Video Article

Defining Substrate Specificities for Lipase and Phospholipase Candidates

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Abstract

Microorganisms produce a wide spectrum of (phospho)lipases that are secreted in order to make external substrates available for the organism. Alternatively, other (phospho)lipases may be physically associated with the producing organism causing a turnover of intrinsic lipids and frequently giving rise to a remodeling of the cellular membranes. Although potential (phospho)lipases can be predicted with a number of algorithms when the gene/protein sequence is available, experimental proof of the enzyme activities, substrate specificities, and potential physiological functions has frequently not been obtained. This manuscript describes the optimization of assay conditions for prospective (phospho)lipases with unknown substrate specificities and how to employ these optimized conditions in the search for the natural substrate of a respective (phospho)lipase. Using artificial chromogenic substrates, such as *p*-nitrophenyl derivatives, may help to detect a minor enzymatic activity for a predicted (phospho)lipase under standard conditions. Having encountered such a minor enzymatic activity, the distinct parameters of an enzyme assay can be varied in order to obtain a more efficient hydrolysis of the artificial substrate. After having determined the conditions under which an enzyme works well, a variety of potential natural substrates should be assayed for their degradation, a process that can be followed employing distinct chromatographic methods. The definition of substrate specificities for new enzymes, often provides hypotheses for a potential physiological role of these enzymes, which then can be tested experimentally. Following these guidelines, we were able to identify a phospholipase C (SMc00171) that degrades phosphatidylcholine to phosphocholine and diacylglycerol, in a crucial step for the remodeling of membranes in the bacterium *Sinorhizobium meliloti* upon phosphorus-limiting conditions of growth. For two predicted patatin-like phospholipases (SMc00930 and SMc01003) of the same organism, we could redefine their substrate specificities and clarify that SMc01003 is a diacylglycerol lipase.

Video Link

The video component of this article can be found at https://www.jove.com/video/54613/

Introduction

Glycerol-based lipids such as triacylglycerols and (glycero)phospholipids constitute important and probably the best-known lipid classes[1]. Triacylglycerols (TAGs) are fats or oils, which usually function as storage lipids, and therefore as potential energy and carbon sources. TAGs can be degraded by lipases, which are frequently secreted by the producing organism to digest external TAGs and make them available as carbon sources. Also, lipases have been widely studied over the years due to their important biotechnological applications[2].

Due to their amphiphilic nature and their near-cylindric shape, (glycero)phospholipids exhibit membrane-forming properties and usually constitute the major lipidic components of a bilayered membrane[3]. In simple microorganisms, such as the bacterium *Escherichia coli*, only three major head group variants, phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylethanolamine (PE) are encountered, although one should be aware that each one of them can be substituted with a considerable number of different fatty acyl chains at the *sn*-1 or *sn*-2 position giving rise to a large number of different molecular species[4]. Other bacteria might have other phospholipids in addition or instead. For example, *Sinorhizobium meliloti*, a soil bacterium, which is able to form a nitrogen-fixing root nodule symbiosis with the legume alfalfa (*Medicago sativa*), contains in addition to PE a second zwitterionic phospholipid, phosphatidylcholine (PC)[5]. Also, lipids not containing phosphorus or glycerol might be amphiphilic and form part of the cellular membrane. For example, upon phosphorus-limiting growth conditions, in *S. meliloti*, (glycero)phospholipids are largely replaced by membrane lipids that do not contain phosphorus, *i.e.,* sulfolipids, ornithine lipids, and diacylglyceryl trimethylhomoserine (DGTS)[6]. In bacteria, DGTS is formed from diacylglycerol (DAG) in a two-step pathway[7] but the source for DAG generation was not clear. Pulse-chase experiments suggested that PC might be a precursor for DGTS[8] and using the methodology described in this manuscript we could identify a phospholipase C (PlcP, SMc00171) that is formed under phosphorus-limiting conditions and which can convert PC into DAG and phosphocholine[8].

In a separate study, we discovered that an acyl-CoA synthetase (FadD)-deficient mutant of *S. meliloti* or of *Escherichia coli* accumulated free fatty acids when entering stationary phase of growth[9]. Although these fatty acids seemed to be derived from membrane lipids, the precise source for the free fatty acids or the enzyme(s) liberating them were not known. Again, employing the strategy outlined in this manuscript, two patatin-like[10] (phospho)lipases (SMc00930 and SMc01003) that contributed to the formation of free fatty acids in *S. meliloti*[11] were predicted.

Surprisingly, SMc01003 used DAG as substrate converting it to monoacylglycerol and finally glycerol and free fatty acids[11]. Therefore, SMc01003 is a DAG lipase (DglA).

Although a number of algorithms exist for predicting potential (phospho)lipases[12,13], their precise function and physiological role is usually not known. Here we outline a protocol, to clone and overexpress predicted or potential (phospho)lipases. This manuscript explains how enzyme assays can be developed and optimized for the overexpressed (phospho)lipase by using artificial chromogenic substrates. We provide examples how with an optimized enzyme assay the real (phospho)lipase substrate can be encountered and how these findings might enrich our understanding of microbial physiology.

Protocol

1. Clone and Overexpress Structural Gene for Predicted Lipase

1. Using polymerase chain reaction (PCR)[14] and specific oligonucleotides (**Table 1**)[15], amplify the gene of interest (*smc01003*, *smc00930*, or *smc00171*), predicted to code for a lipase or phospholipase, from the genomic DNA of the host organism (*i.e., S. meliloti*).
    1. Introduce specific restriction sites (with the designed sequence of the oligonucleotides). Digest the amplified DNA fragment with the corresponding restriction enzymes and clone it into an expression vector such as plasmids of the pET series[16].
    2. After verifying the correct DNA sequence for the cloned gene, transform the vector to an expression strain such as *Escherichia coli* BL21(DE3) pLysS[16].

