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Interaction Mechanism between *Pseudomonas* Species Lipase and Nitro Compounds

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The object of this work was to determine the enzyme kinetics for *Pseudomonas* species lipase catalyzed hydrolysis of substrate 4-nitrophenyl butyrate in the presence of nitro compounds such as nitroglycerin, 2,4,6-trinitrotoluene (TNT), picric acid, styphnic acid, hexahydro-1,3,5-trinitrotriazocine (RDX), octahydro-1,3,5,7-tetranitrotriazocine (HMX) and hexanitrohexaazaisowurtzitane (HNIW) *in vitro*. Kinetically, picric acid and styphnic acid were the mixed-type inhibitors but TNT, RDX and HNIW were the essential activators of the enzyme in the presence of a detergent triton-X 100. Interestingly, HMX and nitroglycerin were neither an inhibitor nor an activator of the enzyme. From chemical structure point of view, hydrophilic nitro compounds such as picric acid and styphnic acid are capable to enter the hydrophilic active site of lipase and become inhibitors of the enzyme, while hydrophobic nitro compounds such as TNT, RDX and HNIW presumably mix with Triton-X-100 and bind to the co-lipase binding site of the enzyme and become the essential activators of the enzyme.

Key Words: Lipase, Enzyme kinetics, Nitro compounds, Enzyme activation.

INTRODUCTION

Nitro compounds such as nitroglycerin, 2,4,6-trinitrotoluene (TNT), picric acid, styphnic acid, hexahydro-1,3,5-trinitrotriazocine (RDX), octahydro-1,3,5,7-tetranitrotriazocine (HMX) and hexanitrohexaazaisowurtzitane (HNIW) are widely used as pharmaceuticals, pesticides, explosives and comprise an important group of environmental pollutants[1-4]. The

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commercial potential of organic syntheses catalyzed by lipases (EC 3.1.1.3) underscores the need for a comprehensive understanding of structure-function relationships of lipase and provided the impetus for many recent investigation[5,6]. Lipases are lipolytic enzymes, which hydrolyze ester bonds of triacylglycerols[7]. However, their substrate specificity is not limited to triacylglycerols. Many X-ray structures of lipases such as *Pseudomonas cepacia* lipase (PCL) and *Candida rugosa* lipase (CRL) have been reported[8-13]. Lipase usually contains a small α-helix or loop, referred to as the lid, which covers the active site pocket[8,10,11,14-17]. This conformation is termed the closed conformation. When the lipase is absorbed to an interface, the lid is displaced so that the active site becomes accessible to substrate[18]. This conformation is termed the open conformation. The structures of the free and bound lipase are believed to represent the start and end conformation in the interfacial activation process[19].

The evidence that simvastatin increases lipase activity *in vivo* suggests that simvastatin can directly affect acylglycerol metabolism by increasing lipase activity and may therefore be suitable for the treatment of combined lipoprotein disorders characterized by elevation of triacylglycerol[20]. Since high blood triacylglycerol level causes many heart diseases, we embark on the study for the lipase activities toward cardiovascular drugs such as nitro compounds. Nitroglycerin is a vasodilator that frequently is used for treatment of angina pectoris[4]. Both nitroglycerin and the lipase substrate, triacylglycerol, have the glycerol backbone in their structures in common. From this point of view, nitroglycerin may act as an inhibitor of lipase. Therefore, we embark on the study of relationships between nitro compounds and the activation of lipase. Thus, the goal of this study is then to characterize interactions between nitro compounds and *Pseudomonas* species lipase (PSL) *in vitro*.

EXPERIMENTAL

Pseudomonas species lipase (PSL), *p*-nitrophenyl butyrate (PNPB) and Triton-X-100 (TX) were obtained from Sigma (USA). Nitroglycerin, 2,4,6-trinitrotoluene (TNT), picric acid (PA), styphnic acid, hexahydro-1,3,5-trinitrotriazocine (RDX), octahydro-1,3,5,7-tetranitrotriazocine (HMX) and hexanitrohexaazaisowurtzitane (HNIW) (Fig. 1) were obtained from Taiwan Army. All other chemicals were of the highest purity available commercially.

