

## Enzyme Reactor System for the Determination of the Quality of Chicken

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An enzyme reactor system was developed for the determination of the quality of chicken over a wide range including both freshness (K-value) and spoilage (diamine content). The system was constructed by fusing a K-value sensor and a diamine sensor in the same channel. In fresh chicken muscle, little diamine is present, and the diamine sensor shows no response. Therefore, the diamine sensor was placed upstream to the K-value sensor and the effects of chemical cross-talk caused by both sensors were examined. Sufficient precision in the measurement was obtained by correcting the K-value for the relationship between the putrescine concentration and cross-talk. One assay required 5 min. The immobilized enzymes were stable over 100 days at 4°C.

### 1. Introduction

With the introduction of hazard analysis and critical control point (HACCP) to food manufacturing, the establishment of a monitoring system for the rapid examination of chicken quality is anticipated. As parameters of freshness, viable count, volatile basic nitrogen,<sup>(1,2)</sup> ammonia,<sup>(3,4)</sup> amines,<sup>(5)</sup> volatile acids,<sup>(6)</sup> pH<sup>(7–9)</sup> and K-value<sup>(10–15)</sup> have been proposed for the conversion of sensory freshness into a numerical value. However, the methods for measuring these parameters have associated problems. Therefore, the freshness of chicken is difficult to measure from these parameters. The development of a reactor system for measuring fish and chicken freshness has been reported.<sup>(16,17)</sup> With this system it is possible to determine K-values (the most common index of freshness) rapidly and simply but not to clarify the spoilage caused by microbial contamination. Therefore, to determine the incipient stage of spoilage, we focused on the amount of diamines (pu-

trepscine and cadaverine) present, which are metabolites produced by growing microorganisms. Considering that the incipient stage of spoilage can be determined by measuring these diamines, we developed an enzyme reactor sensor that can easily measure the amount of diamines present. By combining the proposed reactor sensor with the K-value sensor system, we widened the range of the freshness sensor in order to be able to measure from the reduction of freshness to the incipient stage of spoilage of chicken.

## 2. Materials and Methods

### 2.1 Enzyme and reagents

The enzyme used in the immobilized enzyme reactors in the K-value sensor were alkaline phosphatase (ALP: E.C.3.1.3.1, from calf intestinal mucosa, Biozyme; UK), nucleoside phosphorylase (NP: E.C.2.4.2.1, from calf spleen, Boehringer Mannheim; Germany) and xanthine oxidase (XOD: E.C.1.1.3.22, from cows' milk, Biozyme; UK). The enzyme used in the immobilized reactor in the diamine sensor was putrescine oxidase (PUO: E.C.1.4.3.10, from microorganisms, Toyobo; Japan). Glass porous beads (LCA00500B) and chitosan porous beads (Chitopeal 3001, diameter 0.1 mm) were obtained from Funakoshi and Fuji Spinning (Japan), respectively. Fifty millimolar phosphate buffer, pH 7.8, and 50% glutaraldehyde (GA) were obtained from Kanto Chemical (Japan). Putrescine was obtained from E. Merck (Germany). Cadaverine, tyramine and isoamylamine were obtained from Tokyo Kasei Co. (Japan). Spermidine, ornithine, histidine, lysine and histamine were obtained from Wako Chemical Industries, Ltd. (Japan). Spermine was obtained from Sigma (USA). The various amines used as model samples for diamine sensors were dissolved in aqueous 0.8% NaCl solution. All other chemicals were analytical-reagent grade.

### 2.2 Preparation of enzyme reactors

A pair of enzyme reactors was used for the K-value sensor. To 2.5 ml of chitosan beads, twenty milliliters of phosphate buffer (0.05 M, pH 7.8) and 1060  $\mu$ l of 50% glutaraldehyde solution were added and mixed, then the mixture was shaken for 2 h at 30°C. After activation with glutaraldehyde, the beads were washed well with distilled water and dehydrated by vacuum filtration. To these beads, two milliliter of phosphate buffer was added, followed by the addition of NP (60U) and XOD (20U), then the mixture was shaken for 2 h at 30°C to simultaneously immobilize these two enzymes on the chitosan beads with glutaraldehyde through the formation of a Schiff's base. The immobilized enzymes were preserved in 0.05 M phosphate buffer solution (pH 7.8) at 4°C. The enzyme-bonded beads were packed into a polypropylene reactor tube (50 mm  $\times$  3 mm i.d.) and used as a reactor for measuring inosine and hypoxanthine. To prepare the immobilized enzyme reactor for measuring inosine monophosphate, inosine and hypoxanthine, the chitosan beads with the two immobilized enzymes and 850 mg of glass beads in 20 ml of phosphate buffer in which 14400U of ALP was immobilized were packed in layers at a 1:1 ratio.

