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Histamine oxidase/peroxidase co-immobilized beads for chemiluminometric detection of histamine

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Chemiluminometric flow sensor with histamine oxidase (EC 1.4.3.-.) and peroxidase (EC 1.11.1.7.) was employed for the determination of histamine. Histamine oxidase was found in cells of *Arthrobacter crystallopoietes* KAIT-B-007 isolated from soil. Histamine oxidase and peroxidase were co-immobilized covalently on tresylated hydrophilic vinyl polymer beads and packed into transparent PTFE, which was used as flow cell. One assay for histamine was done at intervals of 2 min without carryover. The calibration curve for histamine was linear from 3 μM to 250 μM. The response was reproducible within 1.25 % of the relative standard deviation when 115-replicate injections of 100 μM histamine was carried out. The sensor system was applied to the determination of histamine in fish meat extracts.

Key Words : histamine, histamine oxidase, chemiluminometric, flow injection, *Arthrobacter crystallopoietes*

1. INTRODUCTION

Histamine is well known as a putrefactive amine which causes an allergy-like food poisoning (histamine poisoning) [1], but it is not present in fresh fish. However, when they are contaminated by the microorganism which has a strong histidine decarboxylase [EC 4.1.1.22.] activity, histamine is produced as a toxic substrate from histidine due to a decarboxylating reaction.Therefore, when fish containing a large amount of histamine is ingested, an allergy-like food poisoning is caused. In order to prevent the food poisoning incidents, it is required to give care in particular to freshness and quality of fish. Then the determination of histamine in fish is important for the prevention of food poisoning.

To date, a fluorometric analysis and a high performance liquid chromatograhy (HPLC) have been mainly used for the histamine determination, but their methods are deficient in the rapidity and simplicity. Therefore the enzymatic method with histamine oxidase (amine oxidase : EC 1.4.3.6.) as a catalytical reagent has been proposed to improve them and for a more selective analysis of histamine [2]. Histamine is oxidized by histamine oxidase to imidazol acetoaldehyde with the simultaneous production of ammonia and hydrogen peroxide. The formed hydrogen peroxide has been measured colorimetrically, electrochemically [2] and chemiluminometrically [3].

We found histamine oxidase having high substrate specificity against histamine in cells of *Arthrobacter crystallopoietes* KAIT-B-007 isolated from soil [4].

$$\text{Histamine} + H_2O + O_2 \xrightarrow{\text{histamine oxidase}} \text{imidazole acetaldehyde} + NH_3 + H_2O_2$$

Recently, chemiluminometric flow sensor on the basis of peroxidase (POD) catalyzed hydrogen peroxide and luminol reaction were developed in an attempt to improve the detection limit of hydrogen peroxide, since the instantaneous luminescence was emitted immediately after the reagents were mixed. [5]

$$2H_2O_2 + \text{luminol} + OH^- \xrightarrow{\text{POD}} \text{3-aminophthalate} + N_2 + 3H_2O + \text{light}$$

We have studied the possibility of utilizing chemiluminometric flow sensor with histamine oxidase and POD by flow-injection (FI) analysis for the assay of histamine. This paper describes the immobilization of histamine oxidase on tresylated hydrophilic vinyl polymer and its application to chemiluminometric flow sensor for histamine in fish meat extracts.

2. MATERIALS AND METHODS

2.1 Materials

Histamine oxidase (histamine : oxygen oxidoreductase, 0.18 U/ml) was produced in our laboratory by fermentation of *A. crystallopoietes* KAIT-B- 007. The enzyme was purified from cell-free extracts of KAIT-B-007. Purification of the enzyme was performed by a procedure involving ammonium sulfate fractionation, column chromatography on DEAE-Sepharose, Phenyl-Sepharose, and gel filtration with Sephacryl S-200. POD (*Arthromyces ramosus*, 250 U mg^{-1}) was purchased from Suntry. Hydrophilic vinyl polymer beads (TSKgel Toyopearl HW-65F, particle size <70 μm) was purchased from Tosoh. The Toyopearl beads were sieved to obtain 50±5 μm sieve fraction. Histamine dihydrochloride and 4-aminoantipyrine were purchased from Wako Pure Chemical Industries. Tresyl chloride (2,2,2-trifluoroehanesulfonyl chloride) was purchased from Tokyo Kasei. A transparent PTFE [poly (tetrafluoro) ethylene] tube was purchased from Valqua. All other substrates and reagents were commercial products of the highest quality.

