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Sensitive fluorescent quantitation of *myo*-inositol 1,2-cyclic phosphate and *myo*-inositol 1-phosphate by high-performance thin-layer chromatography

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Abstract

A non-radioactive micro-assay for the cyclic phosphodiesterase reaction catalyzed by *Bacillus cereus* phosphatidylinositol-specific phospholipase C is described. The assay involves high-performance thin-layer chromatography on silica gel to resolve the substrate (*myo*-inositol 1,2-cyclic phosphate) and the product (*myo*-inositol 1-phosphate), followed by detection with a lead tetraacetate–fluorescein stain. The quantitation of these inositol phosphates in sample spots relative to a series of standards is accomplished by analysis of the fluorescent plate image with a commercial phosphoimager and associated software. The experimental considerations for reliable quantitation of inositol monophosphates in the range of 0.1 to 50 nmol are presented.

Keywords: Enzyme kinetics; Myoinositol phosphate; Cyclic phosphodiesterase; Phospholipase C; Fluorescein; Lead tetraacetate

1. Introduction

Our goal was to develop a sensitive and reliable non-radioactive micro-assay which could be used for kinetics studies of a bacterial enzyme, phosphatidylinositol-specific phospholipase C (PI-PLC). PI-PLCs are produced by a variety of pathogenic gram-positive bacteria including *Bacillus cereus*, *B. thuringiensis*, *Listeria monocytogenes*, *L. ivanovii*, *Staphylococcus aureus*, *Clostriduim novyi*, and *Rhodococcus equii* (reviewed in Ref. [1]). Although PI-PLCs are considered to be potential virulence factors produced by these bacteria, the enzyme is also found in the non-pathogenic species *L. seeligeri*, *Streptomyces antibioticus*, and gram-negative *Cytophaga* sp. Bacterial PI-PLC is also a widely used tool in the study of glycosylphosphatidylinositol-anchored membrane proteins in mammalian cells (reviewed in Ref. [1]). PI-PLC catalyzes two reactions: (1) the rapid cleavage of phosphatidylinositol (PI) to produce *myo*-inositol 1,2-cyclic phosphate (IcP) and diacylglycerol; and (2) a slower hydrolysis of IcP to *myo*-inositol 1-phosphate (IP). A method for the analysis of the second of these two reactions was the focus of this study.

Several methods have been developed for the analysis of inositol phosphates, frequently emphasizing separation of the more highly phosphorylated

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isomers [2–6]. The most common method is probably high-performance liquid chromatography (HPLC) of radiolabeled samples. While HPLC is an excellent analytical tool, the time required for the analysis of each aliquot taken from the assay mixture makes it cumbersome for rapid kinetic analyses. In contrast, ^{31}P nuclear magnetic resonance (NMR) has been successfully used to follow the kinetics of enzyme reactions involving inositol phosphates [7,8]. During NMR analysis, the entire assay mixture is positioned in the probe, data points are taken as repeated scans, and scan times can be short. However, the relative insensitivity of NMR requires the preparation of labor-intensive amounts of both enzyme and substrate, and limits the initial rates that can be measured. As an alternative, high-performance thin-layer chromatography (HPTLC) was explored for this study because it is an established, inexpensive method for resolving inositol phosphates [4]. Multiple samples can be loaded on a TLC plate (approximately 25 samples, for a 20 cm wide plate), and an important feature of TLC is that the samples are analyzed in parallel. In addition, a sensitive fluorescent stain for the detection of *myo*-inositol has been described [9], potentially providing a non-radioactive yet sensitive quantitation of inositol phosphates. The factors examined in the present study were the nature of the staining procedure, the choice of elution solvent, and the effectiveness of the procedure for generating progress curves from actual kinetic runs.

2. Experimental

2.1. Materials

The PI-PLC from *B. cereus* (E.C. number 3.1.4.10) was from recombinant *Escherichia coli* over-expressing the cloned enzyme as described [10] (also available from Molecular Probes, Eugene, OR, USA).