The reactor for diamine measurement was prepared by the same technique. In a mixture of 1 ml of chitosan beads and 10 ml of phosphate buffer, 500U of PUO was immobilized, and the beads were packed into a polypropylene reactor tube (50 mm  $\times$  2 mm i.d.).

### 2.3 Measurement principle and enzyme sensor system

After fish die, ATP-related compounds in the fish decompose to form uric acid as shown in Fig. 1. Saito had reported a high correlation between the percentage of inosine and hypoxanthine in the total ATP-related compounds and the early freshness of fish.<sup>(10)</sup> Therefore, the ratio of inosine and hypoxanthine to the total amount of ATP-related compounds is designated as the K-value and used as an index of fish and chicken freshness in Japan. However, ATP, ADP and AMP are changed to inosine monophosphate in a very short time. Actually, the Ki-value, which represents the ratio of inosine and hypoxanthine to the total amount of inosine monophosphate, inosine and hypoxanthine, is almost equal to the K-value. Therefore, in this study, the Ki-value was measured by the K-value sensor as the indicator of early freshness.

The K-value sensor was assembled with an enzyme reactor system consisting of two types of immobilized enzyme reactors with oxygen electrodes placed behind each reactor, and the K-value was calculated from the amount of oxygen consumed in each immobilized enzyme reactor.

In the measurement of diamines, the amount of hydrogen peroxide produced by the enzyme reaction was detected using three electrodes. The working platinum anode was polarized at 500 mV vs Ag/AgCl.

### 2.4 Preparation of samples

The chicken meat used in the experiment was commercially available white chicken meat (product of Iwate, Japan). The chicken muscle was cut into approximately 1 cm segments and stored under aerobic conditions at 5 and 15°C. Samples (10 g) were arbitrarily gathered at 24 h intervals and physiological saline (90 g) was added to homogenize. The homogenized suspensions were used as is for the measurement of the viable count. For sample preparation in the enzyme reactor, the homogenized suspensions were centrifuged (15000 rpm for 10 min at 4°C).

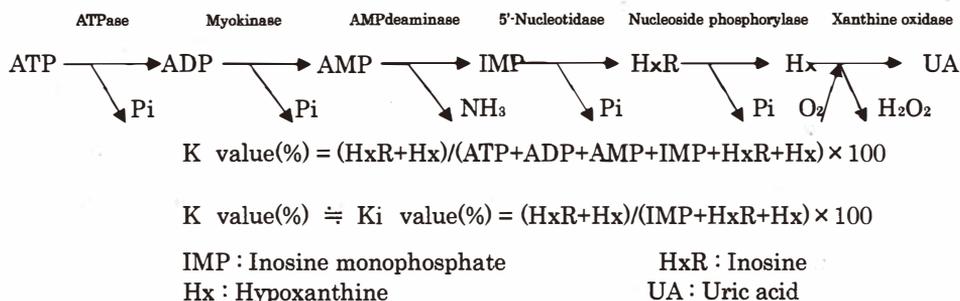


Fig. 1. Changes of ATP and definition of K-value.

### 3. Results and Discussion

#### 3.1 Substrate specificity of the immobilized putrescine oxidase

The PUO used in the diamine sensor shows substrate specificity only to putrescine. Therefore, to simultaneously detect putrescine, cadaverine and spermidine, which was the objective of this study, the glutaraldehyde concentration used in the immobilization of enzymes and the amount of putrescine oxidase were changed, and the optimal immobilization conditions for obtaining equal sensor responses to these three substances were investigated. As the substrate, ten amines adjusted to 0.25 mM were injected into the sensor, and the sensor response to each amine was measured.

