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Chromogenic and Fluorogenic Assays for the Lactonase Activity of Serum Paraoxonases

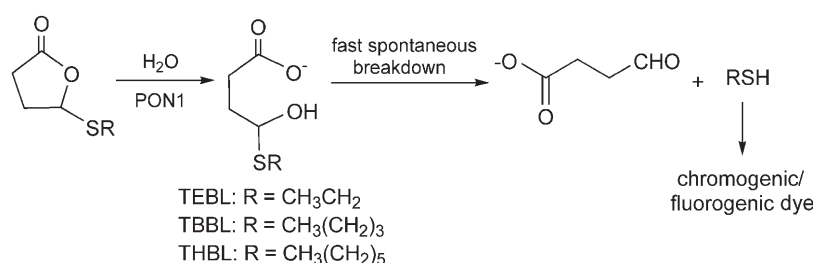
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Serum paraoxonase (PON1) is an high-density lipoprotein (HDL)-associated enzyme with antiatherogenic and detoxification properties that hydrolyzes a wide range of substrates, such as esters, organophosphates (e.g., paraoxon) and lactones. For a long time, PON1 was considered to be an aryl esterase and paraoxonase, and its activity was measured accordingly. However, it recently became apparent that PON1 can catalyze both the hydrolysis^[1,2] and formation^[3] of a variety of lactones. Indeed, structure–reactivity studies^[4] and laboratory evolution experiments^[5] indicate that PON1's native activity is as a lactonase, and that the paraoxonase and aryl esterase are promiscuous activities. Studies of PON1's activation by binding to HDL particles carrying ApoA-I indicate high specificity towards lactone substrates, and lipophilic lactones in particular.^[6] Finally, the lactonase activity is the only activity shared by all other members of the PON family, some of which exhibit no paraoxonase or aryl esterase activity.^[2]

The activity of PON1 in human sera has been the subject of numerous studies that address a possible linkage between polymorphisms of PON1, various environmental factors that modulate its activity and susceptibility to atherosclerosis and other disorders.^[7] The assays, however, use phenyl acetate or paraoxon, which have no physiological relevance. A more relevant assay must address the lactonase activity. However, current methods for measuring lactonase activities with aliphatic lactones are based on pH indicators^[1,4] and HPLC.^[2,3] The latter is highly laborious, while the pH-indicator assay requires repetitive calibrations and gives accurate results only with pure enzyme samples in which the pH and buffer strength can be tightly controlled. We have therefore developed novel aliphatic chromogenic/fluorogenic lactone substrates that would be favoured by PONs, and could be used with biological samples, for example, serum, cells or cell lysates. The development of facile enzymatic assays that are suitable for high-throughput screens is of importance in many fields, including diagnostics, drug

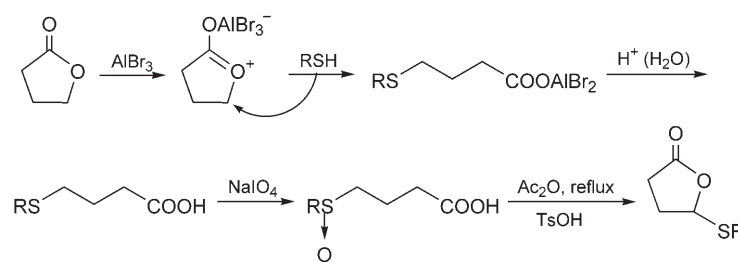
discovery, functional genomics and enzyme engineering and evolution.^[8]

We adopted the general strategy of Reymond et al., by which, the primary enzymatic reaction produces an unstable intermediate that rapidly collapses to release a fluorescent/chromogenic moiety.^[8] In fact, this group has recently developed a fluorescence-based lactonase assay using 6- and 7-member ring lactones substituted with umbelliferone.^[9] However, these substrates significantly differ from the favourable substrates of PON1 that comprise 5- and 6-membered ring oxo-lactones with long alkyl side chains.^[2,4,6] They also exhibit high background rates at the pH optimum for PON1 (~8.0). We therefore designed 5-thioalkyl butyrolactones (TXBLs) that release a thiol moiety upon hydrolysis of the oxo-lactone ring (Scheme 1). The released thiol can be detected with chromogenic or fluorogenic probes such as Ellman's reagent (DTNB)^[10] or CPM.^[11]



Scheme 1. Hydrolysis of 5-thioalkyl butyrolactones (TXBLs), X = ethyl, butyl, or hexyl, and product detection.

