Evaluation of Polymorphism at Codon 192 of Paraoxonase 1 on its Kinetic Behavior

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Evaluation of Polymorphism at Codon 192 of Paraoxonase 1 on its Kinetic Behavior

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Abstract: Human paraoxonase 1 (PON1), a High-Density Lipoprotein (HDL)-associated esterase has been implicated in slowing down the development of atherosclerosis. In the present study, kinetic and inhibition studies on PON1 were conducted to assess three parameters: the Michaelis constant (K_m) and maximal rate of metabolism (V_max) of paraoxonase and inhibition constant (K_i) of phenylacetate. Human paraoxonase 1 (PON1) activity was measured spectrophotometrically at 405 nm, using plasma samples in basal (without added NaCl) and salt-stimulated assays with 1 M NaCl. Inhibition studies were performed using phenylacetate as an inhibitor of PON1 in basal assays, pH 8.0. Estimates of K_m and V_max were obtained from the Lineweaver-Burk plot. Estimates of K_i were obtained from the secondary plot of apparent K_m (K_m-app) versus inhibitor concentration. The parameter values were evaluated for the genotypes PON1_{192QQ, QR, RR}. In salt-stimulated assays, the V_max increased two-fold for the PON1_{192QQ} samples and three to five-fold for the PON1_{192QR} samples compared with basal assays. Similarly, it increased four-fold for PON1_{192RR} samples. Michaelis constant (K_m) was comparable with or without 1.0 M NaCl across the three genotypes. The Lineweaver-Burk and Dixon plots revealed that phenylacetate was a predominantly competitive inhibitor exhibiting linear mixed type inhibition. The K_i values were also comparable across the three genotypes. We conclude that the three kinetic parameters of PON1 in the Malaysian population estimated in the present report were comparable with those reported by other studies on PON1 from Caucasian populations.

Key words: Enzymatic activity, inhibition constant, maximal rate, Michaelis constant

INTRODUCTION

Human paraoxonase 1 (PON1, EC 3.1.8.1) catalyzes the hydrolysis of paraaxon to the nontoxic products, p-nitrophenol and diethylphosphoric acid (Costa et al., 2005). PON1 has been implicated as a predictor of coronary heart disease (Roest and Voorbij, 2008). Human paraoxonase 1 (PON1) activity towards paraaxon (POase), is distributed trimodally (with respect to the polymorphism at codon 192) (Jarvik et al., 2003). In Caucasian populations, the frequency of the PON1_{192R} allele is lower (Hofer et al., 2006) compared to Asian populations such as the Malay, Chinese and Korean populations (Poh and Muniandy, 2007; Zhang et al., 2006). The estimation of enzymatic characteristics of PON1 has been done by kinetic studies. Kinetic and inhibition studies on PON1 have been reported in a number of studies using initial reaction rates to assess three common parameters: the Michaelis constant (K_m), maximal rate of metabolism (V_max) of PON1 and inhibition constant (K_i) of phenylacetate as an inhibitor of paraoxon (Du et al., 2001; Eckerson et al., 1983b). These parameters were usually evaluated based on the PON1 genotypes in previous studies and differences in the three parameters with respect to the genotypes have been reported by Geldmacher-von Mallinckrodt et al. (1979) and Gan et al. (1991). In addition, when sodium chloride was added to the kinetic assay, the V_max of PON1 was consistently higher compared to basal assay conditions without sodium chloride (Smolen et al., 1991). The Q and R isozymes showed differences in K_m values for paraoxon (Smolen et al., 1991). Phenylacetate was shown to act as an inhibitor of PON1 activity towards paraoxon and the inhibition was of the mixed type (Eckerson et al., 1983b) although kinetic studies did not involve secondary plots. In inhibition studies for a one-substrate reaction, it is assumed that catalysis occurs via, the formation of a complex between enzyme, E and substrate, S. This complex breaks down to give product, P and regenerate E. The scheme below assumes that the inhibitor-containing complexes were in equilibrium with each other. K_{ED}, K_{EI}
and $K_m$ represent dissociation constants of ES, ESI and EI, respectively, while $k_2$ represents the conversion of ES to E and P.

\[ \frac{1}{v} = \frac{1}{V_{max}} \left[ 1 + \frac{[I]}{K_{EI}} \right] \left[ 1 + \frac{[I]}{K_{EI}} \right] \left[ 1 + \frac{[S]}{[S]} \right] \]  

(1)

In its reciprocal form, a general kinetic equation was derived as shown in Eq. 1.

