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Signal Interference Effect of Human Paraoxonase 1 using as Substrates N-hexanoyl-L-homoserine Lactone and N-3-oxo-octanoyl-L-homoserine Lactone on Growth of Pathogenic Bacteria[1]

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Abstract—Paraoxonase 1 (PON1) is human lactonase orginally described as enzyme that is capable of hydrolyzing organophosphates. The hypothesis suggested that this enzyme may also participate in attenuation of bacterial virulence through interfering with quorum sensing (QS). Recently, PON1 was shown to hydrolyze over 30 lactones. In the present study, human PON1 (hPON1) was purified using ammonium sulphate precipitation and Sepharose-4B-L-tyrosine-1-naphthylamine hydrophobic interaction chromatography. The purified enzyme had a specific activity of 11.89 U/mg protein and catalyzed the hydrolysis of N-hexanoyl-L-homoserine lactone (C10HSL) and N-3-oxo-octanoyl-L-homoserine lactone (3OC8HSL). The hydrolysis reaction was analyzed with HPLC. The K_M values for hPON1 using 3OC8HSL or C10HSL as subtrate were calculated as 2.71 and 0.80 mM and V_{max} values were detected as 1428.57 and 45.24 μmoles mg^{-1} min^{-1}, respectively. Also, effect of hPON1 on growth of pathogenic bacterial strains using the signal lactone molecules was investigated by microtiter plate assay. Our results demonstrated that hPON1 was responsible for inhibition of QS system by hydrolyzing of signal molecules and effecting bacterial growth.

Keywords: hPON1, N-3-oxo-octanoyl-L-homoserine lactone, N-hexanoyl-L-homoserine lactone, pathogenic bacteria, signal interference

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Many pathogens rely on cell-to-cell communication mechanisms known as quorum sensing (QS) to synchronize microbial activities essential for infection and survival in the host that suggests a promising disease control strategy, i.e. quorum quenching (QQ). As a disease control strategy, QQ approach, also known as antipathogenic or signal interference, which abolishes bacterial infection by interfering QS [1, 2].

Blocking cell-to-cell communication represents a promising research area in designing new targets for antimicrobial activity. QS denotes that a single bacterium in a given population detects the density of the same species and consequently all the cells in the population show a coordinated behavior to produce different virulence determinants. Several pathogen bacteria are now known to communicate by means of that mechanism. The best studied common signalling molecules found in Gram-negative bacteria are N-acylated derivatives of L-homoserine lactone (acyl-HSLs) [3]. It is known that inhibition of QS may provide antibacterial activity.

Interference in the QS mechanism can be achieved in a variety of ways. First, many natural substances can disturb the signal perception by imitating acyl-HSL structure. The acyl-HSL analogues block the acyl-HSL receptor (regulator) protein and, therefore, prevent activation of the target gene expression [4]. Many studies showed that higher plants produce and secrete secondary metabolites that interfere with the microbial QS systems [5, 6]. Synthetic analogues of acyl-HSLs, such as N-acyl-3-amino-5H-furanone, effectively block LuxR protein preventing cognate signal molecules binding. A review concerning the influence of natural and synthetic analogues of acyl-HSLs on QS of Gram-negative bacteria has been recently presented by Geske et al. [7]. In addition, many different bacteria belonging to various genera have been reported to express activities degradating acyl-HSLs. The chemical structure of acyl-HSLs suggests that the degradation of such molecules may occur in 4 different ways. Two of them lead to the degradation of the

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homoserine lactone ring mediated by lactonase or decarboxylase. Acyl-HSL-degrading enzymes have been identified as acyl-HSL lactonases and acyl-HSL acylases.

