Horseradish peroxidase catalyzed and electrochemical oxidations of 2-thiouracil—A comparison

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Horseradish peroxidase (type VIII) catalyzed and electrochemical oxidations of 2-thiouracil have been studied in phosphate buffer of pH 7.20 (μ=1.0 M) at an ambient temperature of 25±2°C. The peroxidase catalyzed oxidation has been initiated by using hydrogen peroxide and electrooxidation carried out at pyrolytic graphite electrode. The UV-absorbing intermediates generated in both the oxidations have been characterized by voltammetry, spectrophotometry and by kinetics of decay and are found identical. Products of enzymic and electrochemical oxidations have been characterized by using GC-MS studies. A tentative redox scheme has been proposed for the enzymic oxidation of 2-thiouracil and compared with that of electrooxidation and has also been found similar. Thus, it has been concluded that oxidation of 2-thiouracil by enzymic and electrochemical methods are, in a chemical sense, identical.

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Nucleic acid bases with sulfur atom have been detected in natural t-RNA1 and therefore have attracted considerable interest during last decade. 2-Thiouracil (I) and its derivatives have been found to induce modifications in thyroid gland and are claimed as an antithyroid drugs2,3. Two well known thiouracil antithyroid drugs, 6-propyl-2-thiouracil and 6-methyl-2-thiouracil, have also been shown to be competitive inhibitors of neuronal nitric oxide synthase4. 2-Thiouracil and its derivatives have also been used as antitumor agents5,6.

It has been reported that electrochemical and horseradish peroxidase catalyzed oxidation of uric acid7 and N-methylated uric acids8 appear to proceed, in a chemical sense, by very similar if not identical mechanisms at around physiological pH. As electrochemical investigations of biologically important compounds provide uniquely invaluable insights about the redox mechanisms of the enzymic reactions, it was considered interesting to study the peroxidase catalyzed oxidation of 2-thiouracil and compare the results with that of electrochemical oxidation9. In particular, we were interested in knowing if the detailed mechanism elucidated from electrochemical studies at pyrolytic graphite electrode was applicable to the enzyme catalyzed oxidation of 2-thiouracil also. It is expected that results of these studies will provide insights into overall chemical pathways followed by the substrate molecule in the enzymic oxidation reaction.

Materials and Methods

2-Thiouracil was procured from Sigma Chemical Co., USA and was used as received. Horseradish peroxidase (HRP) type VIII (Rz >3.0) was obtained from Bangalore Genei Pvt. Ltd., and catalase was supplied by CSIR Centre for Biochemicals, New Delhi, India. Hydrogen peroxide (30% w/v) was a product of Merck. Silylation grade acetonitrile (CH3CN) and N,O-bis[trimethylsilyl]trifluoroacetamide (BSTFA) were supplied by Supelco, USA. Phosphate buffers of ionic strength 1.0 M were prepared from analytical grade chemicals (NaH2PO4 and Na2HPO4) in double distilled water.

Conventional electrochemical equipments11,12 were used to carry out cyclic voltammetric studies. The numbers of electrons (n) involved in electrooxidation reaction were determined by monitoring the exponential decay of the current-time curve as reported by Lingane13. UV/Vis spectral changes during electrooxidation were monitored by electrolyzing 0.2 mM 2-thiouracil in a H-cell using pyrolytic graphite plate (1×6 cm2) as working, cylindrical platinum gauze as auxiliary and Ag/AgCl
as reference electrodes respectively. All potentials are referred to Ag/AgCl electrode at 25± 2°C. The pH of the phosphate buffer was adjusted to 7.2 using Century India Ltd. digital pH-meter (model CP-901) after due standardization. UV studies of the enzymic oxidation were carried out in 1.0 cm optically matched quartz cells using Perkin-Elmer Lambda-35 UV/Vis spectrophotometer. Gas chromatography-mass spectrometry (GC-MS) of the silylated samples were carried out by using Perkin-Elmer (model Clarus-500) mass spectrometer.

Stock solutions of the peroxidase (4 μM), hydrogen peroxide (0.6 mM) and 2-thiouracil (0.2 mM) were prepared fresh each day in a phosphate buffer of pH 7.2. Normally, 1.0 ml of stock solution and 0.5 ml of the peroxidase solution were mixed in a 1.0 cm quartz cell and the enzymic oxidation was initiated by the addition of 0.5 ml H₂O₂ solution to it. Cyclic voltammograms of the resulting solution were recorded by initiating first sweep towards negative potential rather than positive potential and at different time intervals. The course of enzymic oxidation was also monitored by repetitively recording UV spectra of the solution at different times. The reference cell of the spectrophotometric studies contained buffer, hydrogen peroxide and enzyme contained in the sample cell but did not contain 2-thiouracil. In second set of experiment, when absorbance at λmax reduced to ~50%, the enzymic oxidation was terminated by addition of catalase solution (1 mg/ml), also prepared in the phosphate buffer. The kinetics of decay of UV-absorbing intermediate generated during the enzymic oxidation was followed at a single preselected wavelength with the absorbance readings generally taken every 5.0 s. Values of rate constant (k) for the kinetic decay, were calculated from linear log(A–Aₑ) vs time plots.

