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Article

Evaluation of Snake Venom Phospholipase A_2: Hydrolysis of Non-Natural Esters

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Fosfolipase A_2 colhida da serpente *Crotalus durissus terrificus* foi empregada pela primeira vez para testar sua enantiosseletividade na hidrólise de diferentes ésteres não-naturais. Foi observado que a estrutura desta pequena enzima tem sua ação lipase dependente da escolha dos substratos não-naturais. Foram usadas duas formas da enzima; livre e como agregado enzimático formado com ligações cruzadas, CLEA. Com todos os substratos, a enzima livre mostrou atividade similar a da preparada CLEA. A vantagem da fosfolipase CLEA é que permite sua reutilização em várias reações consecutivas sem demonstrar diminuição de atividade e seletividade, propiciando rendimentos bons e superiores, e *ee* similares quando comparados com os da enzima livre.

Phospholipase A_2 from the rattlesnake *Crotalus durissus terrificus* was employed for the first time to test its enantioseletivity on the hydrolysis of different non-natural esters. It was observed that the structure of this small enzyme is restrictive in the choice of its lipase action with non-natural substrates. Two forms of the enzyme were used; free and as its cross-linked enzyme aggregate (CLEA). With all substrates, the free enzyme showed activity similar to the CLEA preparation. The advantage of the CLEA phospholipase is the possibility to reuse it in several consecutive reactions without a decrease of activity and selectivity with good but higher yields and *ee* than with the free enzyme.

Keywords: phospholipase A_2, snake venom PLA_2, cross-linking enzyme aggregate, biocatalysis

Introduction

The phospholipases PLA_2 (EC 3.1.1.4) are calcium-dependent enzymes that catalyze the hydrolysis of 1,2-diacyl-3-*syn*-glycerol phosphoglycerides, liberating the fatty acid from the 2-position. These enzymes possess interesting properties, such as heat stability, the capacity to act in nonaqueous systems and at the surface of lipid particles and monolayers. There are several recent reviews in the literature on these enzymes reporting on different properties of their action.[1] These enzymes are common in pancreatic juice in snake and insect venoms. Mammalian PLA_2 are classified into four groups (I, II, III and IV) based on their origin (extracellular or intracellular), primary structure and disulfide bonding.[2] Snake venom PLA_2 occurs in groups I (Elapidae and Hydrophidae) and II (Crotalidae and Viperidae). Group II contains the catalytically active D49 PLA_2 as well the K49 isoforms, which has low or no activity.[3] In addition to their enzymatic activity, venom PLA_2 may have neurotoxic, myotoxic, hemolytic, anticoagulant, cardiotoxic, and edematogenic activities as well as platelet aggregatory or antiaggregatory actions.[4]

Crotoxin, a heterodimeric complex with phospholipase A_2 activity, is the main neurotoxic component in the venom of the South America rattlesnake *Crotalus durissus terrificus*, accounting for approximately 50% of the venom dry weight. Crotoxin has neurotoxic,[5] myotoxic,[6] hemolytic[7] and platelet aggregating[8] activities. The crotoxin complex consists of a basic PLA_2 and an acidic, nonenzymatic component known as crotapotin,[9] which increases the pharmacologic activity of PLA_2 by acting as a chaperone protein for the enzyme.[10] Crotoxin exists in several isoforms which result from the random association of the PLA_2 with crotapotin[11] following the post-translational modification of a single precursor of crotoxin or the expression of different mRNAs.[12]

The chemical mechanism of the enzymatic catalysis involving PLA_2 is not yet fully explored. In order to obtain more information about the mechanism of the

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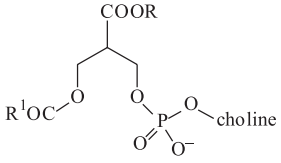
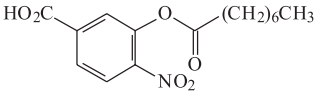
PLA_2-catalyzed reaction, it would be advantageous to use substrates for which structural variations could be correlated with catalytic activity. In the past, Kézdy *et al.*,[13] have explored the activity of the enzyme complex in which the glycerol backbone and the phosphate diester were replaced by simpler structures. Since a major role of the phosphate is to complex Ca^{2+} in the active site, they proposed that a carboxylate group could replace the phosphate in this function. In this case, the distance between the reactive ester and the carboxylate group in the new substrate should be similar to that found in the phospholipids. Also, the glycerol would be replaced by a much better leaving group such as a nitrophenol. They choose 4-nitro-3-(octanoyloxy)benzoic acid **1,** which presents the essential structural features of a phosphatidylcholine (R, R^1 = fatty acid residue). They argued that while the geometries of the molecules are different, the sums of the bond lengths between the reactive ester and the ligand of Ca^{2+} are approximately the same in both molecules. Ester **1** showed the highest specificity toward the enzyme, suggesting that in this compound the distance between the negative charged carboxylate and the reactive ester approximates that found in the lecithin-enzyme complex. All kinetic characteristics of the enzymatic hydrolysis indicated that the reaction occurs by the same mechanism shown in the hydrolysis of lecithins. Also compound **1** is ideally suited for a rapid and sensitive spectrophotometric assay of phospholipases A_2 carried out at pH 8 containing 10 mmol L^{-1} calcium chloride ($CaCl_2$) and 0.1 mol L^{-1} sodium chloride (NaCl) in acetonitrile.