The acyl-HSL lactonase activity has also been reported in mammalian cells [8, 9]. Eukaryotic lactonases, named paraoxonases (PONs), isolated from human airway epithelia behave in a different way than the previously described bacterial enzymes [8–10]. Human serum paraoxonase 1 (hPON1, EC 3.1.8.1) is the best studied member of the family of mammalian enzymes. hPON1 is a calcium dependent serum esterase that has 354 amino acids with a molecular mass of 45 kDa. hPON1 received its name from paraoxon, the toxic metabolite of the organophosphate insecticide parathion, which is one of its most studied substrate [11, 12]. It was originally described as an enzyme capable of degrading paraoxon and other organophosphates [13, 14]. Later, it was found that PONs also play an important role in lipid oxidation and atherosclerosis [12]. More recently, all members of the PON family have been shown to possess lactonase activity [15]. Although the physiological function(s) and natural substrates for the PONs are uncertain, accumulating evidence indicates that the lactonase activity of the PONs may be its natural function [16]. hPON1 hydrolyzes the lactone ring of acyl-HSLs and the lactonase activity of the PONs extends over a number of other QS compounds with various acyl chain lengths [15]. Studies of Ozer et al. [17] have revealed a strong activity of purified hPON1 against *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) of *Pseudomonas aeruginosa*. PONs display the highest degrading activities against long chain acyl-HSLs, such as 3-oxo-C_{12}-HSL, and are less effective with short-chain acyl-HSLs [12].

As many of the human and plant bacterial pathogens employ the acyl-HSL-based QS mechanism for regulation of the virulence factors or biofilm formation for pathogenicity, the application of QQ strategy may be an alternative approach for fighting these microorganisms [18]. hPON1 as a lactonase with acyl-HSL-degrading action and QS inhibitor may be certainly used to disrupt bacterial cell-to-cell communication and to control bacterial infections by signal interference.

In view of the biological interference of acyl-HSLs with QS and the reported lactonase properties of PONs the aim of the study was to examine the in vitro hydrolysis effect of the purified hPON1 on N-hexanoyl-L-homoserine lactone (C10HSL) and N-3-oxo-octanoyl-L-homoserine lactone (3OC8HSL). The enzyme was purified by two-step procedure using ammonium sulfate precipitation and Sepharose-4B-*L*-tyrosine-1-napthylamine hydrophobic interaction chromatography. In addition, we showed that hPON1 acted as an anti-QS agent against pathogenic bacteria by signal interference.

MATERIALS AND METHODS

Chemicals, bacterial strains and growth media. Sepharose-4B, L-Tyr, 1-napthylamine, paraoxon and protein assay reagents were obtained from Sigma-Aldrich (USA). C10HSL and 3OC8HSL and all other chemicals purchased from either Sigma-Aldrich (USA) and Merck (Germany). All experiments were performed at 37°C and included at least 3 independent cultures. Test pathogens including *P. aeruginosa* ATCC27853, *Klebsiella pneumoniae* CCM2318, *Escherichia coli* ATCC11230 and *Staphylococcus aureus* ATCC6538P were maintained in 2% Luria Bertani (LB) broth containing (g/L): tryptone—10.0, 0.5% yeast extract—5.0 and NaCl—5.0 and on 1% LB agar. All cultures were incubated at 37°C for 24 h. Purified hPON1 was prepared as 10, 5, 2.5, 1.0 and 0.1 mg/mL solutions followed filter sterilization using 0.2 μm pore size filters (Sartorius Biotech. Stedim GmbH, Germany).

Purification of hPON1 by hydrophobic interaction chromatography. Human serum was isolated from 50 mL fresh human blood. The blood samples were centrifuged at 15000 g for 15 min and the 10 mL serum was used. hPON1 was isolated by ammonium sulfate fractionation at 60–80% saturation [19]. The precipitate was collected by centrifugation at 15000 g for 20 min, dissolved in 100 mM Tris-HCl buffer (pH 8.0) and subjected to hydrophobic interaction chromatography. The final saline concentration of precipitate was adjusted to 1 M ammonium sulfate, prior to that it was loaded onto the hydrophobic column prepared from Sepharose-4B-L-tyrosine-1-napthylamine [19]. The column was equilibrated with 0.1 M Na_2HPO_4 pH 8.0 including 1 M ammonium sulfate. The hPON1 enzyme was eluted with decreasing ammonium sulfate gradient (from 0 to 1 M) using 0.1 M Na_2HPO_4 (pH 8.0). The purified hPON1 was stored in the presence of 2 mM $CaCl_2$ at +4° C in order to maintain activity.

Determination of protein concentration. The protein concentration was determined by Lowry method with BSA as a standard.