We adopted the method of synthesis of 4-phenylthio-4-butanolide^[12] for the synthesis of 5-thioethyl, thiobutyl and thiohexyl butyrolactones (Scheme 2). First, the γ -butyrolactone ring



Scheme 2. Synthesis of 5-thioalkyl butyrolactones.

was opened with the corresponding thiol.^[13] The resulting 4-(alkylthio)butyric acid was then oxidized with sodium periodate to give 4-(alkylsulfanyl)butyric acid,^[14] which was closed to the corresponding lactone by a Pummerer rearrangement.^[12] We found that this route is generic and can be used to attach thiol side chains of various lengths to 5-butyrolactone.

The kinetic parameters of enzymatic hydrolysis of the three TXBLs by PON1 were determined by detecting the released thiol moiety with DTNB. A typical colorimetric assay of TBBL hydrolysis is shown in Figure 1A, and the kinetic parameters are listed in Table 1. The k_{cat} and K_{M} values for these new sub-

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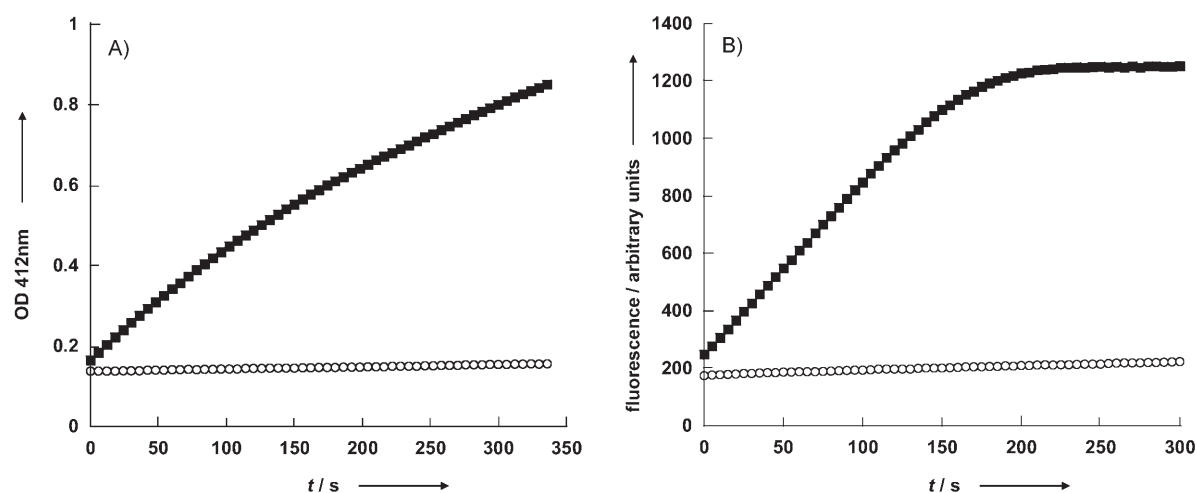


Figure 1. A) Colorimetric and B) fluorogenic detection of the lactonase activity of PON1. A) 0.2 mM TBBL with 0.5 mM DTNB in the presence (■) or absence (○) of PON1 (8.375×10^{-9} M) monitored by absorbance at 412 nm. B) 0.25 mM TBBL with 50 μ M CPM, in the presence (■) or absence (○) of 8.375×10^{-9} M PON1, detected by excitation at 400 nm and emission at 516 nm.