Phosphatidylcholine



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In this study we explore the enzymatic reactivity of the phospholipase A_2 from the component venom of the South America rattlesnake *Crotalus durissus terrificus*, on the hydrolysis of non-natural substrates with structural variations related to **1**. We also studied for the first time the potential of that enzyme to steroselectively resolve esters of secondary alcohols. Considering the low availability of the enzyme we immobilized it in order to reuse the biocatalyst in successive reactions. Within the different methods of immobilization we choose precipitation of the enzyme and cross-linking its aggregates to produce the cross-linked enzyme aggregate (CLEA).[14] Several reports have described the CLEA activity to exceed that of the native enzyme with lipases and other enzymes.[15] The immobilization of enzymes via cross-linking is also attractive because the final preparation is basically pure protein, with a high concentration of enzyme *per* unit of volume.[16]

Experimental

General procedures

Proton and carbon nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) were recorded in $CDCl_3$ solution on Varian INOVA 500 MHz, Bruker Advance DPX 250 MHz and Bruker Advance II 300 MHz spectrometers. Chemical shifts were expressed in ppm using TMS as internal standard. Infrared spectra were recorded in KBr on a BOMEN MB-100 FTIR spectrophotometer from Hartmann & Braun. The gas chromatography-mass spectrometry (GC MS) analyses were obtained on a Agilent 6890 coupled a to HP 5973 mass detector using a Supelco MDN-5S (30 m × 250 μm × 0.25 μm) capillary column. Chiral analyses were performed in a Agilent 6850N GC FID, using a MN Hydrodex®-β-3P (30 m × 250 μm × 0.25 μm), Chrompack Chirasil-dex CB (30 m × 250 μm × 0.25 μm) or Lipodex-E 2,6-Pe-3-Bu-γ-CD (28 m × 250 μm × 0.25 μm) capillary columns. Optical rotations were recorded on a PerkinElmer 341 Polarimeter. The spectrophotometric analyses were recorded on an Agilent HP8453 spectrophotometer or FlashScan 530 Analitic Jena. The melting points were determined on a MQAPF-301 Microquimica apparatus.

The absolute configurations of the alcohols were established by comparison of their chromatograms with the products obtained from known reduction reactions of the corresponding ketones with carrot root. *E* value of the reaction was calculated based on *ee* of the recovered product and the extent of conversion.[17]

Enzyme and chemicals

The phospholipase A_2 were isolated from *Crotalus durissus terrificus* venom and purified by a research group from the Biology Institute of Unicamp (Campinas-SP).[18] All chemicals were purchased from Sigma-Aldrich (São Paulo, Brazil) at least in analytical grade.

CLEA procedure

The CLEA were prepared by dissolving 0.5 mg of phospholipase A_2 in 1 mL of Tris-HCl buffer (10 mmol L^{-1}) at pH 8.0 and then adding 0.5 mL of precipitant (55% ammonium sulphate solution, polyethylene glycol 600 Da, dimethoxyethane or acetone) and stirred for 40 min to form the aggregate. When an additive was used, the enzyme

was dissolved in the presence of 10 mg of Triton-X100 or 0.5 mL of polyethylenediimine (PEI). Then 100 μL of a 25% (v/v) solution of glutaraldehyde was added and the mixture was stirred overnight at room temperature. The solution was centrifuged at 500 rpm and the solid kept in Tris-HCl buffer at 4 °C.

Synthesis of 4-nitro-3-(octanoyloxy)benzoic acid ***1a***

3-Hydroxy-4-nitrobenzoic acid (183 mg, 1.0 mmol) and *N*,*N*-diisopropylethylamine (259 mg, 2.0 mmol) in 2 mL of dry tetrahydrofuran (THF) was added dropwise to a cooled solution (0 °C) of octanoyl chloride (163 mg, 1.0 mmol) in 10 mL of dry THF. The reaction was slowly warmed to room temperature and stirred overnight. The reaction mixture was filtered and washed with ether. The crude product was purified by flash chromatography.