All steady state kinetic data were obtained from an UV-Visible spectrophotometer (Agilent 8453) with a cell holder circulated with a water bath.

Data reduction: Origin (Version 6.0) was used for linear and non-linear least-squares curve fittings.

PSL inhibition and activation: PSL-catalyzed hydrolysis of PNPB in the presence of nitro compounds (Fig. 1) was followed continuously at 410 nm on a UV-Visible spectrometer. The temperature was maintained at 25 ºC by a refrigerated circulating water bath. All reactions were preformed in sodium phosphate buffer (1 mL, 0.1 M, pH 7.0) containing NaCl (0.1 M), CH_3CN (2 % by volume), detergent TX (0.5 % by weight), substrate PNPB (0.5 mM) and varying concentration of the nitro compounds. Requisite volumes of stock solution of substrate and the nitro compound in acetonitrile were injected into reaction buffer *via* a pipette. PSL was dissolved in sodium phosphate buffer (0.1 M, pH 7.0). Picric acid and styphnic acid were characterized as the mixed-type inhibitors of PSL (Fig. 2, **Scheme-I** and eqn. 1), but TNT, RDX and HNIW were the essential activators of the enzyme (Fig. 3, **Scheme-II** and eqn. 2) (Table-1)[21]. Then, the activity of the reaction was derived as eqn. 1 and 2 for inhibition and activation, respectively.

$$\text{Activity} = \nu/\nu_0 = (K_i + (\alpha/\beta)\,[I])/(K_i + [I]) \quad (1)$$

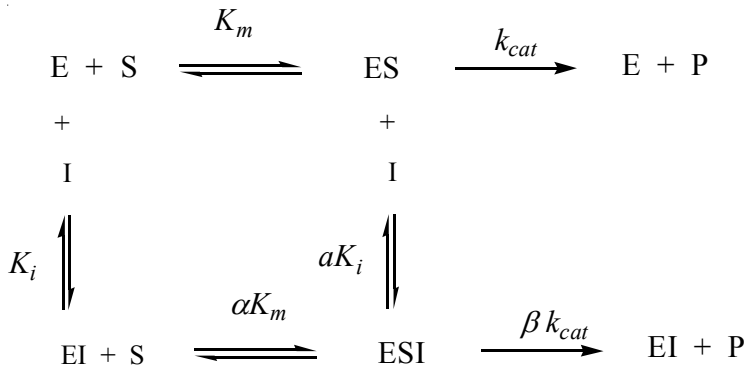
$$\text{Activity} = \nu/\nu_0 = (K_A + (\alpha/\beta)\,[A])/(K_A + [A]) \quad (2)$$

In eqns., ν and ν_0 were initial velocity in presence and absence of an activator or inhibitor. The inhibition constant, K_i (or activation constant, K_A) and α/β values were then obtained from the non-linear least-squares curve fittings of activity *vs.* inhibitor (or activator) concentration ([I]) (or [A]) plot against eqn. 1 or 2 (Figs. 2 and 3) when $K_m >> [S]$. Duplicate sets of data were collected for each activator concentration.

