Spectrophotometry assay of lipase activity using Rhodamine 6G

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Summary. — A method for lipase activity determination by spectrophotometry is described. Rhodamine 6G reagent is used for complexation with the free fatty acids liberated during lipolysis This method uses toluene as the Rhodamine 6G solvent In the presence of fatty acids, a pink coloration appears and absorbance is read at 513 nm It is shown this method is pH independent and gives results similar to those obtained with TCL technique. This method is utilized for lipase activity determination in a yeast strain *Candida steatolytica*.

Key words : Lipase - Activity - Rhodamine 6G - Yeast.

INTRODUCTION

The assay of lipase activity has been a concern of workers in the lipid field for a long period. Usually, the determination of lipase activity is based on the detection of fatty acids released by the hydrolytic action of the lipase on triacylglycerols.

Numerous methods have been proposed to detect the fatty acid liberated [1-3]. Most of these methods are based on colorimetry or titrimetry [4-6]. Lowry and Tinsley [7] improved the method of Ayers [8], based on the formation of a coloured complex of cupricsoaps. This methods involved a tedious separation of two phases by means of centrifugation and is also pH dependent.

A recent method, proposed by Rogel *et al.* [9], uses parinaric acid to monitor the lipase activity. This method is well suited to perform kinetic studies, however interference with calcium was observed.

In this note, we propose to determine rapidly and accuratly the fatty acids released during lipolysis using Rhodamine 6G reagent. The method uses simple chemicals, commonly used in a laboratory and involves the determination of fatty acids in a single apolar phase. This implicates that the method is not pH dependent and that there is no need for the separation of phases.

EXPERIMENTAL

Preparation of the Rhodamine 6G reagent (R6G). The R6G reagent was prepared as indicated by Chakrabarty *et al* [10] with the difference that toluene was substituted for benzene as the rhodamine solvent. The R6G was supplied by Fluka chemical ; toluene was « analytical grade » and obtained from Prolabo. 25 mg of R6G is dissolved in 25 ml of $NaOH/NaH_2PO_4$ buffer (0.2 M − pH = 10) in order to put the R6G in the basic form. When the R6G is completely dissolved, it is immediately extracted with 100 ml of toluene. The organic phase is separated from the water one in a separatory funnel. The R6G reagent is dried over NaOH pellets on a filter and is kept in a flask well protected from light and containing NaOH pellets to eliminate moisture.

Preparation of the enzymatic substrate. The enzymatic reactions were performed in a biphasic oil/water system. The substrate, rapeseed-oil, was emulsified with polyvinyl alcohol (PVA) (1 g oil/20 ml of a 5 % aqueous solution of PVA) with an ultrasonicar apparatus [11].

Determination of lipase activity. The lipase action was monitored under the following conditions. The enzyme is incubated during 1 hour at 37 °C in appropriate buffers in the presence of emulsified rapeseed-oil. The reaction is stopped by addition of acetone/ethanol/H_2SO_4 mixture (50/50/0.1, v/v/v). The free fatty acids (FFA) liberated by the hydrolytic action of the lipase are extracted with hexane and determined by two methods.

Spectrophotometric determination. A 100 µl sample is diluted in 3.4 ml of hexane and 500 µl of R6G is added. A pink colour appears and the absorbance is read with a UV-Vis spectrophotometer at 513 nm. The concentration of FFA is determined with the aid of a standard assay curve.

Photodensitometric determination. Samples were automatically deposited on silicagel 60G plate with a Camag Linomat III apparatus. The migrating solvent was a n-hexane/ethyl ether/acetic acid mixture (70/30/1, v/v/v). The plate was then dried at 120 °C for 10 min and sprayed with a mixture of saturated aqueous solution of copper II acetate/ortho-phosphoric acid (50/50/, v/v). The spots were revealed by charring at 180 °C for 5 min and quantified by photodensitometry with a Camag TCL scanner at 500 nm and the peaks were integrated by a Delsi Enica 10 calculator.

RESULTS

Study of R6G spectrum. We compared the spectrum of R6G dissolved in benzene to the spectrum of R6G dissolved in toluene. When FFA are added, an important absorption peak appears at 513 nm in the R6G/toluene system (Fig. 1). The colour change is more drastic than in the R6G/benzene system (Fig. 2). This shows that R6G/toluene performs as well as R6G/benzene, if not better The major advantage however is toluene being a safer solvent than benzene.

Stability of R6G reagent. The stability of the formed colour was monitored by measuring the absorbance at 513 nm in function of time. The R6G reagent was added on t = 0 and the absorbance was monitored over a period of 10 min for different concentrations of FFA. Figure 3 shows that the colour was stable after 5 min. It is advisable to wait at least 5 min after adding the R6G to measure the absorbance. The colour showed to be stable for at least 2 hours.