Yellow solid, mp 142-145 °C; IR (KBr) ν_{max}/cm^{-1} 3453, 3067, 2959, 2855, 1786, 1696, 1595, 1532. ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3H, *J* 6.7 Hz), 1.2-1.5 (m, 8H), 1.75 (m, 2H, *J* 7.4), 2.71 (t, 2H, *J* 7.5 Hz), 7.92 (s, 1H), 8.10 (d, 1H, *J* 8.2 Hz), 8.15 (d, 1H, *J* 8.2 Hz), ^{13}C NMR($CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 24.4 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 31.6 (CH_2), 34.0 (CH_2), 125.9 (CH), 127.3 (CH), 128.1 (CH), 134.6 (C_0), 144.0 (C_0), 145.1 (C_0), 168.9 (C_0), 171.1 (C_0).

Test of phospholipase activity

To a flask was added 100 μL of a solution 2 mg mL^{-1} of 4-nitro-3-(octanoyloxy)benzoic acid **1** in acetonitrile, 3 mL of Tris-HCl/$CaCl_2$/NaCl (10/10/100 mmol L^{-1}) pH 8.0 buffer. The enzymatic hydrolysis was monitored at 425 nm in a spectrophotometer for 45 min.

General procedure for biocatalysis reactions

The biocatalysis reactions were made by adding the CLEA prepared in 1 mL of Tris-HCl/NaCl/$CaCl_2$ (10/10/100 mmol L^{-1}) pH 8.0 buffer and 1 mg of substrate dissolved in 0.5 mL of acetonitrile and were monitored by GC MS. After each reaction, the CLEA biocatalyst was isolated, washed with water, filtered and kept for further use after testing the phospholipase activity (this test was described above). Reactions with free enzymes were made for comparison. Resolution was stopped before 50% conversion.

General procedure for biocatalysis reactions of acidics compounds

The reactions of 3-(2-bromohexanoyloxy)-4-nitrobenzoic acid and 3-(2-methylhexanoyloxy)-4-nitrobenzoic acid were initially made on polypropylene microplates and analyzed in FlashScan at 425 nm. The substrate (0.05 mg dissolved in 12.5 μL of acetonitrile), 0.045 mg of PLA_2 and the Tris-HCl/$CaCl_2$/NaCl buffer were added to the microplate. The reaction mixtures were stirred at 22 ºC until stabilization.

3-(2-Bromohexanoyloxy)-4-nitrobenzoic acid ***2a***

3-Hydroxy-4-nitrobenzoic acid (257 mg, 1.4 mmol) and *N*,*N*-diisopropylethylamine (363 mg, 2.8 mmol) in 2 mL of dry THF was added dropwise to a cooled solution (0 °C) of 2-bromohexanoyl bromide (362 mg, 1.4 mmol) in 10 mL of dry THF. The reaction mixture was warmed slowly to room temperature and stirred overnight. The reaction mixture was filtered, and the precipitate was washed with ether (10 mL × 3). The filtrate was concentrated under *vacuum*, and the residue was taken up by 50 mL of ethyl acetate. The organic layer was washed with 0.01 mol L^{-1} (50 mL × 2) HCl, dried over anhydrous sodium sulfate (Na_2SO_4), and evaporated. The residue was recrystallized from ethyl acetate-hexane (1:10 v/v).

Yellow solid; mp 125-125.6 °C; IR (KBr) ν_{max}/cm^{-1} 3282, 3065, 2959, 2875, 2560, 1780, 1697, 1594, 1533, 1306, 1215, 1102, 955, 924, 841, 795, 527. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3H, *J* 7.5 Hz), 1.51 (m, 4H), 2.20 (m, 2H), 4.5 (t, 1H, *J* 7.5 Hz), 8.03 (s, 2H), 8.18 (s, 1H), 10.5 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7 (CH_3), 22.0 (CH_2), 29.2 (CH_2), 34.1 (CH_2), 44.4 (CH), 126.1 (CH), 126.7 (CH), 128.6 (CH), 134.9 (C_0), 143.5 (C_0), 144.8 (C_0), 167.3 (C_0), 168.6 (C_0).

