Determination of Fish Freshness with an Enzyme Sensor System

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A new indicator of fish freshness is defined as K_I = ([HxR] + [Hx])/([IMP] + [HxR] + [Hx]) × 100, where [IMP], [HxR], and [Hx] are concentration of inosine 5′-phosphate, inosine, and hypoxanthine, respectively. In order to determine these compounds, an enzyme sensor system was developed by combining a double membrane consisting of a 5′-nucleotidase membrane and a nucleoside phosphorylase–xanthine oxidase membrane with an oxygen electrode. A small anion-exchange resin column was also connected with the enzyme sensor for separation of nucleotides. Each nucleotide concentration was determined as the current decreased. One assay was completed within 20 min. No appreciable decrease of current output was observed during 30 assays. When a sample solution containing 10 mM of IMP was applied, the response was reproducible within 8% of the relative error. Good comparative results were observed between the K_I values determined by the sensor proposed and by the conventional method.

Estimation of fish freshness is very important in food industries. It was estimated from the concentration of nucleotides (Saito et al., 1959), ammonia (Ota and Nakamura, 1952), amines (Karube et al. 1980), and volatile acids (Suzuki, 1953) in fish meat. Catalase activity (Mori and Hata, 1949), pH (Kawabata et al., 1952), electrical resistance (Nagamatsu, 1960), and fluorescence intensity (Ono et al, 1962) were also correlated with fish freshness. However, the determination method of these compounds requires complicated and time consuming procedures. therefore, a simple and rapid method is greatly needed in food industries. Among those indicators mentioned above, nucleotides produced by adenosine triphosphate (ATP) decomposition are considered the most reliable and useful indicator. Immediately after the death of fish, ATP decomposition proceeds according to the sequence

$$\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP} \rightarrow \text{HxR} \rightarrow \text{Hx} \rightarrow \text{X} \rightarrow \text{U} \quad (1)$$

where ADP is adenosine diphosphate, AMP is adenosine 5′-phosphate, IMP is inosine 5′-phosphate, HxR is inosine, Hx is hypoxanthine, X is xanthine, and U is uric acid. The relative concentration of these compounds drastically changes after the death of fish. Therefore, simultaneous determination of each nucleotide is necessary for a rapid estimation of freshness.

Recently, many enzyme sensors consisting of immobilized enzymes and electrochemical devices have been developed for the determination of organic compounds. These sensors are used for the determination of a single compound.

Recently, we have presented an enzyme sensor for the determination of HxR and Hx (Watanabe et al., 1983a). The concentration of HxR and Hx was determined by analyzing responses obtained under two different conditions.

In the present study, the principle of a multifunctional enzyme sensor for the determination of three compounds (IMP, HxR, and Hx) was prosposed for the estimation of fish freshness. Each compound is decomposed to uric acid by a membrane containing 5′-nucleotidase (NT), nucleoside phosphorylase (NP) and xanthine oxidase (XO), according to the reactions

$$\text{IMP} \xrightarrow{\text{NT}} \text{HxR} \xrightarrow{\text{NP, Pi}} \text{Hx} \xrightarrow{\text{XO, O}_2} \text{U} \quad (2)$$

Oxygen consumed may be determined by an oxygen probe attached on the membrane. The sensor consisting of a double membrane and an oxygen electrode was employed for the enzyme sensor system. A tiny anion-exchange column was connected with the enzyme sensor for the separation of nucleotides. The enzyme sensor system was actually applied to the estimation of freshness of several fishes.

MATERIALS AND METHODS

Materials. Xanthine oxidase (EC 1.2.3.2, from bovine spleen) was obtained from Boehringer Mannheim Co. Nucleoside phosphorylase (EC 2.4.2.1. from calf spleen), and 5′-nucleotidase (EC 3.1.3.5. from *Crotalus adamanteus* venom) were purchased from Sigma Chemical Co. Hypoxanthine, inosine, and inosine 5′-phosphate were purchased from Kojin Co. Dichloromethane, 1,8-diamino-4-(aminomethyl)octane, and cellulose triacetate were obtained from Kokusan Kagaku Co., Asahi Kasei Co., and Eastman Kodak Co., respectively. Other reagents were analytical grade or laboratory grade. Distilled water was used throughout the experiments.

Sea bass, *Lateolobrax japonicus*, mackerel, *Scomber japonicus*, Saurel, *Trachurus japonicus*, yellowfish, *Seriola quinqueradiata*, bream, *Pagrosomus unicolor*, and flounder, *Lepidopsetta bilineata* were purchased from a local retail store and stored in ice for up to 2 weeks.

Preparation of Perchloric Acid Extract of Meat. IMP, HxR, and Hx were extracted from fish meats (2 g) with a 10% perchloric acid (PCA) according to Ehira's method (Ehira et al. 1969, 1970). The PCA extract was neutralized to pH 7.7 with 10 N KOH and adjusted to 1 mL with a 10% neutralized PCA solution (pH 7.7).

