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SHORT COMMUNICATION

Enhancement of sequential zymography technique for the detection of thermophilic lipases and proteases

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Abstract Analysis of lipases and proteases present in cell-free fractions of thermophilic *Bacillus* sp. cultures were performed in an enhanced sequential zymography method. After the PAGE run, the gel was electrotransferred to another polyacrylamide gel containing a mixture of glycerol tributyrate, olive oil and gelatin. After transference, this substrate-mix gel was incubated for lipase detection, until bands appeared, and later stained with CBB for protease detection. Assets are, besides detecting two enzymes on a single gel, time and material saving.

Keywords Lipase · Protease · Sequential zymography · Thermophiles

Zymography, a technique derived from electrophoresis, enables the qualitative and quantitative detection of a vast diversity of enzymatic activities. Normally, only one substrate is copolymerized in the gel matrix for the electrophoresis. The sample containing the enzymatic activity to be tested is submitted to electrophoresis. After the electrophoretic run, the enzyme must be activated by incubating the gel in a specific buffer. Eventually the enzyme will degrade the substrate, and detection with a specific staining method may now be performed according to the catalytic reaction executed by the enzyme. For lipases/esterases (EC 3.1.1), typical bands are seen directly on the gel, given the proper incubation conditions (Kwon et al. 2011; Singh et al. 2006). For proteases (EC 3.4), CBB staining is commonly used visualizing translucent bands under a deep blue background (Wilkesman and Kurz 2009). The method is commonly performed for individual enzymes, i.e., separate zymograms are done to detect each enzyme. Here, only one electrophoresis is performed, and the different enzymes separated are sequentially detected. Samples containing the enzymatic activities are submitted to PAGE (non-reducing conditions, SDS present only in sample buffer) and then the gel is electrotransferred to another polyacrylamide gel that contains the copolymerized mixed-substrates. For the sequential detection of lipase and protease, a mixture of glycerol tributyrate, olive oil and gelatin was used. After electrotransference of proteins from the resolving gel to a gel containing both protease and lipase substrates, this two-substrate gel was incubated in a buffer known to activate and support lipase activity until clear bands in a grayish background were visualized. A permanent registration of the image was done immediately, as the next staining step hinders this result. For better images, bands were color-inverted, in order to obtain dark bands. After recording the bands representing active lipases, the same gel was CBB-stained. The presence of protease in the sample being analyzed would be seen as clear bands in a dark blue background. This kind of assay saves working times and reduces the number of gels to be run per assay.

There are several known techniques for zymographic analysis of lipases and proteases separately (Manchenko 2003; Saminathan et al. 2008; Wilkesman and Kurz 2009), but information about simultaneous detection is scarce. Recently, one similar report (Choi et al. 2009a) was published, though glycerol tributyrate and fibrin were used as

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substrates. Instead of using cross-linked fibrin, our proposed method uses gelatin, making it more generally applicable, since gelatin is most commonly used as protease substrate in zymography. Enzyme detection through electrotransference has been reported, where lipases were run in PAGE under non-denaturing conditions (Brahimi-Horn et al. 1991). Pan et al. (2011) describe the use of electrotransference for zymography purposes. Another variation reported, describes protein electrotransference to several polyacrylamide gels, each of them to be incubated and stained differently, according to the enzyme requirements. Thus, cellulose, lipase and protease activity were detected; the method was called 'multiple-layer substrate zymography' (Choi et al. 2009b). Such method, however, creates inconveniences, especially if the amount of protein transferred at the beginning of a transfer is bound to be different from the amount toward the end. The gain of analytical information from this assay (i.e., amount of protein, enzyme activity) is thus complicated.

This study focuses on the sequential detection of lipases and proteases from a thermophilic strain. *Bacillus* sp. were isolated from hot water ponds or sludge, located at Las Trincheras, Venezuela. Four strains, described as CN1, CN1(Zn + Ca), CN2 and CN3, from thermophilic *Bacillus* sp. were treated for the separation of the extra- and intracellular fractions (Jeong et al. 2002; Kim et al. 1998, 2000). Description of the cultures is explained in the Online Resource 1.