Paraoxonase enzyme assay. Paraoxonase activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al. [13]. The reaction was followed for 2 min at 37°C by monitoring the appearance of *p*-nitrophenol at 412 nm in BioTek automated recording spectrophotometer (USA). Final substrate concentration of 2 mM was used during enzyme assay, all measurements were taken in duplicate and corrected for the non-enzymatic hydrolysis. One unit of hPON1 activity (U) was defined as 1 μmoL of *p*-nitrophenol formed per min under assay conditions.

SDS-PAGE. SDS-PAGE was performed in order to verify the purified enzyme. It was carried out in 12 and 3% acrylamide concentrations, containing 0.1% SDS, for the running and stacking gel respec-

tively, according to Laemmli et al. [20] using a Minigel system (Bio-Rad Laboratories, USA). Gels were fixed, stained with Coomassie brilliant blue R250, and destained using standard methods to detect protein bands. Galactosidase (116 kDa), BSA (66.2 kDa), egg albumin (45 kDa), lactate dehydrogenase (35 kDa) REase Bsp981 from E. coli (25 kDa), lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) were used as protein molecular weight standards.

Degredation of C10HSL and 3OC8HSL by HPLC analysis. 100 μM C10HSL and 3OC8HSL were separately prepared in 100 mM HEPES-NaOH (pH 7.4) [17] and 5 mM Tris-HCl (pH 7.4) [16] containing 1 mM $CaCl_2$. Scanning wavelength of each signal molecule was done against to reaction buffer. Absorbance values were used as 220 nm for 3OC8HSL and 210 nm for C10HSL. To analyze acyl-HSL degradation products, 10 mg/mL C10HSL or 100 mg/mL 3OC8HSL was added to microcentrifuge tube containing 10 mg/mL purified hPON1 in the proportion of 1 : 10 and preincubated at 37°C for 60 min. Then according to Yang et al. [21], the reaction was stopped by heating at 95°C for 3 min and the mixtures were sonicated at 0°C for 3 min by use of ultrasonic bath at maximal power (Grupo-Selecta, Spain). The hydrolized C10HSL and 3OC8HSL, as a comparable controls, were performed by incubating C10HSL in 5 mM Tris-HCl buffer (pH 7.4) and 3OC8HSL in 100 mM HEPES-NaOH buffer (pH 7.4) at room temperature for 30 min. Samples (20 μL) were chromatographed at a Agilent 10100 model HPLC system equipped with a UV/visible detector set at 220 nm for 3OC8HSL and 210 nm for C10HSL by use of Apex octadecyl 104 C18 (25 × 0.4 cm ID, with 5-μm packing) column (Agilent Technologies, Germany). Samples were eluted isocratically with water : acetonitrile : acetic acid (20 : 80 : 0.2, vol/vol/vol) at a flow rate of 1 mL/min at 30°C.

Kinetic studies of hPON1 using C10HSL and 3OC8HSL as a substrate. Kinetic studies were performed in 5 mM Tris-HCl buffer (pH 7.4) and 100 mM HEPES-NaOH buffer (pH 7.4) containing 1 mM $CaCl_2$ for different concentrations of C10HSL and 3OC8HSL, respectively. For C10HSL kinetics, 5 different concentrations of the substrate (284, 456, 571, 666 and 857 mM) were used, at which the substrate was completely soluble. For each of these concentration, the substrate was mixed with 10 mg hPON1 (reaction volume of 1 mL) and the activity was detected immediately. A 100 mL sample of the reaction mixture was taken and mixed directly with 100 mL 5 mM Tris-HCl buffer (pH 7.4) in a microtitre plate and the OD_{412} was measured spectrophotometrically. For 3OC8HSL kinetics, the substrate at 5 different concentrations (29, 46, 67, 86, 105 mM, where the substrate was soluble) was incubated with 10 mg hPON1 (reaction volume of 1 mL) for 1 min. The sample was mixed directly with 1 mL 100 mM HEPES-NaOH buffer (pH 7.4) to stop the reaction. The amount of HSL product released was quantified as described above. K_M and V_{max} values of the enzyme for each substrate were determined at pH 7.4 and 37°C by means of Lineweaver-Burk graphs. All kinetic measurements were performed at room temperature and error ranges were derived from at least 3 independent measurements.