Preparation of Immobilized Enzyme Membranes. Membrane for immobilization of enzymes was prepared from cellulose triacetate, glutaraldehyde, and 1,8-diamino-4-(aminomethyl)octane as described in the previous paper (Watanabe et al., 1983a). XO and NP were coimmobilized on the membrane in the same manner as described in the previous paper (Watanabe et al., 1983b). NT was also immobilized on the membrane by the following procedure. The membranes were immersed in a 0.1% glutaraldehyde solution (0.05 M Tris-HCl buffer solution, pH 8.4) for 2 h at 30 °C. After being washed with 0.05 M Tris-HCl buffer solution (pH 8.08), they were placed in 3 mL of a 0.05 M Tris-HCl buffer solution (pH 8.08) containing NT (about 0.5 mg) for 3 h at 25 °C and the enzyme membranes were stored at 5 °C. The relative

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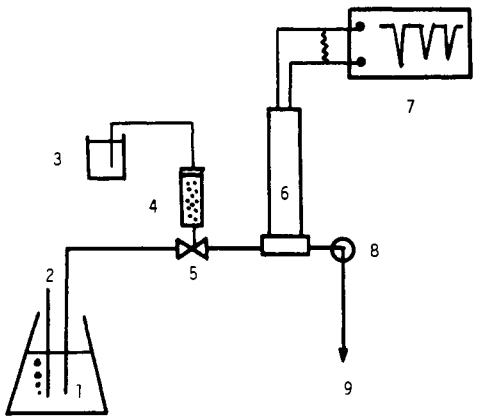

Figure 1. Schematic diagram of the enzyme sensor system for Hx, HxR, and IMP. 1, buffer tank; 2, air; 3, eluent; 4, ion-exchange resin column; 5, injection port; 6, oxygen electrode; 7, recorder; 8, peristaltic pump; 9, waste.

activity of stored membranes were determined every 24 h.

Preparation of Anion-Exchange Column. Since the sensor responds to Hx and IMP, an anion-exchange column was employed for separation of IMP and Hx. Anion-exchange resins (Dowex 1 × 2, Cl type, 100–200 mesh) were packed in a tiny glass column (0.5 i.d. × 2 cm) by slight suction. A needle of syringe was connected to one end of the column. The anion-exchange resin column was equilibrated with a 0.1 M sodium borate solution (pH 7.7).

Assay Procedure. Assay for IMP, HxR, and Hx was performed by the following steps using a flow systems as shown in Figure 1. Solutions used for reaction or elution in the present study were as follows: S-I, 0.05 M Tris-HCl buffer solution (pH 7.8) containing 0.1 mM cysteine; S-II, 0.05 M phosphate buffer solution (pH 7.8); S-III, 0.1 M phosphate buffer solution (pH 7.8) containing 0.07 M KCl; S-IV, 0.25 M phosphate buffer solution (pH 7.8) containing 0.2 M KCl.

S-I transferred continuously to the sensor by a peristaltic pump at a flow rate of 1.5 mL·min^{-1}. After the output current of the sensor became steady, a 20–50-μL aliquot of a sample solution was injected in the flow line. Without phosphate ion, the sensor did not respond to HxR. However, IMP was detected even in the absence of phosphate, because phosphate ions released by the NT reaction were used in NP reaction. Therefore, the current decrease corresponded to the total amount of Hx and IMP (step I). Then, the anion-exchange resin column retained nucleotides connected to the flow line. Consequently, eluting solution (S-II) was transferred to the column and the sensor. After the output current became steady again, 10 mL of another eluting solution (S-III) was transferred to the column. Under this condition, Hx and HxR were eluted, and the output current decreased (step II). When 10 mL of S-IV was passed through the column, IMP was eluted and it was determined by the sensor (step III). From the three peak currents obtained, concentrations of IMP, HxR, and Hx were calculated.

Linear Approximation of the Three-Component Determination. The output current of an oxygen electrode is proportional to oxygen concentration. Oxygen consumption due to enzyme reaction is proportional to the concentration of the substrate.

$$\Delta I_1 = k_{11}[\mathrm{Hx}] + k_{12}[\mathrm{HxR}] + k_{13}[\mathrm{IMP}] \quad (3)$$

$$\Delta I_2 = k_{21}[\mathrm{Hx}] + k_{22}[\mathrm{HxR}] + k_{23}[\mathrm{IMP}] \quad (4)$$

$$\Delta I_3 = k_{31}[\mathrm{Hx}] + k_{32}[\mathrm{HxR}] + k_{33}[\mathrm{IMP}] \quad (5)$$

where ΔI_1, ΔI_2, and ΔI_3 are responses of the sensor obtained in step I, step II, and step III, respectively, k_{ij} (i, j = 1, 2, 3) is constant, and [Hx], [HxR], and [IMP] are concentrations of Hx, HxR, and IMP, respectively. Equations 3–5 can be rewritten as

