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Use of methylumbeliferyl-derivative substrates for lipase activity characterization

Núria Prim, Marta Sánchez, Cristian Ruiz, F.I. Javier Pastor, Pilar Diaz*

Department of Microbiology, Faculty of Biology, University of Barcelona, Avenue Diagonal 645, 08028 Barcelona, Spain

Abstract

Lipases and esterases have been recognized as very useful biocatalysts because of their wide-ranging versatility in industrial applications, their stability, low cost, and non-requirement for added cofactors. The physical properties of lipidic substrates, typically water insoluble, have determined a great difficulty in studying lipolytic enzymes. A method for fast and simple detection of lipolytic activity, based on the use of 4-methylumbelliferone (MUF)-derivative substrates was developed. The system has been used for the detection of lipase activity either from microbial colonies, cell culture suspensions, or from proteins separated on SDS-polyacrylamide or isoelectric focusing gels. The use of MUF-derivative substrates has also been extended to the quantitative determination of lipolytic activity from a variety of assays including optimum pH and temperature determination, growth dependency, kinetics or stability studies, or residual activity quantification after treatment with potential inhibitors. The method has shown to be a useful tool for the characterization of a variety of lipases from microbial origin, including those cloned in heterologous hosts.

Keywords: Lipases; Esterases; MUF-butyrate; MUF-oleate; *Bacillus*; *Saccharomyces*; *E. coli*

1. Introduction

Lipases (glycerol ester hydrolases, E.C. 3.1.1.-) are hydrolases acting on the carboxyl ester bonds present in acylglycerols to liberate organic acids and glycerol. Triacylglycerols are mainly uncharged insoluble lipids, although those with short-chain fatty acids are slightly soluble in water. The physical properties of lipids have caused many difficulties in studying the characteristics of lipolytic enzymes [1].

A variety of lipases from microbial origin with different enzymological properties and substrate specificities have been found and characterized [2,3]. The physiological functions of most microbial lipases or esterases are not clear, some of them being probably involved in metabolic pathways to provide carbon sources, in plant cell-wall degradation, in pathogenicity, or in biocide detoxification [4]. However, lipases and esterases have been recognized as very useful biocatalysts because of their great versatility in industrial applications. Practical use of microbial lipases has determined a great interest concerning the improvement of both the producing strains and the biochemical properties of lipolytic enzymes [5,6].

A large number of methods for measuring lipolytic activity have been reported, most of which differ on the systems used for substrate solubilization, and the type of activity marker employed [1,7]. The choice of a method for enzyme activity determination depends on the type of enzymes being studied and the

* Corresponding author. Tel.: +34-3-4034627; fax: +34-3-4034629.
E-mail address: pdiaz@bio.ub.es (P. Diaz).

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information required. Traditionally, lipases have been assayed by radiometric or titrimetric techniques [7]. Radiometric methods are the most sensitive but require the use of radiolabelled and expensive substrates. On the other hand, titrimetric systems suffer from low sensitivity [7]. For experiments involving a large number of assays or non-purified samples, it is convenient to use chromogenic or fluorogenic substrates which can simply and rapidly be tested. Nevertheless, many chromogenic substrates show low specificity and sensitivity, or become spontaneously hydrolyzed under certain conditions, which represents the major disadvantages of using these type of substrates [1].

Therefore, efforts to improve systems for lipase activity detection from purified or non-purified preparations are desirable. The use of lipidic substrates linked to fluorescent compounds provides a sensitive method for detection and quantification of enzyme activity in biological systems [8,9]. Among the fluorogenic substrates, 4-methylumbelliferone-derivatives, commercially available in a wide range of chain-length, have shown to be useful tools for lipase activity determination [9–11]. In this report we describe the use of MUF-derivative substrates for the complete characterization of several lipases well suited for biotechnological applications, some of which have already been cloned and expressed in heterologous hosts [12–15].