Table 1. Kinetic parameters for rePON1 with 5-thioalkyl butyrolactones and 5-alkyl butyrolactones.				
Substrate	Formula	k_{cat} [s^{-1}]	K_{M} [mM]	$k_{\text{cat}}/K_{\text{M}}$ [$\text{M}^{-1}\text{s}^{-1}$]
5-thioalkyl butyrolactones				
TEBL, thioethyl butyrolactone		161 ± 10	0.36 ± 0.05	$445\,000 \pm 36\,000$
TBBL, thiobutyl butyrolactone		116 ± 4	0.27 ± 0.04	$440\,000 \pm 55\,000$
THBL, thiohexyl butyrolactone		52.4 ± 2.6	0.35 ± 0.03	$150\,000 \pm 9\,300$
5-alkyl butyrolactones ^[a]				
γ -heptanoic lactone		34.0 ± 0.8	0.58 ± 0.03	$58\,000 \pm 3\,000$
γ -nonanoic lactone		31 ± 2	0.39 ± 0.03	$78\,000 \pm 1\,600$
γ -undecanoic lactone		62 ± 2	0.60 ± 0.07	$103\,000 \pm 8\,600$

[a] The kinetic parameters for 5-alkyl butyrolactones are taken from ref. [4].

strates are similar to those observed with the homologous 5-alkyl-substituted butyrolactones. The rates of enzymatic hydrolyses of the 5-thioalkyl lactones were also followed with the fluorogenic thiol-detecting probe CPM^[11] as shown in Figure 1B. The activity of PON1 with the novel TXBL substrates was also stimulated by binding to HDL particles carrying ApoA-I, and the activation was similar to that observed with the homologous 5-alkyl-substituted butyrolactones.^[6]

The developed lactone substrates can also be used to measure PON1 levels in human sera, as shown in Figure 2. To verify that the measured lactonase activity is mediated by PON1 as

opposed to other hydrolases present in the serum, the serum was also preincubated with 2-hydroxyquinoline, a selective competitive inhibitor of PON1's activity,^[4] and EDTA, which chelates the calcium crucial for PON1's activity. In parallel, we determined the activity with phenyl acetate, which is routinely used as a probe for PON1 levels in the serum. The activity with TBBL was comparable to that with phenyl acetate, and was inhibited in the very same manner (Table 2). This clearly demonstrates that the novel lactone substrates can be used in assessing PON levels in human sera, and, since PON2 is not present in human sera, and PON3 is ~ 2 orders of magnitude less abun-

