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A Quantitative Fluorescence-Based Lipase Assay

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Summary

An easy and fast gel diffusion assay for detecting and monitoring lipase activity by quantification of fluorescein is described. By measuring the intensity of fluorescein, it is possible to obtain a calibration curve with a regression coefficient better than by using the radius of fluorescent haloes. Through the quantification of fluorescence intensity of fluorescein released after the hydrolysis of a fluorescent ester, fluorescein dibutyrate, used as substrate in agar plates, commercial and skimmed milk lipase activity were studied. Moreover, with this method, lipase activity can be monitored in reaction medium that contains compounds which are affected by turbidity or cause measurement interference for UV-spectrophotometer and fluorimeter. In this experiment, boiled skimmed milk was dispersed in the agar gel with fluorescein dibutyrate, and it was used as a reaction medium to mimic natural conditions. The development of such an assay has a potential for applications in industries ranging from pharmaceuticals to food production and monitoring.

Key words: agar plate assay, hydrolytic activity, lipase, milk

Introduction

In the search for novel enzymes, it is necessary to apply functional tests during high-throughput screenings that are capable of detecting enzyme activities with high selectivity and sensitivity (*1*). In this regard, several methods have been developed for the measurement of lipases, an important group of ubiquitous biocatalysts which have relevant applications in the food, detergent and pharmaceutical industries (*2*). As reported by Josef *et al.* (*3*), lipases not only catalyze the hydrolysis of triglycerides but also perform esterification, interesterefication, acidolysis, alcoholysis, and aminolysis. Currently, the basic characteristics driving the optimization of any procedure that measures enzymatic activity are that the method is simple, direct, specific, and ready for automation. Agar plate methods, which are useful for studying and quantifying enzymes present in biological extracts (*4*), may provide an alternative to other methods and an interesting starting point from which to develop new assays. Visualization of lipase on gel media can be accomplished by using dyes because the drop in pH due to released fatty acids can be monitored by colour changes of a pH indicator dye (*2–5*). Since there is a linear relationship between the diameter of the fatty acid diffusion spot and the logarithm of the enzyme concentration, the technique is very convenient for detecting lipolytic organisms, even though metabolic acidification of the medium caused by factors other than free fatty acids released by enzymatic activity can give false results. Fluorescence assays can measure the reaction products that become fluorescent upon hydrolysis and can be continuously monitored (*6*). Moreover, these methods are very sensitive and less susceptible to compounds that cause background signals (*7*). Sandoval and Marty (*8*) reported the use of a gel assay that relied on a fluorescent indicator to measure *in vivo* synthetic activity of lipases derived from microorganisms. This protocol is based on the esterification of a long-chain alcohol and different fatty acids contained in a rhodamine B agar plate. It is very sensitive, but it is not suitable for bacterial strains which metabolize fatty acids. To overcome the problems described above, we measured the hydrolytic activity of a commercial prepa-

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ration of lipase and of fresh skimmed milk through quantification of fluorescein, a fluorescent molecule attachable to many substrates (*9*), which is released after enzyme catalysis. Moreover, to avoid complications when scaling up from the laboratory to industrial applications, the enzyme activity of fresh skimmed milk was tested not only in a TBS agar (synthetic buffer), but also using boiled skimmed milk which was dispersed in agar gel to mimic natural conditions.

Materials and Methods

Plates (15 cm in diameter) were filled with 2 % (by mass per volume) agarose (A5093-500G; Sigma-Aldrich, St. Louis, MO, USA) dissolved in microwave oven in TBS (Tris-buffered saline) at pH=7. The solution was cooled to 70 °C, and 50 mM of fluorescein dibutyrate (FDB; Sigma-Aldrich) and 0.2 % Triton X-100 (BDH Chemicals, Poole, UK) were added. To disperse fluorescein dibutyrate, we used Triton even though it is a UV-absorbing detergent and the fluorescent measurement was made under UV excitation.

A volume of 1 mL of 50 mM FDB and 50 mL of gel solution were transferred into plates and cooled at room temperature. The thickness of the resulting gel was 3 mm. The volume and thus the depth of the agar in the plate is an important parameter as it influences signal strength (data not shown).

