Enzymatic Assay of Histamine by Amperometric Detection of H$_2$O$_2$ with a Peroxidase-based Sensor

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Received October 21, 1999; Accepted April 17, 2000

A method for an enzymatic assay of histamine by using histamine oxidase from *Arthrobacter globiformis* in combination with amperometric determination of H$_2$O$_2$ is described. Histamine could be quantified at a level as low as 10$^{-7}$ M. The assay is adaptable to determine histamine in food samples including tuna fish with good sensitivity and selectivity.

**Key words:** histamine; food analysis; histamine oxidase; biosensor; hydrogen peroxide

Seafood processing and quality control requires a rapid and selective assay of histamine, since histamine is one of the putrefactive amines and causes the allergic reaction in seafood poisoning. To fulfill this requirement, an enzymatic assay of histamine combined with electrochemical sensing seems promising. Histamine oxidase (HOX, EC 1.4.3.6) catalyzes the oxidation of histamine with the concomitant formation of H$_2$O$_2$ by the following equation:

$$\text{histamine} + O_2 + H_2O = 2,(4\text{-imidazoyl})\text{acetaldehyde} + NH_3 + H_2O_2$$

HOX from *Arthrobacter globiformis* is of particular interest because this enzyme exhibits high activity with specificity to histamine. In this note, we report a rapid and selective assay of histamine in food samples including tuna fish by using an HOX assay combined with the amperometric determination of H$_2$O$_2$ with a peroxidase-modified carbon paste electrode (POD-CPE). Such an amperometric method with immobilized peroxidase offers the advantage that it enables detection at a low operating potential and base current.

The POD-CPE device was prepared according to Kinoshita et al. with slight modifications. Weighed amounts of peroxidase from horseradish (POD, 100 units/mg, Wako Chemicals), ferrocene, liquid paraffin, and graphite powder were mixed in the ratio of 0.3:1:5:10. The resulting mixture was packed into one end of a glass tube (3.0 mm internal diameter) to make the carbon paste electrode (CPE). The tip of this CPE was immersed in a 0.6% (w/v) polyethyleneimine P-70 solution for 10 min and washed with water. A 30 μl amount of the 10 mg/ml POD solution was put on the electrode surface. After leaving to stand for 10 min, the electrode surface was washed with water and then immediately covered with a dialysis membrane (size 20, Wako Chemicals) to make the POD-CPE device. The device was stored at 4°C in a 0.1 M phosphate buffer (pH 7.5) until needed for use. The amperometric determination of H$_2$O$_2$ was carried out with the POD-CPE in a 5 ml electrolytic cell at 37°C. The test solution was a 0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, unless otherwise stated. A constant potential was applied to the POD-CPE with laboratory-made, PC-controlled three-electrode apparatus, and the current signal was recorded after correction for the base current. All potentials were measured against an Ag/AgCl/0.1 M KCl reference electrode.

The current response measured with the POD-CPE was practically independent of the applied potential in the range of -0.10 to 0.05 V. This response was observed at -0.02 V with the stepwise addition of an H$_2$O$_2$ solution to the test solution. The current reached a steady state 2 min after each addition. The steady-state current increased linearly with the concentration of H$_2$O$_2$ up to 1.5×10$^{-4}$ M. The relative standard deviation was 5.0% (n = 4) from replicated measurements of 1.1×10$^{-4}$ M H$_2$O$_2$ with the same POD-CPE. The calibration curve (steady-state current $I$ vs. H$_2$O$_2$ concentration $c_{H2O_2}$) is expressed by

$$I/A = -((0.98 \pm 0.02) \times 10^{-5})(c_{H2O_2}/M) - ((0.5 \pm 1.5) \times 10^{-11})$$

with a standard deviation of the base current ($\sigma_b$) of 2.5×10$^{-11}$ A. The detection limit, defined by 3$\sigma_b$, $m$ being the slope of the calibration curve, was determined to be 7.8×10$^{-8}$ M.

HOX from *Arthrobacter globiformis*, whose ex-

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**Abbreviations:** HOX, histamine oxidase; POD, peroxidase; CPE, carbon paste electrode; POD-CPE, peroxidase-modified carbon paste electrode
pression vector was presented by Prof. Tanizawa, was expressed in E. coli cells and purified according to Choi et al.8 The enzymatic activity of HOX was determined in a 0.1 M O2-saturated sodium phosphate buffer (pH 7.5) containing 1 mM substrate and 1 mM EDTA at 37°C. The reaction was started by the adding substrate solution to a reaction mixture that had previously been incubated at 37°C for 5 min, and the rate of production of H2O2 was determined by the POD-CPE method.

