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Design of new chromogenic substrates for the spectrophotometric assay of lipolytic activity of lipases

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Abstract

The lipolytic activity of lipases from porcine pancreas (PPL) and *Pseudomonas cepacia* (PCL) on synthetic dialkylglycerol fatty acyl esters was evaluated by titrimetric method and the observed chain length selectivity constituted the basis for the design of new chromogenic substrates [1,2-di-*O*-butyl-*sn*-glycerol-3-(4-nitrophenyl)carbonate and 1,2-di-*O*-octyl-*sn*-glycerol-3-(4-nitrophenyl)glutarate] that were shown to be specific for spectrophotometric assay of PPL and PCL activity, respectively.
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1. Introduction

Lipases are hydrolytic enzymes that are able to catalyze the hydrolysis of triacylglycerols TAG **1** (lipolytic activity) and many other structurally different esters (esterase activity). The determination of the lipolytic activity of lipases is an important goal in analytical biochemistry and several methods are currently available for this purpose. In a recent review, Verger and coworkers have critically examined all the above analytical methods, concluding that none has fulfilled yet the expectations of a completely satisfactory assay [1]. This is certainly due to the fact that the mechanism of lipase-catalyzed hydrolysis of the natural, water-insoluble triacylglycerols is rather complex and several factors may play important roles such as fatty acid selectivity [2] or the interfacial activation, a complex set of chemico-physical interactions between the hydrophobic substrate and the enzyme [3]. Titrimetric method is generally used as reference assay for evaluation of lipase activity and specificity, as well as interfacial phenomenon. However, for routine assays the method is limited by low sensitivity and restricted range of pH values at which lipase activity has to be evaluated [1]. Among routinely used spectrophotometric assays, the method based on the hydrolysis of 4-nitrophenyl esters **2** (Fig. 1) still remains one of the most popular for determination of lipase activity [4]. The method, however, is not specific for lipolytic activity of lipases, since 4-nitrophenyl esters are also substrates for non-specific esterases [5].

Several fluorogenic substrates are also available for lipolytic activity assays [6] and 1,2-*O*-dilauryl-*rac*-3-glutaric-resorufin ester (**3**) has been introduced for continuous assay [7], the hydrolysis taking place at one end of the glutarate moiety with release of a chromogenic anion by an intramolecular mechanism [8] (Scheme 1).

Recently, we started a research project aimed to develop new synthetic substrates for spectrophotometric assay of lipolytic activity of pancreatic lipases, that can be useful in clinical chemistry for the diagnosis of pancreatitis [9]. For this purpose, we designed 4-nitrophenyl derivatives of dialkylglycerols such as compounds **4** that, upon hydrolysis should release 4-nitrophenoxide anion, spectrophotometrically detectable at 400 nm.

In order to select the most appropriate ester moiety in compound **4**, fatty acyl esters of dialkylglycerols (ADAG) were prepared and their hydrolysis compared to that of TAG **1**, taking into account that different polarity of non-hydrolyzable alkyl ethers might influence the interfacial activity. Finally, in order to simplify possible complications arising from the presence of different chain length, compounds **5** should contain alkyl and acyl groups with the same number of carbon atoms (Fig. 2).

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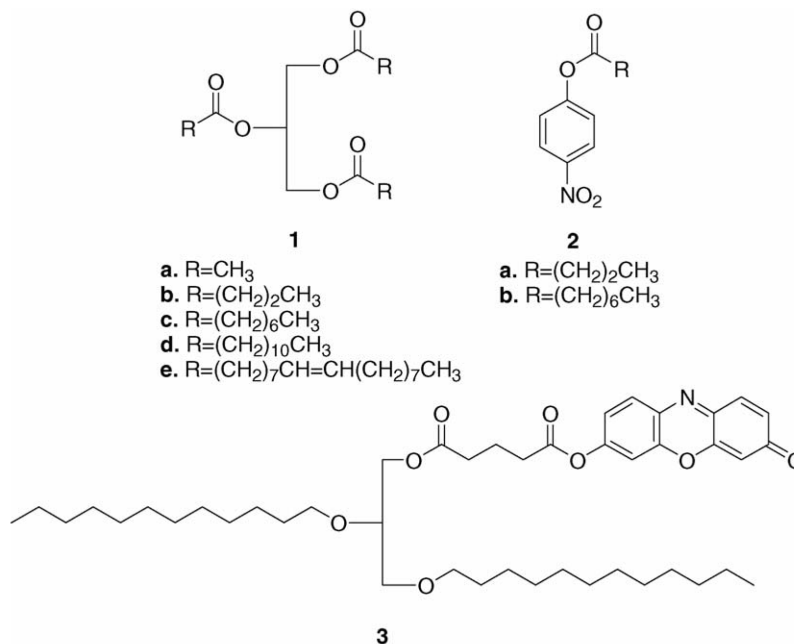
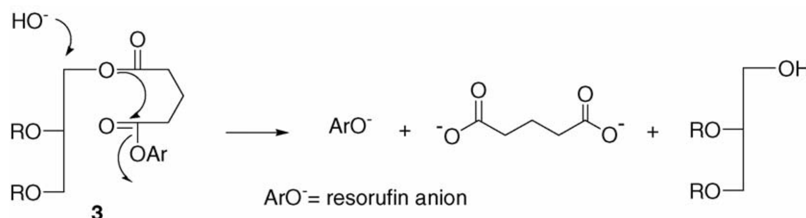