$$\begin{Bmatrix}\Delta I_1\\ \Delta I_2\\ \Delta I_3\end{Bmatrix} = \begin{vmatrix}k_{11} & k_{12} & k_{13}\\ k_{21} & k_{22} & k_{23}\\ k_{31} & k_{32} & k_{33}\end{vmatrix} \begin{Bmatrix}[\mathrm{Hx}]\\ [\mathrm{HxR}]\\ [\mathrm{IMP}]\end{Bmatrix} \quad (6)$$

or in short

$$d = Kc \quad (7)$$

where

$$d = \begin{Bmatrix}\Delta I_1\\ \Delta I_2\\ \Delta I_3\end{Bmatrix} = \text{vector of the responses}$$

$$c = \begin{Bmatrix}[\mathrm{Hx}]\\ [\mathrm{HxR}]\\ [\mathrm{IMP}]\end{Bmatrix} = \text{vector of concentrations of each compound}$$

$$K = \begin{vmatrix}k_{11} & k_{12} & k_{13}\\ k_{21} & k_{22} & k_{23}\\ k_{31} & k_{32} & k_{33}\end{vmatrix} = \text{coefficient matrix}$$

Each component of K was determined from the results obtained under each condition summarized in Table I. Once ever k_{ij} was determined, eq 7 was rewritten as

$$c = K^{-1}d \quad (8)$$

where K^{-1} is the inverse matrix of K, namely

$$K^{-1} = \frac{1}{|K|}\begin{vmatrix}k_{22}k_{33} - k_{23}k_{32} & -k_{12}k_{33} + k_{13}k_{32} & k_{12}k_{23} - k_{13}k_{22}\\ -k_{21}k_{33} + k_{31}k_{23} & k_{11}k_{33} - k_{31}k_{13} & -k_{11}k_{23} + k_{13}k_{21}\\ k_{21}k_{32} - k_{31}k_{22} & k_{11}k_{32} + k_{12}k_{31} & k_{11}k_{22} - k_{21}k_{12}\end{vmatrix}$$

where $|K| = k_{11}k_{22}k_{33} + k_{12}k_{23}k_{31} + k_{13}k_{21}k_{32} - k_{13}k_{22}k_{31} - k_{12}k_{21}k_{33} - k_{11}k_{23}k_{32}$. Using eq 8, we determined each component.

Table I. Operational Conditions for Determination of k_{ij}'s of the Coefficient Matrix

	reaction solution	[Hx]	[HxR]	[IMP]
k_{11}	S-I	V[a]	0	0
k_{12}	S-I	0	V	0
k_{13}	S-I	0	0	V
k_{21}	S-III + S-I	V	0	0
k_{22}	S-III + S-I	0	V	0
k_{23}	S-III + S-I	0	0	V
k_{31}	S-IV + S-I	V	0	0
k_{32}	S-IV + X-I	0	V	0
k_{33}	S-IV + S-I	0	0	V

[a] V = variable.

RESULTS

Time Course of ATP Decomposition and Succeeding Reactions. Concentrations of nucleotides in several fishes were determined. Figure 2 show the results obtained for three different fishes. ATP and ADP rapidly decreased and disappeared around 24 h after the death. AMP also decreased rapidly and the concentration became less than 1 μmol·g^{-1}. On the other hand, IMP sharply increased around 5–24 h after the death and then gradually decreased. Furthermore, HxR and Hx increased when IMP began to decrease. Usually fishes obtained at markets are at least 24 h after the death. Therefore, the freshness indicator was defined as

$$K_I = \frac{[\mathrm{HxR}] + [\mathrm{Hx}]}{[\mathrm{IMP}] + [\mathrm{HxR}] + [\mathrm{Hx}]} \times 100 \quad (9)$$

Response Curves. The enzyme sensor involves three enzymes. Therefore, the response curves of the sensor to