2. Experimental

2.1. Strains, plasmids and growth conditions

Bacillus subtilis MB216 [16], *Bacillus* sp. BP-7 [17], and *Paenibacillus* sp. BP-23 [18] were used as gene donors and grown in nutrient broth at 30 °C. *Escherichia coli* 5K [19], *Saccharomyces cerevisiae* strains 13bxV4 (CECT10837, [*trp1*]) and CENPK 113-11A (*trp1-289 his3-Δ1*) [20,21], and *B. subtilis* strains MB216 and BCL1050 [22], were used as recipient hosts for recombinant plasmids. *E. coli* was grown in LB medium at 37 °C. *S. cerevisiae* strains were routinely grown at 30 °C in YPD [21], and occasionally in Difco Minimal Medium [0.17% YNB w/o amino acids +0.5% $NH_4(SO_4)_2$] plus glucose (2%) or olive oil (1%), supplemented in some cases with histidine [23]. Plasmids pBR322, pUC19 [24], YEplac112 [25], and the pUB110-derivative plasmid pRB473 [26] were used as cloning and expression vectors.

2.2. MUF-staining technique

Stock solutions of MUF-butyrate (25 mM) or MUF-oleate (100 mM; Sigma, USA) in ethyleneglycol monomethylether ($C_3H_8O_2$) in the presence of 50 mM phosphate buffer, pH 7.0 were prepared and used for further assays. Lipase producing microbial strains grown on solid medium were detected under UV illumination after covering the plate surface with a 100 μM solution of MUF-butyrate [11]. Detection of lipolytic activity from cell suspensions or cell fractions was performed by transferring a small aliquot (5 μl) of each sample onto filter paper, and addition of 5 μl of 25 mM MUF-butyrate stock solution, followed by UV illumination of the paper. This procedure was also used to select the most convenient working concentration of MUF-substrate for a given sample, or to determine the amount of enzyme required for hydrolyzing a defined MUF-substrate. Detection of lipolytic activity on MUF-butyrate was achieved in less than 1 min, while hydrolysis of MUF-oleate usually required a 15 min incubation at room temperature [11].

2.3. Electrophoresis and zymograms

SDS-PAGE was performed in 10% (w/v) gels, essentially as described by Laemmli [27]. Isoelectric focusing was performed in a Pharmacia Phast System Unit, using gels with a pH range 3.0–9.0. After the run, gels were soaked for 30 min in 2.5% Triton X-100® at room temperature, briefly washed in 50 mM phosphate buffer, pH 7.0, and covered by a solution of 100 μM MUF-butyrate or 200 μM MUF-oleate in the same buffer [11]. Activity bands became visible in a short time after UV illumination. Following zymogram analysis, both SDS-PAGE and IEF gels were subsequently stained with Coomassie Brilliant Blue R®-250, and protein bands visualized.

2.4. Nucleic acid manipulation and cloning procedures

Plasmid and genomic DNA were purified essentially as described [24]. Restriction nucleases

and DNA-modifying enzymes were obtained from Bio-Labs and used according to the manufacturer's specifications. Northern hybridization analysis (not shown) and DNA manipulations were performed as described [24]. Primer oligonucleotides were purchased at Gifco BRL, and *Pfu* polymerase was from Stratagene. DNA was sequenced as described [28], homology analyzed through BLAST [29], and alignments were performed using ClustalW (1.74) Multalign software (http://www2.ebi.ac.uk/clustalw).

Gene libraries from *Paenibacillus* sp. BP-23 and *Bacillus* sp. BP-7 were constructed as described previously [28,13] and the clones coding for esterases EstA and EstA1, respectively, were isolated for further characterization. *B. subtilis* MB216 lipase A gene was isolated by PCR amplification and cloned in several hosts as previously described [14].

2.5. Activity assays

Determination of lipolytic activity was routinely performed from crude cell extracts or concentrated culture media [12]. Activity was determined by measuring the release of *para*-nitrophenol (*p*NP) or 4-methylumbelliferone (MUF) from *p*NP or MUF-derivative substrates. For colorimetric assays, 0.3% stock solutions of *p*NP-derivatives (0.15% for *p*NP-palmitate and *p*NP-stearate) were prepared in isopropanol, and emulsified by sonication [30]. The reaction mixture consisted of 450 μl of a 1:10 dilution of the substrate stock solution in 50 mM phosphate buffer pH 7.0, containing 0.1% Arabic gum and 0.4% Triton X-100®, and 50 μl of cell extract. This mixture was incubated at 37 °C for 15 min, the reaction terminated by addition of 35 μl of 0.1 M Na_2CO_3, and the released *p*NP determined by measuring the absorbance at 410 nm. One unit of activity was defined as the amount of enzyme that released 1 μmol of *p*NP per minute under the assay conditions described.

