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## Direct Fluorescence-Based Lipase Activity Assay

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Lipases and esterases (glycerol ester hydrolases, E.C. 3.1.1.-) are enzymes acting on the carboxyl ester bonds present in acylglycerols. They have been recognized as useful biocatalysts because of their wide-ranging versatility in industrial applications (2,5). However, the physical properties of lipids have caused a great difficulty in studying lipolytic enzymes (4). Traditionally, lipases have been assayed by radiometric or titrimetric techniques, but these methods require the use of radiolabeled and expensive substrates or they suffer from low sensitivity (8). For experiments involving a large number of

assays or non-purified samples, it is convenient to use chromogenic or fluorogenic substrates, which can be tested simply and rapidly. Unfortunately, many chromogenic substrates show low specificity and sensitivity, or they become spontaneously hydrolyzed (4). On the contrary, the use of fluorogenic substrates like 4-methylumbelliferone (MUF) derivatives provides a sensitive method for determination of enzyme activity (3,10). Here, we describe a fast, simple assay to detect lipolytic activity from purified and non-purified samples, using MUF-derivative substrates.

Identification of lipase producing microbial strains was performed after growth on a solid medium by spreading the plate surface with a 100  $\mu\text{M}$  solution of MUF-butyrate (Sigma, St. Louis, MO, USA) in 50 mM phosphate buffer, pH 7.0, prepared from a 25 mM stock solution in ethyleneglycol monomethylether (Merck, Germany). Under

UV illumination, only strains bearing lipolytic activity showed fluorescence emission due to substrate hydrolysis and MUF release (Figure 1A). Lipolytic activity detection can also be performed from cell suspensions or cell fractions, by transferring a small aliquot (5  $\mu\text{L}$ ) of each sample onto filter paper and adding 5  $\mu\text{L}$  of 25 mM MUF-butyrate stock solution. UV illumination of the paper allows identification of the samples bearing lipolytic activity (not shown). This simple procedure was also used to select the most convenient working concentration of MUF-substrate for a given sample (Figure 1B) or to determine the amount of enzyme required for hydrolyzing a defined MUF-substrate. For most of our samples, detection of lipolytic activity against MUF-butyrate was achieved in less than 30 s, while hydrolysis of MUF-oleate usually required a 15-min incubation at room temperature.

Electrophoretic separation in sodium dodecyl sulfate (SDS)-polyacrylamide gels has widely been used to study lipases and esterases. Activity detection often requires zymographic analysis, usually performed by overlay techniques (11). We standardized a new fluorescence-based assay for lipase activity detection in SDS-polyacrylamide gels (Figure 2). After protein separation, SDS was removed from the gels by soaking them for 30 min in 2.5% Triton® X-100 at room temperature (1). The gels were then briefly washed (1 min) in 50 mM phosphate buffer, pH 7.0 and covered by a solution of 100  $\mu\text{M}$  MUF-butyrate or 200  $\mu\text{M}$  MUF-oleate in the same buffer. After UV illumination, fluorescent activity bands became visible in 30 s for MUF-butyrate and in about 15 min for MUF-oleate. In contrast to other zymographic systems (11), the sensitivity of the method described is extremely high, and allows detection of  $1.5 \times 10^{-7}$  U of Pancrealipase® (USP NF-190; Inquiaroma, LIB, France) using MUF-butyrate as a substrate (Figure 2A, left). Nevertheless, the degree of sensitivity of the system is a function of the properties of the enzyme analyzed and the substrate used. Accordingly, higher amounts of enzyme were needed ( $2.5 \times 10^{-6}$  Pancrealipase U) when MUF-oleate was used