Commercial trypsin from porcine pancreas (Fluka-T6567) and lipase Novozym® 388 (Novozymes) were used as controls. Total protein content of the fractions was determined by the Bradford method (Bradford 1976), employing BSA as standard. Lipolytic activity was tested by spectrophotometry using *4*-nitrophenylpalmitate (Online Resources 1 and 2) (Nawani and Kaur 2000).

Enzymatic active fractions were chosen for PAGE (i.e., intracellular fraction CN1(Zn + Ca) and the extracellular fractions from CN1, CN2 and CN3). Commercial lipase (9.32 μg) and protease (2.48 μg) were dissolved separately in sample buffer under non-reducing conditions [24 mM Tris–HCl (pH 6.8), 0.8 % (w/v) SDS, 10 % (v/v) glycerol, 0.06 % (w/v) bromophenol blue]. Microgram amounts of cell-free extracts from the thermophilic cultures were assayed under same conditions. Two 12 % (w/v) total acrylamide monomer gels were run using a Mini-Protean 3 Bio-Rad unit. Running buffer without SDS (25 mM Tris, 192 mM glycine pH 8.8) was used and 100 V was applied for 90 min at 4 °C. After the run, one gel was stained by the silver-CBB protocol (De Moreno et al. 1985), whilst the other was electrotransferred.

Unless elsewhere stated, electrophoresis under non-reducing conditions (2-mercaptoethanol and sample heating absent) was performed. SDS was present in the sample buffer. Before analyzing the thermophilic fractions, control enzymes were separately assayed. Commercial lipases and protease were submitted to PAGE (12 %) under non-reducing conditions. To avoid lipase inhibition (Kim et al. 2000; Ghori et al. 2011), SDS was absent from running buffer, but present in sample buffer, so that a separation according to MW would be feasible.

Table 1 Preparation of the substrate mixture gel for zymography

Component	Volume (mL)	Final concentration in gel (%)
Acrylamide/*N*,*N*′-methylenebisacrylamide (30:0.8 %)	2.400	12
Glycerol tributyrate	0.120	2
Olive oil (San Juan de los Olivos, Commercial Brand, batch #L1075)	0.040	0.67
Water (DI)	2.677	–
Sonication (5 pulses at 18 % amplitude)		
Gelatin (2 % w/v)	0.700	0.23
Ammonium persulfate (10 % w/v)	0.060	0.10
TEMED	0.003	0.05
Final volume	6.000	–

Table 1 describes the substrate gel preparation. Electrotransference was performed at 15 V for 25 min at 4 °C, employing the Bio-Rad mini Trans-Blot electrophoretic transfer cell unit. Same running buffer as described before was employed as transference buffer. After electrotransference, the substrate gel was removed and incubated for 24 h at 55 °C in activation buffer (Tris–HCl 50 mM, NaCl 25 mM, pH 8.8). This activation at 55 °C is specific for thermostable enzymes, for heat-labile enzymes though, heating is omitted.

Lipase activity was monitored for 24 h until optimal translucent bands under a grayish background were seen. Image was photographed and finally the gel was stained with CBB (Coomassie G-250 0.1 %, methanol 45 %, acetic acid 10 %). Excess stain was washed (methanol 45 %, acetic acid 10 %) until translucent bands were visualized.

Control experiments were run with trypsin and Novozym® 388 employing only one specific substrate, either triglyceride/oil or gelatin, incorporated separately in two different gels. Figure 1Ia shows the polypeptide profile of the controls after the SDS-PAGE run. Figure 1Ib illustrates the zymogram for proteolytic activity when only gelatin was present, and Fig. 1Ic confirms lipase activity when only triglyceride/olive oil was copolymerized. Both zymograms gave typical bands showing substrate hydrolysis. Figure 1Id, e illustrates the results obtained when both substrates were incorporated into the gel matrix and was