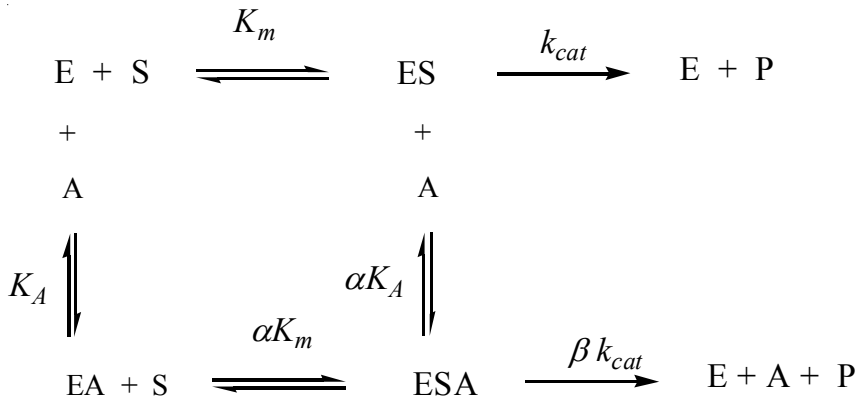
TABLE-1
K_A AND K_i VALUES[a] OF PSL-CATALYZED HYDROLYSIS OF PNPB IN THE PRESENCE OF NITRO COMPOUNDS IN TRITON X-100

Compound	Inhibition	Inhibition	Activation	Activation
	K_i (μM)	α/β	K_A (μM)	α/β
Nitroglycerin	No inhibition	-	No activation	-
TNT	-		6.9 ± 0.8	1.0087 ± 0.0004
Picric acid	8 ± 1.0	0.99932 ± 0.00005	-	-
Styphnic acid	3.3 ± 0.4	0.99839 ± 0.00005	-	-
RDX	-	-	15 ± 2	1.00075 ± 0.00005
HMX	No inhibition	-	No activation	-
HNIW	-	-	5.2 ± 0.6	1.00148 ± 0.00007

[a]Obtained from the nonlinear least-squares curve fittings of activity *vs.* [I] or [A] plot against eqn. 1 or 2.



Scheme-I. Kinetic scheme of the mixed-type inhibition of PSL. S, E, I, EI, ES and ESI are substrate, enzyme, inhibitor, the enzyme-inhibitor adduct, the enzyme-substrate Michaelis complex and enzyme-substrate-inhibitor adduct, respectively



Scheme-II. Kinetic scheme of the essential activation of PSL. S, E, A, EA, ES and ESA are substrate, enzyme, activator, the enzyme-activator adduct, the enzyme-substrate Michaelis complex and enzyme-substrate-activator adduct, respectively

RESULTS AND DISCUSSION

Picric acid and styphnic acid are characterized as the mixed-type inhibitors of PSL (Fig. 2, **Scheme-I**, Table-1 and eq. 1). Thus, the dissociation constant for inhibition (or inhibition constant), K_i, can be determined from nonlinear least-squares curve fittings of the plot of activity (ν/ν_0) on [I] against eqn. 2 (Fig. 2 & Table-1). Like the noncompetitive inhibition[21], that all α/β values are close to one (Fig. 2 and Table-1) indicates that both nitro compounds are noncompetitive inhibitors of PSL[21]. From chemical

structure point of view, picric acid and styphnic acid (Fig. 1) carry hydroxyl groups and make both compounds hydrophilic. Therefore, picric acid and styphnic acid are capable of entering the hydrophilic active site of lipase[7-19] and become inhibitors of the enzyme due to the hydrophilic character of both compounds.