Wells (3 mm in diameter) were obtained using a cork borer with a volume of about 50 μL. To obtain a calibration curve, increasing amounts of fluorescein (Sigma-Aldrich) (0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 μg) dissolved in TBS were loaded into each well. Additionally, increasing quantities (3, 6, 9 and 12 μg of protein) of a commercial lipase from hog pancreas (Fluka, Sigma-Aldrich, 30.1 U/mg) dissolved in TBS and fresh skimmed milk (FSM) (10, 15, 20 and 25 μg of protein) were loaded into each well. Protein quantification was carried out by the Bradford Coomassie method using Pierce Coomassie protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). The calibration curve and enzymatic test of the commercial lipase and of FSM were carried out at the same time and under the same conditions at 39 °C. After an hour, fluorescent haloes were visible under UV light. To monitor the time evolution of the radius and fluorescence, measurements were taken every hour for 4 h. To reproduce the natural turbid conditions of milk, the enzyme activity of FSM was then tested in boiled skimmed milk incorporated into the agarose gel (BSM), heated at 100 °C for 30 min in order to denature constitutive enzymes, especially lipase, and then supplemented with FDB. Images of enzymatic activity were acquired by a digital camera and analyzed with the Gel Doc 1000 system (Bio-Rad, Hercules, CA, USA). Radius and fluorescence measurements were taken using Molecular Analyst® software (Bio-Rad).

The standard curves were obtained from (*i*) a log transformation of increasing fluorescein quantities and the radius of the haloes, and from (*ii*) the increasing quantities of fluorescein and the intensity of fluorescence converted to counts (fluorescence units, FU) by the Molecular Analyst® software. For the regression analysis and *t*-test ($p<0.05$), Excel and CoHort software (Monterey, CA, USA) were used, respectively.

Results and Discussion

Fig. 1 shows the quantification of lipase activity using either the measurement of the radius of the fluorescent halo created by fluorescein, or the intensity of fluorescence. The obtained images were enhanced by Molecular Analyst® and converted into peaks and related measurements. Fig. 1a shows the fluorescence intensity peaks created by increasing doses of fluorescein. In order to

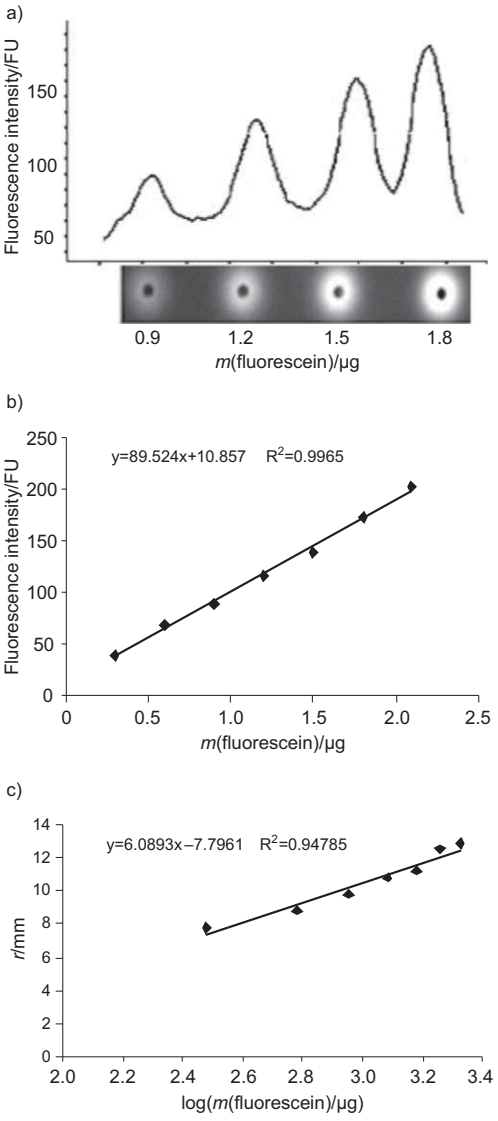

Fig. 1. Quantification of fluorescein: a) an example of densitometric analysis of increasing doses of fluorescein. Image analysis was performed by avoiding the black internal hole of the fluorescent halo. Intensity of fluorescence is converted to counts (fluorescence units, FU) by Molecular Analyst® software. A number of 255 counts represents the value of fluorescence saturation; b) linear regression curve between the intensity of fluorescence, expressed by the measure of the height of the peaks and the fluorescein mass; c) linear regression curve between the logarithm of fluorescein mass and the radius of stained zone formed on agarose plate. All values are means of three trials. Coefficient of variation (CV) is less than 1 %

verify the relationship between the intensity of fluorescence, as measured by the height of the peaks, and the quantity of fluorescein, a model of regression was studied (Fig. 1b). The regression analysis reveals a linear trend. The correlation coefficient, R^2, was found to be 0.9965, which is better than the acceptance criteria of 0.9900. This method of fluorescein quantification was compared with methods proposed by other authors (*4–9*) that consider only the measurement of the diameter or the radius of the haloes. However, as shown in Fig. 1a, the halo borders are not clear. The diffusion of fluorescence does not allow easy measurement of the radius of the halo. The linear relationship (Fig. 1c) between the radius and the log of fluorescein quantity produces a regression coefficient of 0.9478. The value is lower than that obtained using peak intensity and is outside the acceptance criteria. This method is also slower because it requires a log transformation.