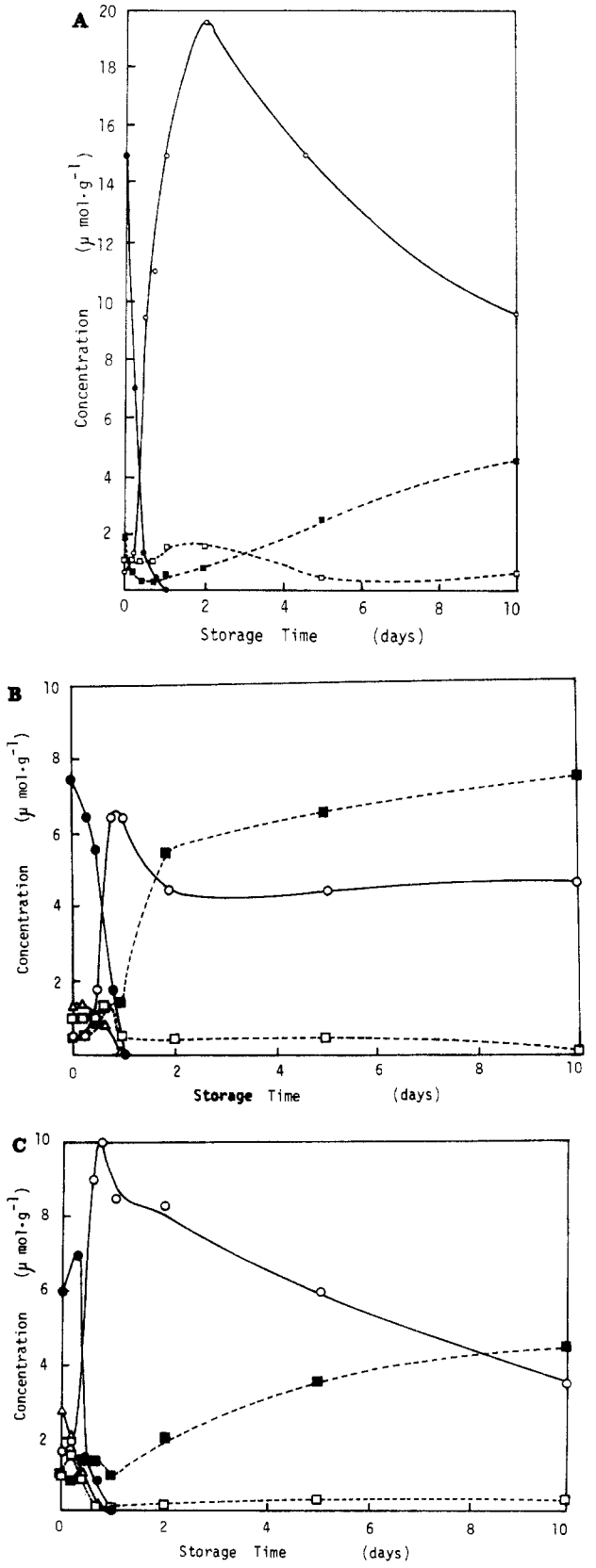

Figure 2. Time course of ATP decomposition and associating reactions. (A) Bream; (B) flounder; (C) sea bass. (●) ATP; (△) ADP; (□) AMP; (○) IMP; (■) HxR + Hx.

IMP, HxR, and Hx were compared. After the output current reached a steady state, an aliquot of each compound was injected into the sensor system. The current decreased and reached the minimum level and then returned to the initial one within 4 min. However, no appreciable difference was observed between the response

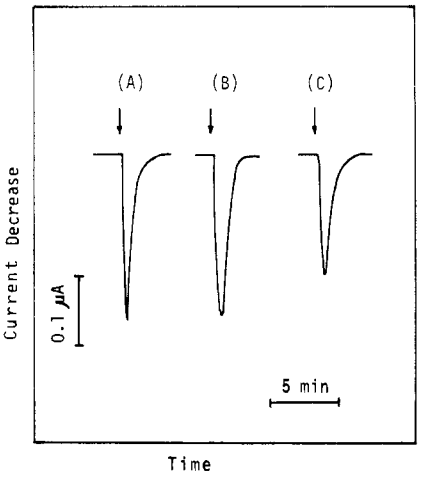

Figure 3. Responses of the enzyme sensor to Hx (A), HxR (B), and IMP (C).

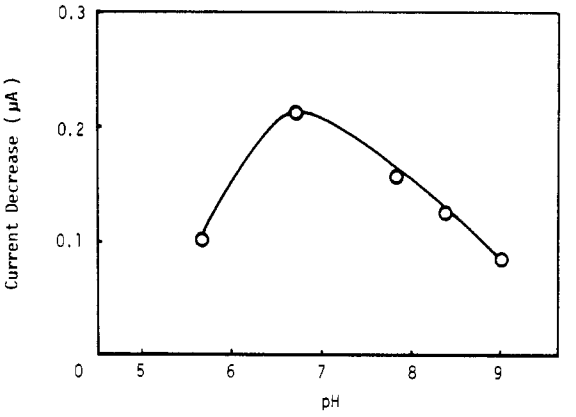

Figure 4. pH dependence of the enzyme sensor.