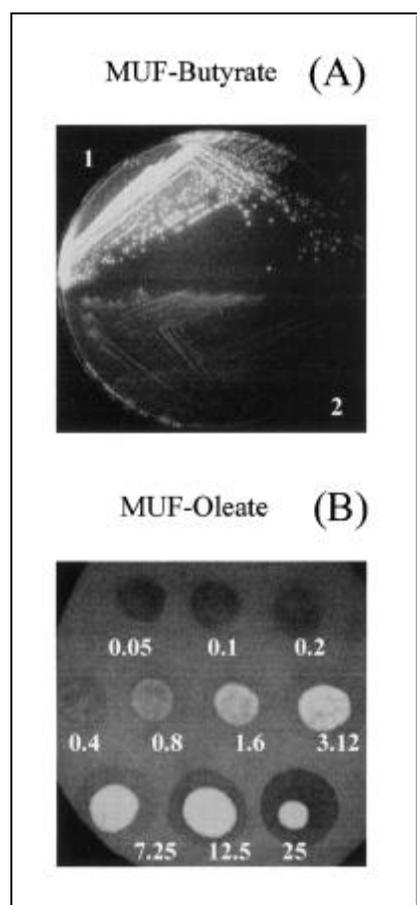
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as a substrate (Figure 2A, right). Traditional overlay zymographic systems usually require incubation periods that allow protein bands to diffuse in the gel. On the contrary, the zymographic technique reported here allows detection of activity in a very short period of time after the electrophoretic separation, thus preventing protein diffusion. Since the appearance of fluorescence is almost immediate (seconds for MUF-butyrates), fluorophore diffusion is negligible. An additional feature of the method developed is that immediately after zymographic analysis, the same gels can subsequently be stained with a

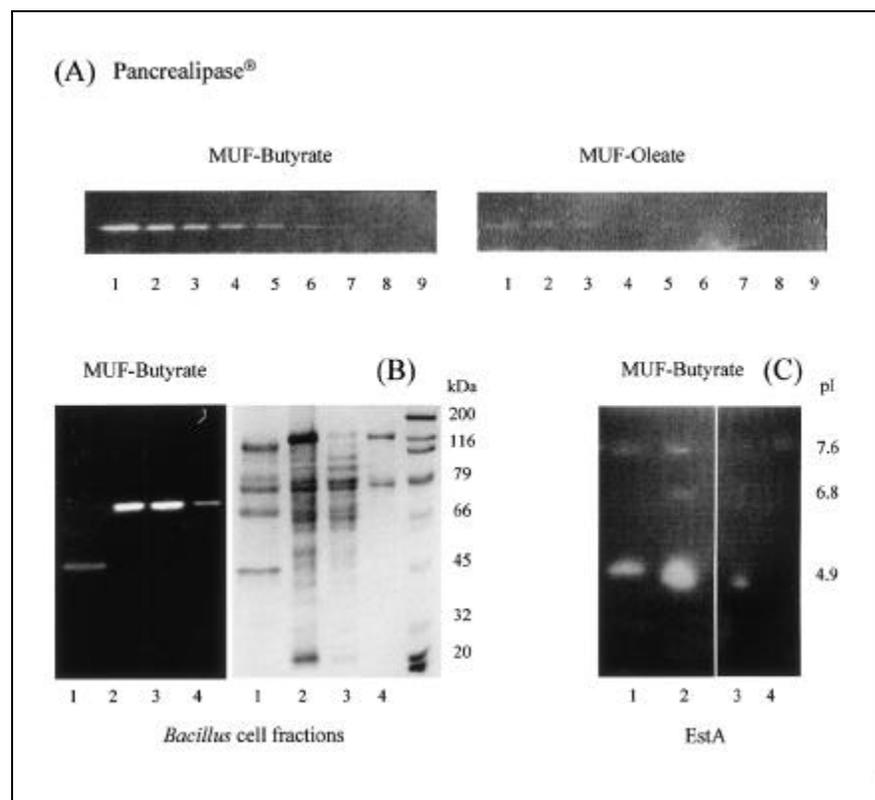
conventional dye. Figure 2B shows the zymogram (left) and protein analysis (right) performed on the same gel, of various cell fractions from the lipolytic strain *Bacillus* sp. BP-23 (1). The fact that the same gel can be used for both the zymogram and protein analysis, combined with the lack of diffusion of the bands visualized in the zymograms, allows great accuracy in the determination of the molecular weight of a given enzyme, which is not provided by the conventional overlay techniques. Zymographic analysis with MUF-derivatives can also be used for determination of the isoelectric point (pI) of enzymes. Figure 2C shows the isoelectric focus-