2.2 Preparation of immobilized enzymes

Histamine oxidase and POD were immobilized on the Toyopearl beads by the method of K. Nilsson et al [6]. The 0.5 g of the beads was washed with 50 ml of dry acetone and suspended in 10 ml of dry acetone-pyridine (1 : 1 v/v). With vigorous magnetic stirring, 1 ml of tresyl chloride was dropwise added to the suspension over 5 min. The reaction was continued for 10 min. The beads were washed with 20 ml of acetone and then with 20 ml of 1mM hydrochloric acid. The tresylated beads were packed into a transparent PTFE (20 cm × 1.0 mm i.d., 1.5 mm o.d., limiting pressure 50 kg cm^{-2}) by slurry-packing method. Both ends of the tube were closed with ceramic filters. The enzyme solution [histamine oxidase (1.3 U) and POD (500 U) in 5 ml of 0.1 M phosphate buffer (pH 7.0)] was circulated through the tube at 0.1 ml min^{-1} for 18 h. The co-immobilized enzymes were washed with 0.1 M Tris-HCl buffer (pH 8.0) to saturate the free linking sites. Then the tube was coiled spirally and used as flow cell as shown in Figure 1.

Histamine oxidase activity was assayed by measuring the amount of hydrogen peroxide produced from histamine as substrate. The formed hydrogen peroxide was determined by measuring the amount of quinoneimine dye formed from the reaction of *N*-ethyl-*N*-(2-hydroxy-3-sulforopyl)-3,5-dimethoxyaniline, 4-antipyrine and POD. The reaction was stopped by addition of sodium dodecyl sulfate solution, and then the absorbance was read at 600 nm with a spectrophotometer (model UV-1200, SHIMAZU Co.,).

2.3 Flow system and procedure

The FI system used in this study is outlined in Figure 2. The luminol solution was pumped by Hitachi L-6000 LC pumps at a flow rate of 0.3 ml min^{-1}. Sample solutions were injected through a Sanuki SVM-6M six-way valve equipped with a 10 μl loop. The chemiluminescence was measured at room temperature (20±2°C), with a Soma S-3400 luminometer, connected to TOA FBR251A recorder.

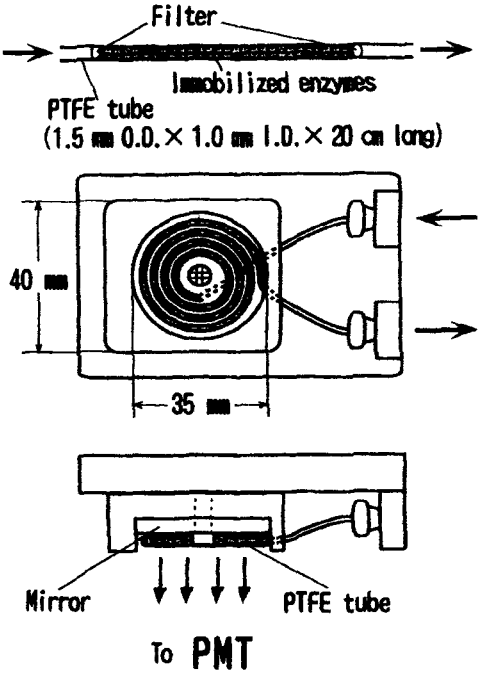

Fig. 1. Schematic diagram of the flow cell reactor. PTFE tube was packed with co-immobilized histamine oxidase/POD beads and coiled spirally and set in front of a photomultiplier tube (PMT) .

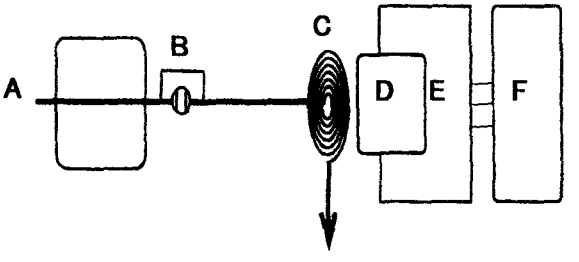

Fig. 2. Flow-injection set-up for the chemiluminometric determination of histamine : (A) 200 μM luminol in 0.1 M CHES-NaOH buffer (pH 10.0), (B) six way valve with a 10 μl loop, (C) flow cell, (D) photomultiplier tube, (E) luminometer, (F) recorder.