Signal interference effect of hPON1 on growth of pathogenic bacteria. Purified hPON1 sterilized by using 0.2 μm pore size filters (Sartorius Biotech. Stedim GmbH, Germany) was tested against standard bacterial strains: *P. aeruginosa* ATCC27853, *K. pneumoniae* CCM2318, *E. coli* ATCC11230 and *S. aureus* ATCC6538P. For each bacterium, 18 wells of a 96-well microtiter plate were filled with 3 layers of bacteria. The each layer consisted of 50 μL of LB broth, 20 μL bacterial culture sample and 150 μL of purified hPON1 in range of 10–0.1 mg/mL. Positive control consisted of 150 μL 5 mg/mL BSA and negative control contained 220 μL LB broth. Microtiter plate was incubated for 24 h at 37°C. After incubation, 10 μL of tetrazolium violet metabolism indicator was added to each well. Then, microtiter plate was again incubated at 37°C for 1–4 h. The bacterial growth, the degradation of bacterial signal molecules by hPON1 was assessed by observation of the appearance of light color in the wells (image not shown). In addition, the each microtiter plate was measured at OD_{600} spectrophotometrically and determined effect of hPON1.

RESULTS AND DISCUSSION

Previous studies on the lactonase activity of the human paraoxonases has established on over 30 different non-acyl-HS type lactones [8–10]. In addition, PONs exhibit a range of other physiologically important hydrolytic activities, including drug metabolism and detoxification of nerve agents [22]. It appears that inactivation of QS signals has now become a new index to the diverse spectrum of the recognized biological functions of PONs. Draganov et al. [23] proposed that PON1 may have evolved to degrade bacterial acyl-HSLs. Hence, a QS blockade by PON1 and other members of the enzyme family may mediate a number of bacterial biofilms, virulence and inflammation processes in host organisms. In order to investigate the effect of hPON1 on acyl-HSLs as signal molecules, the enzyme was purified by ammonium sulfate precipitation and hydrophobic interaction chromatography designed for hPON1 Sinan with coworkers [19]. The enzyme was purified 324.5-fold with a final specific activity 25.41 U/mg protein (table). As seen in Fig. 1, a single band of 45 kDa was obtained, which corresponds to the previous studies [24].

We found that purified hPON1 hydrolyzed the 3OC8HSL and C10HSL (Fig. 2). To determine whether hPON1 acts as a lactonase, C10HSL and

Purification of hPON1

Fraction	Total activity, U/mL	Total protein, mg/mL	Specific activity, U/mg protein	Yield, %	Purification, -fold
Serum, crude enzyme	3315.8	74147	0.0448	100	–
Ammonium sulfate precipitation	1504.58	19215	0.0783	45.37	1.74
Hydrophobic interaction chromatography	394.21	15.51	25.41	11.89	324.52

3OC8HSL degraded by hPON1 was analyzed by HPLC. Fractionation of each of the C10HSL and 3OC8HSL standard revealed one HPLC peak, with a retention time of about 4.37 min and 5.84 min, respectively (Fig. 2a, 2c). To examine the lactone hydrolysis property of hPON1, enzyme was mixed with C10HSL and 3OC8HSL, then sonicated at 0°C for 3 min. Fractionation of hPON1 treated with C10HSL (hydrolyzed C10HSL) and 3OC8HSL revealed one major HPLC peak with a retention time of about 3.79 min and 3.45 min, respectively (Fig. 2b, 2d). hPON1 solution, which was not mixed with C10HSL and 3OC8HSL, displayed no distinct peaks (data not shown). No other peaks were apparent in the chromatograms, and all of the lactone that was hydrolyzed could be accounted for by the formation of the C10HSL acid product and 3OC8HSL acid product. These results indicated that hPON1 works as a lactonase that catalyzes acyl-HSL ring opening by hydrolyzing.