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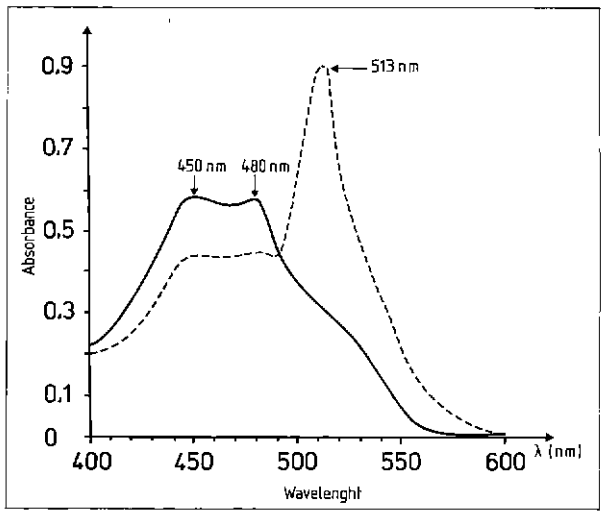

FIG. 1 — Absorption spectrum of R6G/toluene system.
—— without free fatty acids
------ with free fatty acids

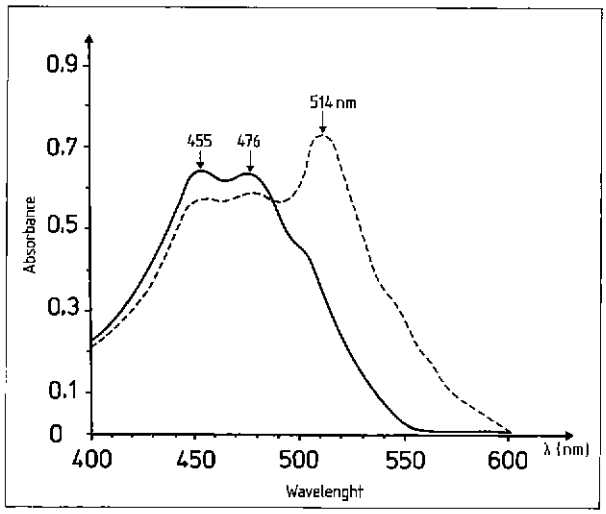

FIG. 2 — Absorption spectrum of R6G/benzene system.
—— without free fatty acids
------ with free fatty acids

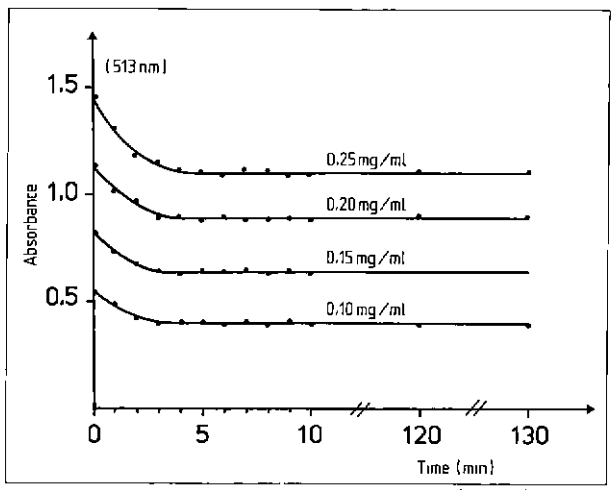

FIG 3 — Stability of R6G/free fatty acids complex absorption.

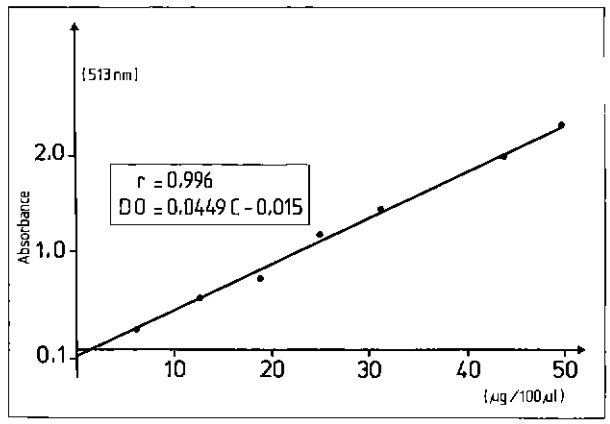

FIG. 4. — Standard curve for free fatty acids determination.

