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Notes & Tips

Zymogram with Remazol brilliant blue-labeled *Micrococcus lysodeikticus* cells for the detection of lysozymes: example of a new lysozyme activity in Formosan termite defense secretions

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The zymogram method is based on the separation of enzymes by gel electrophoresis and a subsequent enzymatic step, in which the proteins are allowed to renature and relevant enzymes are allowed to catalytically convert substrates that have been either incorporated into the separating gel, sprayed onto the gel, or overlaid within an agarose gel. Zymogram assays have been developed for various enzymes [1]. Zymograms have been extensively used in the detection of lysozymes and other cell wall hydrolases in previous studies [2–4]. Generally, inactivated *Micrococcus lysodeikticus* (*M. luteus* is a synonym) cells are trapped in the polymerized polyacrylamide gel as a substrate. Enzymes are separated by electrophoresis under denaturing conditions, which prevent premature interaction of enzyme with substrate. In the following refolding step, enzymes are allowed to renature and hydrolyze the embedded substrate, resulting in clearing zones in otherwise opaque gels. This method permits the simultaneous detection of enzyme activity and estimation of the molecular weight, aiding in the initial identification of the enzyme.

Inactivated *M. lysodeikticus* cells have been traditionally used as a substrate for measuring bacteriolytic activity with a spectrophotometer [5] or in radial diffusion assays (lyso-plate assays) [6]. The common basic principle of these methods lies in the fact that the thick murein layer of bacterial cells forms a turbid suspension, the density of which will be reduced by the activity of a cell wall hydrolase. However sensitivity to experimental

conditions (heterogeneity of the substrate preparation, buffer composition, etc.) compromises the reliability of the assay. For gel-based assays as described herein (lyso-plate, zymogram) the limitation lies mainly in the low contrast between clearing zones and background, often requiring additional time-consuming staining/destaining steps, for example with methylene blue [7,8], to identify clearing zones. The present method increases signal-to-noise and shortens the total time of the assay.

The photometric suspension assay was improved using *M. lysodeikticus* cells stained with the reactive blue dye Remazol brilliant blue R (RBB)¹ [9]. RBB has been shown to bind to hydroxyl groups of sugars [10] present in peptidoglycan, the major constituent of bacterial cell walls. Hydrolysis of RBB dye-labeled *M. lysodeikticus* cells leads to release of soluble blue products and a clearing zone.

Here we present data that whole cells of *M. lysodeikticus* prestained with RBB are an improved substrate for “Lyso”-zymograms, allowing a fast and real-time detection of lysozymes without the need for staining and destaining. We also present data demonstrating the new method used to find lysozyme activity in a protein fraction from the defense secretions of Formosan termites.

Materials and methods

Materials required for gel preparation, lyophilized cells of *M. lysodeikticus*, chicken egg white lysozyme

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¹ Abbreviations used: RBB, Remazol brilliant blue; HEWL, chicken egg white lysozyme; MBP, maltose-binding protein.

(HEWL; 14.3 kDa), bovine serum albumin, Remazol brilliant blue R and all other reagents were purchased from Sigma Chemical (St. Louis, MO). Chemicals were of reagent grade or better.

Preparation of ostrich lysozyme

Ostrich eggs were obtained from Three Fork Creek Ostrich Ranch (Pike Corinth, KY). Ostrich lysozyme (20.5 kDa) (a goose-type lysozyme) was purified as described [11].

Preparation of Formosan termite frontal gland secretion

Defense (frontal gland) secretions were collected from 100 soldier caste termites of the species *Coptotermes formosanus*. The enlarged head of this termite is 1/3 of the body mass and contains the frontal defense gland, from which the secretion was expressed with tweezers directly into ice-cold phosphate buffered saline. The extract was centrifuged for 5 min at 10,000 rpm and the insoluble fraction was discarded. The supernatant was filtered through a 0.2 μ m membrane, and dialyzed (molecular weight cutoff 6–8 kDa) against deionized water overnight, and subsequently lyophilized. Samples were reconstituted in 50 mM sodium phosphate buffer (pH 7.0).

Labeling of *M. lysodeikticus* whole cells with remazol brilliant blue R

M. lysodeikticus whole cells were labeled with the vinyl-sulfone reactive dye RBB according to the procedure for the synthesis of RBB-labeled starch [12] with modifications suggested by Ito et al. [9].

Polyacrylamide gel electrophoresis

PAGE [13] was performed in 12.5% (w/v) polyacrylamide gel (2.5 $\times$ 8.0 $\times$ 0.75 cm 5% stacking gel and a 5.5 $\times$ 8.0 $\times$ 0.75 cm separating gel) containing 0.1% (w/v) blue *M. lysodeikticus* cells. Polyacrylamide gels and buffers contained 0.1% (w/v) SDS. Samples were boiled for 2 min in 2 $\times$ sample buffer without reducing agent (62.5 mM Tris, pH 6.8, 0.006% (w/v) bromophenol blue as tracking dye, 20% (v/v) glycerol, 2% (w/v) SDS). Samples of 10 μ l were used for loading.