Fluorometric assays using MUF-derivative substrates were usually performed as time–drive plots in a Hitachi F-2000 spectrofluorimeter (Hitachi, Japan). The fluorescence release caused by hydrolysis of the fluorogenic substrates was measured at $\lambda_{ex} = 323$ nm and $\lambda_{em} = 448$ nm, established as the maximum under our assay conditions. Stock solutions of MUF-derivative substrates (25 or 100 mM) were prepared as described above and emulsified by shaking vigorously [31]. The reaction mixture consisted of 1.5 ml of 50 mM phosphate buffer pH 7.0 containing 0.1% Arabic gum and 0.4% Triton X-100®, to which 6 μl MUF-derivative stock solution and 60 μl cell extract were added. Activity was determined by measuring the increase of fluorescence emission due to the release of MUF caused by hydrolysis, according to previously generated standard plots [12]. One unit of activity was defined as the amount of enzyme required to release 1 μmol of MUF per minute under the conditions described.

Optimum pH was established using 50 mM sodium succinate (pH 4–6), phosphate buffer (pH 6–7.5), Tris–HCl (pH 7–9), and glycine buffer (pH 9–12) in the standard fluorometric assay. To subtract the effect of pH on fluorescence emission, the reactions were terminated by addition of 30 μl 20% HCl so that fluorescence could be measured under the same conditions for all samples. Determination of the optimum temperature or stability was performed by incubating the reaction mixtures or crude cell extracts at different temperatures prior to determination of MUF release. For inhibition studies, assays were performed on *p*NP-laurate and MUF-butyrate in the presence of several metal ions, enzyme modifiers or chemical agents, and the residual activity was measured with respect to that of untreated samples.

3. Results and discussion

3.1. Screening for lipase activity

MUF-derivative substrates were used for the detection and isolation of lipase or esterase-producing microbial strains. This is routinely one the first steps in the study of microbial lipolytic activity. As shown in Fig. 1A, MUF-derivative substrates provide a fast and simple tool for lipolytic activity detection. Lipase producing microbial strains can easily be identified after growth on agar plates, by addition of the chosen MUF-derivative substrate solution onto the plate. A fluorescent signal is visible only for strains bearing lipolytic activity (Fig. 1A).

Once selected the lipase or esterase producing microorganisms on solid media, fast detection of activity in cell suspensions (Fig. 1B) or purified enzyme solutions (Fig. 1C) can be performed by transferring a

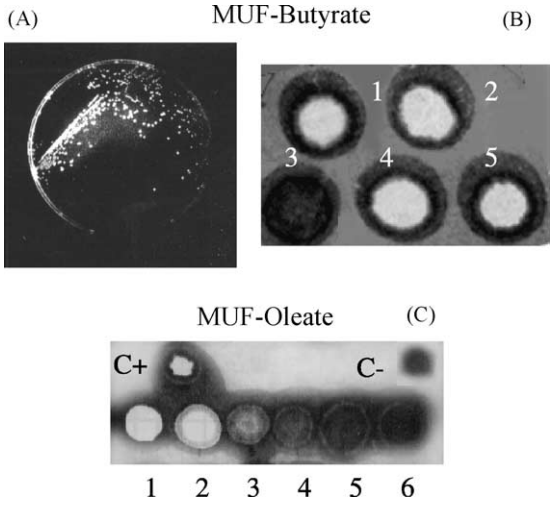

Fig. 1. Activity detection on agar plates and filter paper. (A) Recombinant *E. coli* Xl1 colonies expressing esterase EstA1 from *Bacillus* sp. BP-7. (B) Whole cell suspensions of lipolytic strains *Bacillus* sp. BP-7 (1), *Paenibacillus* sp. BP-23 (2), *Burkholderia cepacia* R6 (4), recombinant *E. coli* 5K expressing EstA from *Paenibacillus* sp. BP-23 (5), and the non-lipolytic strain *E. coli* 5K (3). (C) Several Pancrealipase® dilutions assayed on MUF-oleate; samples 1–6 correspond to 4.9×10^{-6}, 2.45×10^{-6}, 1.22×10^{-6}, 6.1×10^{-7}, 3.0×10^{-7}, and 1.5×10^{-7} enzyme units, respectively. C+ and C− are the control samples.

small aliquot of each sample onto filter paper, and addition of a MUF-derivative solution to the samples. UV transillumination of the paper (Fig. 1B) allows the identification of the lipase or esterase producing cells by appearance of a fluorescent signal. The same system can be used as well for the detection of lipolytic activity in different cell fractions, to determine the amount of enzyme required for activity detection, or as a tool to select the most convenient MUF-substrate concentration for a given sample (not shown). Using the described method, 6.1×10^{-7} units of Pancrealipase® could be detected on MUF-butyrate, while 1.2×10^{-6} units of the enzyme were detected on MUF-oleate (Fig. 1C). In all cases, detection of lipolytic activity was achieved in less than 15 min under UV illumination.