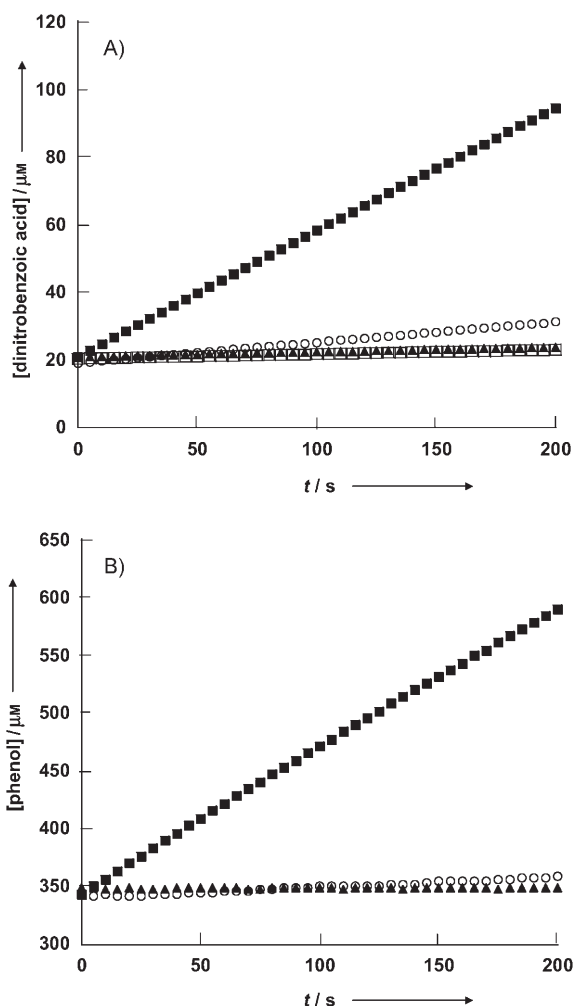


Figure 2. Assays of PON1's A) lactonase and B) aryl esterase activities in human sera. Sera were diluted 1:400 in Tris pH 8.0, and reactions contained: A) 0.5 mM TBBL and 0.5 mM DTNB; B) 1.0 mM phenyl acetate. Shown are the rates observed with no inhibitor (■), with 100 μM 2-hydroxyquinoline (○), or 5 mM EDTA (▲), and the background hydrolysis without serum (□). The hydrolysis of TBBL was detected with DTNB and monitored by absorbance at 412 nm (A). The hydrolysis of phenyl acetate was monitored directly by absorbance at 270 nm (B).

dant than PON1;^[15] > 90% of the observed lactonase and aryl esterase activities stem from PON1. Notably, none of the TXBL substrates was hydrolyzed by phospholipase A2, and the activity (μmol substrate metabolized per minute by 1 mg enzyme) of pig liver esterase was two orders of magnitude lower than the activity of PON1.

Human PON1 exhibits polymorphism at position 192, the two variants being Q192 and R192. We used the novel TXBL substrates, as well as the homologous 5-alkyl-substituted butyrolactones, to probe the polymorphic differences between the two variants in human PON1. The activity of the human PON1 R192 variant with TXBL substrates and with their homologous 5-alkyl-substituted butyrolactones was up to twofold higher than that of Q192. Similar differences were previously obtained with other 5- and 6-member ring lactones.^[11]

We also detected PON1's activity in living cells by using FACS (fluorescence-activated cell sorter) and emulsion droplets that compartmentalize the cells together with the products of the enzymatic reaction.^[16,17] First, *E. coli* cells expressing rePON1 in cytoplasm, as well as GFP (green fluorescent protein) were compartmentalized in the aqueous droplets of a water-in-oil (w/o) emulsion, together with the lactone substrate (TBBL) and the fluorogenic thiol-detecting dye CPM. The w/o emulsion was then re-emulsified, to generate the w/o/w double emulsion with a continuous water phase that is amenable to FACS.^[16] The FACS triggering threshold was set for the emission of GFP, and an appropriate gate was chosen that corresponded to the level of emission of single *E. coli* cells.^[17] The detection of PON1's lactonase activity in the compartmentalized cells was by the fluorescent signal of the thiol-detecting dye at 530 nm. A clear difference (> 20-fold in mean fluorescence) was observed relative to cells bearing no rePON1 (Figure 3).

In conclusion, the data demonstrate that 5-thioalkyl lactones are highly useful and sensitive probes for assaying the lactonase activity of PON1. The rates of PON1 with these substrates

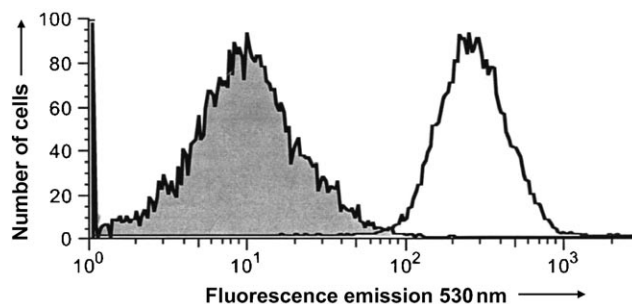


Figure 3. FACS detection of *E. coli* cells expressing PON1 by using a TBBL and w/o/w emulsions. Cells expressing rePON1 in their cytoplasm were emulsified, together with TBBL and the thiol-detecting dye CPM. Shown are representative graphs of the fluorescent emission at 530 nm (the thiol-CPM adduct) for single cells expressing GFP and PON1 (white), and control cells with GFP only (grey).

Table 2. Serum activity with phenyl acetate and TBBL.

