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BAC DNA was then further characterized to verify the purity and the quality of the preparation. First, little or no bacterial genomic DNA was visible on an ethidium bromide-stained agarose gel (Figure 1A). Second, the DNA was easily cut by *Hind*III and *Not*I restriction enzymes (New England Biolabs, Beverly, MA, USA) (Figure 1A). Third, the BAC clones were efficient templates for PCR amplification using Alu primers (Figure 1B). And fourth, the BAC insert ends were directly sequenced (see below). These results illustrate the high quality of the BAC DNA preparations. Furthermore, identical restriction patterns were generated every two weeks over a period of 6 months, indicating the great stability of the BAC DNA preparations (data not shown). BAC end sequences can be used to provide a source of physical markers in genome mapping and walking. Because of the presence of the T7 and SP6 promoters in the BAC vector, we have been able to directly sequence both ends of BAC inserts using the T7- and SP6-specific primers (Figure 2). The double-stranded DNA sequencing was performed by cycle sequencing (Thermo Sequenase™; Amersham Pharmacia Biotech, Oakville, ON, Canada) using an adapted protocol (see Figure 2 legend).

The procedure described here is simple and offers a productive method for isolation of high-quality and stable BAC DNA suitable for numerous applications including restriction analysis, sequencing, PCR, hybridization or fluorescence in situ hybridization.

REFERENCES

1. Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
2. Kim, U.-J., B.W. Birren, T. Slepak, V. Mancino, C. Boysen, H.-L. Kang, M.I. Simon and H. Shizuya. 1996. Construction and characterization of a human bacterial artificial chromosome library. *Genomics* 34:213-218.
3. Shizuya, H., B. Birren, U.-J. Kim, V. Mancino, T. Slepak, Y. Tachiiri and M.I. Simon. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. USA* 89:8794-8797.
4. Thomas, N.R., S. Koshy, M. Simsek and A.K. Abraham. 1988. A precaution when preparing very large plasmids by alkaline lysis procedure. *Biotechnol. Appl. Biochem.* 10:402-407.

D.S. is a scholar of the Fonds de la Recherche en Santé du Québec. This work was supported by the Medical Research Council of Canada. Address correspondence to Dr. Daniel Sinnett, Service d'Hématologie-Oncologie, Centre de Recherche, Hôpital Ste.-Justine, 3175 Côte Ste.-Catherine, Montréal, QC, H3T 1C5, Canada. Internet: sinnettd@ere.umontreal.ca.

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Daniel Sinnett^{1,2}, Chantal Richer¹ and Annie Baccichet¹

¹Hôpital Ste.-Justine

²Université de Montréal
Montréal, QC, Canada

Rapid Zymogram for Lipase

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Microbial lipases have immense use in the detergent, food, agricultural and pharmaceutical industries (5). Recently, the applications of lipases in organic synthesis have increased tremendously because of low costs, stability in organic media, wide versatility, easy use and non-requirement of added cofactors (2).

The most important and fascinating aspect of lipases is perhaps the unique physico-chemical character of the reactions they catalyze. These enzymes are perfectly water-soluble and yet act very efficiently on water-immiscible lipidic substrates that spontaneously self-organize in water as monomolecular films, bilayers, liposomes, emulsions or micelles. This catalysis occurs essentially at the lipid-water interface (8).

To study the conformation and mechanism of lipases, homogeneous preparations are required. Purification of lipases is carried out by most laboratories using conventional techniques, and the electrophoretic separation of protein in polyacrylamide gels is a common analytical technique for protein

identification (4). Detection of lipase in the native state requires an enzyme zymogram. Although native staining of lipases has been referred to by some workers, the exact protocol using Victoria blue has not been mentioned in literature. The heterogeneous state of the substrate for lipase action (triglyceride) causes difficulties during zymogram preparation. To overcome this difficulty, we have developed a rapid method for the detection of native bands by polyacrylamide gel electrophoresis (PAGE). The method involves an overlay staining technique using Victoria blue as a staining dye. The principle of the method is the hydrolysis of triglyceride (which is present in the overlay) into fatty acids that ultimately combine with the functional group of Victoria blue and result in the development of a blue zone in the overlay at places where active lipase is present.