In competitive inhibition, assuming that $K_{EI} = 8$ (the ES complex cannot combine with inhibitor, I nor the EI complex with S), then Eq. 1 is reduced to:

\[ \frac{1}{v} = \frac{1}{V_{max}} \left[ \frac{K_{ES}}{V_{max}} \left[ 1 + \frac{[I]}{K_{I}} \right] \right] \left[ 1 \right] \left[ [S] \right] \]  

(2)

The intercept at the abscissa of this type of plot would then be represented by $-1/K_m \left(1 + [I]/K_{II}\right)$. If inhibition is of the mixed type with predominant competitive traits, the intercept would be:

\[ \frac{1}{v} = \left[ \frac{1 + [I]K_m}{K_m + 1 + [I]} \right] \left[ \frac{V_{max}}{V_{max}} \right] \left[ 1 + \frac{[I]K_{EI}}{K_{EI}} \right] \]  

where, $K_{II}^*$ is the Michaelis constant modified in presence of excess inhibitor, as shown in Eq. 3 below:

\[ \frac{1}{v} = \left[ \frac{1}{V_{max}} \right] \left[ 1 + \frac{[I]K_{EI}}{K_{II}^*} \right] \left[ 1 + \frac{[I]}{K_{II}^*} \right] \left[ 1 + \frac{[I]}{K_{II}^*} \right] \left[ [S] \right] \]  

(3)

In this study, the kinetics of human serum PON1 was investigated by measuring initial rates of hydrolysis to observe the effects of PON1 polymorphisms on the kinetic behavior of PON1.

**MATERIALS AND METHODS**

Kinetic and inhibition studies on PON1 were conducted in the current investigation to assess three parameters: the Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) of PON1 and inhibition constant ($K_I$) of phenylacetate. The velocity of PON1 was determined by measuring the apparent rates of reaction spectrophotometrically (Copeland, 2000). Samples displaying high PONase activity within the highest tertile were selected for the PON1 H374R group, whereas those with low PONase activities well within the lowest tertile were selected for the PON1 H374Q group as suggested by Mueller et al. (1983) in order to clearly define the kinetic values based on genotype.

PON1 catalyses the following hydrolysis reaction:

\[ \text{Paraoxon} \xrightarrow{\text{PON1}} \text{p-Nitrophenol + diethylphosphoric acid} \]

The rate of hydrolysis of paraoxon was monitored by measuring the liberation of the product p-nitrophenyl phosphate at 405 nm.

**Spontaneous hydrolysis:** A blank determination of basal assay mixture (containing TrisCl, pH 8.5 and CaCl$_2$) without plasma was first performed to observe spontaneous hydrolysis of paraoxon. Plastic rectangular cuvettes of 1.0 cm light path were used. All measurements were conducted at room temperature (21.2±1°C). Paraoxon-ethyl-PESTANAL or paraoxon (Sigma Chemical Co., St. Louis, MO) was added to 1.0 mL basal assay buffer (without added NaCl), pH 8.5, in a 1.5 mL microfuge tube to a final concentration of 1.0 mM. Paraoxon was prepared fresh every hour. One milliliter of stock 1.0 mM paraoxon solution was placed in a plastic cuvette containing the assay buffer. The cuvette was covered with a 0.5 square piece of parafilm and inverted 5 times to mix. The cuvette was immediately placed in a UV-Vis spectrophotometer (Varian Cary 50, Varian Inc. Scientific, California). The measurement of activity was performed by using the Kinetics application of the Cary WinUV Version 3.00 software.

**Estimation of $K_m$ and $V_{max}$:** Frozen stored plasma samples were thawed, mixed and centrifuged at 13,000 x g for 1 min to remove particulate matter prior to use in kinetic studies. The substrate dependence of PON1 velocity was determined from the initial rates of hydrolysis run at different paraoxon concentrations. The experiments were run both in the absence and presence of 1 M NaCl, pH 8.5. Substrate: Paraoxon was added to 1.0 mL assay buffer in a 1.5 mL microfuge tube to a final 1.0 mM. The tube was capped securely and shaken vigorously. As before, paraoxon was prepared fresh and used within 1 h.

- **Sample:** The plasma sample was diluted 1:10 in dilution buffer.