The natural substrate for the cyclic phosphodiesterase reaction, IcP, was prepared enzymatically from 0.5 g of soybean phosphatidylinositol (PI; Sigma, St. Louis, MO, USA) by a modification of a published procedure [7]. The PI was dissolved in chloroform and deposited by rotary evaporation in a thin layer on the wall of a round-bottom flask, followed by resuspension in 20 ml of 50 m*M* Tris–HCl, pH 7.2, 4% Triton X-100. A 180-ng amount of PI-PLC was added and the solution incubated for several hours at 37°C, with periodic examination by ^{31}P-NMR [7,8] in order to determine the progress of the reaction. At completion, the reaction mixture was subjected to two cycles of chloroform–methanol phase separation (retaining the upper, aqueous, layer). Isolation of IcP was accomplished by subsequent ion-exchange chromatography using 5 g of Dowex AG 1-X8 (formate form; Bio-Rad, Richmond, CA, USA) and elution with 150 m*M* ammonium formate. Fractions containing IcP but not IP were pooled, lyophilized, reconstituted in water, and re-lyophilized. Storage was at −80°C with care taken to avoid subsequent freeze–thaw cycles.

IP for standards was prepared enzymatically from IcP by allowing the pooled leftover reaction mixtures from the cyclic phosphodiesterase assay (described in Section 2.3) to progress overnight at room temperature in the presence of 0.1% (w/v) NaN_3, followed by preparative thick layer (2 mm) chromatography on 20×20 cm silica gel plates (Fisher Scientific, USA) eluted with chloroform–methanol–30% ammonia (3:10:5, v/v). The migration position of IP was identified by cutting off a 1–1.5 cm strip from the end of the plate for lead tetraacetate–fluorescein staining (described in Section 2.4), then the region corresponding to the IP band was scraped from the remaining plate with a razor blade. After elution from the silica gel in ethanol–water (50:50), aliquots of convenient size were lyophilized and stored at −80°C.

2.2. High-performance thin-layer chromatography procedure; general

Thin layer silica gel 60 plates (10×20 cm) for HPTLC were obtained from EM Sciences (Gibbstown, NJ, USA) and used within 2 weeks of opening the package. Older plates were pre-eluted with chloroform–methanol–30% ammonia (3:10:5, v/v) and stored in a dry box until use. To facilitate spotting samples on an even line parallel to the bottom edge, the plate was then laid onto a bold ink line on a sheet of Al foil such that the line (faintly visible through the plate) served as a guide. Sample

positions at 0.75 cm intervals were also marked in advance by using a clean needle to make tiny notches in the gel layer along the bottom edge of the plate.

Since there can be plate-to-plate differences in the elution and staining steps, plates to be used for the quantitation of inositol phosphates in mixtures or enzyme assays also contained a series of standards of verified concentration and composition. With time, pH variations, and freeze–thaw cycles, inositol phosphates can degrade and in addition can exhibit substantial nonspecific binding to container walls. For redetermining the concentration of stocks at or near the time of use, inositol phosphate stocks were assayed for total phosphate by the acid digestion/ascorbate reduction assay [11]. Stock composition was checked for purity by TLC followed by the lead–fluorescein stain [9] or by an acidic molybdate dip [4]. The latter is less sensitive than the former for IP detection but has the advantage of visualizing free phosphate (an undesirable breakdown component) in the sample. Free phosphate stains blue and remains at the origin in our standard elution solvent system, and samples containing detectable amounts of free phosphate were discarded.

2.3. High-performance thin-layer chromatography procedure; application to enzyme kinetics

For enzyme assays, TLC plates were pre-spotted with 3–4 μl of an enzyme ''stop'' solution consisting of 5 m*M* $HgCl_2$. The presence of the metal does not appreciably alter the elution positions or quantitation of IP or IcP. Reactions with *B. cereus* PI-PLC (typically 2–6 μg/ml) were carried out in a buffer consisting of 100 m*M* PIPES (Sigma), pH 7.0, 1 m*M* EDTA, and 0.02% bovine skin gelatin (Type B, 225 bloom, Sigma). Micro-assays of 20 to 40 μl volume were initiated by the 1:1 (v/v) addition of 2× (relative to the final concentration) enzyme to 2× substrate. The assays were contained in thin-walled 0.2-ml polymerase chain reaction (PCR) tubes (Continental Lab. Products, San Diego, CA, USA) suspended in a temperature-controlled 24°C water bath. Samples of 2 μl volume were taken at regular time intervals using a ''0 to 5 μl'' blunt-tipped Hamilton syringe (Fisher) and spotted onto sequential positions on previously prepared TLC plates. At least five samples of 2 μl each of IP standards prepared in assay buffer (including the IcP substrate to match the assay concentration, but not enzyme) were spotted on adjacent plate lanes. Elution of the thoroughly dry plates was carried out in chloroform–methanol–30% ammonia (3:10:5, v/v) unless specified otherwise (e.g., if the goal is IcP quantitation rather than IP quantitation, the ammonia concentration is decreased, see Section 3.1). It should be noted that the concentration of ammonia in an opened stock bottle tends to drop with time and use, and a change in the ammonia concentration can radically alter the migration distances of inositol phosphate sample spots. Multiple assays (representing different experimental conditions) were usually run in parallel, with staggered start times. In this laboratory a typical experiment consists of 6–12 TLC plates, loaded in sets of 3–4 plates at a time over a period of 1–2 h.