2. Prepare an overnight pre-culture of the expression host *E. coli* BL21(DE3) pLysS, harboring the respective pET vector with the cloned gene or the empty vector, in 100 ml culture flasks containing 20 ml of Luria Bertani broth (LB)[17] plus the required antibiotics. Culture the cells at 30 ℃ (or at the usual growth temperature of the bacterium from which the lipase originates).
    1. Using the overnight pre-cultures, inoculate 500 ml of prewarmed LB medium (plus the required antibiotics) in 2 L culture flasks to obtain an initial optical density at 620 nm (OD_{620}) = 0.05. Follow growth of cultures and at an OD_{620} = 0.3, add isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 100 µM, and incubate under agitation at 30 ℃ for a period of 4 hr.
    2. At the end of the incubation period, transfer each culture to a 500 ml centrifuge tube and centrifuge at 5,000 x g at 4 ℃ for 30 min. Resuspend bacterial cell pellets in 5 ml of suspension buffer (*e.g.*, SMc00930- and SMc01003-expressing cells in 50 mM Tris-HCl pH 8.0 and SMc00171-expressing cells in 50 mM diethanolamine-HCl pH 9.8). Store the cell suspensions at -80 ℃ until use.

2. Prepare Cell-free Protein Extracts and Determine Protein Concentration

1. Defrost bacterial cell suspensions and store on ice. Pass cell suspensions three times through a cold pressure cell at 20,000 lb per in^2. Remove intact cells and cell debris by centrifugation at 5,000 x g for 30 min at 4 ℃.
2. After centrifugation, prepare aliquots of 100 and 500 µl from the supernatant for subsequent analysis and store them at -80 ℃ until use.
3. Use one of the 100 µl aliquot to determine protein concentration of distinct cell-free extracts by a method of choice or as described[18].

3. Use Artificial Substrates for Optimizing Enzyme Activities of (Phospho)lipases

1. For an initial coverage of distinct enzyme activities, use artificial substrates that yield a colored product upon hydrolysis, such as *p*-nitrophenol (*p*-NP).
    1. For enzyme assays optimized already with artificial *p*-nitrophenyl ester substrates (outlined for phospholipase C PlcP (SMc00171), as well as for the predicted patatin-like phospholipases SMc00930 and SMc01003), use pipetting schemes described in **Table 2**.
    2. When exploring a new potential (phospho)lipase, prepare a first standard enzyme assay containing 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 0.05% Triton X-100, 0.5 mM *p*-nitrophenyl-containing compound (*p*-nitrophenyl phosphate, bis-*p*-nitrophenyl phosphate, *p*-nitrophenyl decanoate, or *p*-nitrophenyl palmitate), and cell-free protein extract (check 1, 3, 10, 30, 100, 300, and 1,000 µg) in a total volume of 1 ml in 1 ml plastic cuvettes.
    NOTE: Use alkaline pH (**Figure 1**) when following *p*-nitrophenyl ester hydrolysis in a continuous assay. Alternatively, use single time-point assays for a range of pH values, adding NaOH at the end of the incubation period to terminate the enzyme reaction and to ensure that all *p*-NP is present in the phenolate form.
    3. Follow the time course for an increase of absorbance at 405 nm, due to the formation of *p*-NP, in a spectrophotometer at 30 ℃ over a 5 min period. Quantify the initially linear formation of *p*-NP by determining the initial slope of increase of absorbance per time.
    4. Calculate the change of concentration (Δc) for *p*-NP using the law of Lambert-Beer ($\Delta A = \varepsilon \Delta c\, d$)[1].
    NOTE: ΔA is the linear change of absorbance determined, ε is the molar extinction coefficient at the respective wave length (in units of $M^{-1}\ cm^{-1}$), d is the length of the light path (1 cm), and Δc is the change of concentration (in units of M) to be determined.
        1. Considering that the assay volume is 1 ml, calculate the amount of *p*-NP formed.
        NOTE: Amount = concentration x volume.
        2. Calculate the enzyme activity by dividing the amount of *p*-NP formed by the time in which it is formed. Determine the specific enzyme activity by dividing enzyme activity by the amount of protein (in mg) which was responsible for generating this activity.

    5. Compare absorbance changes provoked by protein extracts in which a candidate gene (*smc00171*, *smc00930*, or *smc01003*) had been expressed with extracts that harbor only an empty vector.
    NOTE: In order to continue with the following steps, the specific activities, caused by protein extracts in which a candidate gene had been expressed, should be at least twice or more than the values obtained for the specific activities caused by protein extracts that harbor only an empty vector.

6. For further experiments, select those conditions in which hydrolysis of the *p*-nitrophenyl-containing compound is minimal with cell-free extracts (*i.e.,* empty vector) and for which the most pronounced formation of *p*-NP and the *p*-nitrophenolate anion (**Figure 1**) can be observed when protein extracts are employed, in which a candidate gene had been expressed.

2. After determining the initial enzyme activity in 3.1, optimize the assay conditions for the respective enzyme by varying pH, type of buffer, buffer strength, concentrations of NaCl, detergents such as Triton X-100, and the absence or presence of different bivalent cations.
   1. For different concentrations of each variable, determine the specific enzyme activity (see 3.1.4.2) (the highest number obtained defines the condition of maximal enzyme activity). Use the combination of the optimal conditions encountered for each variable to define an optimized enzyme assay in which each variable is present in its optimal concentration.

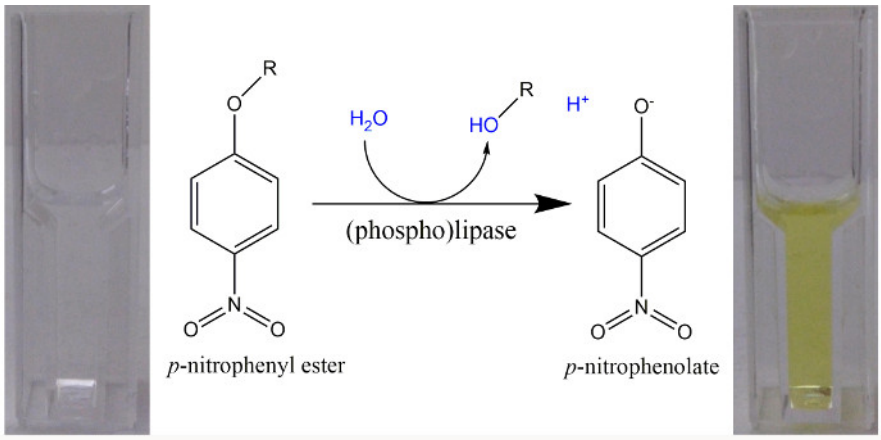

Figure 1. *p*-Nitrophenyl esters as artificial substrates for (phospho)lipases in a spectrophotometric assay. Upon hydrolysis of *p*-nitrophenyl esters, an acid (R-OH) and *p*-nitrophenol (*p*-NP) are formed. Due to the pK_a = 7.2 for the dissociation of the phenolic H^+ from *p*-NP, at a pH > 9.2 more than 99% are in the bright yellow *p*-nitrophenolate form and a molar extinction coefficient of 18,000 M^{-1} cm^{-1} can be used at a wave length of 405 nm for the quantification of free *p*-nitrophenolate[22]. When buffers with a pH of 8.5 were used, the absorbance was determined at 400 nm and a molar extinction coefficient of 14,500 M^{-1} cm^{-1} was employed[23]. Please click here to view a larger version of this figure.