ing (IEF) results obtained for esterase EstA from *Bacillus* sp. BP-23 and the same enzyme cloned and expressed in *Escherichia coli* 5K. A prominent band of pI 4.9 was present in lanes 1–3, while recombinant *E. coli* samples showed minor additional bands of pI 6.8 and 7.6, probably due to microheterogeneity of the cloned enzyme (7). Similar to the case of SDS-polyacrylamide gel electrophoresis (PAGE), IEF gels can subsequently be stained by conventional dyes, and the pI of the active bands accurately determined.

The fluorescence-based lipase activity assay described here provides an excellent tool to identify lipolytic organ-



**Figure 1. Activity detection on agar plates and filter paper.** (A) MUF-butyrates activity detection of (1) recombinant *E. coli* X11 colonies bearing EstA1 from *Bacillus* sp. BP-7 (Reference 6) and (2) the non-lipolytic host *E. coli* X11. The strains were grown at 37°C on LB/agar plates. (B) Paper assay to select the working concentration of MUF-oleate for *Burkholderia cepacia* R6 (Reference 9) cell extracts, prepared as described in Reference 1. Samples (5  $\mu$ L) were assayed with different concentrations (0.05–25 mM) of MUF-oleate (5  $\mu$ L) in ethyleneglycol monomethylether.



**Figure 2. Zymogram analysis.** (A) Pancrealipase dilutions (lanes 1–9:  $9.9 \times 10^{-6}$ ,  $4.9 \times 10^{-6}$ ,  $2.45 \times 10^{-6}$ ,  $1.22 \times 10^{-6}$ ,  $6.1 \times 10^{-7}$ ,  $3.0 \times 10^{-7}$ ,  $1.5 \times 10^{-7}$ ,  $7.5 \times 10^{-8}$  and  $3.7 \times 10^{-8}$  enzyme units, respectively) were loaded without boiling on an 8% SDS-polyacrylamide gel and analyzed for activity using 100  $\mu$ M MUF-butyrates (left) or 200  $\mu$ M MUF-oleate (right) in 50 mM phosphate buffer, pH 7.0. (B) Proteins from various cell fractions of *Bacillus* sp. BP-23 were heated at 100°C for 2 min, separated on an 8% SDS-polyacrylamide gel and analyzed for activity with MUF-butyrates (left) and subsequently stained with Coomassie<sup>®</sup> Brilliant Blue R-250 (right). Samples were prepared as follows: the supernatant (lane 1) from cultures was recovered by centrifugation, and the cells were harvested for further fractionation. Mild treatment of the cells with lysozyme (0.09 mg/mL) rendered the loose-bound envelope protein fraction (lane 4). Cells were then disrupted by sonication, the lysates cleared by centrifugation and the supernatant (cytoplasm fraction) obtained (lane 3). The remaining pellet was considered cell debris (lane 2). (C) MUF-butyrates zymographic analysis performed after IEF of EstA from *Bacillus* sp. BP-23 cell extract (lane 1), and the same esterase obtained from culture medium (lane 2) and cell extract (lane 3) of recombinant *E. coli* 5K expressing the enzyme. A crude cell extract of *E. coli* 5K lacking EstA is shown as negative control (lane 4).

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isms and to study some of the properties of lipolytic enzymes. The remarkable sensitivity, speed and simplicity of this system may help eliminate the difficulties posed by most of the traditional methods for lipase-activity detection as reported in the literature.

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