Electrophoresis was performed using a Mini Protean II cell (Bio-Rad, Hercules, CA) at a constant 120 V for 2 h until the dye front approached the lower gel margin. The gel was washed twice with distilled water for 30 min to remove SDS, incubated in a covered tray with 300 ml of refolding buffer (50 mM NaPO₄, pH 7.0, containing 1% (v/v) Triton X-100), and gently shaken at 37 $^{\circ}$ C.

Standard solutions of HEWL were made in 50 mM potassium phosphate buffer (pH 7.0) by serial dilution.

Broad-range prestained protein markers from New England Biolabs (NEB) or Bio-Rad were used as molecular weight standards. The electrophoretic behavior of the standards is slightly affected by the prestaining and the apparent molecular weight is indicated: (NEB prestained protein markers, broad-range) 175 kDa; maltose-binding protein (MBP)– β -galactosidase, 83 kDa MBP–paramyosin, 62 kDa glutamic dehydrogenase, 47.5 kDa aldolase, 32.5 kDa triosephosphate isomerase, 25 kDa β -lactoglobulin A, 16.5 kDa lysozyme, 6.5 kDa aprotinin; (Bio-Rad prestained SDS–PAGE protein standards, broad range) 208 kDa myosin, 124 kDa β -galactosidase, 101 kDa bovine serum albumin, 55.4 kDa ovalbumin, 35.7 kDa carbonic anhydrase, 29 kDa soybean trypsin inhibitor, 20.9 kDa lysozyme, 6.7 kDa aprotinin.

Digital images of the gels were taken with the NucleoVision 760 Imaging Workstation (Nucleotech, San Carlos, CA) using a UV transilluminator with a white light converter as light source. The presence of a cell wall hydrolase was detected by the formation of transparent lytic zones in the otherwise opaque bluish gel. The areas of the lytic zones were measured using the ImageQuant software program (Molecular Dynamics (Amersham Biosciences), Sunnyvale CA).

Results and discussion

Fig. 1 shows zymograms of hen egg white and ostrich egg white lysozyme. Both enzymes were successfully

Fig. 1. Zymogram of ostrich egg white lysozyme (lane 1) and hen egg white lysozyme (lane 2). Samples at a concentration of 0.7 mg/ml were electrophoresed on a 12.5% SDS–polyacrylamide gel embedded with 0.01% Remazol blue-labeled *M. lysodeikticus* whole cells. The gel was incubated in refolding buffer for 12 h. Lane M contains a prestained protein marker.

renatured and both solubilized the blue *M. lysodeikticus* substrate to produce clearing zones. As a negative control bovine serum albumin failed to yield clear spots indicating that the clearing spots are not artifacts of electrophoresis or refolding.

At high enzyme concentrations, clearing zones become visible after as little as 1 h. A detection limit of 0.1 unit of lysozyme (1.4 ng) was observed after 12 h of incubation. Treatment of the protein samples with a reducing agent prior to electrophoresis had the expected inhibitory effect on renaturation.

After electrophoresis, gels were incubated in refolding buffer to renature proteins. During renaturing incubation enzymes will diffuse in the gel and collide with embedded substrates to form immobilized enzyme–substrate complexes. Hydrolysis of blue-stained *M. lysodeikticus* cells will lead to release of soluble blue-labeled products, and the enzyme will be free to bind to additional substrate sites. Sites of hydrolysis form a readily observable clear reaction zone in a blue-stained gel. The zymogram method can be considered SDS–PAGE with a subsequent quasiradial diffusion assay; however, the diffusion is initiated from a band rather than from a point as in radial diffusion. By analogy with radial diffusion assays, the size of the clearing zone will, in part, be determined by the substrate affinity of the enzyme, its catalytic activity, and its diffusion rate and will lower the accuracy of molecular weight estimation. In radial diffusion assays the diameter of the clearing zone is proportional to the logarithm of enzyme concentration applied, a method which has been applied to lysozyme quantification [6].

Fig. 2 shows a zymogram of a dilution series of HEWL. The areas of clearing were measured and a linear relationship to the logarithm of enzyme concentration was found (Fig. 3). An internal standard with known activity is required.

Fig. 4 shows a zymogram of the frontal defense gland secretion of the soldier caste of *C. formosanus*. The clear zone at approximately 14.5 kDa molecular mass shows a novel protein with lysozyme activity as shown by the zymogram (Fig. 4). A Coomassie blue-stained regular SDS gel showed six to nine bands of proteins (data not shown). Lysozymes have not been reported in termite defense gland secretions, although investigators have shown other glycosyl hydrolase activities, such as sucrase, α -galactosidase, raffinase, amylase, and cellulase [14] postulated to be present due to the salivary gland evolutionary origin of the defense gland [15]. We do not attribute a defense function to this new lysozyme, but report only its presence.