NOTE: After having defined the optimal conditions for the activity of the enzyme of interest, embark on the search for the real/physiological substrate of this lipase. In principle, take two, often complementary, approaches to achieve this goal, an *in vivo* approach or an *in vitro* approach.

4. *In Vivo* Identification of the Physiological Substrate of a Lipase

NOTE: In an *in vivo* approach, express the lipase of interest in a host organism[8,11] in order to register over time whether expression of the lipase alters the host´s lipid profile. In another *in vivo* approach, generate a mutant deficient of the gene of interest[8,11] and study whether its lipid profile is distinct from the wild type version[6,8,11]. In order to obtain a quantitative assessment of an organism's lipid profile, a simple method consists in radiolabeling cellular compounds, extracting the lipids, separating them by chromatography, and quantifying the radioactively labeled separated lipids.

1. Radiolabeling of lipids.
   1. Prepare an overnight pre-culture of an organism of interest (*E. coli* or *S. meliloti*) in 5 ml of the desired culture medium (complex medium or defined minimal medium) and grow at 30 °C.
   2. From the pre-culture, inoculate into 20 ml of the same fresh medium in a 100 ml culture flask to obtain an initial OD_{620} = 0.3 for the culture.
   3. Take an aliquot (1 ml) of the culture under sterile conditions and transfer to a 14 ml sterile polystyrene round-bottom tube.
   4. Add 1 µCi of [1-^{14}C]acetate (60 mCi per mmol) to the 1 ml culture.
   5. Incubate the liquid culture under agitation at 30 °C for a period of 24 hr.
   6. At the end of the incubation period, transfer the culture to a 1.5 ml microcentrifuge tube and centrifuge at 12,000 x g at room temperature for 5 min.
   7. Resuspend the pellet in 100 µl of water. At this point, store the cell suspension at -20 °C or immediately continue with the extraction of polar lipids (section 4.2).

2. Extraction of polar lipids.
   NOTE: The method described here essentially follows the procedure reported by Bligh and Dyer[19].
   1. To the 100 µl of aqueous cell suspension, add 375 µl of methanol:chloroform solution (2:1; vol/vol).
   2. Vortex for 30 sec and incubate for 5 min at room temperature.
   3. Centrifuge 5 min at 12,000 x g at room temperature.
   4. Transfer the supernatant to a new 1.5 ml microcentrifuge tube.
   5. Add 125 µl of chloroform and 125 µl of water, vortex 30 sec.
   6. Centrifuge 1 min at 12,000 x g at room temperature.

7. Transfer the lower chloroform phase to a fresh tube and dry with a stream of nitrogen gas.
8. Dissolve dried lipids in 100 µl of chloroform:methanol solution (1:1; vol/vol).
   NOTE: At this point, an aliquot of 5 µl of the lipid solution can be quantified by liquid scintillation counting.
9. For thin-layer chromatographic (TLC) analysis, dry down the remaining 95 µl with a stream of nitrogen gas and redissolve dried lipids in 20 µl of chloroform:methanol solution (1:1; vol/vol). Use a 3 µl aliquot for TLC analysis.

3. Separation of polar lipids by thin-layer chromatography (TLC).
   NOTE: Depending on the lipid classes to be analyzed, different combinations of solid and mobile phases might be employed for separation. Here a typical separation for charged polar lipids and another, more suited for neutral polar lipids, using high performance thin-layer chromatography (HPTLC) silica gel aluminum sheets as the solid phase, are outlined.
   1. Separation of charged polar lipids by two-dimensional TLC (2D-TLC).
      1. Apply a 3 µl aliquot of lipid sample in one corner of a HPTLC silica gel aluminum sheet (10 x 10 cm), 2 cm from the edge of the plate.
      2. Prepare and mix the mobile phase (140 ml chloroform, 60 ml methanol, and 10 ml water) for separation in the first dimension.
      3. Coat a TLC developing chamber internally with chromatography paper.
         NOTE: This is to ensure that the gas phase of the chamber will be saturated rapidly (within 30 min) after the mobile phase for the first dimension has been added to the chamber and the chamber has been closed with a glass plate.
      4. Prepare and mix the mobile phase (130 ml chloroform, 50 ml methanol, and 20 ml glacial acetic acid) for separation in the second dimension and transfer to a second TLC developing chamber internally coated with chromatography paper and let the chamber saturate.
      5. Carefully transfer the HPTLC silica gel aluminum sheet with the dried lipid sample to the first chamber and develop (*i.e.*, perform chromatography) the plate for 60 min in the closed chamber in the first dimension[5].
      6. Remove the plate from the chamber and let solvents dry off in a flow hood for 30 min.
      7. After turning the plate by 90 degrees with regard to the previous chromatography, transfer the HPTLC silica gel aluminum sheet, on which the lipids have been separated in one dimension, to the second chamber and develop the plate for 60 min in the second dimension[5].
      8. Remove the sheet from the chamber and let the solvents dry off in a flow hood for at least 2 hr.
   2. Separation of neutral polar lipids.
      1. Apply 3 µl aliquots of lipid samples on a HPTLC silica gel aluminum sheet starting 2 cm from the edges of the plate. If multiple samples are analyzed in a one-dimensional chromatography, keep a distance of at least 1.5 cm between the different sample application spots.
      2. Prepare and mix the mobile phase (140 ml hexane, 60 ml diethylether, and 8 ml acetic acid) and transfer to a TLC developing chamber internally coated with chromatography paper and covered with a glass plate to let the chamber saturate (30 min).
      3. Transfer the HPTLC silica gel aluminum sheet with the dried lipid samples to the chamber and develop the plate for 30 min in the closed chamber.
      4. Remove the plate from the chamber and let the solvents dry off in a flow hood for 2 hr.