3-(2-Methylhexanoyloxy)-4-nitrobenzoic acid ***2b***

2-Methylhexanoic acid (221 mg, 1.7 mmol) was dissolved in 17 mL of dry THF. The solution was stirred and cooled to 0 ºC. Then, *N*-methylmorpholine (172 mg, 1.7 mmol) and isobutyl chloroformate (233 mg, 1.7 mmol) were added successively. The reaction mixture was stirred for 30 min at 0 ºC during which a white precipitate was formed. Next, the reaction mixture was allowed to reach room temperature, and a solution of 3-hydroxy-4-nitrobenzoic acid (330 mg, 1.8 mmol) and *N*-methylmorpholine (364 mg, 3.6 mmol) in 10 mL of dry THF was added. The resulting mixture was stirred overnight at room temperature. The reaction mixture was filtered, and the precipitate was washed with ether (10 mL × 3). The filtrate was concentrated in vacuum, and the residue was taken up by 50 mL of ethyl acetate. The organic layer was washed with 0.01 mol L^{-1} (50 mL × 2) HCl, dried over anhydrous Na_2SO_4, and evaporated. The

residue was recrystallized from ethyl acetate-hexane (1:10 v/v).

Pale yellow solid; mp 135-137 °C; IR (KBr) ν_{max}/cm^{-1} 3444, 3096, 2959, 2929, 2863, 1773, 1627, 1594, 1536, 1327, 1242, 1223, 746. ^{1}H NMR ($CDCl_3$) δ 0.948 (t, 3H, *J* 6.9 Hz), 1.35 (d, 3H, *J* 7Hz), 1.41 (m, 4H), 1.58 (m, 2H), 2.78 (m, 1H, *J* 7Hz), 7.95 (s, 1H), 8.23 (m, 2H), 10.53 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 13.9 (CH_3), 16.6 (CH_3), 22.6 (CH_2), 29.3 (CH_2), 32.9 (CH), 39.4 (CH), 125.8 (CH), 127.2 (CH), 128.0 (CH), 134.5 (C_0), 144.0 (C_0), 145.3 (C_0), 168.5 (C_0), 174.0 (C_0).

Synthesis of substrates

General procedure for reduction of ketones

The ketone substrate (5.5 mmol) was dissolved in dry methanol with sodium borohydride ($NaBH_4$) (4.2 mmol) and stirred overnight. Then 20 mL of dilute HCl (pH 4.0) was added and the solution was stirred for 20 min. The solution was extracted with ethyl acetate and the organic layer was dried over anhydrous Na_2SO_4 and evaporated. The alcohol residue was purified by flash chromatography eluting with 20% ethyl acetate in hexane.

*1-Phenylethanol **7***

From acetophenone: colorless oil; IR (KBr) ν_{max}/cm^{-1} 3359, 3063, 3029, 2973, 2927, 2876, 1493, 1451, 1369, 1302, 1204, 1077, 1010, 996, 899. ^{1}H NMR ($CDCl_3$) δ 1.48 (d, 3H, *J* 6 Hz), 2.05 (br, 1H), 4.86 (q, 1H, J 6 Hz), 7.35 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 25.1 (CH_3), 70.3 (CH), 125.3 (CH), 127.4 (CH), 128.4 (CH), 145.7 (C_0); MS *m/z* (%): 122 (M^+, 33); 107 (100); 79 (92); 77 (55).[19]

*(S)-p-Nitrophenyl-1-ethanol **8***

From *p*-nitroacetophenone: yellow oil; IR (KBr) ν_{max}/cm^{-1} 3414, 2978, 2931, 2866, 1735, 1605, 1520, 1347, 1254, 1090, 1046, 855. ^{1}H NMR ($CDCl_3$) δ 1.5 (d, 3H, *J* 6,8 Hz), 2.37 (br, 1H), 5.0 (q, 1H, *J* 6,5 Hz), 7.52 (d, 2H, *J* 8.6 Hz), 8.16 (d, 2H, *J* 8.6 Hz); ^{13}C NMR $CDCl_3$) δ 25.4 (CH_3), 69.4 (CH), 123.7 (CH), 126.1 (CH), 147.1 (C_0), 153.1 (C_0); MS *m/z* (%): 167 (M^+, 0,7), 166 (1), 152 (100), 122 (24), 107 (45), 77 (39).[20]