2.4. Fluorescent quantitation

Eluted TLC plates were dipped in lead–fluorescein stain [9] within 24 h of elution. The stain was prepared from two stocks, one of 0.15 g sodium fluorescein (Matheson, Coleman & Bell, Norwood, OH, USA) in 15 ml of 95% ethanol, and the other of 1 g of lead tetraacetate (J.T. Baker, Phillipsburg, NJ, USA) in 33 ml of glacial acetic acid. These were added sequentially with stirring to 700 ml dichloromethane (dichloroethane can also be used), and the resulting stain used within 3 h. To decrease the required volume of stain, a slab of PTFE (Teflon) was placed into the back of the stain tank to bring the working height of the liquid to about 10 cm. The completely dry TLC plates were dropped vertically into the stain one at a time, and after 10–15 s, were rapidly and evenly removed (a wire loop attached to binder clips facilitated plate handling for this step). Dried plates were stored in the dark and scanned within 2 days. The fluorescent plate images were captured by placing the plates face-down directly on the glass surface of a Storm Phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA). Scanning was performed using a 200 μm pixel size and a typical phosphoimager potential of 625 to 800 V, depending on the intensity of the stained sample spots. Higher scanning voltages increase the sensitivity of the instrumental response until saturation is reached; the

optimal scanning voltage needs to be determined for a given set of stained plates. The fluorescence intensities of the spots on the plates were quantitated using the ImageQuant program (Molecular Dynamics).

3. Results and discussion

3.1. Factors affecting fluorescent staining and quantitation of IPs

Fig. 1a is the fluorescence image of an HPTLC plate containing a series of IcP standards prepared in PIPES buffer and eluted with chloroform–methanol–30% ammonia (3:10:2.5, v/v), followed by lead–fluorescein staining. The PIPES buffer (which is also subject to fluorescein staining; see subsequent text) has the greatest mobility of the components of this sample, and appears as a ''comet'' close to the solvent front. In the solvent system used for the experiment of Fig. 1, IcP migrates to a mid-plate position, and the small amount of IP present in this IcP preparation remains close to the origin (see arrows, Fig. 1a). Although Fig. 1a is a fluorescence image recorded with a phosphoimager, the sample spots can also been seen to a lesser extent in ambient room light, appearing as yellowish spots against the relatively white plate background.

To investigate the role of lead tetraacetate in the sample contrast obtained with the lead–fluorescein stain, the plate of Fig. 1a and a duplicate plate (not shown) were exposed to 10-s dips in fluorescein stain of different composition, and scanned side-by-side on the phosphoimager window. The resulting spot intensities were plotted as a function of IcP content for both stain conditions (Fig. 1b). The stain containing lead tetraacetate at 0.07% gave a plate whose plot of fluorescence intensity vs. nmol IcP yielded a greater slope (greater sensitivity). That is, a sample spot containing 25.6 nmol IcP gave a signal of 57.2 rfu (relative fluorescence units) on the plate exposed to 0.07% lead tetraacetate, compared to 22.3 rfu on the plate exposed to 0.21% lead tetraacetate. In additional experiments, decreasing the lead tetraacetate content of the stain further resulted in progressively higher plate backgrounds. Conversely, increasing the lead content of the stain progressively decreased the intensity of the staining of the IP spots. These results indicate that a key role for the lead tetraacetate in the stain is to mediate the fluorescence from fluorescein in the sample spots compared to that of the fluorescein on the silica gel background.