The herein presented zymogram method allows simultaneous detection and quantitation of multiple distinct enzymes in the same sample, whereas the radial diffusion assay quantifies only total enzyme activity.

Fig. 2. Zymogram of hen egg white lysozyme. Various amounts of hen egg white lysozyme (25, 50, 125, 250, and 500 units per 10 µl, in lanes 1–5, respectively) were electrophoresed on a 12.5% SDS–polyacrylamide gel embedded with 0.01% Remazol blue-labeled *M. lysodeikticus* whole cells. The gel was incubated in refolding buffer for 10 h. The amount of lysozyme activity is related to the size of the clearing zone. Lane M contains a prestained protein marker (NEB).

Fig. 3. Semi-logarithmic plot of lysozyme concentration vs the area of clearing zones observed in the gel shown in Fig. 2.

The appearance and detection of clearing zones is improved by the use of dye-labeled *M. lysodeikticus* cells, leading to increased contrast compared to the reduction of opacity with unlabeled cells. Visual monitoring of easily detectable clearing zones in real time allows effortless and constant following of the reaction process, without the requirement for additional time-consuming staining steps that could disrupt the catalytic reaction.

We are utilizing this technique as a fast and convenient method for the discovery of new lysozymes, as

Fig. 4. Zymogram of Formosan termite defense secretion (lane 1) as shown on a 12.5% SDS-polyacrylamide gel embedded with 0.01% Remazol blue-labeled *M. lysodeikticus* whole cells. The gel was incubated in refolding buffer for 10 h. Lane M contains a broad-range prestained protein marker (Bio-Rad).

demonstrated for the presence of a cell wall hydrolase in the secretion of the Formosan termite frontal gland. This approach may find use in investigations of other cell wall hydrolases, which could include *N*-acetylmuramidases, *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases [16].

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References

- [1] O. Gabriel, D.M. Gersten, Staining for enzymatic activity after gel electrophoresis, I, *Anal. Biochem.* 203 (1992) 1–21.
- [2] P. Audy, J. Grenier, A. Asselin, Lysozyme activity in animal extracts after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, *Comp. Biochem. Physiol. B* 92 (1989) 523–527.
- [3] D. Leclerc, A. Asselin, Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis, *Can. J. Microbiol.* 35 (1989) 749–753.
- [4] C. Potvin, D. Leclerc, G. Tremblay, A. Asselin, G. Bellemare, Cloning, sequencing and expression of a *Bacillus* bacteriolytic enzyme in *Escherichia coli*, *Mol. Gen. Genet.* 214 (1988) 241–248.
- [5] D. Shugar, The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme, *Biochim. Biophys. Acta* 8 (1952) 302–309.
- [6] E.F. Osserman, D.P. Lawlor, Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia, *J. Exp. Med.* 124 (1966) 921–952.
- [7] G. Bernadsky, T.J. Beveridge, A.J. Clarke, Analysis of the sodium dodecyl sulfate-stable peptidoglycan autolysins of select gram-negative pathogens by using renaturing polyacrylamide gel electrophoresis, *J. Bacteriol.* 176 (1994) 5225–5232.
- [8] S.R. Watt, A.J. Clarke, Role of autolysins in the EDTA-induced lysis of *Pseudomonas aeruginosa*, *FEMS Microbiol. Lett.* 124 (1994) 113–119.
- [9] Y. Ito, H. Yamada, T. Imoto, Colorimetric assay for lysozyme using *Micrococcus luteus* labeled with a blue dye, Remazol brilliant blue R, as a substrate, *Chem. Pharm. Bull.* 40 (1992) 1523–1526.
- [10] O.A. Stamm, Zur Reaktion von Reaktivfarbstoffen mit Cellulose: II. Natur der Binding, *Helv. Chim. Acta.* 46 (1963) 3008.
- [11] J. Jolles, J.P. Perin, P. Jolles, The ostrich (*Struthio camelus*) egg-white lysozyme, *Mol. Cell. Biochem.* 17 (1977) 39–44.
- [12] H. Rinderknecht, P. Widding, B.J. Haverback, A new method for the determination of α -amylase, *Experientia* 23 (1967) 805.
- [13] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [14] S. Mishra, Presence of carbohydrases in defensive secretion of soldiers of *Odontotermes distans* (Holmgren), *Indian J. Entomol.* 45 (1983) 199–200.
- [15] C. Noirot, in: K. Krishna, F.M. Weesner (Eds.), *Biology of Termites*, vol. I, Academic Press, New York, 1969, pp. 89–123.
- [16] J. Ghuysen, D. Tipper, J. Strominger, Enzymes that degrade bacterial cell walls, *Methods Enzymol.* 8 (1968) 685–699.