4. Quantification and visualization of separated polar lipids.
   1. Once the developed TLC sheet is dry, incubate it with a photostimulable luminescence (PSL) screen in a closed cassette for 3 days.
   2. Expose the incubated screen to a PSL scanner and acquire a virtual image of the separated radiolabeled lipids.
   3. Perform quantification using PSL software[20].

5. Visualization and isolation of individual polar lipids classes.
   1. Incubate developed TLC sheet for 10 min in a chromatography chamber in the presence of 1 g of iodine crystals.
      NOTE: Separated lipidic compounds will dissolve the iodine and appear as brownish spots.
   2. Circle the spots with a pencil, compare them to the relative mobility (R_f) of standard compounds (*i.e.,* 1,2-dipalmitoyl-*sn*-glycerol, dipalmitoyl-L-α-phosphatidylcholine, DL-α-monopalmitin, or palmitic acid), and identify to which lipid class they might belong.
   3. In a fume hood, let the iodine evaporate from the TLC sheet.
   4. With the help of a spatula, scrape the silica gel containing the compound of interest from the sheet, and extract the compound from the silica gel with a mixture of 100 µl of water and 375 µl of methanol:chloroform solution (2:1; vol/vol).
   5. Continue with extraction according to Bligh and Dyer as outlined (4.2.2 onwards).
   6. Store purified lipid class in 100 µl of chloroform:methanol solution (1:1; vol/vol) at -20 °C until use.

5. *In Vitro* Identification of the Physiological Substrate of a Lipase

NOTE: In an *in vitro* approach, study whether the lipase of interest can convert a mixture of isolated lipids or individual pure lipids to the corresponding hydrolysis products under the conditions defined as optimal in 3.2.

1. Use pipetting schemes for enzyme assays as per **Table 3** for PC-specific phospholipase C SMc00171 (see 5.2), phospholipase A (see 5.3), and DAG lipase SMc01003 (see 5.4) activity.
2. Determination of PC-specific phospholipase C activity (**Table 3**).
   1. To a 1.5 ml microcentrifuge tube, add 5,000 counts per minute (cpm) of total ^{14}C-labeled PC and a solution of Triton X-100.
   2. Mix and dry under a stream of nitrogen.
   3. Add diethanolamine-HCl, pH 9.8 buffer, as well as NaCl and $MnCl_2$ solutions and bidistilled water to obtain a final volume of 99.5 µl. Vortex for 5 sec.
   4. Add 0.5 µl of enzyme (5 µg protein) (*i.e.,* a cell-free extract in which overexpressed SMc00171 is present) to initiate the reaction. Mix briefly.

5. Incubate at 30 °C for 4 hr.
6. Stop the reaction by the addition of 250 µl of methanol and 125 µl of chloroform.
7. Extract lipids as described previously (see 4.2).
8. Separate lipids by one-dimensional (1D)-TLC (see 4.3.2 and 4.4), and analyze them by PSL imaging.

3. Determination of phospholipase A activity (**Table 3**).
    1. To a 1.5 ml microcentrifuge tube, add 5,000 cpm of total ^{14}C-labeled phospholipids and a solution of Triton X-100.
    2. Mix and dry under a stream of nitrogen.
    3. For a final 100 µl assay, add Tris-HCl, pH 8.5 buffer, NaCl solution and water. Vortex for 5 sec.
    4. Add 5 µl of enzyme (50 µg protein) (*i.e.*, a cell-free extract in which overexpressed SMc00930 or SMc01003 is present).
    5. Incubate at 30 °C for 5 hr.
    6. Stop the reaction by the addition of 250 µl of methanol and 125 µl of chloroform.
    7. Extract lipids as described previously (see 4.2), separate them by 1D-TLC using 130 ml chloroform, 50 ml methanol, and 20 ml glacial acetic acid as the mobile phase, and analyze them by PSL imaging.

4. Determination of diacylglycerol (DAG) lipase activity.
    1. Preparation of ^{14}C-labeled DAG.
        1. Radiolabel *S. meliloti* cultures (see 4.1) and extract polar lipids (see 4.2) as described. Separate *S. meliloti* total lipid extracts by 1D-TLC in chloroform:methanol:acetic acid (130:50:20; vol/vol) using conditions described for separation in second dimension in 4.3.1.
        2. Visualize PC by iodine staining and use a pencil to mark the localization of phosphatidylcholine (PC).
        3. Isolate radiolabeled PC as described in 4.5.
        4. Quantify extracted PC by scintillation counting.
           NOTE: About 320,000 cpm PC is expected.
        5. Treat PC (250,000 cpm) with 0.1 U of phospholipase C from *Clostridium perfringens* in 50 mM Tris-HCl, pH 7.2, 0.5% Triton X-100 and 10 mM $CaCl_2$ for 2 hr in a total volume of 100 µl and stop the reaction by adding 250 µl of methanol and 125 µl of chloroform.
        6. Extract lipids as described previously and separate by them by 1D-TLC (see 4.3.2).
        7. Isolate diacylglycerol from the silica plate and quantify by scintillation counting (as described in 4.2)

    2. Diacylglycerol lipase assay (**Table 3**).
        1. To a 1.5 ml microcentrifuge tube, add 5,000 cpm of ^{14}C-labeled DAG and a solution of Triton X-100.
        2. Mix and dry under a stream of nitrogen.
        3. For a final 100 µl assay, add Tris-HCl (pH 9.0) buffer, a NaCl solution and bidistilled water. Vortex for 5 sec.
        4. Initiate the reaction by adding 5 µl of enzyme (50 µg protein of cell-free extract).
        5. Incubate at 30 °C for 4 hr.
        6. Stop the reaction by the addition of 250 µl of methanol and 125 µl of chloroform and extract lipids as described previously (see 4.2).
        7. Analyze neutral polar lipids by 1D-TLC (see 4.1.3.2) and subsequent PSL imaging.