There are evidently two lead tetraacetate reactions taking place on these TLC plates. One is the quenching of fluorescein fluoresence, which is due to a reversible complex formation between the lead and the fluorescein [12,13]. The second is an irreversible oxidation of the inositol derivatives in the sample spots, which consumes lead tetraacetate in the immediate vicinity and thus frees fluorescein from the lead–fluorescein complex. Although the application of this pair of reactions for the specific purpose of quantitation of *myo*-inositol in biological samples has been described [9], the reaction is actually not specific for *myo*-inositol, or even the inositol monophosphates. On TLC plates eluted and stained as described here, PIPES buffer reacts with the stain, as do the common buffers HEPES and Tris (not shown), and a variety of saccharides [14,15]. This is consistent with the known oxidation reactions of lead tetraacetate with free (not esterified) alcohols and amines [16,17]. The chemistry predicts that IcP should react with lead tetraacetate at a somewhat slower rate than with IP because two of the inositol hydroxyl groups are protected by participation in the cyclic phosphate diester. In contrast, there is only one esterified hydroxyl group in IP. Nevertheless, we find that IcP reacts sufficiently rapidly to allow quantitation (e.g., Fig. 1a). Due to the lack of hydroxyl groups available for reaction with the lead tetraacetate, the more highly phosphorylated inositols are poor candidates for the lead–fluorescein stain procedure. Consistent with this, the lead–fluorescein stain did not react with HPTLC sample spots containing 5–10 nmol of either inositol bisphosphate $I(1,4)P_2$ or inositol trisphosphate, $I(1,4,5)P_3$ (not shown). However the procedure is clearly applicable to IP quantitation, as in the course of these experiments we were able to obtain useful data for sample spots containing from 0.1 to 50 nmol of IP.

Quantitation of the fluorescence intensity of sample spots on HPTLC plates proved to be most reproducible if the spots to be quantitated were located in approximately the central region of the plate, clearly removed from both the solvent front