Fig. 1. Structure of triacylglycerols **1a**–**e**, 4-nitrophenyl esters **2a**,**b**, and 1,2-*O*-dilauryl-*rac*-3-glutaric-resorufin ester (**3**).



Scheme 1. Release of spectrophotometrically detectable anion from substrate **3**.

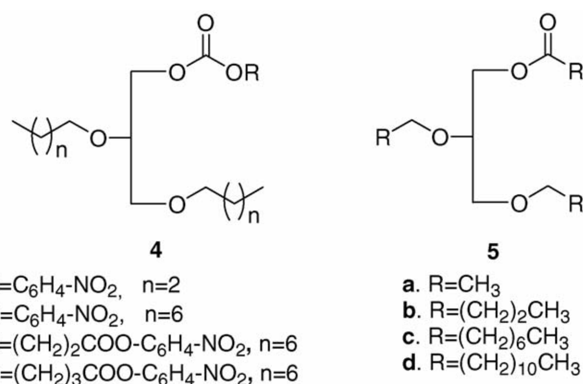


Fig. 2. Structures of dialkylglycerol chromogenic **4** and dialkylglycerol acyl esters **5**.

2. Results and discussion

2.1. Lipolytic activity of PPL

2.1.1. Hydrolysis of triacylglycerols *1a–e*: fatty acid selectivity

Titrimetic determination of PPL activity by pH-stat method was preliminarly carried out with TAG **1**. Triacetin (TAcG, **1a**), tributyrin (TBG, **1b**), tricaprylin (TOG, **1c**), and triolein (TOlG, **1e**) were selected as substrates under experimental conditions that were a compromise between the described [10] activation of the enzyme (calcium chloride and sodium taurocholate) and stabilization of the required emulsion (Triton X-100). The titrimetric assay of TAG indicated that the PPL specific activity with TBG **1b** was higher than for TOG **1c**, in accordance with other observations which suggest that TBG **1b** is one of the preferred substrate for the PPL activity [10]. TAcG **1a** was a poor substrate comparable to TOlG **1e** (Table 1), although emulsified TOlG **1e** is often proposed for titrimetric evaluation of lipase activity [11].

2.1.2. Hydrolysis of dialkylglycerol esters *5a–d*: comparison with triacylglycerols *1a–d*

On the basis of the results with TAG, we excluded oleyl ester and prepared ADAG esters **5a–c** following a general synthetic protocol that started from commercially available (*R*,*S*)-1-(benzyloxy)propane-2,3-diol [12]. Titrimetric analysis of ADAG esters [diethylglyceryl acetate, DEGA, **5a**; dibutylglyceryl butanoate, DBGB, **5b**; dioctylglyceryl octanoate, DOGO, **5c**] showed that PPL activity was higher for DBGB **5b** than for the corresponding octanoate **5c** or acetate **5a** (Table 1).

Table 1
Lipolytic activity of PPL on triacylglycerols **1a**–**c**, triolein **1e** and dialkylglycerol esters **5a**–**c**

Substrate[a]	Specific activity[b] (μeqH$^+$/min per mg of enzymatic preparation)
TAcG **1a**	n.d.[c]
TBG **1b**	0.6
TOG **1c**	0.02
TOlG **1e**	0.0038
DEGA **5a**	0.0041
DBGB **5b**	0.3
DOGO **5c**	0.03

[a] Substrate dispersion in 0.9 M NaCl, 155 mg/ml Triton X-100, 43 mM sodium taurocholate.
[b] Enzyme preparation in 0.1 M phosphate buffer pH 8.0 containing 10 mM $CaCl_2$.
[c] Activity not detectable.