3.2. Zymogram analysis

Separation of proteins in polyacrylamide or isoelectric focusing gels is a common analytical technique

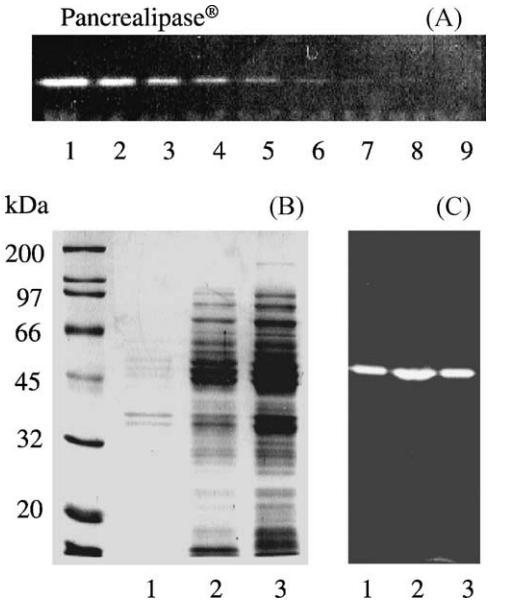

Fig. 2. Zymogram analysis performed on SDS-PAGE gels. (A) Zymogram from an SDS-polyacrylamide gel of different Pancrealipase® dilutions analyzed with MUF-butyrate. Samples (1–9) correspond to 9.9×10^{-6}, 4.9×10^{-6}, 2.45×10^{-6}, 1.22×10^{-6}, 6.1×10^{-7}, 3.0×10^{-7}, 1.5×10^{-7}, 7.5×10^{-8}, and 3.7×10^{-8} enzyme units, respectively. (B) SDS-PAGE of cell fractions from *E. coli* 5K bearing *Paenibacillus* sp. BP-23 esterase A, analyzed for lipolytic activity using MUF-butyrate (right) and subsequently stained with Coomassie Brilliant Blue R®-250 (left) to determine the molecular mass of the active proteins. The samples loaded correspond to peryplasm (1), cytoplasm (2), and membrane-bound proteins (3).

for protein and enzyme characterization. Conventional staining methods allow the identification of purified lipases, but non-purified enzymes have to be detected through zymograms, usually performed by overlay techniques [32]. MUF-derivative substrates were used for the rapid detection of lipases and esterases separated on SDS-PAGE and IEF gels. Fig. 2A shows a zymogram analysis performed with MUF-butyrate of several Pancrealipase® dilutions separated on a 10% SDS-polyacrylamide gel. In contrast to other zymographic systems [32], the sensitivity range of this technique is extremely high, allowing detection of 1.5×10^{-7} units of Pancrealipase® on MUF-butyrate in less than 15 min. The short time required for activity detection on gels greatly contributes to prevent protein diffusion, thus allowing a most accurate determination of the protein molecular weight. Nevertheless, the degree

of sensitivity of the system is a function of the properties of the enzyme analyzed and the substrate used. According to this, higher amounts of enzyme were needed (2.5×10^{-6} units of Pancrealipase®) when MUF-oleate was used as a substrate in the zymogram (not shown).

An important advantage of the zymographic technique described is that after activity detection, the same gels can subsequently be stained with a conventional dye in order to determine the molecular mass of the active proteins (Fig. 2B and C). This fact and the lack of protein diffusion due to the short reaction time allow a great accuracy in the determination of the molecular weight of a given enzyme, not provided by the conventional overlay systems or by analysis of proteins and activity in separate gels. Fig. 2B and C show the same gel containing different cell fractions of lipolytic strain *Paenibacillus* sp. BP-23, analyzed as a zymogram (Fig. 2C), and subsequently stained with Coomassie Brilliant Blue R®-250 (Fig. 2B). The active bands detected by the zymogram are not visible at the Coomassie Blue stained gel due to their low protein contents. Zymographic analysis with MUF-derivatives was also used for the determination of the isoelectric point of several lipolytic enzymes (not shown). Like in the case of PAGE, IEF gels can subsequently be stained by conventional dyes, and the pI of the active proteins established with great accuracy. Traditional zymographic analysis of lipases are less sensitive, time consuming and more expensive procedures. For this reason, the higher effectiveness of the method described makes it a very valuable tool for straightforward detection of lipolytic enzymes even from non-purified or non-concentrated samples [11].

