Analytical Methods for Lipases Activity Determination: A Review

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Abstract: The increasing industrial demand for lipases has lead to the identification of new sources of enzymes with unique properties and generated a great interest in applying rapid, reliable, specific, selective, and sensitive analytical methods for evaluating their catalytic activity. This work offers a professional look on the achievements and the emerging strategies for lipases activity determination, involving volumetry, spectrometry, radioactive assays, immunoassays, conductimetry, chromatography, and biosensors. The principle, the advantages, and the deficiencies of each of these methods are addressed in detail.

Keywords: Analytical methods, biosensors, catalytic activity, conductimetry, chromatography, immunoassays, lipase, radioactive assays, spectrometry, volumetry.

1. INTRODUCTION

Lipases (EC 3.1.1.3 triacylglycerol acylhydrolase) are a group of water soluble enzymes, which exhibit the ability of acting at the interface between aqueous and organic phases. They primarily catalyze the hydrolysis of ester bonds in water insoluble lipid substrates. However, some lipases are also able to catalyze the processes of esterification, interesterification, transesterification, acidolysis, aminolysis and may show enantioselective properties [1]. The unique features and biotechnological potential of lipases have lead to their use in food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [2, 3]. Efficient, energy-saving, and environmental friendly biodiesel production by lipase catalyzed transesterification was recently suggested as a promising alternative to the conventional chemical catalysis [4].

Lipases are of plant, animal, and microbial origin, but only bacterial and fungal lipases such as: *Candida antarctica* (Novozym 435), *Candida Rugosa* (Lipase AY), *Pseudomonas cepacia* (Lipase PS), *Pseudomonas fluorescens* (Lipase AK), *Pseudomonas aeruginosa*, and *Thermomyces lanuginose* (Lipozime TL), among others are produced at industrial scale [5]. The catalytic properties and potential applications of *Bacillus* lipases are extensively reviewed by Guncheva & Zhiryakova [6]. Among the available lipase producing microorganisms, filamentous fungi belonging to various species of genera *Aspergillus* [7, 8], *Rhizopus* [9, 10] *Penicillium* [11-13], and *Trichoderma* [14, 15] have been described as potentially the most useful for industrial purposes lipases producers. A review on microbial lipases production with emphasis on lipases engineering and use of mathematical models for process improvement and control is provided by Treichel *et al.* [16].

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The growing industrial demand for lipases has lead to the identification of new and prospective highly lipase productive microbial strains together with rational enzyme properties design to achieve high levels of activity and substrate specificity, and has generated a great interest in applying rapid, reliable, specific, selective, and sensitive analytical methods for lipases activity evaluation. Most of these are comprehensively reviewed by Jensen, 1983 [17], Thomson *et al.*, 1999 [18], Beisson *et al.*, 2000 [19], Gupta *et al.*, 2003 [20], Starodoub, 2006 [21], and Hasan, 2009 [1]. The current review provides recent information on the achievements and the emerging strategies for lipases activity quantification, involving volumetry, spectrometry, radioactive assays, immunoassays, conductimetry, chromatography, and biosensors. The principle, the advantages, and the deficiencies of each of these methods are addressed in detail.

2. METHODS FOR LIPASES ACTIVITY DETERMINATION

2.1. Volumetry

The method is based on the titrimetric determination of the free fatty acids released from triacylglycerols by lipase catalyzed hydrolysis. The techniques applied involve sample incubation and end-point alkaline titration of the liberated acids or continuous titration of the generated products in a pH-stat titrator. Results are dependent on lipase activity. The commonly used titrant is NaOH. The end-point method has been known since 1932 when it was applied for serum lipase activity evaluation, using phenolphtalein as indicator [22]. However, the 24 h incubation time at 40^{o}C, as well as the rapid separation of the substrate emulsion/enzyme solution mixture limited its experimental utilization and lead to its modification aiming the reduction of the incubation time and experimental conditions optimization [23]. Ideal substrates are the long-chain triacylglycerols triolein and olive oil [24, 25]. Triolein is a highly specific lipase substrate; nevertheless, the high content of triolein in olive oil and its lower cost