3. RESULTS

3.1 Effect of pH and luminol concentration on the chemiluminescence intensity

The effect of pH on the chemiluminescence intensity was examined in the pH range from 6.0 to 10.0 using each buffer (0.1 M phosphate buffer , 0.1 M CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid)-NaOH buffer and 0.1M carbonate buffer) by injecting 10 μl of 50 μM histamine. The optimum pH for activity of the flow cell was found to be at about 10.0 when CHES-NaOH buffer was used, as shown in Figure 3. The influence of luminol concentration on the chemiluminescence intensity was examined from 200 μM to 500 μM in 0.1M CHES-NaOH buffer (pH 10.0). A 500 μM luminol solution in 0.1M CHES-NaOH buffer (pH 10.0) was used in this study as shown in Figure 4.

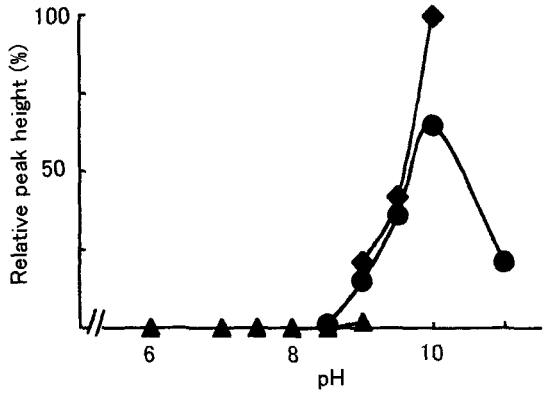

Fig. 3. The effect of pH on the chemiluminescence intensity. The intensity was measured using the system shown in Figure 2 (see "MATERIALS AND METHODS").
▲ : phosphate buffer, ● : carbonate buffer,
◆ : CHES-NaOH buffer

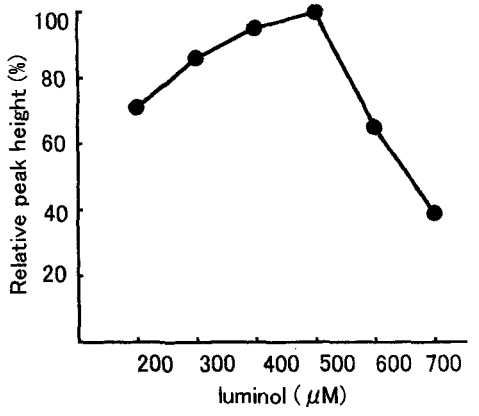

Fig. 4. The influence of luminol concentration. The intensity was measured using the system shown in Figure 2. (see "MATERIALS AND METHODS").

3.2 Substrate specificity of immobilized enzyme

The substrate specificity of immobilized histamine oxidase was examined using various monoamines, diamines and polyamines listed in Table 1. Immobilized enzyme showed powerful activity toward histamine, whereas was inactive toward putrescine, cadaverine, spermine and spermidine. The relative activities for hexylamine, tyramine, phenylethylamine and dopamine were 41 %, 36 %, 44 %, and 51 %, respectively.

Table 1. Substrate specificity of immobilized histamine oxidase. The relative activities for various substrate (100 μM) were measured using the system shown in Figure 2 (see "MATERIALS AND METHODS").

Substrate	Relative rate(%)
Histamine	100
Methylamine	0
Ethylamine	0
Propylamine	7
Butylamine	22
Phenethylamine	32
Hexylamine	41
iso-Butylamine	0
2-Ethanolamine	0
Benzylamine	0
Tyramine	36
Dopamine	51
Tryptamine	15
Phenylethylamine	44
Agmatine	4
Putrescine	0
Cadaverine	0
Spermine	0
Spermidine	0

3.3 Calibration curve for histamine

A calibration curve was prepared using 10 μl of histamine as a standard solution (3-250 μM). The plot of chemiluminescence intensity against histamine concentration was linear from 3 μM to 250 μM as shown in Figure 5. Therefore, a sample solution containing histamine above 250 μM must be diluted with buffer.

3.4 Precision and reproducibility

The reproducibility of the response for the chemiluminescence intensity was examined with 115-replicate injection of 100 μM histamine. The relative standard deviation was 1.25 %. The system gave satisfactorily precise and reproducible results.