We found that the signaling activity of the 3OC8HSL and C10HSL QS signal molecules was lost when exposed to purified hPON1. To characterize the structural changes associated with this loss of activity, HPLC was used. Analysis of HPLC on a C18 column was used to determine whether exposure to hPON1 did in fact hydrolyze the 3OC8HSL and C10HSL lactone ring. In previous studies [21, 25], it was demonstrated that the lactonase(s) in mammalian sera contribute to acyl-HSL inactivation. The serum lactonase(s) opened up the lactone ring of 3OC12HSL and produced a single product identical to that hydrolyzed by the known bacterial acyl-HSL lactonase encoded by *aiiA* [21]. Also, it is known that alkaline pH promotes hydrolysis of acyl-HS lactone rings [26], so we used neutral pH to determine only effect of hPON1.

The kinetics of interaction of signal molecules with the purified hPON1 was studied. Kinetic parameters were determined for the hPON1-catalyzed conversion of C10HSL and 3OC8HSL using the Lineweaver–Burk graphs. K_M and V_{max} values were detected by means of these graphs. The K_M values of 3OC8HSL and C10HSL were calculated as 2.71 and 0.80 mM, V_{max} values were determined as 1428.57 and 45.24 μmoles mg^{-1} min^{-1}, respectively. The kinetic studies with purified hPON1 have shown that the enzyme degraded these signaling molecules quite efficiently. Previous studies showed that PONs display the highest degrading activity against long chain acyl-HSLs molecules, such as 3-oxo-C12-HSL, and are less effective with short-chain acyl-HSLs [21, 27]. Results obtained indicate that affinity of hPON1 against C10HSL signal molecule was significantly higher than towards 3OC8HSL.

It was found that paraoxonase 1 (PON1), a mammalian lactonase with an unknown natural substrate, hydrolyzed the *P. aeruginosa* acyl-HSLs and acyl-HSLs produced by other pathogenic bacterial genera, such as *Burkholderia, Yersinia, Serratia*, and *Aeromonas*. Serum PON1 prevents *P. aeruginosa* biofilm formation and bacterial growth by inactivating the QS signal demonstrating its antimicrobial role [15, 28–30]. In our study, there was a significant decrease of bacterial growth in the presence of hPON1 in range of 10–0.1 mg/mL in

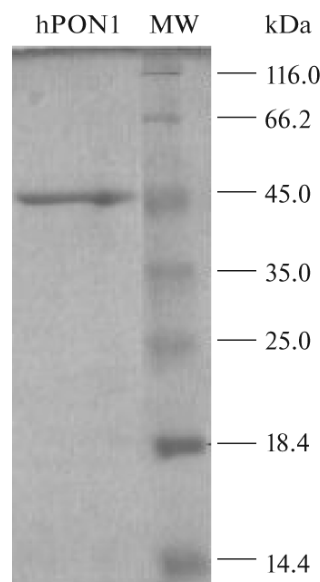

Fig. 1. SDS-PAGE of the purified hPON1. MW—protein molecular weight standards.