Representative Results

Activity of PC-specific Phospholipase C SMc00171 with Bis-*p*-nitrophenyl Phosphate

Cell-free extracts obtained from *E. coli* BL21(DE3) x pLysS, which had *smc00171* expressed, were studied for their ability to hydrolyze bis-*p*-nitrophenyl phosphate esters, using a spectrophotometric enzymatic assay, measuring the *p*-NP formed. No hydrolytic activity was present in cell-free extracts obtained from *E. coli* BL21(DE3) x pLysS harboring an empty pET9a vector (data not shown) when bis-*p*-nitrophenyl phosphate was employed as substrate.

Time courses for the *p*-NP formed indicate that there is a strong dependence on the amount of cell-free extract (from *E. coli* BL21(DE3) x pLysS, which had *smc00171* expressed) added to the assay (**Figure 2A**). If the amount of *p*-NP product formed would depend only on enzyme concentration, a linear correlation between enzyme (protein) concentration employed and product formed after a certain time should be observed. Clearly, this is not the case for cell-free extracts obtained from *E. coli* BL21(DE3) x pLysS, which had *smc00171* expressed (**Figure 2B**). Although there might be several reasons for an exponential dependence of enzyme activity on protein concentration[21], a frequent reason is that upon dilution of an enzyme-containing protein extract, other components in the extract which may be limiting for enzyme activity, *i.e.*, potential cofactors, are diluted as well. In such cases, unexpectedly low enzyme activities are registered with diluted cell-free extracts. By including a saturating concentration of 3 mM $MnCl_2$ in the enzyme assay, the *p*-NP product formed after a certain time only depends on the amount of enzyme added (data not shown) indicating that $MnCl_2$ was a limiting component for SMc00171 activity in cell-free extracts of *E. coli*.

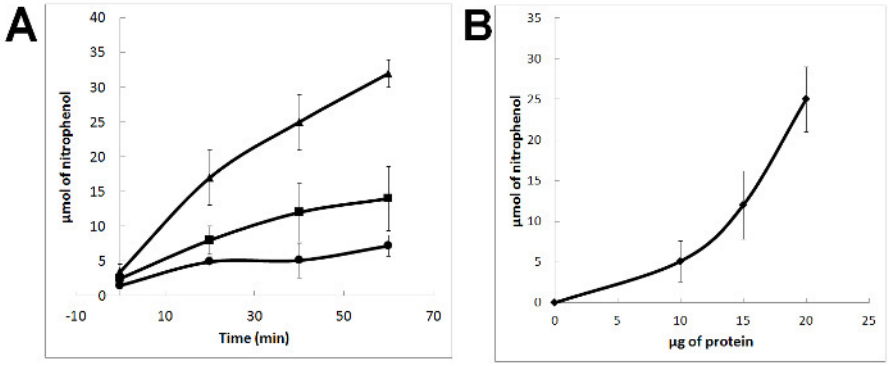

Figure 2. Determination of SMc00171 (phospholipase) activity using the artificial substrate bis-*p*-nitrophenyl phosphate. The time course for *p*-NP formation is shown employing cell-free proteins extracts (● 10 µg/ml, ■ 15 µg/ml, ▲ 20 µg/ml) in which SMc00171 had been expressed (**A**). *p*-NP formed after 40 min of incubation with different amounts of cell-free proteins extracts in which SMc00171 had been expressed (**B**). Error bars indicate standard deviation. Please click here to view a larger version of this figure.

Activities of Predicted Patatin-like Phospholipases SMc00930 and SMc01003 with *p*-Nitrophenyl Acyl Esters of Different Chain Lengths

After expression of *smc00930* or *smc01003* in *E. coli* BL21(DE3) x pLysS, cell-free extracts were obtained and studied for their ability to hydrolyze *p*-nitrophenyl fatty acyl esters. During the enzymatic assay, the *p*-NP formed was determined in a spectrophotometer. Minor hydrolytic activities were present in cell-free extracts obtained from *E. coli* BL21(DE3) x pLysS with an empty pET17b vector (**Table 4**) when *p*-nitrophenyl acyl esters of different chain lengths were employed as substrates. When *smc01003* was expressed, the extracts obtained showed an increased hydrolysis of *p*-nitrophenyl esters of different chain lengths. SMc01003 degraded the medium chain *p*-nitrophenyl decanoate as well as the long-chain *p*-nitrophenyl palmitate and *p*-nitrophenyl stearate (**Table 4**). As SMc01003 is a major contributor for the formation of long-chain free fatty acids in *S. meliloti* during stationary phase[11], it was surprising that SMc01003 seemed to act equally well on medium-chain than on long-chain *p*-nitrophenyl acyl esters (**Table 4**). Unexpectedly, SMc01003 also acts on short-chain substrates such as *p*-nitrophenyl butyrate or *p*-nitrophenyl octanoate (data not shown). However, these latter substrates are also degraded by activities present in cell-free extracts of *E. coli* (*i.e.,* harboring the empty vector pET17b) without expressing any extra gene and with short-chain substrates (C4), half of the substrate is already degraded by *E. coli* intrinsic enzymes (data not shown). Clearly, SMc01003 needs to be purified in order to clarify whether SMc01003 works equally well on short-, medium-, and long-chain substrates. In general, *p*-nitrophenyl acyl esters seem not to be good substrates for SMc01003 which might be understandable knowing that SMc01003 is in reality a DAG lipase[11]. Cell-free extracts, in which *smc00930* had been expressed, show much higher enzyme activities with *p*-nitrophenyl esters (**Table 4**). Also, SMc00930 is able to hydrolyze *p*-nitrophenyl acyl esters of different chain lengths (C10, C12, C14, C16 and C18), though *p*-nitrophenyl palmitate (specific activity 5.5 mmol NP min^{-1} mg $protein^{-1}$) is clearly the best substrate for SMc00930. To date, the physiological substrate for SMc00930 is not known.

Exploring Whether Polar Lipids Can Function as Substrates for Predicted (Phospho)lipases

Once a (phospho)lipase enzymatic assay has been optimized with artificial substrates, radiolabeled lipid mixtures or pure lipids can be tested as potential substrates.

When assaying SMc00171-containing cell-free extracts with ^{14}C-labeled PC under optimized conditions, we could show that SMc00171 degrades PC to DAG, a result that was confirmed by mass spectrometric studies[8]. SMc00171 also degrades ^{32}P-labeled PC to phosphocholine and therefore acts as a PC-specific phospholipase C (PlcP)[8], that is induced under phosphorus-limiting conditions of growth.