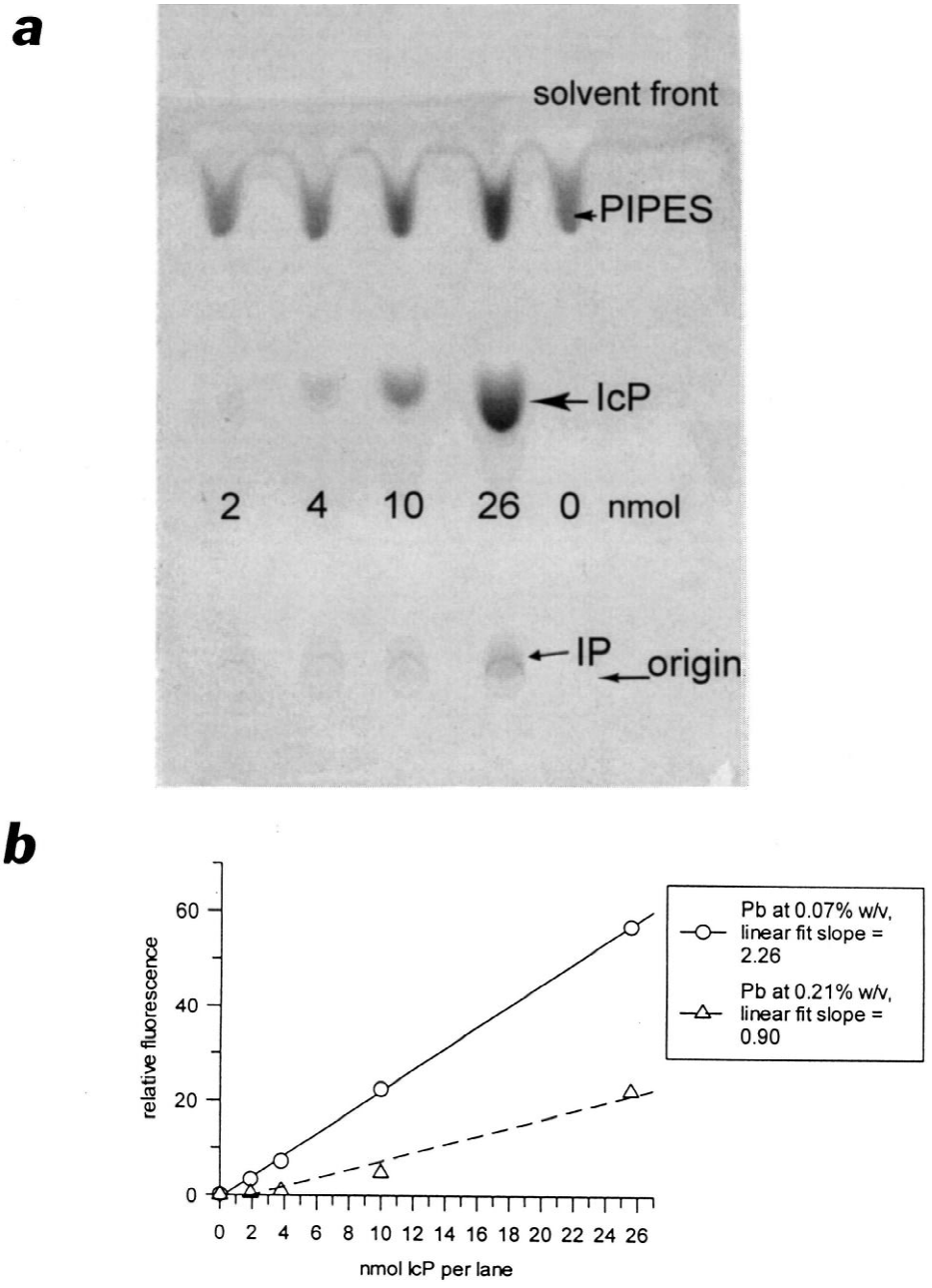

Fig. 1. (a) HPTLC optimized for quantitation of IcP. IcP standards were spotted on the plate and eluted in chloroform–methanol–30% ammonia (3:10:2.5, v/v), then exposed to lead–fluorescein stain. In this elution solvent mixture, the IcP runs in the center portion of the plate (ideal for quantitation) while the IP remains near the origin. (b) Quantitation of the fluorescence of the plate shown in (a) and of a duplicate plate (not shown) demonstrate first the linearity of the fluorescence response to IcP concentration in the sample, and second, the effect of stain composition on the sensitivity of the assay. The plate shown in (a) was exposed to 0.21% lead tetraacetate and on quantitation yielded a slope of 0.90 fluorescence units per nmol cIP (standard error 0.09); the duplicate plate exposed to 0.07% lead tetraacetate yielded a slope of 2.26 (standard error 0.03). The desirable concentration of lead in the stain is a balance between quenching the fluorescein fluorescence on the background and preserving the fluorescence intensity of the sample spots. For subsequent experiments, a lead tetraacetate concentration of 0.13% was selected. A phosphoimager scanning voltage of 675 V was used for both plates.

and the origin. The elution conditions for the optimal quantitation of the IcP substrate and the IP product of the second reaction catalyzed by PI-PLC were found to differ slightly, consistent with the difference in polarity of these two compounds. The chloroform–methanol–30% ammonia (3:10:2.5, v/v) used for the experiment of Fig. 1a brings IcP to the central portion of the plate, while IP has a low R_F, too close to the origin for reliable quantitation. Increasing the ammonia concentration in this basic