*1,2,3,4-Tetrahydronaphthalen-1-ol **12***

From 1-tetralone: colorless oil; IR (KBr) ν_{max}/cm^{-1} 3285, 3041, 2946, 2928, 2855, 2835, 1491, 1453, 1431, 1286, 1267, 1069, 42. ^{1}H NMR ($CDCl_3$) δ 1.60-1.70 (m, 2H), 1.70-2.10 (m, 3H), 2.71 (m, 2H), 4.78 (t, 1H), 7.13 (m, 1H), 7.18 (t, 1H, *J* 3.5 Hz), 7.23 (t, 1H, *J* 3.5 Hz), 7.44 (t, 1H, *J* 4.5 Hz); ^{13}C NMR ($CDCl_3$) δ 18.8 (CH_2), 29.2 (CH_2), 32.3 (CH_2), 68.1 (CH), 126.2 (CH), 127.6 (CH), 128.7 (CH), 129.0 (CH), 137.1 (C_0), 138.8 (C_0); MS *m/z* (%):148 (M^+, 31), 147 (35), 130 (100), 120 (76), 115 (29), 105 (29), 91 (54).[21]

Esterefication procedure for the alcohols

The esterification of the alcohols with acetyl chloride and propionyl chloride was carried out as described by Vogel's Textbook.[22]

*1-Phenylethyl acetate **5***

From 1-phenylethanol **7,** colorless oil; IR (KBr) ν_{max}/cm^{-1} 3034, 2982, 2934, 1744, 1495, 1453, 1371, 1241, 1209, 1065, 1029, 944, 699. ^{1}H NMR ($CDCl_3$) δ 1.53 (d, 3H, *J* 6 Hz), 2.07 (s, 3H), 5.88 (q, 1H, *J* 6 Hz), 7.35 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 21.3 (CH_3), 22.2 (CH_3), 72.3 (CH), 126.1 (CH), 127.8 (CH), 128.5 (CH), 141.7 (C_0), 170.3 (C_0); MS *m/z* (%): 164 (M^+, 21), 149 (1), 122 (95), 104 (100), 91 (2), 77 (36).[21]

*1-Phenylethyl propionate **5b***

From 1-phenylethanol **7,** colorless oil; IR (KBr) ν_{max}/cm^{-1} 3063, 3034, 2982, 2941, 2880, 1738, 1495, 1454, 1368, 1273, 1189, 1064, 1029; ^{1}H NMR ($CDCl_3$) δ 1.14 (t, 3H, *J* 7 Hz), 1.53 (d, 3H, *J* 6,5 Hz), 2.35 (m, 2H *J* 7.5 Hz and 3.5 Hz), 5.89 (t, 1H, *J* 6.5 Hz), 7.32 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 9.0 (CH_3), 22.2 (CH_3), 27.8 (CH_2), 72.0 (CH), 126.0 (CH), 127.8 (CH), 128.4 (CH), 141.8 (C_0), 173.7 (C_0); MS *m/z* (%): 178 (M^+, 25), 122 (92), 105 (100), 77 (28).[23]

*p-Nitrophenyl-1-ethyl acetate **6a***

From *p*-nitrophenyl-1-ethanol **8**, white solid; mp 49-51 °C; IR (KBr) ν_{max}/cm^{-1} 2965, 2923, 2856, 1731, 1602, 1518, 1347; 1248, 1069, 856. ^{1}H NMR ($CDCl_3$) δ 1.55 (d, 3H, *J* 6.8 Hz), 2.11 (s, 3H), 5.92 (q, 1H, *J* 6.7 Hz), 7.51 (d, 2H, *J* 8.5 Hz), 8.21 (d, 2H, *J* 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 20.8 (CH_3), 22.4 (CH_3), 71.0 (CH), 123.8 (CH), 126.9 (CH), 148.9 (C_0), 170.0 (C_0); MS *m/z* (%): 209 (M^+,6); 167 (100); 150 (23); 119 (16); 103 (25); 91 (18); 77 (19).[24]

*p-Nitrophenyl-1-ethyl propionate **6b***

From *p*-nitrophenyl-1-ethanol **8**, white solid; mp 36-37 °C; IR (KBr) ν_{max}/cm^{-1} 3066, 2989, 2946, 1736, 1605, 1524, 1333, 1184, 1086, 1065, 855. ^{1}H NMR ($CDCl_3$) δ 1.15 (t, 3H, *J* 7.6 Hz), 1.55 (d, 2H, *J* 6.7 Hz), 2.39 (qd, 2H, *J* 7.58 Hz and *J* 1.3 Hz), 5.93 (q, 1H, *J* 6.7 Hz), 7.50 (d, 2H, *J* 8.8 Hz), 8.19 (d, 2H, *J* 8.8Hz); ^{13}C NMR ($CDCl_3$) δ 9.0 (CH_3), 22.4 (CH_3), 27.7 (CH_2), 71.8 (CH), 123.9 (CH), 126.7 (CH), 149.2 (C_0), 173.5 (C_0); MS *m/z* (%): 223 (M^+, 11); 167 (100); 150 (68); 119 (24); 103 (36); 91 (25).