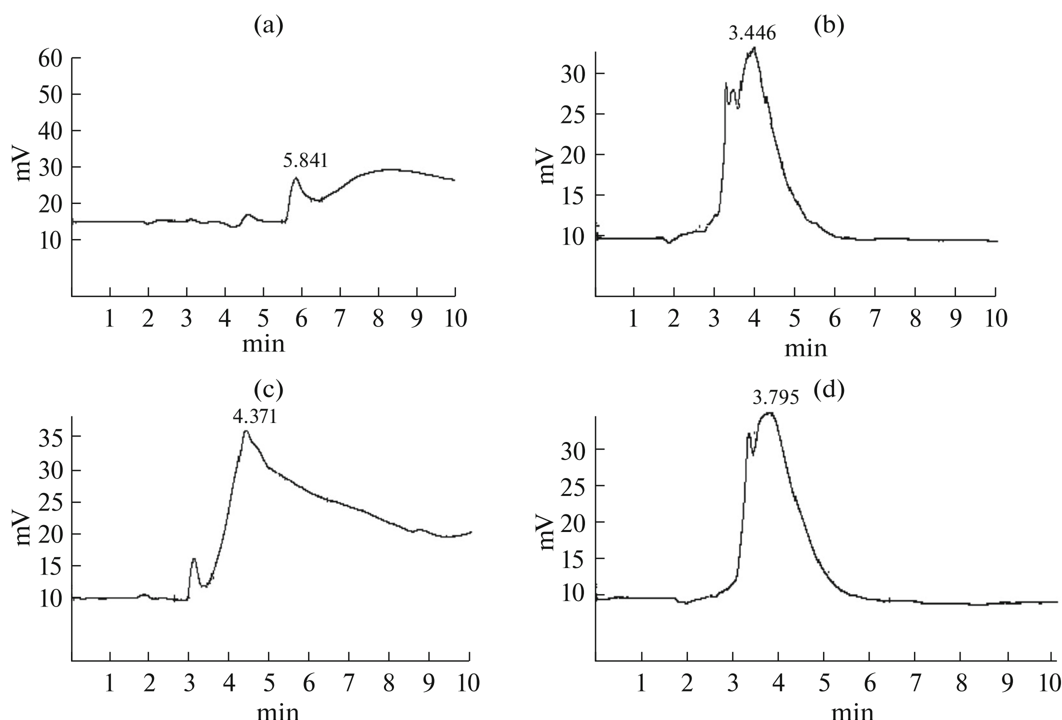

Fig. 2. HPLC chromatograms showing standard 3OC8HSL peak (a), standard C10HSL peak (c) and product peaks of 3OC8HSL (b) and C10HSL (d) incubated in the presence of purified hPON1.

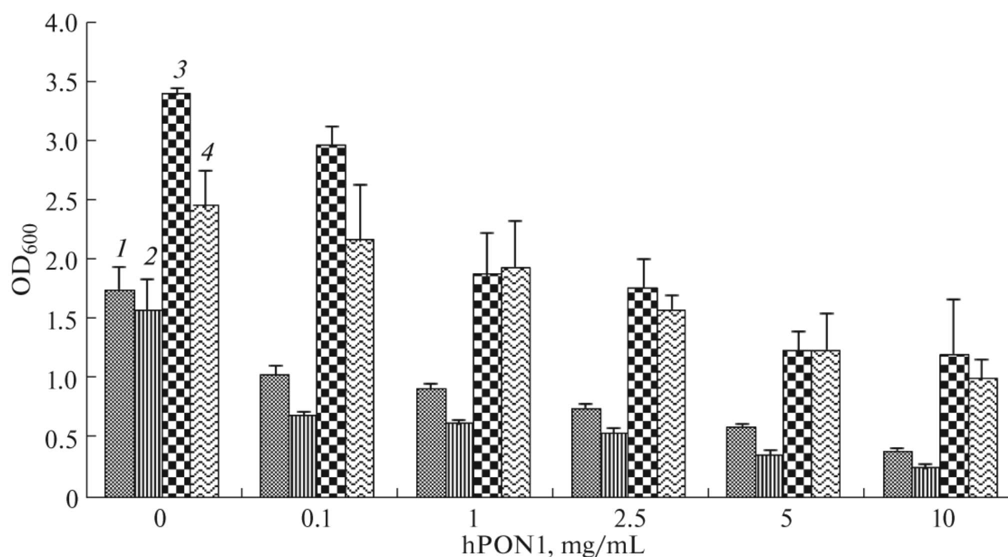

Fig. 3. Effect of hPON1 on pathogenic bacterial growth. 0.5% BSA was used as a control (0 concentration of hPON1). *1*—*K. pneumoniae* CCM2318; *2*—*S. aureus* ATCC6538P; *3*—*P. aeruginosa* ATCC27853 and *4*—*E. coli* ATCC11230. The average ±SD for 3 samples is presented in each column.

comparison to the control. The use of hPON1 resulted in a reduction on bacterial growth biomass in all used strains (Fig. 3). The acyl-HSLs hydrolyzing capability of hPON1 by reduction on bacterial growth suggests that this enzyme may function as a quorum quencher in pathogenic bacteria.

Continued research on the acyl-HSLs lactonase activities of the hPON1 will improve our understanding of the mechanisms by which the host defends against pathogenic bacteria and may result in the identification of hPON1 as an important therapeutic target.

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