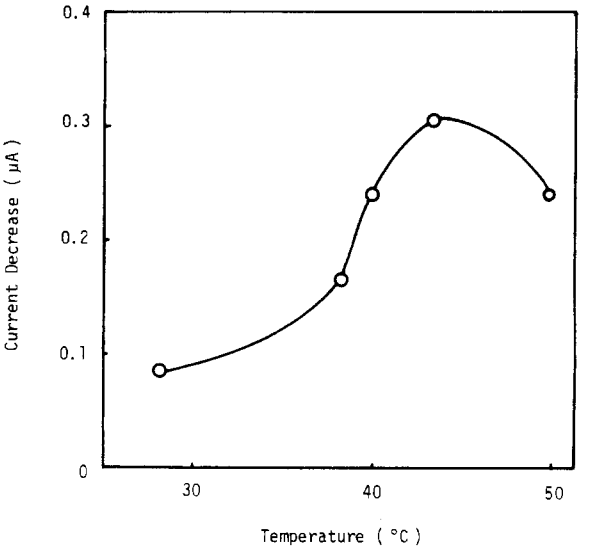

Figure 5. Temperature dependence of the enzyme sensor.

times for IMP, HxR, and Hx as shown in Figure 3. From these results, the response of the sensor was defined as the difference of the initial current and the minimum current.

Effects of Assay Conditions on the Response of the Sensor. Optimum conditions of the present sensor were surveyed by using IMP as a substrate. The pH and temperature dependences are shown in Figures 4 and 5. Maximum responses were obtained at pH 6.7 and 44 °C. Under these conditions, however, the enzyme activity was immediately lost during repeated assays. However, the

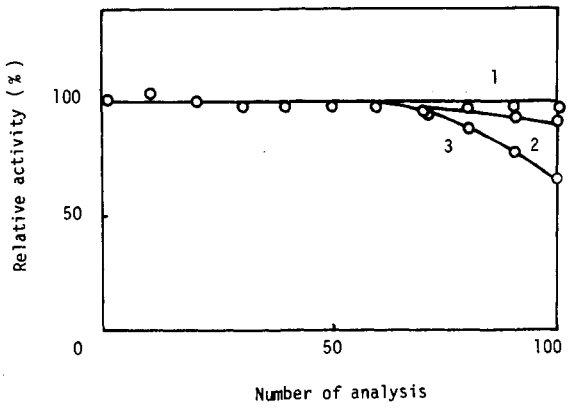

Figure 6. Effect of assay conditions on the stability of the enzyme sensor. 1, 30 °C, pH 7.8; 2, 30 °C, pH 6.7; 3, 35 °C, pH 7.8.

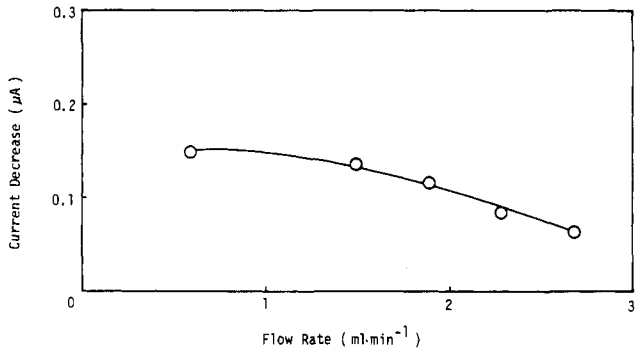

Figure 7. Influence of the flow rate on the response of the sensor.

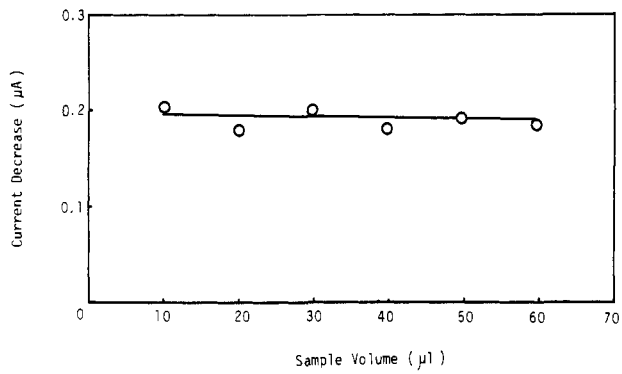

Figure 8. Effect of the sample volume on the response of the sensor.

enzyme sensor could be used for 100 assays without any loss of enzyme activity at pH 7.8 and 30 °C (Figure 6). Therefore, these conditions were employed in the following experiments. Effects of the flow rate are shown in Figure 7. The sensor showed a constant response around 0.5–1.5 mL·min^{-1}. Therefore, the flow rate was adjusted at 1.5 mL·min^{-1}. Similarly, the optimum sample volume was fixed at 20 μL based on the results shown in Figure 8.