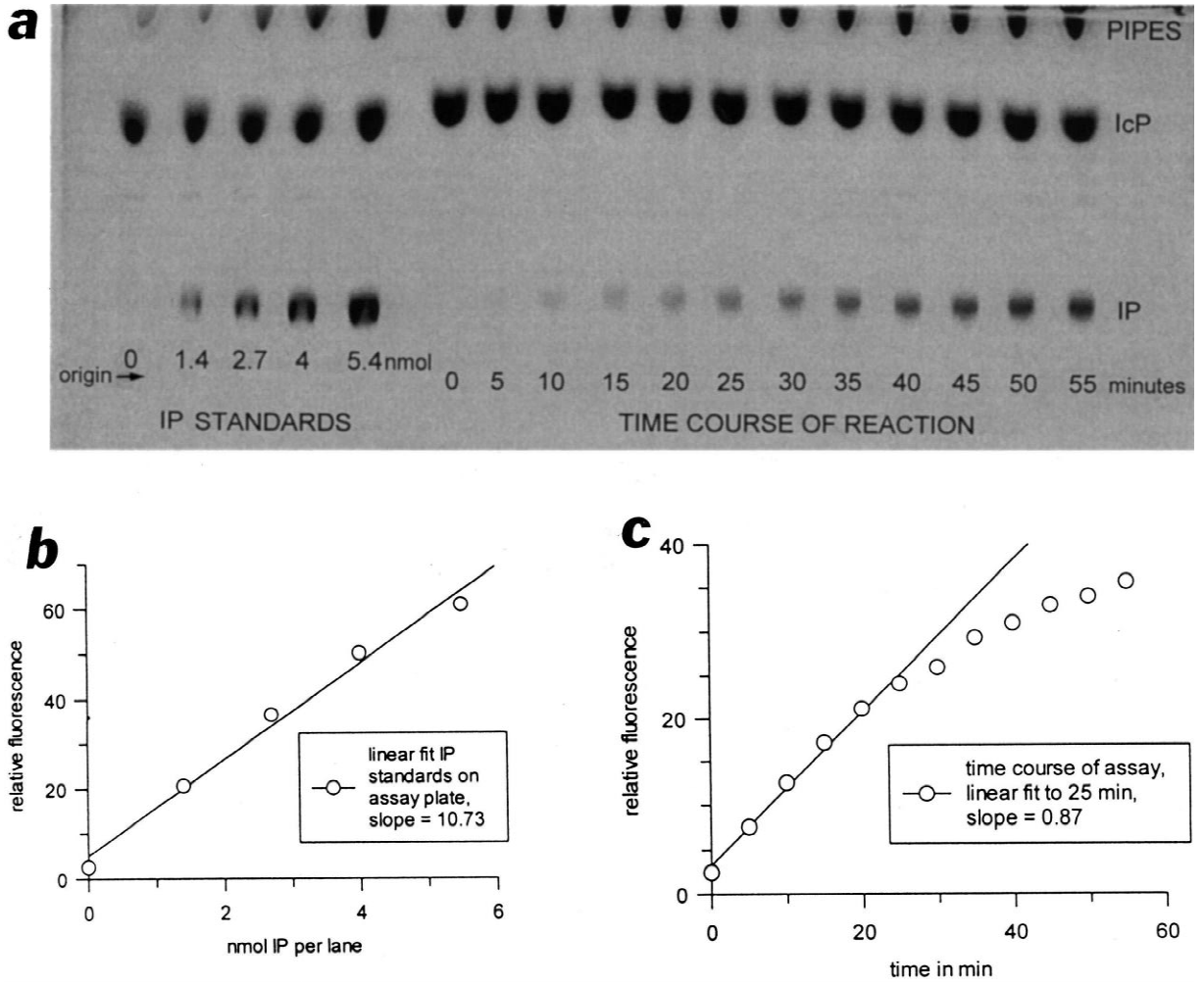

Fig. 2. (a)–(c) Examples of an application of the HPTLC assay to enzyme kinetics. (a) Fluorescence image of an HPTLC plate containing both the IP standards (on the left) and successive 2 μl aliquots of a *B. cereus* PI-PLC enzyme assay mixture taken at 5-min intervals (right two-thirds of the plate, ''time course of the reaction''). The assay was carried out in 40 μl initial volume and consisted of 6 μg/ml PI-PLC, 8 m*M* IcP (the substrate), pH 7.0, 24°C. The IP standards (used for quantitation of the IP product of the reaction) were prepared in assay buffer containing 8 m*M* IcP substrate. The dried plate was eluted with chloroform–methanol–30% ammonia (3:10:5, v/v), stained, and the fluorescence image recorded with a phosphoimager. The scanning voltage used was 675 V. (b) Quantitation and linear fit of the intensity of the IP standard spots to obtain a slope of fluorescence intensity vs. nmol IP, which in this example was 10.73 fluorescence units/nmol (standard error 0.71). (c) Quantitation of the IP spots from the assay lanes to give a progress curve for the generation of IP product from IcP substrate, with linear fit of the early portion to obtain an initial rate of 0.87 fluorescence units/min (standard error 0.04). These two slopes are used to calculate a reaction rate (see text).

elution solvent composition induced greater mobility of the IP spot. This is shown by the HPTLC plate of Fig. 2a, in which IP standards and an enyzme assay mixture containing both IcP substrate and IP product were eluted in chloroform–methanol–30% ammonia (3:10:5, v/v). In this solvent system the average R_F values for IcP and IP were 0.7 and 0.2, respectively, sufficient to allow quantitation of either component. (For comparison, this solvent system gave R_F values of 0.08, 0 and 0.17 for IP_2, IP_3 and *myo*-inositol, respectively; not shown). Since it is usually preferable to follow the time course of a reaction by monitoring the appearance of product as compared to the decrease in the amount of substrate, it is this latter solvent system that was chosen for the kinetic analysis of the PI-PLC second reaction.