3.3. Characterization of lipases using MUF-derivative substrates

Biochemical characterization of enzymes requires the use of systems that allow accurate quantification of activity. We extended the use of MUF-derivative substrates to the determination of lipolytic activity in samples from different origin and under a variety of conditions.

Growth dependency of enzyme production by microbial strains or by recombinant microorganisms bearing lipase genes was evaluated by spectrofluorometry assays, as described in the experimental section. Fig. 3 shows the production of lipolytic activity along growth of *Paenibacillus* sp. BP-23 strain (Fig. 3A), and the production of lipase A in parental strains and recombinant clones of *S. cerevisiae* bearing *B. subtilis lip*A gene (Fig. 3B). Fig. 3A shows that there is a growth-dependent expression of lipases in strain *Paenibacillus* sp. BP-23, as the highest amount of lipolytic activity appeared after 24 h incubation. On the contrary, expression of *B. subtilis* lipase A in recombinant *S. cerevisiae* strains is mainly constitutive, with a maximum peak of production after 24 h growth. Activity was found at the cell extract fractions of these clones from the beginning of the exponential phase for both MUF-butyrate (Fig. 3B) and MUF-oleate (Fig. 3C) substrates. These results are in agreement with the use of a constitutive yeast promoter in the recombinant constructions [14]. As expected, parental yeast strains lacking lipase A, used as control samples, showed negligible lipolytic activity.

Lipases exhibit different kinetic behaviors depending on the properties and concentration of the substrate they hydrolyze. An interfacial activation of true lipases occurs at high substrate concentration or when long chain-length substrates are used. On the contrary, esterases and other lipolytic enzymes display a Michaelis–Menten behaviour, without interfacial activation [1,3]. Therefore, we used the MUF-derivative substrate-based spectrofluorometric method to assay the substrate specificity and kinetic parameters of lipases. Table 1 shows the results obtained for *Paenibacillus* sp. BP-23 EstA, *Bacillus* sp. BP-7 EstA1, and *B. subtilis* LipA, assayed from crude cell extracts of the corresponding clones in *E. coli* 5K. In all cases, activity was higher on MUF-butyrate, indicating a preference of these enzymes for short chain-length substrates. A significant decrease of activity was observed as the length of the fatty acid chains increased, a behavior similar to that described for true esterases [1]. The kinetic parameters of the cloned enzymes were analyzed on MUF-butyrate and MUF-oleate. For both substrates, a standard Michaelis–Menten plot was obtained (not shown), with the apparent constants shown in Table 1. According to the proposed model for esterases and true lipases, the three enzymes displayed the typical properties of esterases, showing no interfacial activation by substrate concentration. The results obtained are not surprising for *Paenibacillus* sp. BP-23