(R)-1,2,3,4-Tetrahydronaphthalen-1-yl acetate ***11a***

Yellow oil; IR (KBr) ν_{max}/cm^{-1} 3064, 3023, 2940, 2859, 2838, 1731, 1492, 1455, 1371, 1238, 1210, 1154, 1030, 1016, 882. ^{1}H NMR ($CDCl_3$) δ 1.70-1.95 (m, 4H), 2.10 (s, 3H), 2.85 (m, 2H), 6.02 (t, 1H, *J* 4.2 Hz), 7.25 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 18.8 (CH_2), 21.5 (CH_3), 29.0 (CH_2), 29.1 (CH_2), 70.0 (CH), 126.1 (CH), 128.1 (CH), 129.1 (CH), 129.4 (CH), 134.6 (C_0), 137.9 (C_0), 170.8 (C_0); MS *m/z* (%): 190 (M^+, 1), 148 (19), 130 (100), 115 (32); 91 (20).[25]

1,2,3,4-Tetrahydronaphthalen-1-yl propionate ***11b***

Colorless oil; IR (KBr) ν_{max}/cm^{-1} 3023, 2979, 2941, 2870, 2840, 1731, 1492, 1455, 1271, 1185, 1152, 1080, 766. ^{1}H NMR ($CDCl_3$) δ 1.17 (t, 3H, *J* 6.8 Hz), 1.80-2.05 (m, 4H), 2.36 (q, 2H, *J* 6.8 Hz), 2.82 (m, 2H), 6.01 (t, 1H; *J* 4.3 Hz), 7.12-7.29 (m, 4H); ^{13}C NMR($CDCl_3$) δ: 9.2 (CH_3), 18.8 (CH_2), 28.2 (CH_2); 29.0 (CH_2), 29.1 (CH_2), 69.8 (CH), 126.0 (CH), 128.0 (CH), 129.0 (CH), 129.4 (CH), 134.8 (C_0), 138.0 (C_0), 174.3 (C_0); MS *m/z* (%): 204 (M^+, 0.8), 148 (27), 130 (100), 115 (29), 91 (24).[26]

Determination of absolute configurations

Bioreduction of α*-tetralone with Daucus carota*

α-Tetralone (20 mg, 0.14 mmol) dissolved in 1 mL of ethanol was added to a suspension of freshly cut carrot root (30 g) in 80 mL of distilled water, and the reaction mixture was incubated on an orbital shaker (180 rpm) at 30 °C for 6 days. Finally, the suspension was filtered, and the carrot root was washed three times with water. Filtrates were extracted with ethyl acetate (3 × 125 mL), the organic phase was dried over anhydrous Na_2SO_4 and then evaporated. The residue was analyzed by GC MS and GC FID with a chiral column. The product (*S*)-1,2,3,4-tetrahydronaphtalen-1-ol **12** was obtained in 64% yield and *ee* 65% with $[\alpha]_D^{20}$ +15 (*c* 0.59 in THF).

Bioreduction of p-nitroacetophenone with daucus carota

Using a procedure similar to the describe above, 100 mg (0.60 mmol) of *p*-nitroacetophenone was reacted with 30 g of carrot root. Following separations and GC analysis the product (*S*)-*p*-nitrophenyl-1-ethanol **8** was obtained in 81% yield and *ee* 96% with $[\alpha]_D^{20}$ −24 (*c* 1.48 in THF).

Results and Discussion

Considering that snakes in general produce only small amounts of venom, we attempted to immobilize the purified phospholipase A_2 in order to reuse it in successive batch reactions. CLEAs were prepared varying several parameters like precipitant and additives, such as surfactants and then, their activities were investigated (Table 1). The activities of the resultant CLEA were compared with those of the free enzyme, which was considered as 100%. The kinetics of hydrolysis of 4-nitro-3-(octanoyloxy)benzoic acid **1** were monitored on a UV-spectrophotometer at 425 nm at pH 9. This compound is currently used to assay PLA_2 activity since it shows the highest specificity toward the Ca^{2+} dependent phospholipase.[29] The preparation of CLEA was taken from the simplified procedure of Sheldon *et al.*,[30] by combining the aggregation and cross-linking steps. The precipitants ammonium sulfate, PEG 600, 1,2-dimetoxyethane or acetone were added simultaneously with the surfactant and then the glutaraldeyde. The results (Table 1) show the highest activity was obtained without addition of any surfactant and the most effective precipitant was ammonium sulfate (entry 1). In further experiments we adopted this procedure (Table 1, entry 1) since this CLEA has hydrolysis activity comparable with that of the free enzyme.