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makes it most suitable in lipase activity assays. Another commonly used substrate is tributyrin; it is not as specific as triolein, but exhibits the property of forming aqueous dispersions in the absence of emulsifiers [26]. Butyric acid and sodium butyrate released are water soluble, in contrast to triolein, olive oil, and the products of their lipolysis. The long-chain triacylglycerols need to be emulsified, typically using gum arabic or various detergents. The substrate used in the recommended European Pharmacopeia standard lipase activity assay is olive oil, in a gum arabic stabilized emulsion. It has been demonstrated that the increased oil/water interface leads to an increase in the enzymatic hydrolysis rate [27]. The complete titration of the oleic acid released is achieved at pH 9, because of its high pK_a value, but butyric acid could be titrated at pH 6.

Despite of its deficiencies: long analysis time (2 determinations/h), low sensitivity (1 μmol mL^{-1}), tedious measurements, and errors due to incomplete titration, the titrimetric method for lipases activity determination remains in use as a reference method.

2.2. Spectrometric Methods

2.2.1. Colorimetry

Colorimetric assays as the titrimetric ones are based on the quantification of the lipolysis products. The released free fatty acids are converted in a blue color soap of cupric complexes of the fatty acids. The colored end products are extracted into an organic solvent and evaluated by spectrophotometric measurements [28, 29]. The numerous attempts made to improve the method resulted in reduction of the analysis time (4 determinations/h) by shortening the incubation period and by eliminating the phase transfer steps [30-36]. Sensitivity increase was achieved by selecting appropriate color developing reagents and suitable organic solvents [35-37]. The method is specific for fatty acids and more sensitive in comparison to titrimetric method. Redding [38] for instance performed lipase activity determinations in the range 0.02-0.4 nmol mL^{-1}. The drawbacks of the method are related to the use of toxic organic solvents.

2.2.2. Visible Spectrophotometry

The spectrophotometric methods for lipase activity determination make use of synthetic lipase substrates transformed upon enzyme catalyzed hydrolysis into products able to be detected spectrophotemetrically. The predominant substrates are p-nitrophenyl and naphthyl esters of the long chain fatty acids, and thioesters.

The lipolysis of the p-nitrophenyl esters (laurates, palmitates, oleates) gives rise to the yellow colored p-nitrophenol, measured at 405-410 nm [39-51]. The deficiency of the method is related to the pH dependence of the p-nitrophenol absorption coefficient and the total absence of absorption at acidic pH values. In addition, p-nitrophenol esters could undergo a non-enzymatic hydrolysis [19].

The cleavage of the naphthyl esters (naphthylcaprylate, naphthylacetate, naphthylpropionate) yields naphthol, in which the red colored complex with diazonuim salts is monitored at 560 nm [52-55].

Thioesters produce thiols which are coupled with the Ellman's reagent (5, 5'dithiobis 2-nitro benzoate, DTNB) to obtain yellow colored TNB anions, evaluated at 412 nm [56-58]. These processes have been employed for the development of rapid and sensitive (40-1600 mU mL^{-1}) lipase assay kits [59, 60], using dimercaptopropanol tributyrate (BALB) as a substrate.

Another lipase assay kit, based on the method developed by Panteghini [61] is proposed by Randox [62]. The lipase substrate used is the resorufin ester 1, 2-o-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) transformed upon enzymatic hydrolysis into methyl resorufin, which is quantified at 570 nm. The sensitivity achieved is 4 mU mL^{-1}. However, as established by Beisson [19], the hydrolytic activity measured in a plant homogenate with the resorufin ester could not be attributed to a true lipase activity. In addition, resorufin ester is poorly hydrolyzed by many lipases [19].

A common drawback of the mentioned spectrophotometric methods is the low substrate specificity of the enzyme towards the synthetic substrate analogues.

Alternative methods are based on the spectrophotometric quantification of the glycerol formed as a result of the triacylglycerols lipolysis, according to the following scheme: acylglycerol cleavage; periodic glycerol oxidation to formaldehyde; formaldehyde conversion with chromotropic acid to a violet chromophore which is then spectrophotometrically quantitated [63].

Other assays [64, 65] make use of a series of coupled enzyme reactions to convert the released glycerol and formation of a quinoneimine dye monitored at 545 nm. Such a test assay kit was developed by Sigma [66].