Bulk electrolysis of 10-12 mg of 2-thiouracil in the phosphate buffer pH 7.2 [100 ml] was carried out at a potential 100 mV more positive than peak potential of peak Iₐ, observed during the voltammetric studies. The cell and electrodes used for these studies were essentially similar to the ones used for UV studies. The progress of electrolysis was monitored by recording cyclic voltammograms at different time intervals. When oxidation peak (Iₐ) disappeared in the CV, the electrooxidized solution was filtered and lyophilized. The freeze-dried material was extracted with methanol (AR grade, 2×10 ml) and the methanolic extract was concentrated and dried under reduced pressure. For identification of major products formed on enzymic oxidation, 4.0 ml of stock solution was mixed with 2.0 ml of HRP and 2.0 ml of H₂O₂ solutions, in a small beaker (10 ml). The solutions were mixed thoroughly with the help of a glass rod. The resulting solution was left as such with occasional stirring for 1 h and later worked up as described for the electrooxidized sample.

Silylation of the oxidized samples were carried out in Reacti-vials. Approximately 50 μg of oxidized sample, acetonitrile (50 μl) and BSTFA (50 μl) were taken in a vial. The vial was carefully sealed using Teflon coated stopper and heated at 120°C in an oil bath for 10-15 min with constant shaking. After cooling to room temperature, 5.0 μl of the silylated product was injected in GC-MS and spectrum was recorded.

**Results and Discussion**

**Voltammetric study**

**Electrochemical oxidation**

A typical cyclic voltammogram of 0.2 mM solution of 2-thiouracil in the phosphate buffer is shown in Fig.1A. It exhibits a single well defined oxidation peak (Iₐ) at potential 315 mV during initial sweep and at a scan rate of 100 mV/s. In the reverse sweep cathodic peak (IIₑ) was observed at -245 mV potential. Peak potentials (Eₚ) of the peaks Iₐ and IIₑ were linearly dependent on pH according to the relationships:

$$Eₚ(1.95-11.08) = (816.17-44.34 \, \text{pH}) \, mV \, vs \, Ag/AgCl$$

... peak Iₐ

$$Eₚ(1.95-7.2) = (46.59-37.28 \, \text{pH}) \, mV \, vs \, Ag/AgCl$$

... peak IIₑ

The linear regression analysis indicated correlation coefficients of 0.9771 and 0.9634 respectively. If the initial direction of sweep is kept negative, peak IIₑ is not observed. This observation indicates that the peak IIₑ is due to the reduction of species generated in peak Iₐ reaction. It has been reported⁹ that the peak current of peak Iₐ was linear with increase in concentration of 2-TU in the range 0.1-1.8 mM, whereas at higher concentrations the peak current became constant. The variation of peak current function (Iₚ/ACₚ已经有了) with increase in sweep rate (5 to 500 mV/s) further revealed strong adsorption of 2-TU at the surface of PGE. Thus, it is believed that at 0.2 mM concentration of 2-thiouracil, the process will be purely diffusion controlled.
Fig. 1—Cyclic voltammograms at PGE of 0.2 mM 2-thiouracil at pH 7.2. [Curve (A), electrochemical oxidation and curve (B) enzymic oxidation using 4 μM horseradish peroxidase; Sweep rate: 100 mVs−1. Potential was scanned from 0 → −500 → +500 → −400 mV vs Ag/AgCl].

In coulometric experiments, the number of electrons (n) involved in the electrooxidation of 2-TU at pH 7.2, were calculated from the graphical integration of the current–time curve. The plots of peak current vs time were exponential for first 15 min of electrolysis and thereafter deviation from straight line was noticed. This deviation clearly indicates that for the first 15 min the electrode reaction followed a simple path and thereafter subsequent chemical reactions played an important role. Similar observation has also been reported by Cauquis and Parker for charge transfer reactions coupled with follow-up chemical reactions, i.e. EC reaction. The average experimental value of n at pH 7.2 was 6 ± 0.25.