Table 1. CLEA enzyme activities with several different precipitant and surfactants in the hydrolysis of 4-nitro-3(octanoyloxy)benzoic acid **1**

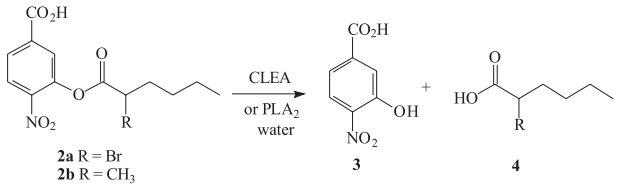
entry	Precipitant	Surfactant	Activity[a] of CLEA, %
1	$(NH_4)_2SO_4$	Without	95
2		Triton X-100	40
3		PEI[b]	13
4	PEG 600	Without	60
5		Triton X-100	7
6		PEI	0
7	DME[c]	Without	25
8		Triton X-100	20
9		PEI	6
10	Acetone	Without	16
11		Triton X-100	0
12		PEI	6

[a]Compared to activity of the same amount of free PLA_2 enzyme; [b]polyethylenediimine; [c]1,2-dimethoxyethane.

One of our objectives was to investigate the capability of the phospholipase A_2 of the rattlesnake *Crotalus durissus terrificus* to produce chiral compounds through stereoselective hydrolysis. With this in mind, we prepared two substrates closed related to acid **1**, with a substituent at α-position of the ester moiety. We choose the commercially available 2-bromohexanoic acid **4a** and 2-methylhexanoic acid **4b** to prepare the substrates 3-(2-bromohexanoyl)-4-nitrobenzoic acid **2a** and 3-(2-methylhexanoyl)-4-nitrobenzoic acid **2b**. Initially, we tested the hydrolysis of both substrates in a blank reaction (acetonitrile 3%, water in Tris-HCl/$CaCl_2$/NaCl at pH 8) to be sure that **2a** and

2b are stable in that reaction medium. The spontaneous hydrolysis of **2a** was very fast in the reaction medium, avoiding carrying out the enzymatic hydrolysis with this substrate, but slow with **2b.**

The enzymatic hydrolysis of **2b** in 3% acetonitirle (v/v) in a buffer of Tris-HCl/$CaCl_2$/NaCl at pH 8 with the free phospholipase at 25 °C gave a 25% yield and 7% *ee* after 4 days (Scheme 1).



Scheme 1.

When the reaction was carried out at 45 °C the yield increased to 52% but the stereoselectivity decreased to 3% *ee*. With CLEA, the yield increased as is shown in Table 2. When compared with substrate **1** for which the reaction is fast, about 2 h, the hydrolysis of **2b** was much slower (3-4 days) and gave good yields and low *ee*. We assume that the decrease of reactivity is a consequence of the presence of the methyl group near the reaction center of the hydrolysis. The absolute configuration of the 2-methylhexanoic acid **4b** was not determined considering that it was isolated with low enantioselectivity (closed to a racemate). The yield of **4**b with CLEA increased when compared with the free enzyme (entries 3 an 4 of Table 2), but the *ee* was very low.

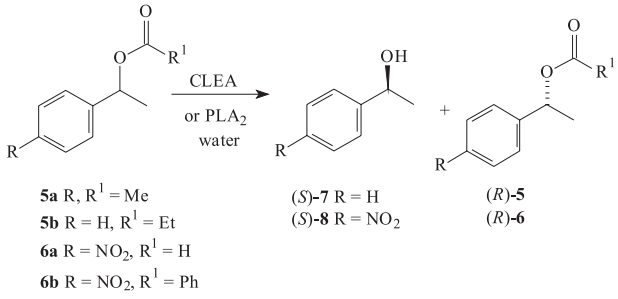
Table 2. Hydrolysis of 3-(2-methylhexanoyl)-4-nitrobenzoic acid **2b** with free PLA_2 and with CLEA-phospholipase

entry	Enzyme	Temperature (°C)	Yield (%)	*ee* (%)
1	Free PLA_2	25	25	7
2	Free PLA_2	45	52	3
3	CLEA	25	55	9
4	CLEA	45	76	5