When assaying SMc00930- or SMc01003-containing cell-free extracts with ^{14}C- or ^{32}P-labeled total polar lipids under optimized conditions, we could show that none of the phospholipids is degraded by SMc00930 or SMc01003 under conditions where *p*-nitrophenyl acyl esters functioned as substrates[11]. In contrast, a commercial phospholipase A_2 from snake venom was able to degrade such a mixture of phospholipids[11]. These results were surprising and unexpected as both, SMc00930 and SMc01003, were predicted to be patatin-like phospholipases.

When assaying SMc00930- or SMc01003-containing cell-free extracts with ^{14}C- labeled diacylglycerol (DAG) under optimized conditions, we could show that DAG is not degraded by SMc00930 or by cell-free extracts of *E. coli*, whereas SMc01003 degrades DAG and forms compounds that migrate like free fatty acids (**Figure 3**). Recently we could show that SMc01003 acts as a DAG lipase that can degrade DAG or monoacylglycerol to glycerol and free fatty acids[11] (**Figure 4**).

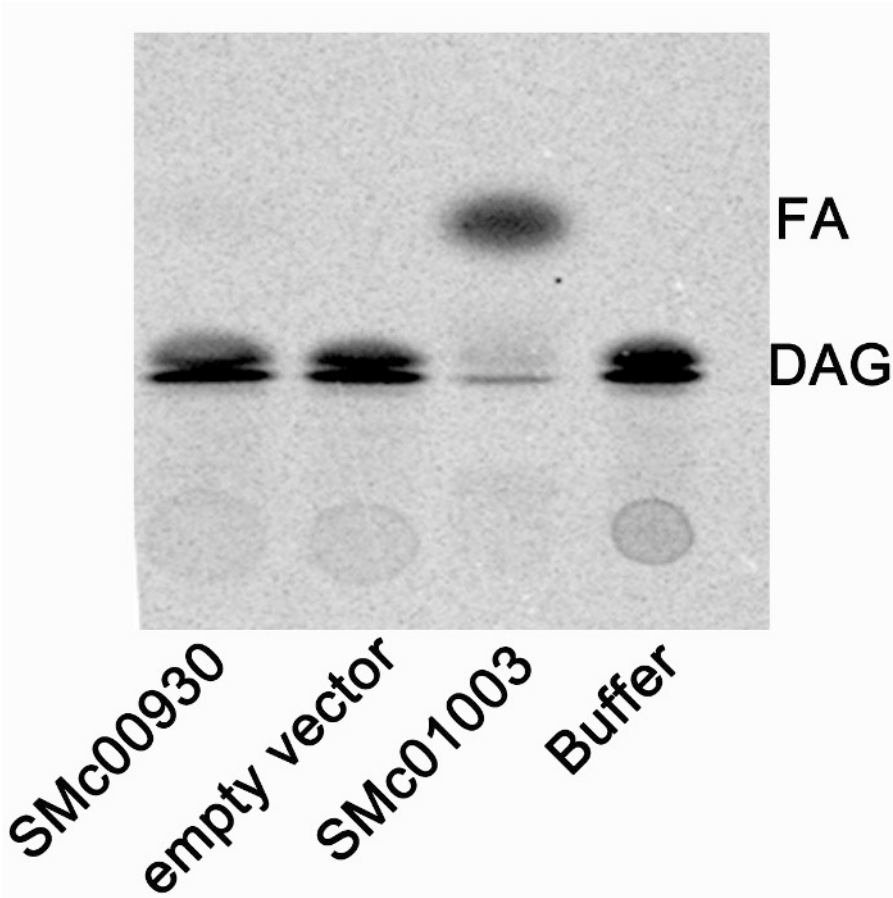

Figure 3. SMc01003 degrades diacylglycerol. Cell-free extracts of *E. coli* BL21(DE3) × pLysS expressing SMc01003, SMc00930 or containing the empty pET17b vector, or buffer were incubated with ^{14}C-DAG (obtained from *S. meliloti*) for 24 hr. At the end of the respective incubation periods, radiolabeled lipids were extracted and separated by 1D-TLC using the mobile phase for separating neutral polar lipids (see 4.3.2). FA, fatty acids; DAG, diacylglycerol. This figure has been modified from [Sahonero-Canavesi *et al.* 2015][11]. Please click here to view a larger version of this figure.

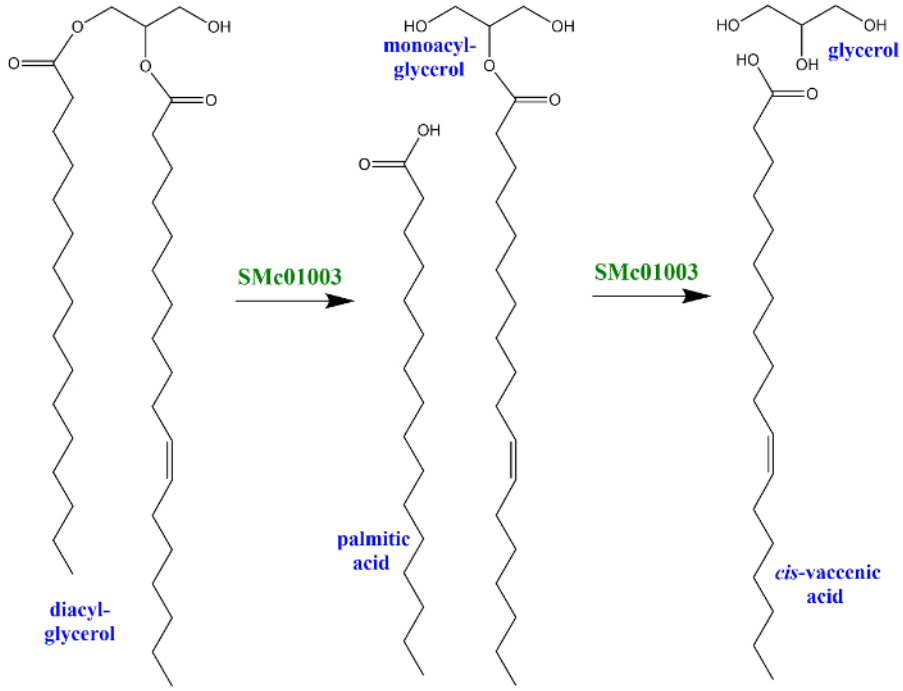

Figure 4. Enzymatic function of diacylglycerol lipase DglA (SMc01003). Diacylglycerol (DAG) lipase SMc01003 degrades DAG to monoacylglycerol and one fatty acid, and then degrades monoacylglycerol further to glycerol and another fatty acid. Please click here to view a larger version of this figure.