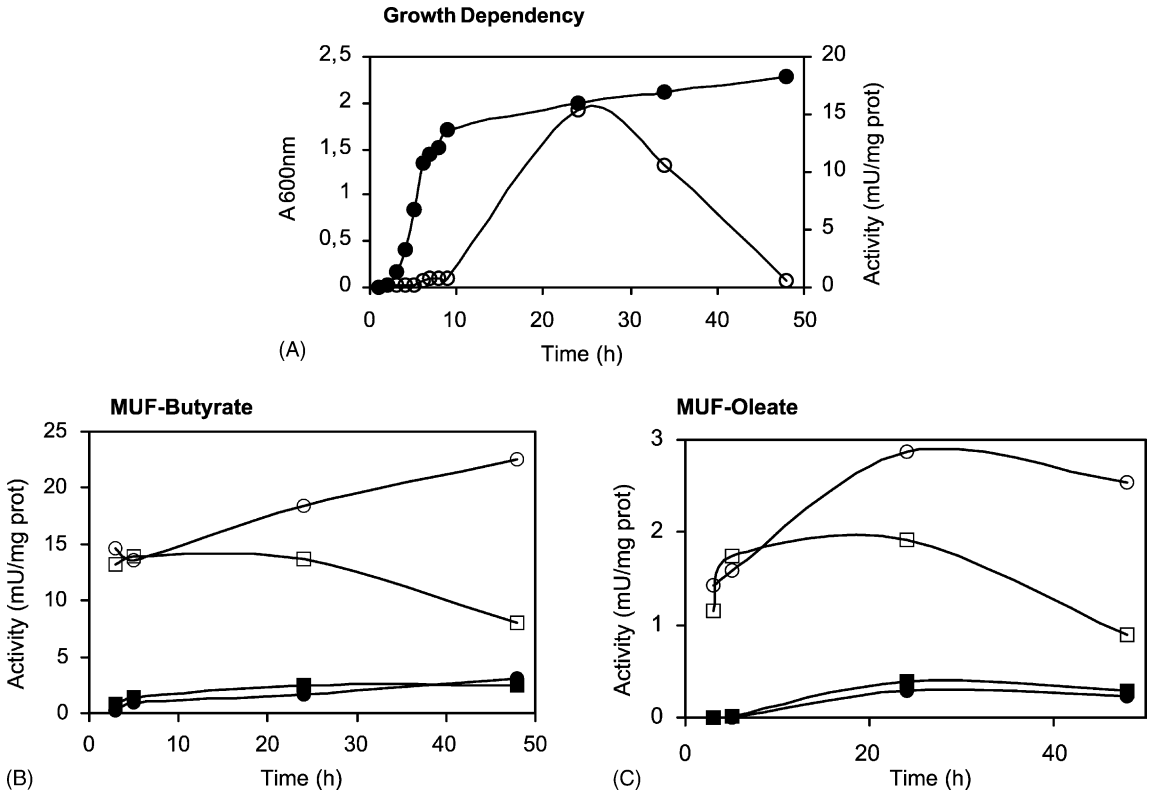

Fig. 3. Use of MUF-derivative substrates for lipase activity determination. Growth dependency of lipase activity production in *Paenibacillus* sp. BP-23; (●) absorbance at 600 nm, (○) secreted activity, measured with MUF-butyrate (A). Cell extract lipase activity found on MUF-butyrate (B) or MUF-oleate (C) for *S. cerevisiae* strains 13bxV4 (squares) and CENPK113-11A (circles) transformed with plasmid YE[pACT1-ssInv-LipA-T] (empty symbols) or YEplac112 (control samples, solid symbols), grown in YPD medium.

EstA or *Bacillus* sp. BP-7 EstA1, previously described as esterases [12,13]. Although *B. subtilis* LipA has for a long time been considered a true lipase, our results agree with recent reports indicating that no interfacial activation is required for activity, as the enzyme lacks a structural α-helix fold that acts to prevent true lipases from displaying activity in the absence of an interface [4,33,34].

A number of enzyme activity inhibitors were also studied by fluorometric techniques using MUF-derivatives. Fig. 4B shows an example of enzyme inhibition caused by phytic acid on Pancrealipase®. Increasing concentrations of phytic acid were added to the reaction mixture, and residual activity was measured using MUF-butyrate (Fig. 4B) or MUF-oleate (not shown). A concentration of 0.31% phytic acid

Table 1
Specific activity and K_m values of tested lipases for different MUF-derivative substrates

	Paenibacillus sp. BP-23 EstA		*Bacillus* sp. BP-7 EstA1		*Bacillus subtilis* LipA	
	Units (mg)	K_m (mM)	Units (mg)	K_m (mM)	Units (mg)	K_m (mM)
MUF-butyrate	9.5	0.0155	0.28	0.026	0.13	0.031
MUF-oleate	1.2×10^{-4}	0.029	3×10^{-3}	0.055	0.02	0.044

Activity values are the mean of three independent assays.