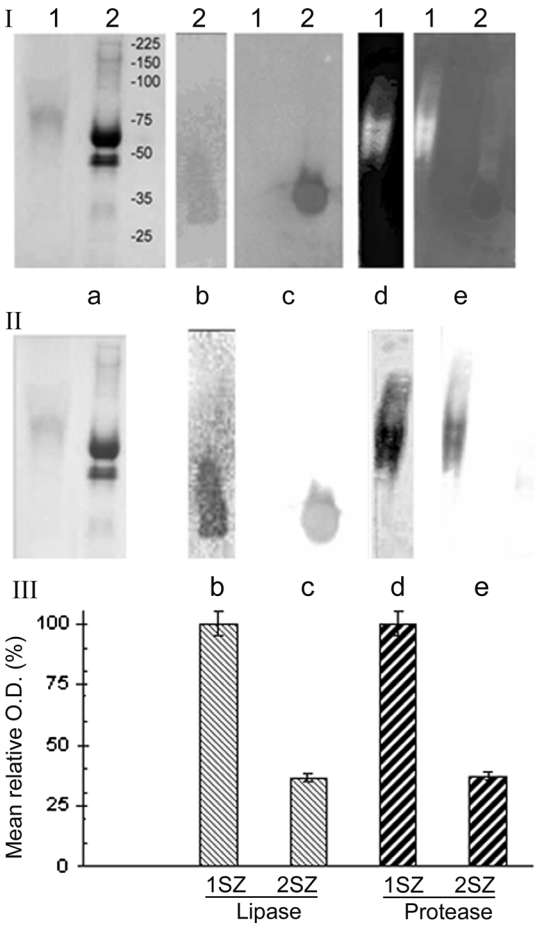

Fig. 1 Study of controls for mixed-substrate zymography. **I**, (*a*) Electropherogram showing protein profiles in SDS-PAGE (12 %) under non-reducing conditions. (*b*, *d*) One-substrate zymography (1SZ). (*c*, *e*) Two-substrate zymography (2SZ) (glycerol tributyrate, olive oil, gelatin) by electrotransference. Sequential visualization of the enzymatic activities was performed. *Lanes* (*1*) 2.5 μg control protease (trypsin); (*2*) 9.3 μg control lipase (Novozym®388). (*b*) Control lipase by 1SZ. (*c*) Control lipase by 2SZ. Gel was incubated for 24 h at 55 °C in lipase activation buffer. (*d*) Control protease by gelatin 1SZ. (*e*) Control protease by gelatin 2SZ stained with CBB. **II**, Optimized image for densitometric analysis. **III**, Histogram of the mean relative optic density (%)

sequentially developed. Figure 1Id shows control lipase activity obtained after addition of incubation buffer. The same gel was then stained with CBB (Fig. 1Ie). In brief, all bands corresponding to lipase or protease activity were equally detected either with a one-substrate gel or with the substrate-mix gel.

Furthermore, the image was optimized and densitometric analysis was performed (Fig. 1II, III). A percentage decrease of the mean relative optical density is observed, when comparing the bands obtained in two-substrate zymogram with the one-substrate zymograms. As they belong to two different staining methods, and background optimization is different for each gel, densitometric analysis for bands in different gels is affected. Nevertheless, no apparently negative influence of the substrate over the enzymatic activity was observed, i.e., though a ~60 % reduction of the mean relative optical density is registered, enzyme activity is not inhibited during the electrotransference procedure. Though a similar result has been reported (Choi et al. 2009a), our results show the behavior of enzyme controls run separately and mixed, as well as a densitometric analysis for quantification purposes, which so far, has not been reported in the literature for this method.

Interestingly, the trypsin revealed an atypical R_f, giving an unusual higher MW (~60 kDa instead of ~23 kDa). Though Bittar et al. (2003) reported a dimeric form of trypsin, it is assumed that a low concentration of SDS for denaturation purposes might had affected the R_f observed. In our case, where SDS was present only in the running buffer, it is evident that there was not enough SDS to fully denature the protein, thus resulting in an unusually high MW for trypsin. This may not be necessarily the case for other proteins, but suggests that care must be taken when assigning MW values to the bands, as probably the SDS–protein complex was inappropriately formed.