2.1.3. *Synthesis of 4-nitrophenylcarbonate **4a** and spectrophotometric assay of lipolytic activity*

The above results show that also with synthetic ADAG esters **5a**–**c** PPL is more active with short chain substrates. Therefore, a chromogenic ester of dibutylglycerol should not obligatorily contain glutarate as spacer arm of the hydrolizable part of the compound. Consequently, 4-nitrophenylcarbonate (NPC, **4a**) was prepared from dibutylglycerol (80% yield after purification) [13]. The enzymatic hydrolysis of the substrate **4a** was evaluated at 400 nm following the formation of 4-nitrophenoxide anion generated by the spontaneous decomposition of the intermediate carbonic monoester (Scheme 2).

Interestingly, PPL activity with 4-nitrophenyl butanoate (PNB, **2a**) or octanoate (PNO, **2b**) could not be determined in a reproducible manner. On the other hand, PPL activity was satisfactorily evaluated with NPC **4a**. In aqueous acetonitrile, the enzymatic activity was higher than that in the medium used for titrimetric assays. The activity of the enzyme with NPC **4a** was higher with respect to that determined with resorufin ester **3** (Table 2), suggesting that the rational design of a chromogenic substrate for the lipolytic activity of a lipase should carefully evaluate the alkyl chain length and/or the ester spacer arm, without excluding the possibility that the size of the chromogenic moiety could play an important role.

In line with this observation, a lipase selective for medium–long chain fatty acids could exhibit a lower activity for the carbonate **4a** with respect to long chain alkyl resorufin derivative **3**. In order to verify this hypothesis, we selected the lipase from *Pseudomonas cepacia* (PCL) that had shown to be selective for medium–long chain fatty acids TAG [14].

Table 2
PPL activity by spectrophotometric assay: NPC **4a** vs. resorufin ester **3**

Substrate	Specific activity [μeq 4-nitrophenoxide (or resorufin anion)/min per mg of enzymatic preparation]
PNB **2a**[a]	—[b]
Resorufin ester **3**[c]	0.0064
NPC **4a**[d]	0.047
NPC **4a**[e]	0.029

[a] Similar results were obtained with PNO **2b**.
[b] Non-reproducible results.
[c] For the condition see ref. [5].
[d] 10 mM solution in acetonitrile; PPL solution in Tris–HCl buffer.
[e] Substrate dispersion in 0.9 M NaCl, 155 mg/ml Triton X-100, 43 mM sodium taurocholate. The enzyme preparation in 0.1 M phosphate buffer contained 10 mM $CaCl_2$.

2.2. *Lipolytic activity of PCL*

2.2.1. *Spectrophotometric evaluation of PCL activity*

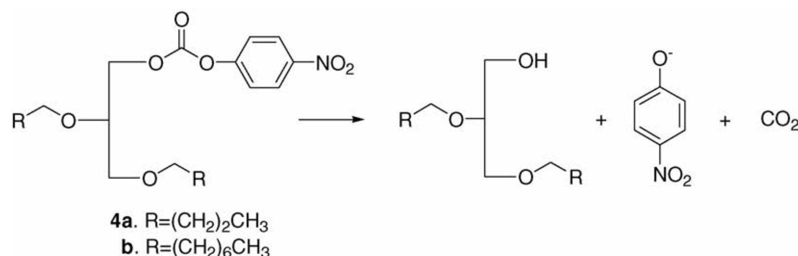
Differently from PPL, spectrophotometric assay of PCL with 4-nitrophenyl esters PNB **2a** or PNO **2b** could be evaluated and activity with PNO **2b** was found to be higher (1.4 μeq versus 0.3 μeq of *p*-nitrophenoxide hydrolized/min per mg enzymatic preparation). Spectrophotometric determination of PCL activity with NPC **4a** and the resorufin ester **3** showed that no clear activity could be observed towards NPC **4a**, whereas with resorufin ester **3** the activity of PCL was superior to that of PPL (Table 3).

Starting from this observation, the design of a 4-nitrophenyl ester **4b** was envisaged, where a spacer arm such as a glutarate

Table 3
Lipolytic activity of PPL and PCL towards chromogenic substrates

Enzyme	Substrate	Specific activity (μeq 4-nitrophenoxide/min per mg of enzymatic preparation)
PPL	Resorufin ester **3**	0.006
PCL	Resorufin ester **3**	0.03
PPL	NPC **4a**	0.029
PCL	NPC **4a**	n.d.[a]

[a] Activity not detectable.