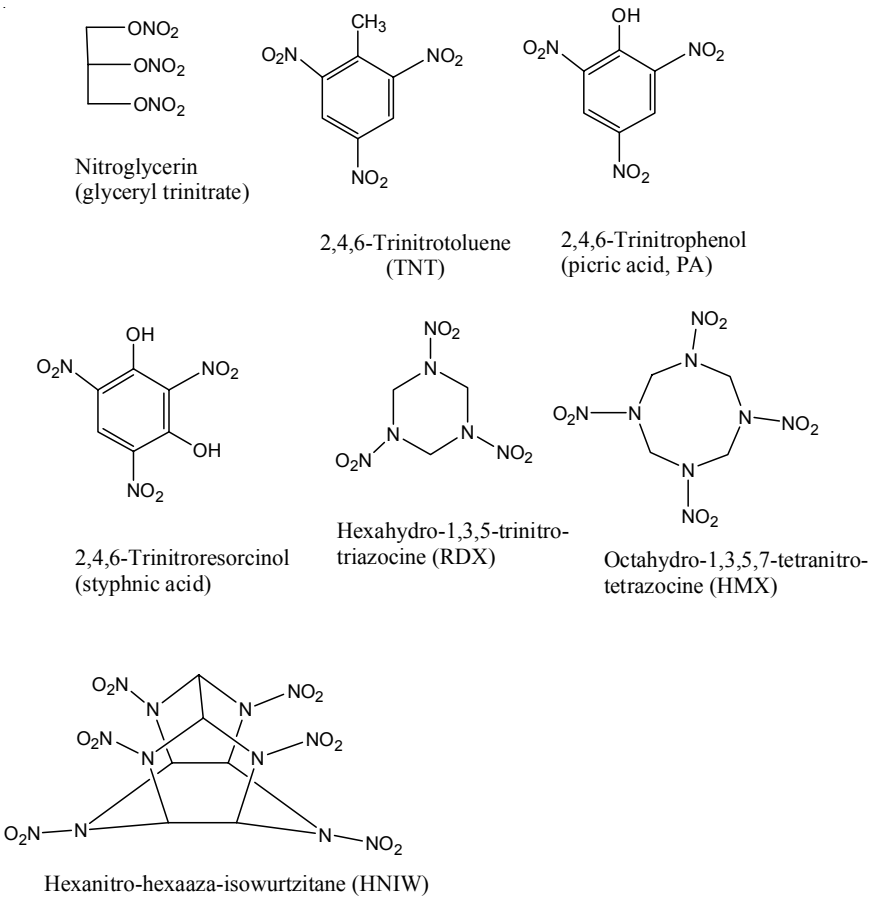


Fig. 1. Chemical structures of nitro compounds

On the other hands, TNT, RDX and HNIW are the essential activators[21] of PSL (Fig. 3, **Scheme-II**, Table-1 and eqn. 2). The characteristic for the essential activation to an enzyme reaction is analogous to that for the mixed type inhibition to an enzyme reaction[21]. Thus, similar to the mixed type inhibition, the dissociation constant for activation (or activation constant), K_A, can be determined from nonlinear least-squares curve fittings of the plot of activity (v/v_0) on [A] against eqn. 1 (Fig. 3 & Table- 1). Like the noncompetitive inhibition[21], that all α/β values are close to one (Fig. 3 and Table-1) indicates that these compounds are non-competitive,