While many protein bands are seen on the SDS-PAGE, only one active band (~30 kDa) of the lipase control was present on the zymogram, corresponding to reported values (~29 kDa) (Derewenda and Derewenda 1992), though other values for lipase *Rhizomucor mihei* have been reported (~39.55 and 21.59 kDa) (Boel et al. 1988).

Figure 2 shows the sequentially stained zymogram, analyzing the extra- and intra-cellular fractions from the thermophilic microorganisms. Lipase activity was seen after 2 h incubation (Fig. 2d), and monitored for 24 h (Fig. 2e). Afterwards, CBB staining was performed and proteolytic activity was detected (Fig. 2f). CN1, CN1(Zn + Ca) and CN2 showed the presence of both enzymatic activities at different R_f on the zymogram, whilst CN3 showed scarce lipase activity and proteolytic activity was absent, probably caused by low protein amount applied.

For comparison purposes, the image was cross-referenced with molecular weight markers run separately. CN1 gave a lipolytic band of ~60 kDa and proteolytic band of ~75 kDa, while CN1(Zn + Ca) and CN2 gave both lipase bands of ~30 kDa, and both with proteolytic bands of ~100 kDa. Interestingly, these three samples gave each one unique band for both enzymatic activities. One may infer that under changing activation buffer conditions, other bands might be detected as well. Differences in R_f for lipase and protease activity are noticeable, as CN1 is an extracellular fraction, and CN1(Zn + Ca) is an intracellular fraction, both with different lipase activity (Online resource 2).

Other zymograms performed with SDS inhibited lipase activity (results no shown). Further analysis of lipase stability towards SDS must be performed, as thermophilic enzymes tend to be more resistant toward chemical

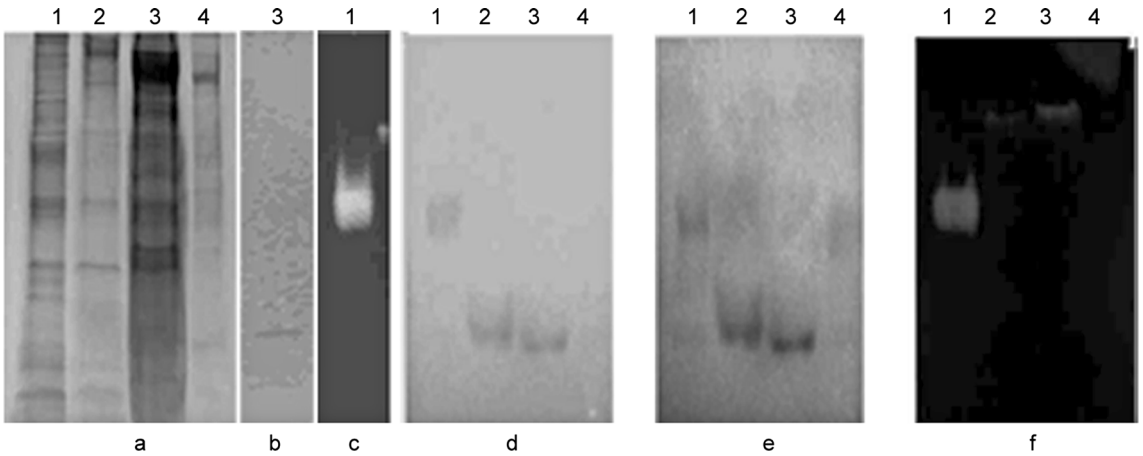

Fig. 2 Analysis of the thermophilic lipases and protease samples by electrophoresis. *Lanes* (*1*) intracellular fraction CN1 (Zn + Ca) (106 μg); (*2*) extracellular fraction CN1 (35 μg); (*3*) extracellular fraction CN2 (35 μg); (*4*) extracellular fraction CN3 (5 μg). (*a*) First an SDS-PAGE (12 %) under non-reducing conditions was run and stained. An unstained replica of gel (*a*) was electrotransferred to a substrate gel containing glycerol tributyrate, olive oil and gelatin and treated for lipase activity. Gel was incubated in activation buffer for 2 h and image was taken (*d*). The same gel was further incubated for 24 h (*e*) and photographed again. (*f*) Finally, the gel was CBB-stained for protease detection. As arbitrary controls, (*b*) a separate lipase detection in 1SZ and (*c*) a separate protease detection in 1SZ were run