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• **Procedure**: Stock 0.01 to 0.2 mL of 1.0 mM paraaxon solution was first placed in a plastic cuvette containing 0.89 to 0.70 mL of the assay buffer. The diluted plasma was then introduced to the paraaxon-assay buffer solution, mixed and measured spectrophotometrically. The initial rate of hydrolysis was measured at 405 nm at room temperature, with readings continuously taken for 2 min. The initial linear rates were used in calculations for POase activity. The accuracy of the computerized calculations was evaluated by the method of Richter et al. (2004). One unit of enzyme activity is expressed as 1 μmol of product/min and reported as U/L. PON1 velocity at different substrate concentrations were used to generate the double reciprocal Lineweaver-Burk plot of 1/v versus 1/[S], where [S] is the concentration of paraaxon in mM and v is the velocity in U/L. Estimates of K_M and V_max were obtained directly from the plot. As paraaxon is a potent cholinesterase inhibitor, all paraaxon-containing waste was hydrolyzed in 10 M NaOH solution overnight and washed down with copious amounts of water (Mueller et al., 1983).

**Inhibition studies**: Pheny lacetate was used in inhibition studies of PON1. As NaCl inhibits arylesterase activity, a blank determination without both salt and plasma was performed to observe the spontaneous hydrolysis of the diluted phenylacetate solution (Senti et al., 2001). Inhibition studies were then performed using phenylacetate (Sigma Chemical Co., St. Louis) as an inhibitor of PON1. To prepare the inhibitor stock solution to a final concentration of 10 mM, phenylacetate was dissolved in basal assay buffer, pH 8.0 by continuous stirring for 2 h. Stock solutions of phenylacetate (1, 0.5, 0.3 and 0.2 mM) were then prepared. Stock phenylacetate inhibitor solution was placed in a plastic cuvette containing the assay buffer. Paraaxon solution (1.0 mM) was then added. Finally, the diluted plasma was introduced to the assay mixture, mixed and monitored spectrophotometrically. PON1 activity with paraaxon as the substrate in the presence of phenylacetate as an inhibitor was calculated. The extinction coefficient at pH 8.0 was 17 mM/cm as used in the calculations (Richter et al., 2004). The calculated PON1 activity was used to generate the Lineweaver-Burk plot to yield apparent K_M (K_M,app) values at each inhibitor concentration. The k value was determined from a secondary plot of K_M,app versus inhibitor concentration. The mode of inhibition was determined by using the Dixon plot (Dixon and Webb, 1964) of 1/v versus inhibitor concentration.

**Statistical analysis**: Comparisons of three or more subgroups were performed by using either one-way Analysis of Variance (ANOVA) or the nonparametric Kruskal-Wallis test. Analysis of Variance (ANOVA) was used in the analysis of data when equal variance was assumed whereas Kruskal-Wallis test was used for data when unequal variance was assumed. In case of significant difference between groups, the post hoc analysis was used to identify, which group(s) of samples differed significantly from the others. Tukey's post hoc test was performed if the variances were similar, whereas Dunnett's T3 post hoc test was used when variances were different. Statistical analyses in general were performed using Statistical Package for the Social Sciences (SPSS) 11.0 for Windows software (SPSS, 2002). A p-value of <0.05 was taken to be statistically significant.

**RESULTS AND DISCUSSION**

**Spontaneous hydrolysis**: Table 1 shows that rates of spontaneous hydrolysis for paraaxon and phenylacetate were negligible in a typical experiment.

**Estimation of K_M and V_max**: The increase in absorbance due to PON1 activity was linear for at least 2 min of the assay duration. The linearity allowed for the calculation of activity based on initial rates. POase activities for typical samples from the PON1_192QQ-OR and 1R genotypes are shown in Fig. 1. The selected PON1_192RR samples had high POase activity, whereas PON1_192QQ samples had low POase activity. PON1_192Q samples had intermediate activity.

The double reciprocal Lineweaver-Burk plots for estimation of K_M and V_max for PON1_192QQ, PON1_192OR, and PON1_192RR samples are collectively shown in Fig. 2 for comparison.

**Effect of 1.0 M NaCl on K_M and V_max**: The K_M values obtained from assays with or without 1.0 M NaCl were comparable as the differences were not significant (paired samples t test, p = 0.510). There was significant correlation between the basal and salt-stimulated K_M

<table>
<thead>
<tr>
<th>Type of substrate</th>
<th>Rate of spontaneous hydrolysis, Mean±SD (U L⁻¹)</th>
<th>Rate of hydrolysis in presence of NaCl (U L⁻¹)</th>
<th>Difference* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylacetate</td>
<td>0.0020±0.00025</td>
<td>73</td>
<td>6.2×10⁻⁴</td>
</tr>
<tr>
<td>Paraaxon</td>
<td>0.00033±0.00014</td>
<td>700</td>
<td>4.7×10⁻⁴</td>
</tr>
</tbody>
</table>

*The percentage of difference is calculated as follows: (Rate of Hydrolysis in presence of NaCl - Rate of spontaneous hydrolysis)/Rate of Hydrolysis × 100%
Table 2: \( K_m \) values in comparison with three genotype