Assay of a Mixed Sample of IMP, HxR, and Hx. According to the assay procedure described above, a sample containing IMP, HxR, and Hx was applied to the sensor system. When an aliquot of the sample was injected into the flow, the output current decreased within 30 s as shown in Figure 9. Peak A corresponds to the summation of Hx and IMP concentrations. Then the anion-exchange resin column was connected to the flow line and three different eluting solutions (S-II, S-III, S-IV) were transferred to the column. Two peaks, B and C, were obtained as shown in Figure 9, when the eluting solution of S-III and S-IV was passed through the column. Peak B corresponds to the summation of Hx and HxR concentrations,

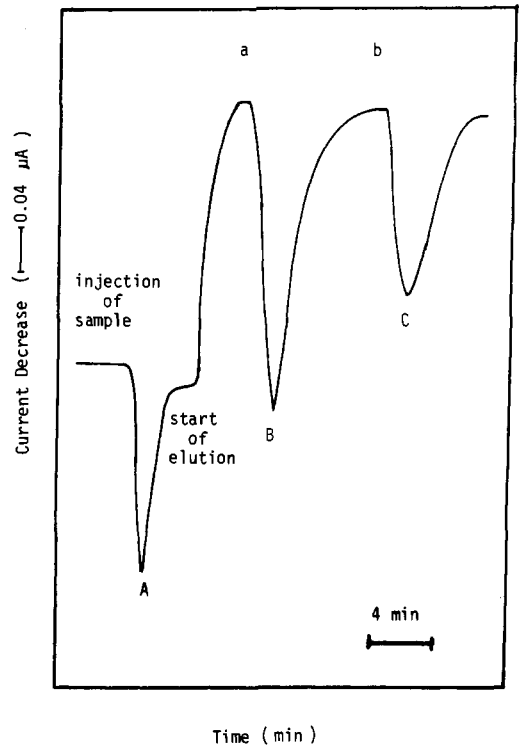

Figure 9. Response curves of the enzyme sensor to a mixture of Hx, HxR, and IMP. (A) Hx + IMP; (B) Hx + HxR; (C) IMP.

while peak C corresponds to IMP concentration. One assay was completed within 20 min.

Determination of Coefficient Matrix. Each component of the coefficient matrix K was determined by applying standard solutions containing one of above three compounds under different conditions summarized in Table I. Figure 10A shows a linear relationship obtained for Hx, when S-I and S-IV + S-I were used. Concentration ranges were 0.1–0.5 and 0.8–3 mM, respectively. When S-III + S-I was used, no response was observed in these concentration ranges. Figure 10B shows a correlation between the response of the sensor and HxR concentration in S-III + S-I. In neither S-I nor S-IV + S-I was response obtained for HxR. Figure 10C shows relationships obtained for IMP either in S-I or S-IV + S-I. Linear relationships were observed in the range of 5–15 and 8–30 mM, respectively. In S-III + S-I, no response was obtained for IMP. From these results, components of K were determined: $k_{11} = 0.3$ (μA·mM^{-1}), $k_{12} = 0$, $k_{13} = 0.02$, $k_{21} = 0.06$, $k_{22} = 0.015$, $k_{23} = 0$, $k_{31} = 0$, $k_{32} = 0$, and $k_{33} = 0.008$. Thus, the inverse matrix of K was calculated as

$$K^{-1} = \begin{vmatrix} 3.3 & 0 & -8.3 \\ -13.3 & 66.6 & 33.3 \\ 0 & 0 & 125 \end{vmatrix}$$

Application of the Sensor to the Estimation of Fish Freshness. The enzyme sensor system was applied to the estimation of freshness of several fishes. PCA extracts prepared from meats of sea bass, mackerel, saurel, and yellowfish were applied to both the sensor system and a conventional assay method. Good comparative results were observed between K_I values determined by both methods (Figure 11). The correlation coefficient was 0.95 for 12 assays.

Reusability of the Electrode. The activity of enzyme sensor decreases generally on repeated runs. In IMP sensor, on appreciable decrease of the activity was observed during 30 assays under the optimum conditions. The response was reproducible within ±8% when samples con-