3.2. Application of the TLC assay to enzyme kinetics

To test the feasibility of the HPTLC assay as a method for quantitation of small-volume enzyme assays, the experiment shown in Fig. 2 was performed as described in Section 2.3 and in the caption to Fig. 2. Fig. 2a shows the fluorescent image of the resulting plate after elution and staining, while Fig. 2b and c present the quantitation results for the IP standards and for the IP produced in the course of the reaction, respectively. Performing a linear fit of the IP standard data gave a conversion factor (the slope) which related fluorescence intensity to nmol IP for the plate. Performing a linear fit for the first portion of the time course of the kinetic run (quantitating the IP product spot intensity as a function of time) gave an initial rate in fluorescence intensity units/min. Dividing the initial rate of the reaction by the conversion factor gave a rate expressed in nmol IP/min per 2 μl spot, which can be converted to the rate expressed as a change in the concentration of product with time (in this example, 0.04 m*M*/min).

The basic approach described here is not necessarily limited to PI-PLC, as there are other enzymes which either act on or produce *myo*-inositol or IPs. Examples include *myo*-inositol dehydrogenase (E.C. 1.1.1.18) [18,19], and *myo*-inositol monophosphatase (E.C. 3.1.3.25), the latter of which is implicated in depression in humans [20].

4. Conclusion

In this study we explored HPTLC combined with fluorescent sample detection as a nonradioactive micro-assay for studies of the kinetics of the second reaction catalyzed by bacterial PI-PLC. The procedure described here involved extending and developing the *myo*-inositol quantitation procedure reported by Stepanek [9] into a method suitable for the quantitation of both IP and IcP. Practical considerations for optimizing quantitation include the choice of solvent systems and the lead content of the stain. The result is an inexpensive, sensitive and quantitative assay that does not require radiolabeled samples and can be used in conjunction with commercially available instrumentation.

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References

[1] O.H. Griffith, M. Ryan, Biochim. Biophys. Acta 1441 (1999) 237.
[2] I.M. Bird, in: I.M. Bird (Ed.), Methods in Molecular Biology, Humana Press, Totwa, NJ, 1998, p. 11, Chapter 2.
[3] S. Palmer, M.J.O. Wakelam, Biochim. Biophys. Acta 1014 (1989) 239.
[4] F. Hatzack, S.K. Rasmussen, J. Chromatogr. B 736 (1999) 221.
[5] A.K. Singh, Y. Jiang, J. Chromatogr. B 671 (1995) 255.
[6] A.H. Guse, A. Goldwich, K. Weber, G.W. Mayr, J. Chromatogr. B 672 (1995) 189.
[7] C. Zhou, Y. Wu, M. Roberts, Biochemistry 36 (1997) 347.
[8] J.J. Volwerk, M.S. Shashidar, A. Kuppe, O.H. Griffith, Biochemistry 29 (1990) 8056.
[9] J. Stepanek, J. Chromatogr. 257 (1983) 405.
[10] M. Ryan, M.P. Smith, T.K. Vinod, W.L. Lau, J.F.W. Keana, O.H. Griffith, J. Med. Chem. 39 (1996) 4366.
[11] B.C. Cogan, G.B. Birrell, O.H. Griffith, Anal. Biochem. 271 (1999) 29.
[12] B. Agarwal, B.V. Agarwala, A.K. Dey, J. Ind. Chem. Soc. 57 (1980) 130.

[13] Sh.T. Talipov, L.E. Zel'tser, A.T. Tashkhodzhaev, Izv. Vyssh. Ucheb. Zaved., Khim. Khim. Tekhnol. 16 (1973) 299.
[14] A.S. Perlin, Adv. Carbohydr. Chem. 14 (1959) 9.
[15] P.M. Collins, R.J. Ferrier, in: Monosaccharides, Wiley, Chichester, 1995, p. 406.
[16] G.M. Rubottom, in: Oxidation in Organic Chemistry, Vol. 5D, Academic Press, New York, 1982, p. 1.
[17] J. Zỳka, Pure. Appl. Chem. 13 (1966) 569.
[18] R. Stein, C. Schnarrenberger, W. Gross, Phytochemistry 46 (1997) 17.
[19] F. Kwok, S.C. Lo, J. Biochem. Biophys. Methods H94 (1994) 173.
[20] S.J. Pollack, J.R. Atack, M.R. Knowles, G. McAllister, C.I. Ragan, R. Baker, S.R. Fletcher, L.L. Iversen, H.B. Broughton, Proc. Natl. Acad. Sci. 91 (1994) 5744.