The linear range of Fig. 1 corresponds to a very low quantities of fluorescein (0.3–2.1 μg), but due to the contrasting effect of white fluorochrome signal and the black background under UV light, enhanced by the Molecular Analyst software, it is possible to load even lower concentrations of fluorescein, increasing the sensitivity of the method (data not shown). Besides, the calibration curve of Fig. 1b, encompassing a low change in concentration, shows how small quantities of fluorescein produce great variations of intensity, further demonstrating the high sensitivity of the method. Sensitivity of an assay is essential in most applications where the aim is the identification of dilution of an enzyme, and therefore weak enzymatic activity (*6*). The linear trend of the calibration curve is observed until the maximum value of 255 counts (FU) that represents, for the Molecular Analyst software, the saturation limit of fluorescence. The main advantage of fluorimetric assays besides their sensitivity is the possibility to continuously follow the reaction kinetics (*2*).

There are many synthetic enzymatic substrates that release fluorescein upon hydrolysis. In this experiment, a synthetic ester, FDB, was chosen to study lipase activity. The lipolytic activity of increasing doses of lipases of different origin was studied by using agar plates in which FDB, detergent and TBS were present. Table 1 shows the lipolytic activity of the commercial enzyme and of FSM during 4 h of incubation (T1, T2, T3 and T4). As shown in Table 1, the lipase activities as estimated by the intensity of fluorescein released after the hydrolysis and as predicted by the radius of the halo are not statistically significantly different as determined by the *t*-test ($p<0.05$). Both methods show an increase in the lipolytic activity of both the commercial lipase and the FSM over time. The coefficient of variation is higher if the enzyme activity is estimated as a function of the radius of the halo. This is due to the diffusion of fluorescence around the border that makes it difficult to measure, as shown in Fig. 2a, which shows the haloes of fluorescein released by increasing amounts of FSM-derived lipases over time. It is likely that some FDB is incorporated into micelles and that the lipase molecules that diffuse out of the well sample may hydrolyze FDB in the micelles.

Fluorescence assays have not only got the advantage to be very sensitive but also they are less susceptible to interference by compounds causing background signals. For instance, enzymatic activity can be measured in crude lysates and even in turbid solutions (*8,10*).

It is known that synthetic esters, such as FDB, are insoluble in aqueous buffers and in the presence of surfactants. Turbidity caused by such synthetic esters has been considered an interfering factor in techniques such as spectrophotometric reading (*11*). Thus, the screening method presented here, which is in gel media, allows the calculation of enzyme activity even if insoluble compounds that produce turbidity or interference are present. This method is specific for the quantification of the analyte without interference, because the estimation of

Table 1. Comparison of lipase activities using TBS and BSM as reaction media

Buffer	Sample	Time	Fluorescence activity	CV	*r*(fluorescein halo)	CV
			FU	%	mm	%
TBS	lipase	T1	0.86±0.084	9.7	0.978±0.322	32.9
		T2	1.212±0.141	11.6	1.001±0.309	30.8
		T3	2.034±0.155	7.6	1.89±0.76	40.2
		T4	2.548±0.392	15.4	2.321±0.89	38.34
TBS	FSM	T1	0.07±0.011	15.0	0.051±0.014	27.5
		T2	0.092±0.004	3.9	0.079±0.021	26.5
		T3	0.164±0.25	15.0	0.178±0.078	43.8
		T4	0.350±0.018	5.2	0.321±0.112	34.9
BSM	FSM	T1	0.03±0.005	16.8	0.025±0.011	44.0
		T2	0.054±0.008	14.8	0.044±0.018	40.9
		T3	0.064±0.013	20.2	0.057±0.025	43.9
		T4	0.081±0.015	18.5	0.078±0.028	35.9

Evolution of radius of fluorescein halo and fluorescence activity was monitored every hour for 4 h (T1–T4). To check the precision of the method, the corresponding coefficients of variation (CV) were calculated at the fluorescein mass of the used range. All values are means of three determinations

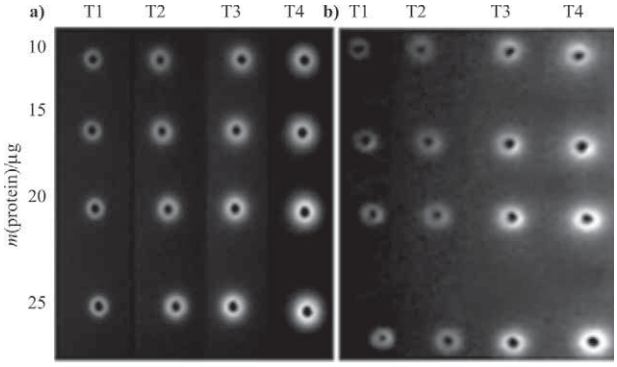

Fig. 2. Lipolytic activity of increasing quantities of fresh skimmed milk over time, as measured on plates in the presence of: a) TBS and b) BSM as reaction media. See Table 1 for definitions of T1, T2, T3 and T4

the fluorescence intensity and haloes of fluorescein are not affected by turbidity.