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Basal*</th>
<th>1.0 M NaCl†</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ</td>
<td>0.576±0.182</td>
<td>0.328±0.175</td>
</tr>
<tr>
<td>QR</td>
<td>0.335±0.092</td>
<td>0.314±0.028</td>
</tr>
<tr>
<td>RR</td>
<td>0.454±0.224</td>
<td>0.584±0.357</td>
</tr>
</tbody>
</table>

Values are Mean±SD (range). One-way ANOVA, *p = 0.383, †p = 0.512

Table 3: \( V_{max} \) in comparison with three genotype

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Basal*</th>
<th>1.0 M NaCl†</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ</td>
<td>12.1±25</td>
<td>25.3±29</td>
</tr>
<tr>
<td>QR</td>
<td>19±43</td>
<td>74±29</td>
</tr>
<tr>
<td>RR</td>
<td>294±102</td>
<td>1197±247</td>
</tr>
</tbody>
</table>

Values are Mean±SD (range). One-way ANOVA, *p = 0.028, †p<0.001

was not significant (one-way ANOVA, p = 0.383 and 0.512 for basal and salt-stimulated assays, respectively).

The \( V_{max} \) value was increased significantly according to the PON1 Q2R polymorphism in the following order (Table 3). QQ<QR<RR, in both basal and salt-stimulated assays. The salt-stimulated assay showed a higher degree of increment in activity compared to the basal assay.

**Inhibition studies:** In the Lineweaver-Burk plot, straight lines at different fixed inhibitor (phenylacetate) concentrations intersected at a common intersection point at the y intercept (Fig. 3a-c) for all three PON1 Q2R genotypes, a characteristic signature of a competitive inhibitor.

The Lineweaver-Burk plots were used to estimate \( K_m \) to build the secondary plot in the determination of \( K_i \) as shown simultaneously in Fig. 4 for all three genotypes.

The mean \( K_i \) values for PON1 Q2Q2, Q2R, R2R genotypes determined from secondary plots of \( K_m \) versus phenylacetate concentration were as follows: 0.25±0.14 (0.13-0.44) mM, 0.27±0.22 (0.12-0.42) mM and 0.40±0.08 (0.31-0.49) mM, respectively (Fig. 5). The difference in \( K_i \) between genotypes was not significant (ANOVA, p = 0.308).

Dixon plot (1/v versus phenylacetate concentration) lines at different fixed paraxox concentrations intersected in the second quadrant (Fig. 6a-c).

A replot of the Dixon plot slopes vs. phenylacetate concentration passed close to the origin for all three PON1 Q2Q2, Q2R, R2R genotypes (Fig. 7).
Kinetic studies may be an approach that may be used to characterize PON1, based on quantitative measurements of the rate of hydrolysis of paraoxon (Dixon and Webb, 1964). Inferences may be made about the mechanism of PON1 action.

**Spontaneous hydrolysis:** Spontaneous hydrolysis of paraoxon and phenylacetate apparently were shown to be insignificant in this study. Thus, assays for PON1 activity were not routinely corrected for spontaneous hydrolysis. In this respect, it is relevant to note that Eckerson et al. (1983a) showed that spontaneous or nongenotypic hydrolysis may be considered negligible except in samples having extremely low activity.

**Estimation of $K_m$ and $V_{max}$ values:** When the effect of NaCl on $K_m$ was explored, it was seen that the apparent $K_m$ value was comparable with or without 1.0 M NaCl for all three genotypes of PON1 $192_{QQ}$ polymorphism. Although $K_m$ values for low PON1 activity samples (QQ) were on average slightly higher than for high (RR) or intermediate (QR) activity samples (QR), the apparent difference in $K_m$ values between genotypes was not significant. In
addition, the difference within the genotype was not significant either. This is in agreement with Mueller et al. (1983), who demonstrated that $K_m$ was constant for the homozygotes for both low- and high-activity alleles. A slight increase in salt-stimulated $K_m$ of PON1_{192RR} samples compared to the basal counterpart was also observed. On the other hand, a slight decrease in $K_m$ was observed in the PON1_{192QQ} samples. The slightly higher $K_m$ values of PON1_{192QQ} samples compared with PON1_{192RR} samples concurred with the study by Mueller et al. (1983). However, Eckerson et al. (1983a) showed that $K_m$ values of PON1_{192QQ} samples were lower compared to PON1_{192RR} samples. Hence, various studies have reported minor differences in both genotypes with respect to $K_m$ as shown in Table 4. Although, Sotloen et al. (1991) concluded that the Q and R alleles showed differences in $K_m$ values for paraoxon, the observation was not supported by statistical analysis.