In BioVision's Lipase Assay Kit [67] glycerol is quantified enzymatically by monitoring a linked change in the Oxi-Red probe absorbance (λ=570nm). Lipase activity is detected at levels as low as 0.02 mU per well.

The spectrophotometric methods allow performing 4 determinations/h, but their number could be increased up to 16-96 determinations/h using automated systems and multi cuvettes holders or 96-well microplate readers.

2.2.3. IR Spectrophotometry

The method is based on the analysis of the Fourier Transform Infrared spectrum monitoring lipases catalyzed hydrolysis of triacylglycerols [68, 69]. Fatty acid esters and free fatty acids can be evaluated on the basis of their molar absorption coefficients and Beer's law. Because of the pH dependence of the carbonyl absorption frequency of the free fatty acids, lipolytic activity should be quantified preferentially using the specific carbonyl ester absorption frequency at 1730–1750 cm^{-1} [70]. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) was applied for monitoring lipase activity on surface-attached substrate films [70].

2.2.4. Fluorometry

Fluorometry is a sensitive analytical technique allowing continuous monitoring of enzyme activity. The numerous fluorometric methods for lipase activity determination could

be classified as methods using chromogenic substrates, and methods based on the quantification of the fatty acids released after their conversion into chromogenic products.

A variety of fatty acid esters derived form parinaric acid [71-74], coumarin (umbelliferone) [75-79], pyrenic compounds [80-83], resorufin [884], fluoresceine [85], etc. are used as chromogenic substrates.

Parinaric acid is a naturally occurring chromophore, providing fluorescence at 432 nm with an excitation wavelength at 320 nm. It was successfully applied to pancreatic lipase activity measurement with a limit of detection of 0.1 ng at pH 8. Significant increase of the free parinaric acid fluorescence was observed in the presence of detergents [19]. A drawback of the assay is parinaric acid oxidation by the atmospheric oxygen [19].

Numerous coumarin fatty acid esters were synthesized and tested as chromogenic lipase substrates [79, 86]. The sensitivity reached is 0.1-5 nmol [78]. However, these substrates undergo spontaneous hydrolysis (at pH ≥ 8.8).

When using pyrenic acylglycerol derivatives, the free fatty acids released upon lipase catalyzed hydrolysis cause a shift in the peak fluorescence intensity. Peak intensity increase with time is correlated to lipase activity. According to the procedure developed by Marker Gene Technologies, lipase activity measurement could be performed using the fluorescent substrate 1, 2-dioleoyl-3-(pyren-1-yl) decanoyl-rac-glycerol. Its cleavage gives the fluorescent fatty acid pyrenedecanoic acid [87]. Nevertheless, the pyrenic acylglycerol derivatives are also poorly hydrolyzed by lipases [74, 82].

Another lipase assay kit offered by Marker Gene Technologies is based on the use of the long wavelength fluorescent substrate resorufin oleate. The release of resorufin upon cleavage is quantified by fluorometry [88].

Another method based upon lipase catalyzed hydrolysis involves conversion of free fatty acids into chromogenic products using rhodamine B and measuring the emitted fluorescence at 535 nm with an excitation wavelength at 485 nm [89-91]. The method is rapid, could be automated, and allows the simultaneous determination of a large number of samples using various substrates.

2.2.5. Turbidimetry and Nephelometry

Turbidimetry is a method for determining the concentration of a substance in a solution by measuring the change of intensity of light in the direction of propagation of the incident beam, with reference to a standard solution. Lipase activity quantification is performed monitoring the decrease with time in the absorbance of a triacylglycerol emulsion, due to its de-emulsification with free fatty acids release [92-99]. The turbidimetric method of Neumann [93, 94] was marketed by Boehringer-Mannheim.

Another technique involved the use of Tweens as substrates with activity determined by increase in absorbance due to precipitation of the lipolysis products as calcium salts [100, 101]. The later method is 36 times more sensitive than the titrimetric assay with Tween 20, and 4 times more sensitive than the spectrophotometric assay with p-nitrophenyl palmitate [20]. Nevertheless, Tweens are not specific lipase substrates.