3.5 Application

The sensor system shown in Figure 2 was applied to the determination of histamine in fish meat extracts.

The mackerel meat extracts were used as samples in this study. Histamine in mackerel was extracted by adding 0.1M phosphate buffer (pH 7.0) and heating at 90°C according to the method of Nomura et al [2]. Histamine in 4 fish meat extracts was measured by chemiluminometric flow sensor shown in Figure 2.

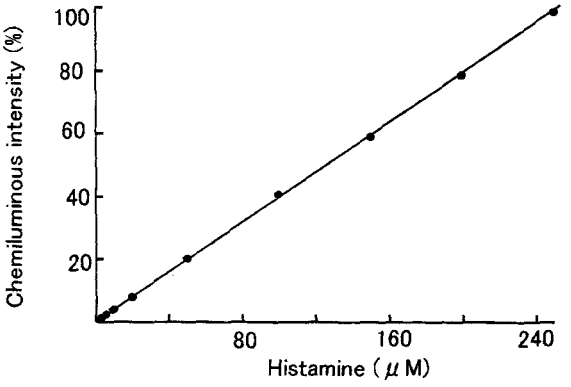

Fig. 5. The calibration curve for histamine. The intensity was measured using the system shown in Figure 2 (see "MATERIALS AND METHODS").

The results were compared with those obtained by HPLC. The results of these comparisons are shown in Figure 6. The calculated coefficient was 0.97 and the regression equation was $Y = 0.985\ X + 0.021$. The present method gave good agreement with values obtained by HPLC. Therefore, chemiluminometric flow sensor with immobilized histamine oxidase and POD is a suitable method for the continuous analysis of histamine in fish meat.

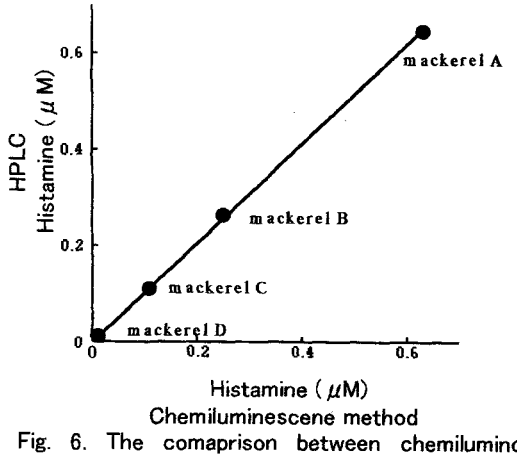

Fig. 6. The comaprison between chemiluminometric method and HPLC for the assay of histamine in fish meat extracts. The intensity was measured using the system shown in Figure 2 (see "MATERIALS AND METHODS").

4. DISSCUTION

Immobilization of histamine oxidase from *A. crystallopoietes* KAIT-B-007 on tresylated Toyopeal beads was effected with a 95 % yield. The immobilized enzyme was 2.5 U/g and stable for 2 weeks. We have been prepared immobilized histamine oxidase by using aminated inorganic support with the glutaraldehyde activation method and obtained immobilized histamine oxidase of 1.6 U/g [7]. In comparison on the immobilized enzyme on inorganic support, the activity was 1.5 times higher and the stability was twice higher. This may be attributed to the presence of more activated and hydrophilic groups on Toyopearl beads.

Chemiluminometric flow sensor system for the sensitve assay of histamine can be prepared by using a flow cell packed with co-immobilized histamine oxidase and POD. Since the optimum pH for histamine oxidase activity was close to that of POD-catalyzed luminol reaction, it became feasible to couple directly the enzyme reactions. The coupling of the reactions resulted in the elevation in the sensitivity of the chemiluminometric method, because the hydrogen peroxide produced in the histamine oxidase reaction is consumed *in situ* with POD reaction and the histamine oxidase reaction shifts to the right. The sensor was stable enough to permit measurements of more than 500 samples. We have developed the amperometric histamine sensor with immobilized histamine oxidase column reactor in FI system [7]. The detection limit was 1 mM. Comparing this chemiluminometric sensor with the amperometric one, the sensitivity is 100 times higher and maximum throughput is three times higher. This chemiluminometric flow sensor system was applicable to quantification of histamine in fish meat extracts..

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