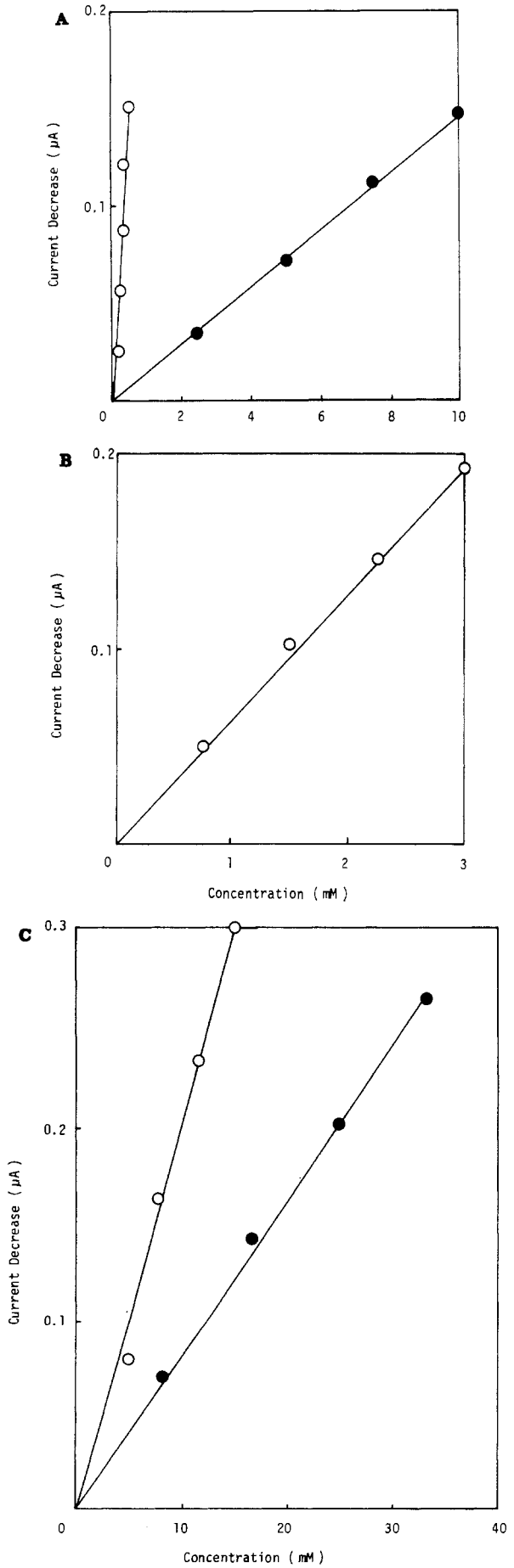

Figure 10. Standard curves. (A) Hx assayed in S-I (○) and S-III + S-I (●); (B) HxR assayed in S-III + S-I; (C) IMP assayed in S-I (○) and S-IV + S-I (●).

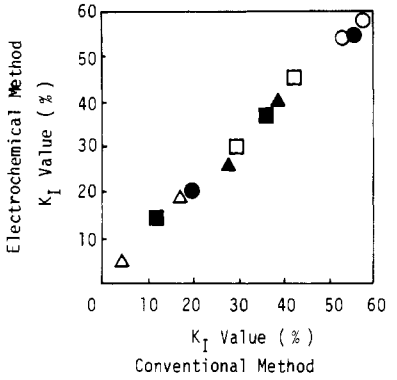

Figure 11. K_I values in several fish muscles. (○) Flounder; (●) sea bass; (△) bream; (▲) mackerel, (□) saurel; (■) yellowfish.

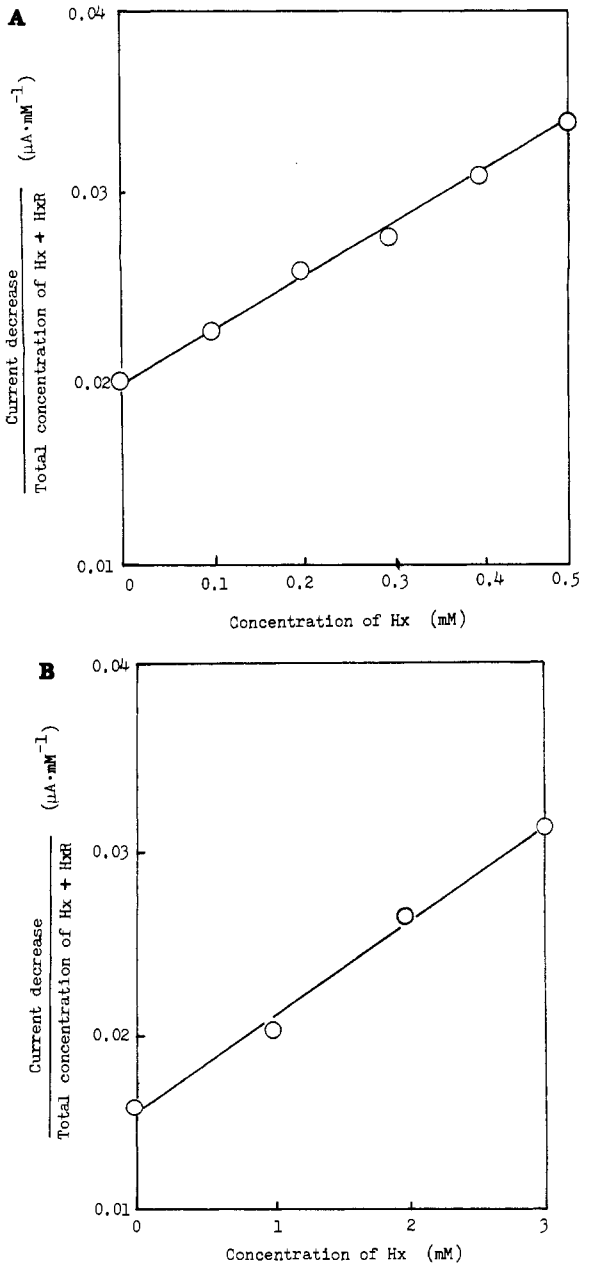

Figure 12. Linear relationships between the response of the sensor and the relative concentration of one component in the mixture of two components. (A) Hx + IMP = 10 mM assayed in S-I; (B) Hx + HxR = 8 mM assayed in S-III + S-I.

taining 10 mM of IMP were employed. No appreciable decrease of the output current was observed for more than 30 days at 5 °C.