Since this method monitors only fluorescence, the lipolytic activity of FSM was studied when boiled skimmed milk was incorporated into the agarose gel (Fig. 2b). This allowed the study of the lipolytic activity under chemical and physical conditions similar to natural turbid conditions of milk. As shown in Fig. 2b, the intensity and radius of haloes of fluorescein increased as a function of time and quantity of the loaded sample, although the use of BSM in the reaction medium causes slight background noise. These findings are quantified in Table 1. The coefficient of variation (CV) is higher when BSM is used in the reaction medium and when the halo radius is considered. However, there is not statistically significant difference between the two methods of quantification (*t*-test, $p<0.05$), and the interference caused by BSM in the reaction medium is very low and does not prevent the estimation of lipolitic activity of FSM. The lipase activity of FSM, calculated with BSM in the reaction medium, was lower than that detected in TBS, probably due to the competition of two substrates, FDB and endogenous lipids in boiled skimmed milk (*12*).

Conclusion

The method proposed here is a simple and fast assay for measuring lipase and esterase activities. To our knowledge, this is the first report on the use of gel diffusion assay for determining these enzymes by using fluorescein dibutyrate, fluorescent substrate with ester linkage, based on the activity quantification by the measure of fluorescence intensity of the halo. It is useful for measuring a large number of samples simultaneously, allowing for easy automation. This is an alternative to high-throughput methods such as microplate-based fluorescent lipase assay that have many wells per plate, and has the advantage of not requiring a plate reader or fluorometer, which may not be broadly available in many laboratories.

Another advantage is that it allows the determination of enzyme activity even in the presence of insoluble compounds that produce turbidity or cause measurement interference for UV-spectrophotometer and fluorimeter. In our laboratory this method allowed us to compare the lipolytic activity of milk of different mammals. Moreover, it should be possible to extend this method to other enzyme assays based on fluorescent substrates. The development of an assay such as this holds great promise for applications in industries ranging from pharmaceuticals to food production and monitoring.

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References

1. J. Grognux, D. Walher, E. Nyfeler, J.L. Reymond, Universal chromogenic substrates for lipases and esterases, *Tetrahedron Asymmetry, 15* (2004) 2981–2989.
2. F. Hasan, A.A. Shah, A. Hameed, Methods for detection and characterization of lipases: A comprehensive review, *Biotechnol. Adv. 27* (2009) 782–798.
3. B. Josef, P.W. Ramteke, G.O. Thomas, Cold active microbial lipases: Some hot issues and recent developments, *Biotechnol. Adv. 26* (2008) 457–470.
4. F. Zocca, G. Lomolino, A. Lante, 3,4-Dihydroxyphenylalanine gel diffusion assay for polyphenol oxidase quantification, *Anal. Biochem. 383* (2008) 335–336.
5. H. Scholze, H. Stütz, F. Paltauf, A. Hermetter, Fluorescent inhibitors for the qualitative and quantitative analysis of lipolytic enzymes, *Anal. Biochem. 276* (1999) 72–80.
6. J.P. Goddard, J.L. Reymond, Recent advances in enzyme assays, *Trends Biotechnol. 22* (2004) 363–370.
7. D. Gilham, R. Lehner, Techniques to measure lipase and esterase activity *in vitro*, *Methods, 36* (2005) 139–147.
8. G. Sandoval, A. Marty, Screening methods for synthetic activity of lipases, *Enzyme Microb. Technol. 40* (2007) 390–393.
9. M. Schmidt, U.T. Bornscheuer, High-throughput assays for lipases and esterases, *Biomol. Eng. 22* (2005) 51–56.
10. L.F. Hernández, J.C. Espinosa, M. Fernández Gonzáles, A. Briones, β-Glucosidase activity in a *Saccharomyces cerevisiae* wine strain, *Int. J. Food Microbiol. 80* (2003) 171–176.
11. P. Jain, S. Jain, M.N. Gupta, A microwave-assisted microassay for lipases, *Anal. Bioanal. Chem. 381* (2005) 1480–1482.
12. H.C. Deeth, Lipoprotein lipase and lipolysis in milk, *Int. Dairy J. 16* (2006) 555–562.