Sample	Serum activity [μM product per min] (% of uninhibited activity)					
	uninhibited	with 0.5 mM TBBL		uninhibited	with 1 mM phenyl acetate	
		HQ ^[a] [100 μM] ^[a]	EDTA [5 mM]		HQ ^[a] [100 μM] ^[a]	EDTA [5 mM]
1	21.0 \pm 0.4	1.80 \pm 0.01 (8.6%)	0.06 \pm 0.01 (0.3%)	79 \pm 6	3.9 \pm 0.3 (4.9%)	~0 (0%)
2	21.3 \pm 0.1	2.09 \pm 0.04 (9.8%)	0.04 \pm 0.01 (0.2%)	80 \pm 3	5.9 \pm 0.4 (7.4%)	~0 (0%)

[a] HQ: 2-hydroxyquinoline.

are similar to aliphatic 5-alkyl substituted lactones that are favourable substrates of PON1 and might well resemble its native substrates.^[2] The 5-thioalkyl lactones can be used with complex biological samples such as HDL preparations, or intact cells and sera, and thus provide a novel, physiologically relevant mean of testing the levels of PON1 in human serum in a high-throughput manner. These substrates also provide a powerful means of screening for lactonase activity by using FACS and double emulsions, that enables the screen of libraries of $>10^7$ enzyme variants in few hours, for directed evolution and functional genomics.^[17,18] Finally, the novel 5-thioalkyl lactones can be used with enzymes other than PON1, in particular with other PON family members for which no chromogenic/fluorogenic substrates exist. For example, the lactonase activity of PON3 could be assayed with TEBL and TBBL, both in purified enzyme samples and crude cell lysates (data not shown). The lactonase activity of other enzymes (e.g., *Pseudomonas diminuta* phosphotriesterase) could also be detected.^[19]

Experimental Section

General: Chemicals were purchased from Aldrich, Fluka and Acros Chemicals. CPM dye (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin) was purchased from Molecular Probes (Eugene, OR). Kinetics were performed with recombinant PON1 variant rePON1-G2E6 expressed in fusion with a thioredoxin and 6xHis tag, and purified as described.^[20] The polymorphic differences between the Q192 and R192 variants were measured with human PON1, kindly provided by Prof. Michael Aviram (Technion, Israel). Pig liver esterase and phospholipase A2 were purchased from Sigma. All the data presented are the averages of at least three independent experiments, and standard deviations were calculated by using Microsoft Excell 2003.

The typical synthesis of 5-thioalkyl-substituted butyrolactones is given for 5-thiobutyl butyrolactone (TBBL).

4-(butylthio)butyric acid: γ -Butyrolactone (12.9 mmol, 1.11 g) was added dropwise to a mixture of AlBr_3 (2.2 equiv, 28.38 mmol, 7.56 g) and butanethiol (~20 mL). The resulting mixture was stirred for 2 h at room temperature, and then slowly poured into water (~50 mL). The aqueous mixture was extracted with CH_2Cl_2 (2×50 mL), and the organic phase was washed with NaCl brine and dried over Na_2SO_4 . The solvents were evaporated, and the product was dried under vacuum to yield 1.84 g, 80.9%. $^1\text{H NMR}$ (250 MHz, CDCl_3) δ = 0.89–0.94 (t, 3H), 1.36–1.50 (m, 2H), 1.53–1.62 (m, 2H), 1.86–1.97 (m, 2H), 2.46–2.60 ppm (m, 6H).

4-(butylsulfinyl)butyric acid: 4-(Butylthio)butyric acid (1.84 g, 10.4 mmol) was added to an aqueous solution of sodium periodate (0.5 M, 21 mL, 10.5 mmol) at 0 °C, and the reaction mixture was stirred overnight at 0 °C. The precipitate was removed by filtration, and the filtrate was evaporated. The resulting solid was extracted with CH_2Cl_2 (3×50 mL, 15 min extractions), and the solvent was removed by evaporation to yield 4-(butylsulfinyl)butyric acid (1.88 g, 94%). $^1\text{H NMR}$ (250 MHz, CDCl_3) δ = 0.92–0.98 (t, 3H), 1.42–1.53 (m, 2H), 1.68–1.80 (m, 2H), 2.07–2.16 (m, 2H), 2.49–2.64 (t, 2H), 2.69–2.94 ppm (m, 4H).