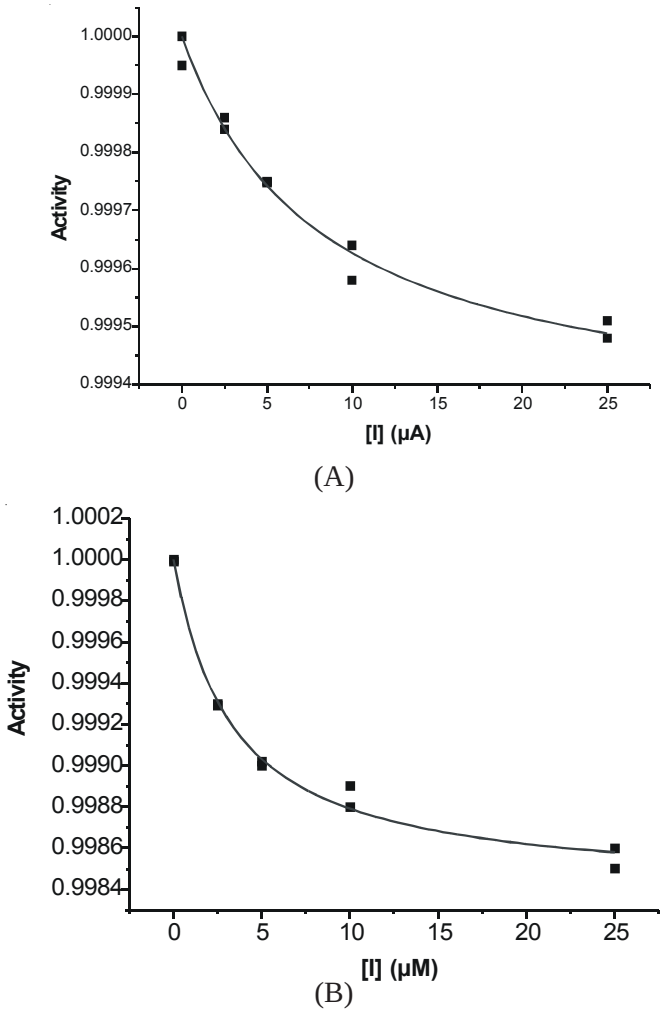


Fig. 2. Nonlinear least-squares curve fittings of activity *vs.* inhibitor concentration ([I]) plot against eqn. 1 for PSL inhibition by (A) picric acid (parameters of the fit were K_i = 8 ± 1 μM, α/β = 0.99932 ± 0.00005 and R^2 = 0.98005) and (B) styphnic acid (parameters of the fit were K_i = 3.3 ± 0.4 μM, α/β = 0.99839 ± 0.00005 and R^2 = 0.99163) in the presence of Triton-X-100

essential activators. From chemical structure point of view, TNT, RDX and HNIW are more hydrophobic than picric acid and styphnic acid (Fig. 1). Therefore, TNT, RDX and HNIW presumably mix with Triton-X-100 and bind to the co-lipase binding site of PSL and become the essential activators of PSL (Fig. 4). Moreover, the interfacial activation of PSL by the essential activators in the presence of detergents is proposed according to the lipase-colipase mechanism (Fig. 4)[22,23]. Colipase is a small protein