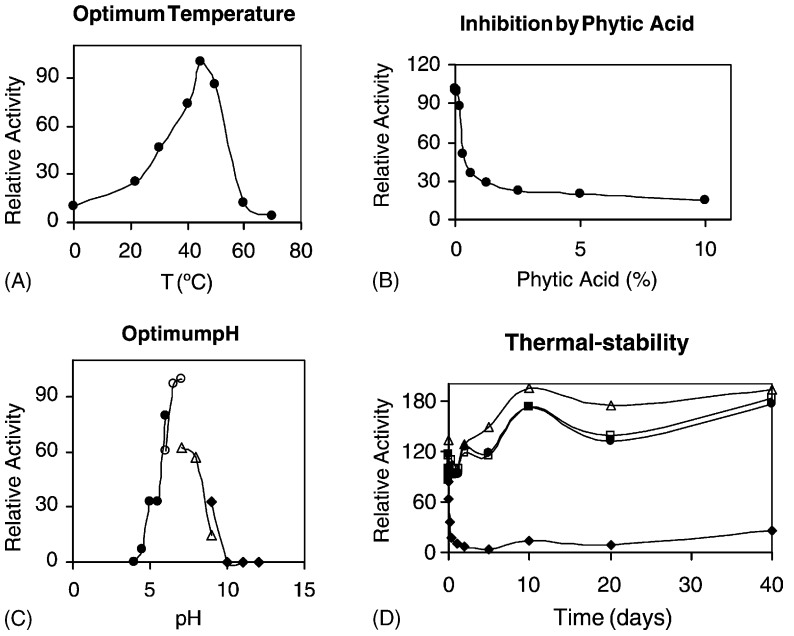

Fig. 4. Characterization of lipases using MUF-derivative substrates. (A) Optimum temperature determination for *E. coli* 5K cell extracts bearing EstA1 from *Bacillus* sp. BP-7, using MUF-butyrate. (B) Inhibition of Pancrealipase® activity caused by phytic acid, measured with MUF-butyrate. (C) Optimum pH determination for *E. coli* 5K cell extracts bearing EstA1 from *Bacillus* sp. BP-7, using MUF-butyrate. The reaction was performed at different pH in the presence of succinate (●), phosphate (○), Tris–HCl (△), and glycine (◆) buffers. (D) Thermal stability of *Bacillus megaterium* lipase A cloned in *E. coli* 5K, measured using MUF-butyrate. Temperatures assayed were: 4 °C (△), 20 °C (□), 30 °C (●), and 45 °C (◆). The highest relative activity value for each parameter analysed was set at 100.

caused a 50% reduction in Pancrealipase® activity on MUF-butyrate. However, we must point out that when inhibition assays are performed using MUF-derivative substrates, quenching of fluorescence [35], potentially caused by the added substances or their solvents, must be taken into account, and accurate standard plots must be performed for each compound as well as for the solvents used. In all our determinations, standard plots for both the compound and the solvent were constructed and used to calculate the quenching effect.

Determination of optimum pH and temperature for lipase activity was also performed using MUF-derivative substrates. However, both parameters cause important effects on fluorescence emission [35]. For this reason, a simple modification of the standard protocol was established, based on stopping the reaction with HCl and bringing all samples to the same pH and temperature conditions before measuring the fluorescence emission. Fig. 4 shows the determination of optimum temperature (Fig. 4A) and pH (Fig. 4C) for *Bacillus* sp. BP-7 EstA1 esterase cloned in *E. coli* 5K, performed using MUF-butyrate. As shown in the plots, maximum activity of the enzyme was achieved at pH 7.0 and 45 °C. The system described was also successfully used for measuring the thermal (Fig. 4D) or pH stability of lipases and to study the need for cofactors of these enzymes (not shown). Lipase A from *B. megaterium* [15] displayed good thermal stability in the temperature range from 0 to 30 °C, being fully active for at least 40 days (Fig. 4D). When incubated at 45 °C, a rapid decrease of activity was detected, reaching 50% of the initial activity after 2.5 h incubation. All the activity determinations were performed in triplicate, and control plots were obtained for each sample. The reliability of the method was studied using *para*-nitrophenyl-derivatives as alternative substrates. The results obtained following the protocol described in the experimental section [12] (not shown) confirmed the reproducibility of the MUF-based system.

The results obtained indicate that detection and determination of lipase activity using MUF-derivative substrates provides a fast, sensitive and accurate system to gain information about the biochemical and molecular properties of lipases, allowing thus to take rapid and valuable decisions about the potential biotechnological applications of the lipases studied.

4. Conclusions

The fluorescence-based lipase activity assay described here provides an excellent tool to identify lipolytic microorganisms and to study the biochemical properties of lipolytic enzymes. The remarkable sensitivity, speed and simplicity of this standarized system may contribute to eliminate the difficulties posed by most of the traditional methods for lipase assay reported in the literature, and may help in the search for new biotechnologically useful enzymes.