Enzymic oxidation

As peak IIc in the electrooxidation was assigned to the reduction of species generated in peak Ia reaction, an attempt was made to observe peak IIc during the enzymic oxidation of 2-thiouracil. For this purpose voltammograms of the enzymic oxidation solution were recorded at different time intervals by initiating the negative sweep. Cyclic voltammograms recorded from 0 V to -500 mV did not show peak IIc. However, the peak at -245 mV on CV recorded during the enzymic oxidation from positive to negative potentials increased with increasing duration of the oxidation. The Epc of peak IIc obtained using a solution after 2 min oxidation with peroxidase, was very similar to that obtained during electrochemical oxidation of 2-thiouracil (compare Fig. 1B and 1A). Thus, a species reducible at -245 mV in peak IIc is evidently identical with the species generated by the enzymic oxidation of 2-thiouracil. After 10 min of addition of hydrogen peroxide, peak Ia completely disappeared indicating that 2-thiouracil taken in the cell has been completely oxidized.

Spectrophotometric study

Changes of UV-absorption spectra recorded as a function of time during the electrochemical and enzymic oxidation of 0.2 mM 2-thiouracil in the phosphate buffer were used to monitor formation of UV-absorbing intermediates and their decay.

Electrochemical oxidation

At pH 7.2, a 0.2 mM solution of 2-thiouracil exhibited two well defined absorption bands at λmax=270 and 210 nm and a shoulder at 285 nm, which is represented by curve 1 (Fig. 2A). Upon application of a potential 100 mV more positive than peak potential of peak Ia, absorbance in the region 320-250 nm and 225-200 nm systematically decreased while, absorbance in the region 225-250 nm systematically increased. Curve 10 (in Fig. 2A) was recorded at 60 min. of electrolysis; the peak at 225 nm completely disappears and a new band at 230 nm appears. No clear isosbestic point was noticed during spectral changes.

In another set of experiments, after electrolyzing the solution of 2-thiouracil for 30 min (corresponding to curve 5 in Fig. 2A), potentiostat was open circuited and UV spectra were recorded at different time intervals. It was observed that, absorbance in the region 320-200 nm decreased systematically due to decay of intermediate generated during electrooxidation. Thus, the intermediate is generated and decayed due to involvement of subsequent chemical reactions. Kinetics of decay of the intermediate was monitored by recording absorbance at a selected wavelength (270, 240 and 210 nm). Time versus absorbance profile showed an exponential decay (Fig. 3A) and the plots of log(A−A∞) vs time were linear (A∞ and A are absorbance at infinite time and at reaction time respectively). Values of rate constant (k) were calculated from the slope value of
linear best fit equation for the log$(A-A_{oo})$ vs time plots.

Enzymic oxidation

Horseradish peroxidase catalyzed oxidation of 2-thiouracil was carried out in a conventional 1 cm quartz spectrophotometer cell. When a UV spectrum was recorded after addition of hydrogen peroxide to the mixture of stock solution and the peroxidase, two absorption bands at $\lambda_{max}$ =270 nm and 210 nm and a shoulder at 285 nm (curve 1 in Fig. 2B) were noticed. Curves 2-8 (in Fig. 2B) were recorded after different time intervals of enzymic oxidation. It was noticed that during the enzymic oxidation, absorbance in the region 320-250 nm systematically decreased. A systematic increase in absorbance in the region 225-200 nm was also noticed. The absorbance region 200-220 nm was distorted due to absorption of HRP-H$_2$O$_2$ complex. This has also been reported by Zaton and de Aspuru. When the absorbance at 270 nm reached ~50%, enzymic reaction was quenched by addition of catalase(1 mg/ml) and kinetics of decay of the UV-absorbing intermediate was studied. The change in absorbance was monitored at different wavelengths (270, 240 and 210 nm). Time versus absorbance profile showed an exponential decay (Fig. 3B) similar to that observed during electrooxidation. The plots of log$(A-A_{oo})$ versus time were linear. Values of rate constant $k$ were calculated from the slope value of log$(A-A_{oo})$ versus time plots. Values of $k$ observed for the decay of the intermediate of 2-thiouracil generated during both the oxidations is represented in Table 1.

From Table 1 it is clear that the rate constant values for both, electrochemical as well as enzymic, oxidation of 2-thiouracil are same. The non-absorbance of $k$ value at 210 nm during enzymic oxidation is probably related to the fact that composition of the enzyme solution was somewhat different. In the case of the enzymic oxidized solution, significant amounts of HRP and catalase were present which absorb strongly at 210 nm$^{6,7}$.