Scheme 2. Proposed mechanism for release of 4-nitrophenoxide anion from substrate **4a**,**b**.

or succinate should be bound to the selected dialkylglycerol and 4-nitrophenol. This prompted us to reinvestigate the titrimetric activity of PCL with TAG and ADAG esters, in order to define the selectivity of the lipase with respect to chain length of alkyl and acyl moieties.

2.2.2. Hydrolysis of dialkylglycerol esters *5a–d* and comparison with triacylglycerols *1a–e*

Titrimetric assay of PCL activity using TAcG **1a**, TBG **1b**, TOG **1c**, trilaurin (TDG, **1d**) and TOlG **1e** as representative of different chain length substrates did not require activators (calcium chloride and sodium taurocholate). The titrimetric assay of TAG **1a–e** indicated that the PCL activity was higher for longer acyl chain (TDG, **1d** > TOG, **1c** > TBG, **1b**), in agreement with previous observations [14].

Activity of PCL with ADAG esters **5a–d** was lower than that with the corresponding TAG. The chain length selectivity was less evident with ADAG esters, since activity with DDGD **5d**, DOGO **5c** and DBGB **5b** were all in the range of 7 μmol/min of liberated fatty acid, a value that corresponds to about the same activity of PCL for TBG **1b** (Table 4).

2.2.3. Synthesis of 1,2-di-O-octyl-sn-glycerol-3-O-(4-nitrophenyl) glutarate NPG (*4d*) and spectrophotometric evaluation of PCL activity

Above results suggested that dioctyl glycerol could be the appropriate backbone for the design of a chromogenic ester. 1,2-Di-*O*-octyl-*sn*-glycerol-3-*O*-(4-nitrophenyl) carbonate (**4b**) was initially prepared, but spectrophotometric assay with PCL afforded unreproducible results. This observation was an indication that a spacer arm between the ester moiety to be hydrolyzed and the chromogenic group to be liberated could be required for a spectrophotometric assay of PCL. Due to its instability, 1,2-Di-*O*-octyl-*sn*-glycerol-3-(4-nitrophenyl) succinate (**4c**) could not be used as substrate, whereas the preparation of 1,2-di-*O*-octyl-*sn*-glycerol-3-*O*-(4-nitrophenyl) glutarate (NPG, **4d**) was readily accomplished from 1,2-di-*O*-octyl-*sn*-glycerol [15]. PCL activity with the resorufin ester **3**, NPC **4a** and NPG **4d** was measured and results show the highest activity for NPG **4d** (Table 5).

Table 4
Lipolytic activity of PCL on triacylglycerols **1a–e** and dialkylglycerol esters **5a–d**

Substrate[a]	Specific activity (μeqH$^+$/min per mg of enzymatic preparation)
TAcG **1a**	0.05
TBG **1b**	6.2
TOG **1c**	26.6
TDG **1d**	60.0
TOlG **1e**	1.91
DEGA **5a**	1.25
DBGB **5b**	6.7
DOGO **5c**	7.1
DDGD **5d**	7.7

[a] Substrate dispersion in 0.15 M NaCl, 155 mg/ml Triton X-100. The enzyme preparation in 0.1 M phosphate buffer pH 7.7.

Table 5
Lipolytic activity of PCL towards chromogenic substrates

Substrate[a]	Specific activity [μeq 4-nitrophenoxide (or resorufin anion)/min per mg of enzymatic preparation]
Resorufin ester **3**	0.03
NPC **4a**	n.d.[b]
NPG **4d**	0.60

[a] 10 mM solution in acetonitrile; PCL solution in 0.1 M Tris–HCl buffer pH 8.0.
[b] Activity not detectable.

The higher activity of PCL with NPG **4d** with respect to resorufin ester **3**, that mainly differs for the chromogenic moiety, confirmed the importance of the structure of the chromogen in the design of a suitable substrate for lipase spectrophotometric assay.

3. Conclusions

We have shown that optimization of spectrophotometric assay of lipolytic activity of lipases is possible through the synthetic design of a chromogenic substrate. Titrimetric analysis of synthetic dialkylglycerol esters has allowed to establish chain length as a structural basis for the synthesis of 4-nitrophenyl carbonic or glutaric esters. In the specific case, whereas for PPL the carbonate **4a** has proven to be a substrate superior to resorufin ester **3**, for PCL the glutarate spacer arm was necessary and the chromogenic ester NPG **4d** was synthesized. PCL activity with NPG **4d** was higher than with resorufin ester **3**, thus showing that information about molecular properties of the chromogenic moiety were required to more properly design a substrate suitable to a specific spectrophotometric assay.

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