The Michaelis constant (Km) and rate constant (kcat) of HOX used in this study were Km = (1.3 ± 0.2) × 10−4 M and kcat = 87 ± 4 s−1. These kinetic parameters are comparable with Km = (5.8 ± 1.2) × 10−5 M and kcat = 29.7 s−1 at pH 8.0 reported by Choi et al.9 The substrate specificity for major putrefactive amines, including putrescine, cadaverine, tyramine, spermidine, spermine and histamine, was studied. The initial velocity obtained for each of the substrates at 1.0 mM was determined and used as an indicator of the substrate specificity. The result is summarized in Table 1, indicating that the major putrefactive amines, except for histamine and tyramine, were inert as substrates, whereas tyramine was a poor substrate.

Enzymatic assay of histamine. The enzymatic assay of histamine was carried out by an end-point assay method. The reaction mixture containing an appropriate amount of the sample solution was subjected to an enzymatic reaction in a 0.1 M O2-saturated sodium phosphate buffer (pH 7.5) containing 1 mM EDTA at 37°C. The enzymatic reaction was started by adding an HOX solution to the reaction mixture at 0.015 unit/ml and was terminated after an appropriate time (usually 3 min) by heating the reaction mixture at 80°C for 3 min. This heating inhibited the catalase activity in the reaction mixture and prevented the consequent degradation of H2O2. An aliquot was then withdrawn from the mixture and added to the test solution in the electrocyclic cell to determine H2O2 with the POD-CPE device.

Figure 1 shows the current response obtained from successive addition of the HOX reaction mixture containing 2.0 × 10−3 M histamine to the test solution. The steady-state current increased linearly with the concentration of histamine up to 0.95 × 10−8 M. The inset to Fig. 1 shows the calibration curve (steady-state current I vs. concentration of histamine chistamine) which is expressed by

\[ I/\Delta = -(1.2 \pm 0.0) \times 10^{-3} (c_{\text{histamine}}/\text{M}) + (0.4 \pm 0.7) \times 10^{-11} \]

with σ of 2.7 × 10−11 A. A detection limit of 6.9 × 10−8 M was obtained.

Determination of histamine in fish. Histamine in tuna fish was extracted according to the method of

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>histamine</td>
<td>100</td>
</tr>
<tr>
<td>putrescine</td>
<td>0</td>
</tr>
<tr>
<td>cadaverine</td>
<td>0</td>
</tr>
<tr>
<td>tyramine</td>
<td>35</td>
</tr>
<tr>
<td>spermine</td>
<td>0</td>
</tr>
<tr>
<td>spermidine</td>
<td>0</td>
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Fig. 1. Current Response of the POD-CPE Device to Successive Addition of Small Amounts of the HOX Reaction Mixture to the Test Solution.

Nomura et al.10 Tuna fish (5 g) was homogenized in 30 ml of a homogenizing buffer, 0.1 M sodium phosphate and 3 × 10−3 M NaN3, in a small mortar. The homogenate was heated at 90°C for 15 min, cooled to room temperature, and diluted to 50 ml with the homogenizing buffer (final pH 7.5). It was then centrifuged at 4,000 × g for 20 min, the resulting supernatant being used for the histamine assay as already described. The enzymatic reaction was terminated after 7 min when the current response had reached a stationary value under the condition that the reaction mixture containing a 5% (v/v) supernatant solution was incubated with 0.03 unit/ml of HOX.

The concentration of histamine in tuna fish sample was determined by the method of standard addition (Fig. 2), the analytical results being given in Table 2. The detection limit was determined to be 5 mg/100 g of wet tissue. The histamine contents in the tuna fish samples were found to be 35 ± 19 and 55 ± 32 mg/100 g of wet tissue (n = 3, respectively) after storage at 37°C for 8 days. The hazardous level of histamine in
tuna is considered to be 50 mg/100 g of wet tissue,\(^1\) and the U.S. Food and Drug Administration (FDA) has set 50 ppm (5 mg/100 g) as the defect action level of histamine in tuna, mahi mahi, and related fish.\(^1\) Our experimental results indicate that the POD-CPE method could potentially provide a simple and rapid analysis of histamine in food without the need for a time-consuming pretreatment.

In conclusion, our enzymatic analysis of histamine with HOX from *Arthrobacter globiformis* in combination with the amperometric determination of \(\text{H}_2\text{O}_2\) by a membrane-covered POD-CPE device may be useful for quantifying the histamine content in fish and other foods. The detection limit for the enzymatic assay with the POD-CPE device is estimated to be as low as \(10^{-7}\) M in a test solution. Among the major putrefactive amines, tyramine may interfere with this assay.

**Acknowledgment**

We indebted to Professor Katsuuki Tanizawa of Osaka University for generously presenting the HOX expression vector.

**References**


9) Choi, Y.-H., Matsuzaki, R., Suzuki, S., and