Product identification

Electrochemical oxidation

Attempts to separate different constituents (TLC $R_f$, 0.30, 0.45 and 0.52) and to determine molar mass of
Table 1—A comparison of rate constant (k) values for the decay of UV-absorbing intermediate generated during electrochemical and enzymic oxidations of 0.2 mM 2-thiouracil at pH 7.2

<table>
<thead>
<tr>
<th>λ, nm</th>
<th>Electrochemical</th>
<th>Enzymic</th>
</tr>
</thead>
<tbody>
<tr>
<td>270</td>
<td>0.26 ± 0.02</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>240</td>
<td>0.27 ± 0.02</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>210</td>
<td>0.26 ± 0.04</td>
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</tr>
</tbody>
</table>

*Average of at least three replicate determinations*

the concentrated methanolic extract of exhaustively electrolyzed solution of 2-thiouracil were unsuccessful. This is probably due to non-volatile nature of its constituents. Hence, the product mixture was converted to their thermally stable, volatile trimethylsilyl derivatives. GC-MS analysis of silylated sample of electrooxidized product exhibited three major peaks. The peak at 5.24 min having molar mass of m/z 249 (M+1; 30%) indicates formation of a monosilylated derivative of 4-hydroxypyrimidine-2-sulfonic acid. The peaks at R_t ~10.79 and ~16.42 min with molar masses of 375 (M+1; 20%) and 431 (M+1; 30%) respectively may be identified as a monosilylated S-S linked dimer with O=S=O and S=O groups and a bisilylated S-S linked dimer (Scheme 1) with O=S=O group respectively.

![Scheme 1](image)

**Enzymic oxidation**

It was interesting to observe that in GC-MS analysis of the silylated enzymic oxidation products, three major peaks were again noticed comparable to that of electrooxidation. Molar mass of these products were same, however the retention times noticed were slightly different as tabulated in Table 2. Disturbance in the background of gas-chromatogram was noticed in this case which may be due to the presence of traces of enzyme and its silylated products.

![Scheme 2](image)

**Redox mechanism**

The voltammetric studies have indicated that enzymic and/or electrochemical oxidation of 2-thiouracil is a 6e, 6H⁺ process to yield three major products (VI), (VII) and (VIII) as shown in Scheme 2. First step of the oxidation seems to be a 1e, 1H⁺ process to give a free radical (II). A 1e, 1H⁺ oxidation of thiols has been well reported in literature and it has also been established that removal of proton occurs from the –SH group. Combination of two moles of the radical (II) will rapidly give S-S linked dimer (III). This dimer (III) can further undergo

Table 2—Fragmentation pattern observed in GC-MS spectrum for the enzymic oxidation products of 2-thiouracil

<table>
<thead>
<tr>
<th>Products</th>
<th>Retention time (R_t)</th>
<th>Mol. ion peak (M+1)</th>
<th>Fragmentation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>5.30</td>
<td>249</td>
<td>222(15%), 207(35%), 193(30%), 147(85%)</td>
</tr>
<tr>
<td>VII</td>
<td>11.59</td>
<td>359</td>
<td>345(5%), 285(10%), 271(25%), 251(10%), 207(40%), 193(30%)</td>
</tr>
<tr>
<td>VIII</td>
<td>16.40</td>
<td>431</td>
<td>419(5%), 359(45%), 345(15%), 327(5%), 285(20%), 206(55%)</td>
</tr>
<tr>
<td></td>
<td>10.85</td>
<td>375</td>
<td>359(55%), 285(25%), 271(25%), 251(10%), 207(75%)</td>
</tr>
</tbody>
</table>
oxidation at sulfur atoms giving S=O linked compounds. Formation of 2-thiouracil dimers having S-S linkage with S=O group in aqueous solution is known in literature. A 2e, 2H⁺ oxidation of dimer (III) will give compound (IV) which can undergo hydrolysis as well as further oxidation to give compound (VII). It is thus revealed that, a competition between hydrolysis and oxidation occurs in compound (IV). The k value observed during kinetic studies represents hydrolysis of compound (IV) to give compound (V). Compound (V) has a SOH group which will easily oxidize in a 4e, 4H⁺ step to give 4-hydroxypyrimidine-2-sulfonic acid (VI) which has been characterized in GC-MS studies.

On the other hand 2e, 2H⁺ oxidation of compound (IV) will give compound (VII) in which sulfur atom will further oxidize to give O=S=O group. Compound (VII) has been identified in oxidation products of 2-thiouracil during electrochemical and enzymic oxidations. As compound (VII) has another sulfur atom, its oxidation to compound (VIII) in a 2e, 2H⁺ process appears to be a slow reaction. However, formation of (VIII) has also been confirmed by identification of its monosilylated derivative in GC-MS.

Appearance of peak IIe in the first negative going sweep in the cyclic voltammogram recorded during enzymic oxidation of 2-thiouracil clearly indicates that a species similar to electrooxidation is generated during the enzymic oxidation which reduces in peak IIe reaction. The most likely candidate for such reduction is dimer (III), which can easily reduce in a 2e, 2H⁺ process to give starting material (I) as the product. The nature and number of products identified during the enzymic and electrochemical oxidations of 2-thiouracil clearly indicate that both the oxidations proceed by an identical mechanism (Scheme 2).

Acknowledgement

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