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References

[1] K.E. Jaeger, S. Ransac, B.W. Dijkstra, C. Colson, M. Heuvel, O. Misset, FEMS Microbiol. Rev. 15 (1994) 29.
[2] J.L. Arpigny, K.E. Jaeger, Biochem. J. 343 (1999) 177.
[3] K.E. Jaeger, B.W. Dijkstra, M.T. Reetz, Annu. Rev. Microbiol. 531 (1999) 315.
[4] V. Khalameyzer, I. Fischer, U.T. Bornscheuer, J. Altenbuchner, Appl. Environ. Microbiol. 65 (1999) 477.
[5] K.E. Jaeger, M.T. Reetz, Trends Biochem. Biotech. 16 (1998) 396.
[6] F.D. Gunstone, J. Sci. Food Agric. 79 (1999) 1535.
[7] L.R. Reynolds, W.N. Washburn, R.A. Deems, E.A. Dennis, Methods Enzymol. 197 (1991) 3.
[8] H.S. Hendrickson, Anal. Biochem. 219 (1994) 1.
[9] M. Miller, A. Palojarvi, A. Rangger, M. Reeslev, A. Kjoller, Appl. Environ. Microbiol. 64 (1998) 613.
[10] T.G. Warner, L.L. Tennant, M.L. Veath, J.S. O'brien, Biochem. Biophys. Acta 572 (1979) 201.
[11] P. Diaz, N. Prim, F.I.J. Pastor, Bio. Tech. 27 (1999) 696.
[12] N. Prim, A. Blanco, J. Martínez, F.I.J. Pastor, P. Diaz, Res. Microbiol. 151 (2000) 303.
[13] N. Prim, F.I.J. Pastor, P. Diaz, Curr. Microbiol. 42 (2001) 237.
[14] M. Sánchez, N. Prim, F. Rández-Gil, F.I.J. Pastor, P. Diaz, Biotechnol. Bioeng. 78 (2002) 339.
[15] C. Ruiz, F.I.J. Pastor, P. Diaz, FEMS Microbiol. Lett. 217 (2002) 263.
[16] J.O. Lampen, F.I.J. Pastor, M. Hussain, in: L. Leive, P.F. Bonventre, J.A. Morello, S.D. Silver, H.C. Wu (Eds.), Microbiology-1986, American Society for Microbiology, Washington, 1986, p. 279.
[17] C. López, A. Blanco, F.I.J. Pastor, Biotechnol. Lett. 20 (1998) 243.
[18] A. Blanco, F.I.J. Pastor, Can. J. Bacteriol. 39 (1993) 1162.
[19] N. Godessart, F.J. Muñoa, M. Regué, A. Juárez, J. Gen. Microbiol. 134 (1988) 2779.
[20] F. Rández-Gil, P. Sanz, Appl. Microbiol. Biotechnol. 42 (1994) 581.
[21] A. Monfort, A. Blasco, P. Sanz, J.A. Prieto, J. Agric. Food Chem. 47 (1999) 803.
[22] V. Dartois, J.Y. Coppée, C. Colson, A. Baulard, Appl. Environ. Microbiol. 60 (1994) 1670.
[23] F. Sherman, G.R. Fink, J.B. Hicks, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1986.
[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
[25] R.D. Gietz, A. Sugino, Gene 74 (1988) 527.
[26] R. Brückner, Gene 122 (1992) 187.
[27] U.K. Laemmli, Nature 227 (1970) 680.
[28] A. Blanco, P. Diaz, J. Martínez, T. Vidal, A.L. Torres, F.I.J. Pastor, Appl. Microbiol. Biotechnol. 50 (1998) 48.
[29] S. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Nucl. Acids Res. 25 (1997) 3389.
[30] G.B. Oguntimein, M. Kordel, R.D. Schimid, Fat. Sci. Technol. 9 (1992) 345.
[31] H.G. Hoppe, Marine Ecol. 11 (1983) 299.
[32] R.P. Yadav, R.K. Saxena, R. Gupta, W.S. Davidson, Bio. Tech. 24 (1998) 754.
[33] S. Ransac, M. Blaauw, E. Lesuisse, K. Schank, C. Colson, J. Mol. Biol. 238 (1994) 857.
[34] G. van Pouderoyen, T. Eggert, K.E. Jaeger, B.W. Dijkstra, Mol. Biol. 309 (2001) 215.
[35] T.J. Johnson, Spectrofluorometry: An Introduction to the Theory and Practice, A.G. Kontron, Switzerland, 1983.