denaturation (Lee et al. 1999). This issue would imply that there might be incompatibility between lipase and protease detection when applying the method for the analysis of hydrolytic activity of other biological source of the enzymes. The detergent effect over the enzyme activity must be previously assayed in order to determine the viability of employing substrate-mix gel zymography, otherwise data interpretation might be misleading. These facts have not been approached by former publications (Choi et al. 2009a; Pan et al. 2011).

SDS is a denaturing agent able to form a complex with the proteins and allowing separation according to molecular mass in the PAGE (Bhuyan 2010). However, many factors affect the detergent–protein interactions, like pH, temperature, CMC, hydrophobic interaction and the protein tertiary structure may affect both detergent-loading levels and polypeptide-SDS-PAGE migration rates (Rath et al. 2009). It has been reported that SDS may be omitted from the gel matrix, as long as it is present in the sample buffer and running buffer. Another possibility would be to resolve the proteins using standard non-reducing SDS-PAGE, transferring the proteins to the two-substrate gel, and then replacing the SDS by incubation with Triton X-100. This would be of advantage as many proteases are also sensitive to SDS. If lipase activity turns out to be sensitive to Triton X-100 too, then other detergents could be used to replace the SDS. Though SDS is important in electrophoresis, its presence may be substituted by cationic detergents (i.e., CTAB or 16-BAC) (Westermeier 2011).

It must be stated that the enzyme activation buffer used in this case supports both lipase and protease activity. According to the type of protease to be detected (Cys-, Ser-, Asp- or metallo-protease) incubation buffer must meet other requirements. Though no further experiments were done in order to test the type of protease activity present (normally performed with specific inhibitors), we do consider it is feasible to still incubate the gel in another buffer to promote the protease activity after recording the lipase zymogram image. Though changing buffer conditions implies extra incubation times and would possibly lead to broader bands depending on initial enzyme concentration in the sample, it is recommended to test optimal incubation time for the lipase visualization. Thus, incubation for protease activation under a different set of conditions may be initiated as soon as 2 h after lipase activity visualization (Fig. 2). However, it is advised to determine optimal incubation times for lipase activity, as not all lipase bands may be efficiently visualized (Choi et al. 2009a).

This protocol reduced working times by ~150 min, being thus a fast and non-expensive approach for multiple enzyme detection. In summary, sequential detection of lipase and protease on polyacrylamide gel supports is readily performed. Electrotransference as a technique for the movement of the previously separated enzymes to the substrate gel is effective and efficient. The presence of the protease substrate does not hinder lipase detection and vice versa. The procedure is potentially very useful especially for laboratories analyzing enzymes that may be difficult to obtain. As the method reduces the number of electrophoretic lanes by half and the number of gels to be transferred by half, it is also suitable for analysis of very large numbers of samples. Further work still needs to be improved to gain quantitatively information, regarding enzyme activity according to bands intensity (Leber and Balkwill 1997), since factors, including incubation and staining times, background subtraction, may jeopardize final data

interpretation. Nevertheless, we anticipate the proposal of new protocols for the sequential—if not simultaneous—detection of more than two different enzymes, belonging to different enzymatic classifications. A higher expectation, which might need more time for protocol handling, may be achieved by the introduction of 2D zymography for multi-enzymatic screening.

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Conflict of interest The authors declare no competing financial or commercial conflicts of interests.

Ethical standard It is stated that no human or animal studies were performed in this research. Bacterial management of non-pathogenic strains was performed according to Venezuelan legislation. Ethics guidelines were followed according to Venezuelan legislations.

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