Considering that the substituents of the alkyl moiety of substrates **2a** and **2b** were not suitable for our objective to produce enantioselective products by phospholipase hydrolysis, we choose esters with different structures to test enzyme reactivity. The compounds **5** and **6** (Scheme 2) were prepared by esterification of the parent alcohols which were prepared from the corresponding ketones by reduction with sodium borohydride. We employed the same methodology used for **2**. The results of the hydrolysis are summarized in Table 3. For most cases, the reaction using CLEA gave good yields (> 60%) but superior than with the free enzyme. As before the enantioselectivity of the hydrolyzed products was poor nearly racemic. Only in two examples did the enantioselectivity gave better *ee*, compound **5a** with 16% *ee* for product **7** (entry 2) and **6a** with 19% *ee* for product **8** (entry 5). A low *E* value (*E* = 3) for **5a and** 6**a** after 43% conversion were calculated for both reactions. The *E*-value and the *ee* of **5a**, **5b**, **6a** and **6b** were the same after each cycle, that is, < 3 and 20%, respectively. The absolute configuration of the alcohols (*S*)-**7** and (*S*)-**8** was determined by comparison of GC of the alcohols from authentic samples obtained by carrying out the known bioreduction of acetophenone and *p*-nitroacetophenone with *Daucus carota*.[31]

Table 3. Hydrolysis of esters **5** and **6** with free PLA_2 and with CLEA-phospholipase

entry	Substrate	Enzyme	Product, Yield (%)		Product, *ee* (%)	
1	**5a**	free PLA_2	**7**, 40	**5a**, 35	**7**, 3	**5a**, 2
2	**5a**	CLEA	**7**, 67	**5a**, 53	**7**, 16	**5a**, 3
3	**5b**	free PLA_2	**7**, 20	**5b**, 20	**7**, 3	**5b**, 20
4	**5b**	CLEA	**7**, 64	**5b**, 48	**7**, 6	**5b**, 28
5	**6a**	free PLA_2	**8**, 55	**6a**, 68	**8**, 19	**6a**, 24
6	**6a**	CLEA	**8**, 83	**6a**. 79	**8**, 5	**6a**, 4
7	**6b**	free PLA_2	**8**, 34	**6b**, 35	**8**, 10	**6b**, 15
8	**6b**	CLEA	**8**, 72	**6b**, 70	**8**, 6	**6b**, 7

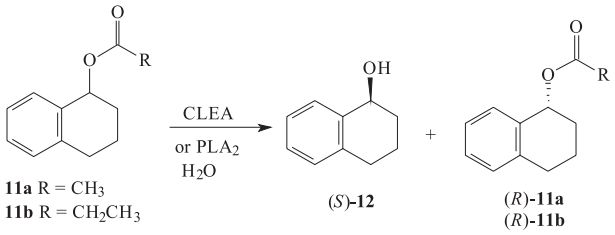


Scheme 2.

Finally, we carried out the hydrolysis of α-tetralol esters **11a** and **11b** with free phospholipase and CLEA. The results are summarized in Table 4. For both substrates, (*S*)-α-tetralol **12** was isolated in good yields (> 50%) but with CLEA phospholipase the yields were superior to those with the free enzyme. Also, the acetyl derivative **11a** gave higher yields than the propyl ester **11b**. Only with acetyl derivative **11a** a modest enantioselectivity was obtained, with 16% *ee* and a low *E* value (*E* = 3) (entry 2) when CLEA was employed (Scheme 3). The enantiomeric ratio *E* was very low reflecting the low especificity of the enzyme, both free and immobilized (CLEA).

Table 4. Hydrolysis of esters **11a** and **b** with free PLA_2 and with CLEA-phospholipase

entry	substrate	enzyme	Product, yield (%)		Product, *ee* (%)	
1	**11a**	PLA_2 free	**12**, 53	**11a**, 55	**15**, 3	**11a**, 3
2	**11a**	CLEA	**12**, 85	**11a**, 86	**15**, 16	**11a**, 10
3	**11b**	PLA_2 free	**12**, 28	**11b**, 20	**15**, 1	**11b**, 1
4	**11b**	CLEA	**12**, 60	**11b**, 70	**15**, 3	**11b**, 3



Scheme 3.

As in the case of 1-phenylethanol **7**, the absolute configuration of the (*S*)-α-tetralol **12** was determined by comparison the GC of the alcohol obtained with an authentic sample prepared by carrying out the known bioreduction of α-tetralone with carrot root (Scheme 3).[26]

Conclusions

For the first time, the phospholipase A_2 from rattlesnake *Crotalus durissus terrificus* has shown some enantioseletivity in the hydrolysis of different esters. It was observed that this small protein is restrictive in the choice of its lipase action with non-natural substrates. With all substrates the free enzyme showed reactivity similar to the CLEA preparation. The advantage of the CLEA phospholipase is to reuse it in several consecutive reactions without a decrease of activity, having a higher yield and similar *ee* when compared with the free enzyme.

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