The dye Victoria blue has been used over a long period of time for the sensitive detection of lipase in plate assay procedures (1). Activity staining of lipase on polyacrylamide gels was carried out by Gilbert et al. (3) using an agarose overlay attached to 1850-101 GelBond® Film (Amersham Pharmacia Biotech, Uppsala, Sweden) for 24 h. However, our method is more rapid (4-12 h), less cumbersome, less expensive (GelBond Film is not required) and more sensitive in terms of the quantity

Figure 1. (A) Polyacrylamide gel (12.5%) showing active lipase from *A. terreus* stained with Victoria blue. Lane 1: 0.312 U/10 µL of purified lipase; lane 2: 0.625 U/10 µL of purified lipase; lane 3: 0.05 U/10 µL of crude culture filtrate. (B) SDS polyacrylamide gel (12.5%) showing pure lipase from *A. terreus* (41 kDa) in lane 1, along with BSA as marker (66 kDa) in lane 2. Bands are stained with Coomassie Brilliant Blue R-250.

of dye used (10 µg/mL as opposed to 100 µg/mL).

Victoria blue B used in the present investigation was purchased from the Central Drug House (Delhi, India) and was of analytical grade. Sephadex® G-100 and Q Sepharose® were obtained from Amersham Pharmacia Biotech. All other chemicals were of analytical grade and were purchased locally (E. Merck Ltd., Bombay, India).

During the course of our extensive work on fungal lipases, we have isolated a strain of *Aspergillus terreus* that produces a highly thermostable and regio-specific lipase (7,10,11). This lipase is capable of catalyzing transesterification between sugar alcohols and natural triglycerides, leading to the production of biosurfactants (9). Experimentally, lipase from the culture filtrate of *A. terreus* was estimated using titri-

metry according to the procedure of Nahas (6) and purified by ammonium sulfate precipitation (85% saturation), desalted by dialysis and subjected to acetone precipitation (25% and 35%). The enzyme was then purified using successive chromatographic separation on Sephadex G-100 and Q Sepharose and further concentrated. A 10-µL sample containing 30 µg of protein (including lipase) was loaded on a polyacrylamide gel (12.5%) (Model AE-6450; Atto, Tokyo, Japan) at 4°C. The gel was run at 80 mV for stacking and 120 mV for separation. At the end of the run, the gel was removed and properly washed with double-distilled water (3–4 times). Then the gel with overlay was incubated at $37^{\circ} \pm 1^{\circ}\text{C}$ for 12 h in a humid plastic tray. Care should be taken to ensure that the plastic tray is tightly covered with aluminum foil to

Figure 2. (A) Polyacrylamide gel (8%) stained with Coomassie Brilliant Blue R-250. Lane 1: porcine pancreatic lipase (0.66 U/10 µL); lane 2: potato tuber lipase (0.66 U/10 µL); lane 3: *Aspergillus* sp. lipase (0.66 U/10 µL); and lane 4: *A. terreus* lipase (0.66 U/10 µL). (B) Polyacrylamide gel (8%) showing active lipases in an overlay containing Victoria blue (incubation of 6 h). Lane 1: porcine pancreatic lipase; lane 2: potato tuber lipase; lane 3: *Aspergillus* sp. lipase; and lane 4: *A. terreus* lipase. (C) SDS polyacrylamide gel (8%) stained with Coomassie. S: standard markers (66 kDa: BSA and 43 kDa: ovalbumin); lane 1: porcine pancreatic lipase; lane 2: potato tuber lipase; lane 3: *Aspergillus* sp. lipase; and lane 4: *A. terreus* lipase. (D) Polyacrylamide gel (8%) showing porcine pancreatic lipase stained with Coomassie. Lane 1: 0.66 U/10 µL; lane 2: 0.33 U/10 µL; and lane 3: 0.17 U/10 µL. (E) Polyacrylamide gel (8%) showing active porcine pancreatic lipase in an overlay containing Victoria blue (incubation of 12 h). Lane 1: 0.66 U/10 µL; lane 2: 0.33 U/10 µL; and lane 3: 0.17 U/10 µL.

Benchmarks

prevent low humidity in the system. The efficiency of the technique is enhanced by the presence of a film of phosphate buffer (0.2 M, pH 8.0) between the overlay and the gel during incubation. The location of lipase on the polyacrylamide gel is detected by the presence of a distinct blue zone in the overlay (Figure 1A). Figure 1B shows sodium dodecyl sulfate (SDS)-PAGE containing pure lipase from *A. terreus* (41 kDa) in lane 1 and bovine serum albumin (BSA) (66 kDa) in lane 2, both stained with Coomassie Brilliant Blue R[®]-250. For preparation of the overlay, 3.0 g of gum acacia were added to 30 mL of phosphate buffer (0.2 M, pH 8.0) and brought to a final volume of 50 mL. Olive oil (20 mL) and 10 g of crushed ice were added, and this mixture was homogenized. Homogenization was carried out in a top-drive homogenizer (Model RU-56-1 Metrex; Remi Motors, Bombay, India) for 5 min. The emulsion obtained was filtered through glass wool, finally diluted to 100 mL using phosphate buffer and maintained

at 50°C. Victoria blue (10 µg) was dissolved in 1 mL of 70% ethanol. Molten 1% agarose in phosphate buffer (0.2 M, pH 8.0) was added into the emulsion along with the solution of Victoria blue (maintained at 50°C) and mixed well. The overlay was prepared in the same apparatus (Model AE-6450; Atto) by pouring the emulsion between the plates at 4°C.