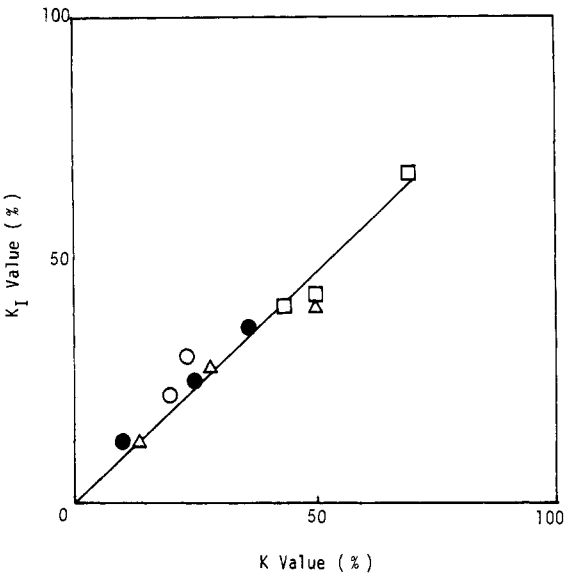

Figure 13. A correlation between K_I and K values. (○) Sea bass; (●) saurel; (△) mackerel; (□) yellowfish.

DISCUSSION

Linear Approximation. Concentrations of Hx, HxR, and IMP were estimated by analyzing the three peaks obtained under three different conditions, as shown in Figure 9. The first and the second peaks corresponded to the summation of Hx and IMP concentrations and Hx and HxR concentrations, respectively. These two peaks are expressed by the formulas

$$\Delta I_1 = a_1[\mathrm{Hx}] + b_1[\mathrm{IMP}] + f_1([\mathrm{Hx}],[\mathrm{IMP}]) \quad (10)$$

$$\Delta I_2 = a_2[\mathrm{Hx}] + b_2[\mathrm{HxR}] + f_2([\mathrm{Hx}],[\mathrm{HxR}]) \quad (11)$$

where a_i and b_i (i = 1, 2) are constants, [Hx], [HxR], and [IMP] are concentrations of Hx, HxR, and IMP, respectively, and f_i (i = 1, 2) is a nonlinear function of [Hx] and [IMP] or [HxR]. Subscript i respresents the condition of reaction solution i = 1 for S-I and i = 2 for S-III + S-I.

Under the condition that the summation of [Hx] and [IMP] was constant (c_1), eq 10 is rewritten as

$$\Delta I_1 = (a_1 - b_1)[\mathrm{Hx}] + b_1c_1 + f_1([\mathrm{Hx}],c_1 - [\mathrm{IMP}]) \quad (12)$$

According to eq 12, ΔI_1 was plotted vs. [Hx]. As shown in Figure 12A, a linear relationship was obtained between ΔI_1 and [Hx] in the range 0–0.5 mM.

In the same manner, eq 11 is rewritten as

$$\Delta I_2 = (a_2 - b_2)[\mathrm{Hx}] + b_2c_2 + f([\mathrm{Hx}],c_2 - [\mathrm{HxR}]) \quad (13)$$

when [Hx] + [HxR] = c_2 (constant). As shown in Figure 12B, a linear relationship was observed in the range 0–3 mM.

Therefore, the assumption that response of the enzyme sensor is expressed by a linear combination of three compounds is reasonable in the concentration range mentioned above.

Feasibility of the Present Sensor as a Freshness Sensor. The freshness estimated with the enzyme sensor is based on the concentrations of Hx, HxR, and IMP. However, ATP, ADP, and AMP remain in some species of fish even after 2 weeks. In that case, these components should be considered for the freshness estimation. Therefore, the K_I values defined by eq 9 were compared with the K values defined by the equation

$$K = \frac{[\mathrm{HxR}] + [\mathrm{Hx}]}{[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}] + [\mathrm{IMP}] + [\mathrm{HxR}] + [\mathrm{Hx}]} \times 100 \quad (14)$$

As shown in Figure 13, a linear correlation was obtained between K_I and K values for sea bass, saurel, mackerel, and flounder. The correlation coefficient was estimated as 0.95 by the least mean square method.

As suggested from Figure 2, time cources of ATP, ADP, and AMP concentrations depended on fish species. Therefore, freshness of fish cannot be simply shown by K_I values. Our developmental research is directed toward the incorporation of a pattern-displaying system for the estimation of freshness by the enzyme sensor.

Registry No. Inosine, 58-63-9; inosine 5′-phosphate, 131-99-7; hypoxanthine, 68-94-0; EC 3.1.3.5, 9027-73-0; EC 2.4.2.1, 9030-21-1; EC 1.2.3.2, 9002-17-9.

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