5-(thiobutyl) butyrolactone: Acetic anhydride (3 equiv, 10 mmol, 1 g) and a catalytic amount of *p*-toluenesulfonic acid were added to a solution of 4-(butylsulfinyl)butyric acid (630 mg, 3.2 mmol) in toluene. The resulting solution was refluxed for few hours, and the

solvents were evaporated to dryness. The residue was dissolved in ethyl acetate/hexane (1:3) and purified by flash chromatography (silica gel, ethyl acetate/hexane (1:3)) to give 5-(thiobutyl) butyrolactone (130 mg, 23.3%). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ = 0.86–0.92 (t, 3H), 1.40–1.48 (m, 2H), 1.62–1.71 (m, 2H), 2.06–2.18 (m, 2H), 2.49–2.80 (m, 4H), 5.64–5.72 ppm (t, 1H); $^{13}\text{C NMR}$ (400 MHz, CDCl_3) δ = 15.0, 23.3, 29.4, 30.0, 32.8, 33.0, 78.1–79.6 ppm; ESI-MS: m/z 174 $[M]^-$.

Kinetic measurements: The rates of enzymatic hydrolysis of the thioalkyl-substituted lactones were determined in activity buffer (Tris, (50 mM, pH 8.0) with CaCl_2 (1 mM) and NaCl (50 mM)). The enzyme stocks were kept in activity buffer containing 0.1% tergitol, and the enzyme concentration used was 8.375×10^{-9} M. Stocks of substrates (100–400 mM) were prepared in acetonitrile and diluted with the reaction buffer immediately before initializing the reaction. 5-(Thiohexyl)butyrolactone (THBL) was dissolved in buffer with Triton X-100 detergent at a final concentration of 0.03–0.24%. The substrate concentrations were varied in the range of $0.3 \times K_M$ up to $(2-3) \times K_M$. The cosolvent percentage was kept at 1% in all reactions. The DTNB dye (Ellman's reagent, 5',5-dithiobis (2-nitrobenzoic acid) was used at a final concentration of 0.5 mM. An $\epsilon_{412\text{nm}} = 7000$ OD/M value was used to calculate the activity. Product formation was monitored spectrophotometrically in 200 μL reaction volumes in 96-well plates, on a microtiter plate reader (Synergy HT multi-detection microplate reader with time-resolved fluorescence; optical length ~0.5 cm). Initial velocities (v_0) were determined at eight different concentrations for each substrate. The v_0 values were corrected for the background rate of spontaneous hydrolysis in the absence of enzyme, which was in all the cases lower than 5% of the enzymatic hydrolysis rate. Kinetic parameters (k_{cat} , K_M , k_{cat}/K_M) were obtained by fitting the data to the Michaelis-Menten equation [$v_0 = k_{\text{cat}}[E]_0[S]_0/([S]_0 + K_M)$] by using the program Kaleidagraph 5.0.

For fluorescent detection, TBBL was incubated for 3 min with the CPM dye (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin) at final concentration of 50 μM in activity buffer with 0.1% triton. Product formation was monitored in 200 μL reaction volumes in 96-well plates on a microtiter plate reader (excitation: 400 nm filter, emission: 450 and 516 nm filters).

Serum activity with TBBL and phenyl acetate. The serum was used at a final dilution of 1 to 400 in activity buffer. The reaction mixtures contained TBBL (0.5 mM) and DTNB (0.5 mM), or phenyl acetate (1 mM) from 500 mM stock in methanol. All the reaction mixtures contained a final 1% DMSO. 2-Hydroxyquinoline was used from 500 mM stock in DMSO, and EDTA was used from 0.5 M stock in water. The serum was incubated with the inhibitors for 5–10 min before the initiation of the reaction.

Detection of PON1 activity with TBBL by FACS. The emulsification of the *E. coli* cells and FACS analysis were performed as previously described.^[15]

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Keywords: enzyme fingerprinting · fluorescent probes · lactones · phenotyping · PON1

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