The Lipase type II (from porcine pancreas) and tributyrin were from Aldrich. Triolein, polyvinyl alcohol, n-butyric and n-caproic acids were from Sigma. All other chemicals of analytical grade were from S. D. Fine Chemical Co. (Boisar, India). Free fatty acid phase GC column was from Chromatopak (Mumbai, India).

Butyric acid estimation by GC

Gas chromatography column – 10% free fatty acid phase (FFAP) on Chromosorb W (AW), SS; 3 m length, 2.2 mm ID, carrier – nitrogen 40 ml/min, hydrogen 30 ml/min, air 300 ml/min, column temperature 180 °C, injector temperature 200 °C, flame ionization detector (FID) 220 °C. A precolumn of 4 cm was fitted before the column. Linearity of the FID was determined by injecting different amounts of butyric acid dissolved in 147 mM orthophosphoric acid, as standard solutions for GC analysis.

Effect of emulsifiers and sonication time

Ten ml of tributyrin were emulsified in 90 ml of emulsifier solution (cold water soluble or hot water soluble polyvinyl alcohol (PVA) or gum acacia 20 g/l) by sonication. The sonication time with cold water soluble PVA was varied from 5 seconds to 10 min and the emulsion stability was studied over 72 h by microscopy.

Assay by titrimetry

Twenty g cold water soluble polyvinyl alcohol was dissolved in 1000 ml distilled water and substrate was prepared by emulsifying 10 ml tributyrin with 90 ml of this solution. The reaction mixture composed of 5 ml substrate emulsion, 4 ml Tris HCl (0.1 M, pH 7.5) and 1 ml lipase solution (Amano PS 0.5 mg/ml or Lipase type II 5 mg/ml) was incubated at 30 °C for 30 min. The reaction was stopped by adding 20 ml acetone:ethanol (1:1 v/v). The liberated fatty acids were titrated with 0.01 M NaOH. One unit of activity was defined as the amount of enzyme which liberated 1 μM butyric acid per min under standard conditions.

Assay by gas chromatography

The assay was carried out in 1.5 ml microcentrifuge tubes. The reaction mixture composed of 250 μL tributyrin emulsion (as above), 250 μL Tris HCl buffer (0.1 M, pH 7.5) and 400 μL lipase solution (as above) was incubated at 30 °C in shaking water bath at 180 rpm for 30 min. The reaction was stopped by adding 100 μL orthophosphoric acid (14.5 M) and tubes were centrifuged at 10,000 *g* for

10 min. After centrifugation, 500 μL of the aqueous phase was withdrawn, mixed with 125 μL caproic acid (4 mg/ml) as internal standard and 5 μL of this mixture were injected. The enzyme activity was measured in terms of free butyric acid produced.

Results and discussion

Butyric and caproic acid peaks were well resolved during GC analysis. The estimation of butyric acid had a linear response upto 100 μg (regression coefficient 0.999) and the internal standard method had a high precision (standard deviation 0.057 μg). Caproic acid is a suitable internal standard as it elutes close to butyric acid and has similar response factor.

Among the emulsifiers tested, cold water soluble PVA was found to be the best followed by gum acacia and hot water soluble PVA. They gave 302, 289 and 107 U lipase activity/mg protein respectively, with 0.5 mg/ml AmanoPS lipase preparation. The tributyrin emulsion using cold water soluble PVA made by 4 min sonication was stable for 24 h. It is therefore necessary to use freshly prepared emulsion for assay. Results of the titrimetric and gas chromatographic assays with two commercial enzymes were comparable (Table 1).

The conventional olive oil based assays give true lipase activities but require large substrate and sample volumes, longer processing time for titration and are therefore unsuitable for large number of samples. The spectrophotometric assays are quick, need small sample volume but are not specific for lipase assay because the substrates can be cleaved even by esterases (Jaeger *et al*., 1994). It was found that the enzymes that hydrolyzed triolein always hydrolyzed pNPP and pNPB but hydrolysis of pNPP and pNPB did not predict lipolytic activity with triolein (Vorderwülbecke *et al*., 1992). In addition, some of these substrates are useful in a narrow pH range. Gas chromatographic fatty acid analysis methods published earlier require cumbersome and expensive derivatization techniques that make them unsuitable for quantitative, routine analysis of lipase activity.

Table 1 Comparison of lipase activity by two different assay methods.

Enzyme	*Method*	*Estimated lipase activity (U/mg protein)**
AmanoPS	GC	292
	Titrimetry	257
Lipase type II	GC	6.7
	Titrimetry	5.9

* Average results of three assays

The present assay requires a small enzyme sample, uses a substrate that is closer to a true lipid as compared to chromogenic substrates, can be used in a wide pH and temperature range and takes only six minutes for estimation of lipase activity after enzyme reaction. The assay has excellent accuracy and precision. Moreover, it can be automated by use of auto-injectors and chromatography software.

Acknowledgment

The authors are thankful to the Council of Scientific and Industrial Research, New Delhi, India for a Senior Research Fellowship to Mrs. Neelima Kulkarni.

References

Jaeger, K.E., Ransac, S., Dijkstra, B., Colson, C., Heuvel, M. and Misset, O. (1994). FEMS Microbiol. Rev. 15:29–63.

McKellar, R. C. (1986). J. Dairy Res. 53:117–127.

Omar, I.C., Nishio, N., and Nagai, S. (1987). Agric. Biol. Chem. 51:2153–2159.

Vorderwülbecke, T., Kieslich, K. and Erdmann, H. (1992). Enzyme Microb. Technol. 14:631–639.

Winkler, U.K. and Stuckmann, M. (1979). J. Bacteriol., 138:663–670.

Received: 26 May 1998
Revisions requested: 12 June 1998
Revisions received: 30 June 1998
Accepted: 30 June 1998