This technique was also applied to other sources of lipases and found to be extremely sensitive. Figure 2 shows porcine pancreatic lipase (Boehringer Mannheim GmbH, Mannheim, Germany), potato tuber lipase, *Aspergillus* sp. and *A. terreus* sp. lipases (prepared in our laboratory) stained with Coomassie Brilliant Blue R-250 (Figure 2A) and with Victoria blue (Figure 2B). Panel C shows the SDS-PAGE profile of the four lipases along with bovine serum albumin and ovalbumin (66 and 43 kDa, respectively) as markers. Further, Panels D and E portray gels with different concentrations of porcine pancreatic lipase stained with Coomassie and Victoria blue, respectively.

Hence, the present technique can be used for the detection of lipase in crude as well as purified forms obtained from all kinds of sources: microbial (fungal or bacterial), animal and plant.

The sensitivity range of our technique is approximately 0.005–6.25 U/10 µL of sample. However, if the native polyacrylamide gel is loaded beyond this limit, diffusion is observed after 12 h. This diffusion can be retarded by observing the gel in lesser time periods of 4–8 h, depending on the concentration of lipase loaded in the gel. In addition, even at low concentrations of protein (unstained by Coomassie Brilliant Blue R-250) in native PAGE, sensitive staining of the active lipase with Victoria blue can be achieved (Figure 2, A and B, lanes 3 and 4).

This technique is highly efficient and rapid. It confers the ability to estimate the approximate molecular weight of the lipase in its native form. The technique eliminates the difficulties posed in zymogram preparation using oil.

REFERENCES

1. Alford, J.A. and E.E. Steinle. 1967. A double layered plate method for the detection of mi-

crobial lipolysis. *J. Appl. Bacteriol.* 30:488-494.

2. Ghosh, P.K., R.K. Saxena, R. Gupta, R.P. Yadav and S. Davidson. 1996. Microbial lipases: production and applications. *Sci. Prog.* 79:119-157.
3. Gilbert, E.J., A. Cornish and C.W. Jones. 1991. Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2. *J. Gen. Microbiol.* 137:2223-2229.
4. Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
5. Macrae, A.R. and R.C. Hammond. 1985. Present and future applications of lipases. *Biotechnol. Genet. Eng.* 3:193-217.
6. Nahas, E. 1988. Control of lipase production by *Rhizopus oligosporus* under various growth conditions. *J. Gen. Microbiol.* 134:227-233.
7. Parmar, V.S., H.N. Pati, R.P. Yadav, A. Kumar, K.S. Bisht, R. Gupta, S. Davidson, Poonam and R.K. Saxena. 1998. Utility of a novel lipase from *Aspergillus terreus* in deacetylation reactions. *Biocatal. Biotransform.* 16:17-25.
8. Verger, R., C. Riviere, H. Moreau, Y. Gargouri, E. Rogalska, S. Nury, A. Moulin, F. Ferrato et al. 1991. Enzyme kinetics of lipolysis, p. 105-116. In L. Alberghina, R.D. Schmid and R. Verger (Eds.), *Lipases: Structure, Mechanism and Genetic Engineering*. VCH Publishers, New York.
9. Yadav, R.P., R.K. Saxena, R. Gupta and S. Davidson. 1997. Production of biosurfactant from sugar alcohols and natural triglycerides by *Aspergillus terreus* lipase. *J. Sci. Indus. Res.* 56:479-482.
10. Yadav, R.P., R.K. Saxena, R. Gupta and S. Davidson. Lipase production from *Aspergillus* and *Penicillium* spp. *Folia Microbiol.* (In press)
11. Yadav, R.P., R.K. Saxena, R. Gupta and S. Davidson. Purification and characterization of a regiospecific lipase from *Aspergillus terreus*. *Biotechnol. Appl. Biochem.* (In press)

The authors are thankful to the Department of Biotechnology, India, for financial support. Address correspondence to Dr. R.K. Saxena, Head, Department of Microbiology, University of Delhi, South Campus, Benito Juarez Road, New Delhi-110021, India. Internet: micro@disc.ernet.in

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Raman P. Yadav, Rajendra K. Saxena, Rani Gupta and W. Sheba Davidson
University of Delhi
New Delhi, India