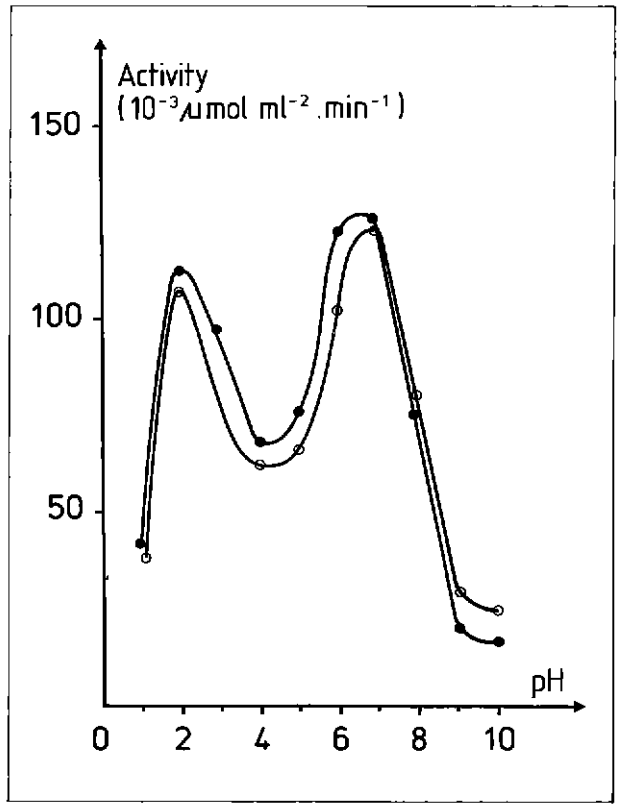

FIG 5. — Activity of *Candida steatolytica* against pH.
x————x photodensitometric method (TLC)
○————○ spectrophotometric method (R6G)

Standard curve study. 25 g of rapeseed-oil are hydrolyzed with 150 ml of ethanolic potassium hydroxyde (1N). The mixture is refluxed during 1 hour ; the reaction is stopped and the mixture is acidified with HCl4N. The reaction products are extracted with 3 × 100 ml of hexane. The solvent is eliminated under vacuum (Rotavapor).

0.1 g of FFA from rapeseed-oil were emulsified in 20 ml of a 5 % aqueous solution of PVA. Samples were prepared in order to obtain a final concentration of FFA in the hexane layer varying from 0 to 0.75 mg · ml^{-1}. The standard assay curve is presented in figure 4. There is a good correlation between optical density and FFA concentration.

Application for lipase activity determination. The R6G method was used to determine the pH optimum of lipase activity produced by *Candida steatolytica* CBS n° 5839 The reaction conditions were those described in « Experimental » for lipase activity determination.

Buffers were prepared with a pH range from 1 to 10 [12] ; the reaction mixture was incubated for 1 hour at 37 °C. The FFA released by lipase were assayed by both methods : spectrophotometric method (R6G) and photodensitometric method (TLC). The profile obtained with the two methods are similar, although R6G method gives values about 7 % higher than the photodensitometric method (Fig. 5).

DISCUSSION

The R6G method has shown to be a rapid and convenient method to monitor lipase activity. The method is flexible enough to assay low activities as well as larger quantities of FFA, by varying the amount of hexane used to extract the FFA and the amount of extract added to the R6G.

However, the preparation of the R6G is difficult to completely master, especially the extraction with toluene. This makes it necessary to elaborate a standard assay curve on each new R6G reagent

All the assays were done with rapeseed-oil used as substrate, but the method can be applied to other FFA, derived from other vegetable oils.

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RÉSUMÉ

Détermination spectrophotométrique de l'activité lipasique à l'aide de Rhodamine 6G.

P. VAN AUTRYVE, R. RATOMAHENINA, A. RIAUBLANC, C. MITRANI, M. PINA, J GRAILLE et P GALZY, *Oléagineux*, 1991, **46**, N° 1, p 29-31.

Une méthode spectrophotométrique de détermination de l'activité lipasique est décrite. Un réactif à base de Rhodamine 6G, préparé dans le toluène, est utilisé pour la complexation des acides gras libres libérés au cours de la lipolyse. En présence d'acides gras, apparaît une coloration rose dont l'absorbance est lue à 513 nm. On montre que cette méthode est indépendante du pH et qu'elle donne des résultats identiques à ceux obtenus avec les méthodes CCM Cette méthode est appliquée pour la détermination de l'activité lipasique d'une souche de levure *Candida steatolytica.*

RESUMEN

Determinación por espectrofotometría de la actividad de lipasas empleando Rhodamina 6G.

P. VAN AUTRYVE, R. RATOMAHENINA, A. RIAUBLANC, C MITRANI, M. PINA, J. GRAILLE y P. GALZY, *Oléagineux*, 1991, **46**, N° 1, p. 29-31.

Se describe un método espectrofotométrico de determinación de la actividad de lipasas. Para complejar ácidos grasos libres liberados en la lipólisis se emplea un reactivo a base de Rhodamina 6G, preparado en tolueno. En presencia de ácidos grasos aparece una coloración rosa cuya absorbancia se lee a 513 nm. Se demuestra que este método es independiente del pH, y que da resultados idénticos a los que proporcionan los métodos CCM. Se aplica este método para establecer la actividad de lipasas de una cepa de levadura *Candida steatolytica.*