Nephelometry involves measurement of the intensity of the scattered light. The nephelometric determination of lipase activity could be performed within 2-4 min [100-102].

In general, turbidimetry and nephelometry are very convenient methods for the fast evaluation of the lipase activity. The arising problems and some solutions have been reviewed by Tietz [98].

2.3. Radioactive Assays

The two main techniques applied involve: (i) use of labeled lipase substrates and quantification of the liberated products, and (ii) use of unlabeled substrates and determination of the free fatty acids labeled once released.

The radioactive substrates commonly used are oleoylglycerols labeled with ^{14}C or ^{3}H, as well as iodine-131-labeled triglyceride analogues [103-106]. The detectable quantity of the product was found to be of the order of nanograms [17].

Fatty acids labeling is performed by employing ^{63}Ni [107].

Radioactive assays are specific and sensitive analytical methods. However, they do not allow continuous monitoring and are time-consuming, because of the implicated extraction steps to remove the fatty acids. In addition, they make use of radioactive substances.

2.4. Immunoassays

Immunoassays are known as highly specific and sensitive techniques for lipases activity determination. These methods are of primarily importance for clinical diagnostics and are applied for lipases quantification in serum and plasma, tissues and cell cultures lysates, and duodenum [108-122]. A number of ELISA-based clinical test kits have been developed, and are useful in the range up to 500 ng mL^{-1} lipase [123-129]. These techniques are not suitable for the evaluation of the activity of lipases originated from various sources, because they require the selection of a wide range of specific antibodies. In addition, the enzyme could form aggregates [130], which limits the accuracy of the immunological assays.

2.5. Conductimetry

Conductimetric evaluation of lipase activity is based on the measurement of the variation of the solution conductance due to electrical charge concentration change as a result of the release of free fatty acids. Reliable results were obtained using triacetin [131-133]. Triacetin is a suitable substrate due to its water solubility. The limiting equivalent conductivity of the liberated acetate anions is higher in comparison with that of the long-chain fatty acids, increasing the sensitivity of the determinations. The drawback of the technique is that triacetin is not a specific lipase substrate and conductimetric measurements suffer from high temperature dependence.

2.6. Chromatography

Chromatography is a powerful technique, well suited for testing complex matrices. The related methods currently applied for measuring lipids and released upon lipolysis free fatty acids are: thin layer chromatography, gas chromatography, and high performance liquid chromatography (HPLC).

The present state of lipid analysis by thin layer chromatography was recently reviewed by Fuchs *et al.* [134]. The method is sensitive and allows detecting picomols of fatty acids [135].

Simple and rapid gas chromatographic methods for lipase activity determination using tributyrin as substrate are described by Kulkarni and Li [136]. The techniques involve straightforward treatment procedures, short incubation period, and only 6 min analysis time after enzyme reaction.

HPLC methods for monitoring lipase reactions were designed by Ergan and André [137]. Direct HPLC monitoring of lipase activity in reverse micellar media were performed by Mingarro *et al.* [138].

A novel *in vitro* lipase assay which overcomes the substrate and pH limitations of conventional techniques was developed by Hao *et al.* [139]. It is based on the quantitation of fatty acids by liquid chromatography-mass spectrometry Oleic acids enzymatically released from triolein substrates were isolated from the reaction mixture by reverse-phase chromatography, ionized in negative mode electrospray mass spectrometry and quantitated with the aid of C^{13}-oleic acid internal standard. The enzymatic activity was measured by monitoring oleic acid productions at multiple time points. Nevertheless, chromatographic determinations remain expensive and time consuming. In addition, they require experienced personnel and sophisticated laboratory equipment.