When the effect of NaCl on $V_{max}$ was examined, it was shown that NaCl significantly increased $V_{max}$ by two- to five-fold. This observation has been attributed to either an increased number of active sites or increased turnover number of the enzyme, but not to a change in the binding constant or concentration of paraoxon as the effect of salt has a rapid onset and is also rapidly reversible (Eckerson et al., 1983a).

**Inhibition studies:** In purely competitive inhibition, the Lineweaver-Burk plots show straight lines at different fixed inhibitor concentrations intersecting at a common point at the y intercept. However, as the mode of inhibition of phenylacetate is of the mixed type, the apparent $K_m$ was increased by a factor:

$$
\frac{1 + \left(\frac{[I]K_m}{V_{max}}\right)}{K_m + \left(\frac{[I]}{K_i}\right)}
$$

The inhibitor, in effect, causes the formation of the EI or PON1-phenylacetate complex. The slopes of the lines, given by $K_{m,ref}/V_{max,ref}$ vary among the lines because of the effect imposed on $K_m$ by the inhibitor the degree of perturbation of $K_m$ depending on the value of $K_i$.

Initial evaluation suggested that the type of inhibition was predominantly competitive. This was
Table 4: Comparison of $K_m$, $V_{max}$ and $K_v$ values

<table>
<thead>
<tr>
<th>Authors</th>
<th>Samples</th>
<th>$K_m$ (mM) (pH 8.5)</th>
<th>$V_{max}$ (UL⁻¹) (pH 8.5)</th>
<th>$K_v$ (mM) (pH 8.0)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>Salt</td>
<td>Basal</td>
<td>Salt</td>
</tr>
<tr>
<td>Eckerson et al. (1983a, b)</td>
<td>High activity (B)</td>
<td>0.460</td>
<td>0.460</td>
<td>744</td>
<td>1670</td>
</tr>
<tr>
<td></td>
<td>Low activity (A)</td>
<td>0.430</td>
<td>0.430</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mueller et al. (1983)</td>
<td>High activity</td>
<td>0.420 (0.3-0.4)</td>
<td>-</td>
<td>420</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Low activity</td>
<td>0.460 (0.35-0.75)</td>
<td>-</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>Smolen et al. (1991)</td>
<td>High activity</td>
<td>0.271</td>
<td>0.21</td>
<td>659</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Low activity</td>
<td>0.503</td>
<td>0.47</td>
<td>344</td>
<td>-</td>
</tr>
<tr>
<td>Gan et al. (1991)</td>
<td>High activity</td>
<td>0.271</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Davies et al. (1986)</td>
<td>Low activity</td>
<td>0.503</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>High activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1790</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>977</td>
</tr>
<tr>
<td></td>
<td>Low activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>328</td>
</tr>
<tr>
<td>Billecke et al. (2000)</td>
<td>High activity, R</td>
<td>-</td>
<td>741&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Low activity, Q</td>
<td>-</td>
<td>870&quot;)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zhu et al. (2006)</td>
<td>Serum-purified hPON1</td>
<td>0.54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PON1 variants from E. coli</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>This study</td>
<td>High, RR</td>
<td>0.45</td>
<td>0.58</td>
<td>294</td>
<td>1197</td>
</tr>
<tr>
<td></td>
<td>Intermediate, QR</td>
<td>0.35</td>
<td>0.31</td>
<td>194</td>
<td>742</td>
</tr>
<tr>
<td></td>
<td>Low, QQ</td>
<td>0.58</td>
<td>0.53</td>
<td>121</td>
<td>253</td>
</tr>
</tbody>
</table>

shown by the extrapolating lines of different fixed paraoxon concentrations in the Dixon plot. These intersected in the second quadrant, thus excluding competitive or uncompetitive mechanisms and in agreement with the earlier studies by Eckerson et al. (1983b) and Gan et al. (1991).

Further analysis revealed that the replot of the Dixon plot slopes vs. the reciprocal of paraoxon concentration passed slightly over origin, confirming the previous observation that the inhibition was not purely competitive but of the linear mixed type. Gan and Eckerson’s studies support the presence of a single enzyme in the human serum which accounts for both PONase and arylerase activities.

The $K_v$ determined in the present study for phenylacetate was relatively low when compared to other studies as shown in Table 4. Gan et al. (1991) reported $K_v$ in the range of 0.401-0.885 mM whereas Eckerson et al. (1983b) reported 0.55-0.77 mM. A trend that was apparent was that the $K_v$ values decreased from high- to low-activity genotypes although the differences were not statistically significant.

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