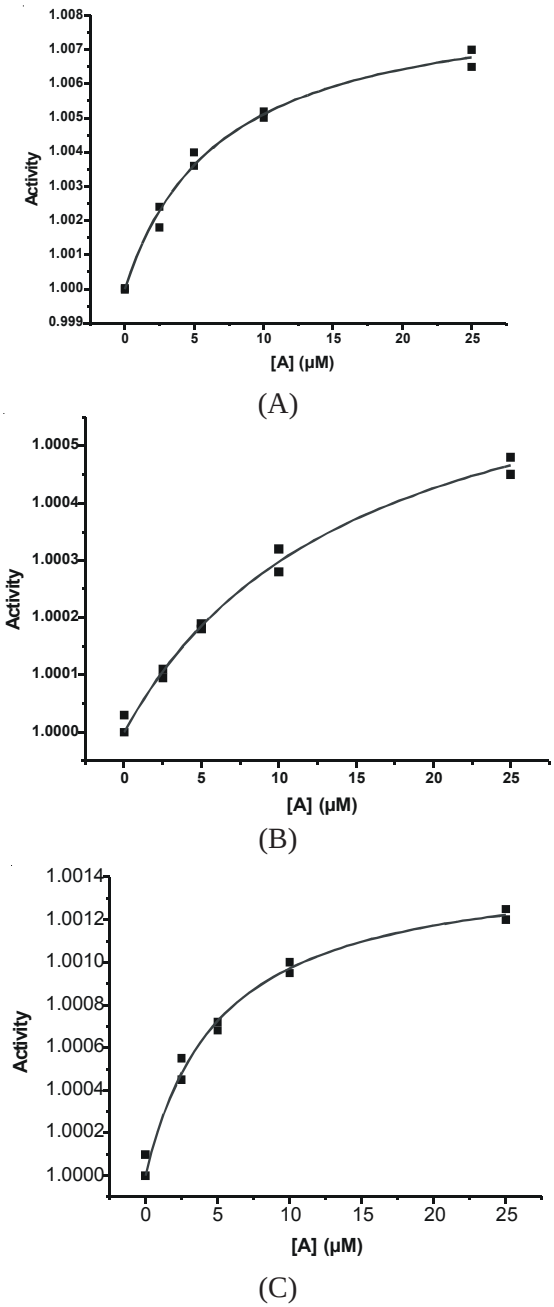


Fig. 3. Nonlinear least-squares curve fittings of activity *vs.* activator concentration ([A]) plot against eqn. 2 for PSL activation by (A) TNT (parameters of the fit were $K_A = 6.9 \pm 0.8$ μM, $\alpha/\beta = 1.0087 \pm 0.0004$ and $R^2 = 0.99016$), (B) RDX (parameters of the fit were $K_A = 15 \pm 2$ μM, $\alpha/\beta = 1.00075 \pm 0.00005$ and $R^2 = 0.99054$) and (C) HNIW (parameters of the fit were $K_A = 5.2 \pm 0.7$ μM, $\alpha/\beta = 1.00148 \pm 0.00007$ and $R^2 = 0.98761$) in the presence of Triton-X-100

that fully opens the active site of lipase by removing the half-open lid of lipase to full-open (activation) conformation after colipase binds to the colipase binding site of lipase. Thus, the essential activators may act like colipase and fully open the active site of PSL by removing the half-open lid of ES to form ESA (full-activation) after the essential activators bind to the pseudo-colipase binding site of PSL.

Interestingly, HMX and nitroglycerin are neither an inhibitor nor an activator of the enzyme. Therefore, HMX and nitroglycerin do not bind to the active site of PSL due to the hydrophobic characters of HMX and nitroglycerin. Moreover, HMX does not bind to the co-lipase binding site of PSL (Fig. 4) probably due to huge dimension of HMX and weak interactions between HMX and detergent Triton-X-100. With the glycerol backbone, nitroglycerin has more stable conformations than other nitro compounds and therefore interacts loosely with the detergent.

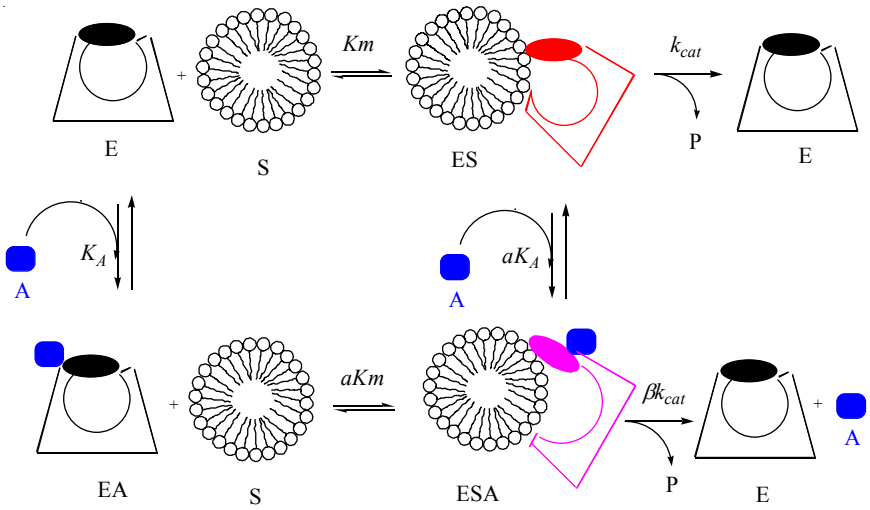


Fig. 4. Schematic representation of PSL activation by the cardiovascular drugs in the presence of detergent Triton-X-100 (Ref. 22). S, E and A are the mixed micelle of a detergent and substrate, enzyme and activator, respectively. All other notations are the same with that of **Scheme-II**. The mixed micelle of the detergent and substrate induce a conformational change of the enzyme forming the ES complex. The activator may bind outside the active site of the enzyme forming the EA complex with the activation constant, K_A. The αK_A step involves a further conformational change of the enzyme from half-open (or *meta*-activated) ES complex to fully open (or fully activated) ESA complex

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