2.7. Biosensors Based Methods

The biosensors, according to the IUPAC definition, are "devices that use specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" [140]. Various biosensors were developed for lipases substrates and lipolysis products determination, using immobilized enzymes. They are comprehensively reviewed by Starodub, 2006 [21]. The novel biosensors for triglycerides determination recently designed include: (i) amperometric biosensors, constructed by co-immobilization of lipase, glycerol kinase, and glycerol-3-phosphate oxidase onto various supports: cellulose acetate [141], PVC [142], polyvinyl alcohol [143], egg shell [144], or chitosan and zinc oxide nanoparticles composite film [145] deposited on the surface of a Pt electrode. In all cases the amperometric response is the current of H_2O_2 oxidation; (ii) impedimetric biosensor using as a support for lipase immobilization an electrophoretically deposited polyaniline nanotubes (PANI-NT) film onto indium-tin-oxide coated glass surface. Fatty acid molecules produced during triglyceride hydrolysis provoked a change in charge transfer resistance of PANI-NT film depending on triglyceride concentration [146]; (iii) potentiometric and micromechanical biosensors, based respectively on electrolyte-insulator-semiconductor capacitor, and a polysilicon microcantilever [147]; and (iv) enzyme field effect transistor, based on lipases immobilized via magnetic nickelferrite nanoparticles [148]. Nevertheless, only few biosensors were applied to lipase activity determination. Such a sensor is the capacitive one with a sandwich-like structure: $Au/S(CH_2)_{17}CH_3$/substrate/electrolyte [149]. Enzyme activity is quantified by monitoring the rate of desorption of the lipolysis products. Desorption is evaluated measuring the increase of the electrode capacitance. An amperometric biosensor for lipase activity evaluation was suggested by Rejeb *et al.* [150]. It involves glycerol dehydrogenase/NADHoxidase immobilized on the surface of a Prussian Blue modified screen printed electrode. The principle of the determination is based on the following reactions:

$$\text{triacylglycerols} + 3H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3 \text{ fatty acids}$$

$$\text{glycerol} + NAD^+ \xrightarrow{\text{glyceroldehydrogenase}} \text{dihydroxyacetone} + NADH + H^+$$

$$NADH + H^+ + O_2 \xrightarrow{\text{NADHoxidase}} NAD^+ + H_2O_2$$

The analytical signal is the current of H_2O_2 oxidation, determined using Prussian Blue as a mediator, and proportional to the glycerol concentration, that is dependent on the lipase activity. The measurements are performed at low electrode potential, thus avoiding interferences. The sensitivity of the analysis is inferior to that achieved by gas chromatography, but sufficient for determination of triacylglycerols levels in biological sera and food preparations.

A very convenient method for lipase activity determination was recently developed by QSENSE, using quartz crystal microbalance with dissipation (QCM-D) [151]. Triolein, used as lipase substrate, was deposited onto the surface of the gold electrode of the QCM-D. The enzyme degradation of the triolein film was continuously monitored measuring the changes in frequency and dissipation and was correlated to lipase activity.

Biosensors are considered as promising analytical tools, because of the rapidity and sensitivity of the determinations, and the low cost of the equipment.

2.8. Other Methods

A chemiluminescence method, allowing the completion of a single assay within 5 min, and displaying a sensitivity and a repeatability superior to those of the titration method was suggested by Arima *et al.* [152]. It uses the lauric acid ester of 2-(4-hydroxyphenyl)-4,5-diphenylimidazole (HDI-laurate) as a proenhancer substrate, and is based on the chemiluminescence reaction of luminol-horseradish peroxidase-hydrogen peroxide with HDI, which is liberated from the substrate by enzymatic hydrolysis. The method was applied to the determination of lipase activity in pharmaceutical preparations.

A novel technique for differential activity-based gel electrophoresis of lipolytic enzymes, combined with fluorescence imaging, and identification of the tagged proteins by MS/MS was developed by Morak *et al.* [153]. The study

represents the first application of this technology for comparative analysis of lipases and esterases.

In this group are also included the methods designed for lipase activity detection and lipolysis kinetics investigation, rather than for quantifying lipase activity. These involve: numerous plate assays [91, 154, 155], surface tension measurements [156, 157], electron microscopy [158, 159], atomic force microscopy [160, 161], and nucleic magnetic resonance [162] based methods.

3. CONCLUSION

In this work are discussed the basic principles, the advantages, and the deficiencies of the numerous established and emerging methods for lipase activity determination. Taking into consideration that none of them fulfills totally the analytical requirements, the method of choice should be selected based upon the purpose of the analysis, the available laboratory equipment, and qualified personnel. Current efforts are devoted to rapidity, sensitivity, and selectivity improvements.

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