Oligonucleotide name	Sequence (5'-3')	Gene of interest	Enzyme restriction site added	Vector for expression
oLOP149	AGGAATA**CATATG**CTGAACTGGACATTCACG	*smc01003*	*Nde*I	pET17b
oLOP150	ACGG**CTCGAG**TCAACGGGACAGCGGTTC	*smc01003*	*Xho*I	pET17b
oLOP151	AGGAATA**CATATG**ACGGAGGTGGCGATGG	*smc00930*	*Nde*I	pET17b
oLOP152	AAA**GGATCC**TTATATCCCTTTCCTCCACC	*smc00930*	*Bam*HI	pET17b
cgpho171u	AGCT**CATATG**GCGGCGGCATCGGCACCCCACAG	*smc00171*	*Nde*I	pET9a
cgpho171d	ACGT**GGATCC**TCAGGCGGCCTGCGGTGCGAACC	*smc00171*	*Bam*HI	pET9a
Letters in bold indicate restriction sites.				

Table 1. Oligonucleotides used to amplify and clone predicted phospho(lipase) genes into pET17b or pET9a vector. Primer oligonucleotides were designed using the described software[15].

		Assay for SMc00171	Assay for SMc01003	Assay for SMc00930
Stock	**Component**			
2 M	Tris-HCl, pH 8.5			25 µl
2 M	Tris-HCl, pH 9.0		25 µl	
1 M	diethanolamine-HCl, pH 9.8	50 µl		
1 M	NaCl	40 µl	150 µl	150 µl
1%	Triton X-100		200 µl	200 µl
50 mM	*p*-nitrophenyl palmitate		12.5 µl	12.5 µl
200 mM	bis-*p*-nitrophenyl phosphate	2.5 µl		
cell-free extract (1 mg protein/ml) with candidate gene expressed		20 µl	100 µl	1 µl
	bidistilled water	887.5 µl	512.5 µl	611.5 µl
Final Volume		1 ml	1 ml	1 ml

Table 2. Pipetting schemes for optimized enzyme assays with artificial *p*-nitrophenyl ester substrates.

		Assay for *smc00171*-encoded Phospholipase C	Assay for *smc01003*-encoded DAG lipase	Optimized assay for Phospholipase A activity
Stock	**Component**			
2 M	Tris-HCl pH 8.5			2.5 µl
2 M	Tris-HCl pH 9.0		2.5 µl	
100 mM	$MnCl_2$	3 µl		
1 M	Diethanolamine-HCl pH 9.8	5 µl		
1 M	NaCl	4 µl	15 µl	15 µl
1%	Triton X-100	2 µl	20 µl	20 µl
	^{14}C-labeled lipid (phospholipids)			20 µl (5,000 cpm)
	^{14}C-labeled lipid (PC)	20 µl (5,000 cpm)		
	^{14}C-labeled lipid (DAG)		20 µl (5,000 cpm)	
10 mg/ml	Protein extract with expressed candidate genes	0.5 µl	5 µl	5 µl
	bidistilled water	87.5 µl	77.5 µl	77.5 µl
Final volume		100 µl	100 µl	100 µl

Table 3. Pipetting schemes for optimized enzyme assays for (phospho)lipases with radiolabeled lipid substrates.

activity is given in units (µmol nitrophenol/mg protein x min)					
Bold numbers indicate acyl chain length of *p*-nitrophenyl ester substrate	**10**	**12**	**14**	**16**	**18**
E. coli extract (background)	16 ± 0.3	10 ± 1.1	13 ± 0.3	11 ± 0.8	10 ± 0.6
SMc00930 expressed in *E. coli* extract	1,839 ± 283	1,839 ± 283	2,873 ± 117	5,517 ± 394	3,402 ± 65
SMc01003 expressed in *E. coli* extract	43 ± 2.1	29 ± 2	20 ± 0.7	25 ± 1.5	22 ± 0.9
SMc00930 minus background	1,823 ± 283	1,829 ± 283	2,860 ± 117	5,506 ± 394	3,392 ± 65
SMc01003 minus background	27 ± 2.1	18 ± 2	7 ± 0.7	14 ± 1.5	12 ± 0.9

Table 4. *p*-Nitrophenyl acyl esters as substrates for patatin-like lipases SMc00930 and SMc01003.

Discussion

Over the past 20 years, genomes of many organisms have been sequenced and although a wealth of genome sequence data has been generated, functional interpretation is lagging behind and therefore hampers our understanding of genome function. Gene functions in genomes are often assigned based on similarity to genes of known function or occurrence of conserved motifs. However, the precise function of a given gene is often not known. Especially, predicted structural genes for enzymes cannot be easily explored by omic techniques due to the fact that most enzymes catalyze complex reactions involving two substrates and two products. Presently, it seems more feasible to explore substrate specificities for groups of enzymes where at least one substrate is fixed and where the other can be varied. For example, in eukaryotes most phosphorylation motifs in proteins are known and consensus peptides for kinases have been spotted on solid supports in order to generate peptide arrays. Using such arrays and radiolabeled ATP, substrate specificities and activity levels of distinct kinases of an organism can be determined permitting the establishment of a kinome profile and defining substrate specificities[24]. Hydrolases compose another big group of enzymes for which one substrate, water, is fixed but for which the precise nature of the (other) substrate to be hydrolyzed is often not known. The fact that for a new enzyme the optimal assay conditions are not known either, converts the detection of a new enzyme activity in a multi-variable problem. It therefore may help to be able to fix the substrate to be hydrolyzed in order to define under which conditions the enzyme works best.

In the present manuscript, we describe the optimization of assay conditions for prospective (phospho)lipases with unknown substrate specificities and elaborate how to employ these optimized conditions in the search for the natural substrate of the respective (phospho)lipase. The significance of the present technique consists in the fact that the hydrolysis of fluorogenic or chromogenic, artificial substrates, that mimic natural substrates to some extent, can be followed by spectrophotometry[25] and that these assays are easily applicable to large scale studies[26]. Due to the sensitivity of such assays and their simplicity to monitor the reaction, they can be easily optimized for a given enzyme. Obviously, for artificial substrates one might expect less favorable kinetic constants (*i.e.,* high K_M, low k_{cat}) for a specific enzyme than for natural substrates[1,21]. In practice, however, the facile availability and detectability of artificial substrates functioning as (bad) substrates for whole groups of enzymes often more than compensate the disadvantages. Once having encountered the conditions for an enzyme to work (with the artificial substrate), it will do so with the natural/physiological substrate as well. As the preparation and isolation of potential lipid substrates can be laborious and time-consuming, it is important to have the assurance that an enzyme is ready to work when combining and incubating it with valuable substrates. Importantly, the procedure of first optimizing the conditions for an enzyme to work, will permit a more rapid identification of the physiological substrate for a new (phospho)lipase. As a proof of concept, we have successfully employed this method for the characterization of substrate specificities of lipases SMc00171, SMc00930, and SMc01003[8,11]. In contrast, many traditional, alternative methods establishing assays for (phospho)lipase activities involve reactions where substrate and product are previously known.

Obviously, modifications of assays to detect optimal enzyme activity can include the use of other fluorogenic, chromogenic, or otherwise marked artificial substrates, variation of enzyme concentration, type and concentration of buffers, or other additives (*i.e.,* detergents or bivalent cations). If no enzyme activity is detected with artificial substrates, the respective enzyme simply might not work with this substrate, but troubleshooting in such cases certainly should include to have the assurance that all the precautions had been taken that might have been necessary to maintain enzyme activity intact (*i.e.,* storing cell-free extracts at 4 °C or lower temperatures until use and, if needed, adding cocktails of protease inhibitors to cell-free extracts in order to avoid proteolytic degradation of the enzyme of interest)[27].

Limitations of the technique presented in this manuscript are due to the fact that the artificial substrate and the natural substrate are different from each other and in consequence the bioenergetics for the catalyzed reaction will be as well[1]. The various parameters for the optimal conditions of enzyme activity should be established also for the natural substrate (by varying pH, type of buffer, buffer strength, concentrations of NaCl and detergents such as Triton X-100, the absence or presence of different bivalent cations), as they might turn out to be slightly different from the optimal conditions encountered for the artificial substrate. Another important limitation is that probably not all (phospho)lipases are detectable by the use of artificial chromogenic substrates. For example, the artificial *p*-nitrophenyl residue of the ester substrates simply might be too bulky, or exhibit other chemical properties that prevent that such a substrate can get access to the active site of an enzyme. It was noted that the DAG lipase SMc01003 displayed low activities with all *p*-nitrophenyl ester substrates of different chain lengths[11]. With the knowledge

that DAG is the natural substrate for SMc01003 and that DAG has a relatively small polar head group, consisting of glycerol only[11], it is easily imaginable that the bulky *p*-nitrophenyl residue is simply too big for an easy access to the active site of SMc01003. However, the molecular details why *p*-nitrophenyl esters are not good substrates for SMc01003 are not known at this point.

Critical steps within the protocol include all provisions taken to ensure that the enzyme studied has access to the respective substrate. For example, in the search for the physiological lipid substrate, it is critical that the lipid substrate is provided in a solubilized form. Therefore, when setting up such enzyme assays (described in 5 of the protocol), lipidic substrates, usually dissolved in mixtures of methanol:chloroform, should be mixed with a watery solution of a detergent, *i.e.*, Triton X-100, before drying down the mixture with nitrogen gas. This procedure ensures that, after adding the remaining ingredients for the assay, the potential lipid substrate is embedded in detergent micelles and as such accessible for the enzyme during the assay.

Principally, the technique presented here should be widely applicable in the future for the discovery of new (phospho)lipases and for the definition of their natural substrates. The technique is easily expandable for other classes of hydrolases, but it might be more complicated to develop assays with artificial substrates for new enzymes that require two, usually unknown, substrates to complete their catalytic cycle.

For predicted (phospho)lipases or phosphatases neither the correct substrate is known nor the assay conditions in which the enzyme works well. Therefore, it might be useful to study whether some compound from a battery of artificial compounds, such as distinct *p*-nitrophenyl-containing compounds (*p*-nitrophenyl phosphate, bis-*p*-nitrophenyl phosphate, *p*-nitrophenyl decanoate, or *p*-nitrophenyl palmitate), might function as substrate. The discovery of initial enzyme activities with artificial substrates has paved the way to resolve that SMc00171 is a PC-specific phospholipase C[8], that SMc01003 is a DAG lipase[11], and helped to define the conditions under which SMc00930 acts as a hydrolase[11]. Obviously, the natural substrate of SMc00930 is presently still unknown and the physiological contexts of SMc00930 and SMc01003 are still to be resolved. Therefore, the task of proposing a physiological function for a predicted lipase is challenging and not always immediately met with success. Using mutants deficient of the predicted lipase gene and comparing them to the respective wild type strain might give experimental evidence that the lipase acts with the specificity in its native strain background that has been postulated in part 4) of the protocol. Clearly, the claim of a new lipase activity should be supported by *in vitro* evidence that a certain substrate is converted by hydrolysis into defined products. Sometimes the postulated physiological function might fill gaps in metabolic pathways or explain the physiological behavior of the producing organism, but in other cases there simply still might not be enough biochemical knowledge to make sense of some activities. In the course of our studies, we identified several enzymes that act on intrinsic polar membrane lipids of bacteria degrading them and, in some cases, converting them into a player in more complex lipid cycles. For example, combining the new knowledge that SMc00171 is a PC-specific phospholipase C (PlcP)[8] that is induced under phosphorus-limiting conditions of growth, with preexisting data, one can postulate a novel DAG cycle that might exist in many environmental proteobacteria and which gives rise to the formation of phosphorus-free membrane lipids when phosphorus is growth-limiting. In contrast, when phosphorus supplies are abundant, DAG is rephosphorylated to phosphatidic acid entering *de novo* phospholipid biosynthesis, thereby closing this lipid cycle and ensuring that mainly (glycero)phospholipids are present in the membrane[8,28].

Disclosures

The authors have nothing to disclose.

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