The results are shown in Table 1. As the enzyme amount increased, the relative responses to cadaverine and spermidine compared to the response to putrescine increased, and this increase became marked upon treatment with glutaraldehyde. This sensor responded only to these three amines. Based on this observation, the enzyme immobilization conditions used in this sensor system were set to 2.5% glutaraldehyde concentration and 500U putrescine oxidase.

The calibration curves for the determination of putrescine, cadaverine and spermidine were determined. Linear relationships were observed between the sensor response and all three compounds up to 1 mM. The lower limit of detection for the compounds was 0.01 mM.

#### 3.2 Application of the diamine sensor to the detection of the incipient stage of spoilage of chicken

Changes in the diamine concentration in chicken extracts during storage at 5 and 15°C are shown in Fig. 2. The response to diamine rapidly increased after 4 days and 1 day at 5 and 15°C, respectively, and the viable counts at these points were  $10^6$ ~ $10^7$  cells/g. There-

Table 1

Effects of putrescine oxidase concentration on relative sensor response (relative activity).

Substrate	Immobilized enzyme (glutaraldehyde: 2.5%)					Native
	5U (GA 0%)	10U	100U	300U	500U (GA 0%)	
Putrescine	100 (100)	100	100	100	100 (100)	100
Cadaverine	14.5 (7.1)	22.7	78.4	94.8	96.9 (33.8)	1.9
Spermidine	30.6 (11.4)	46	92.8	104.7	102.4 (48.7)	14
Lysine	0 (0)	0	0	0	0 (0)	0
Histamine	0 (0)	0	0	0	0 (0)	0
Tyramine	2.41 (1.05)	2.16	1.03	2.37	1.09 (1.61)	0
Isoamylamine	0 (0)	0	0	0	0 (0)	0
Ornithine	0 (0)	0	0	0	0 (0)	0
Spermine	0 (0)	0	0	1.12	1.09 (0)	0.38
Histidine	0 (0)	0	0	0	0 (0)	0

Substrate: 0.25 mM

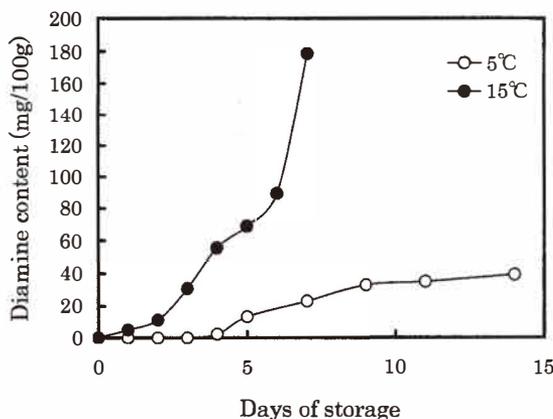


Fig. 2. Changes in diamine content of chicken muscle during storage at 5 and 15°C. ○: Chicken extract during storage at 5°C. ●: Chicken extract during storage at 15°C.

fore, the incipient stage of spoilage was detected. The levels of diamines further increased very rapidly when the samples were stored at 15°C in comparison to 5°C. These results depended on the optimum growth temperature of the microorganisms. Microorganisms generally have optimum growth temperatures of around 30°C. Therefore, the growth velocity of the microorganisms was greater in chicken muscle stored at 15°C than in those stored at 5°C. Accordingly, the microbial metabolic velocity increased, resulting in an increase in the concentration of diamines, which are metabolic products.

The sensor response was observed in chicken muscle stored for more than 4 days at 5°C. Therefore, it is difficult to monitor the early stage of the reduction of freshness by this sensor alone.

### 3.3 Determination of the freshness of chicken meat by the K-value sensor

The results of K-value determination in chicken muscle stored at 5 and 15°C are shown in Fig. 3. The K-value increased more rapidly in the chicken muscle stored at 15°C than in that stored at 5°C because the velocity of the reactions of the enzymes that decompose ATP-related compounds is greater in the chicken muscle stored at 15°C. Accordingly, the velocity of the decomposition of the ATP-related compounds was increased in the meat stored at 15°C, resulting in a rapid increase in the K-value. However, regardless of the storage temperature, after the K-value rose by almost 70%, it did not increase further. This finding showed that the early reduction of freshness can be measured by determining the K-value, but spoilage thereafter is difficult to determine from the K-value.

### 3.4 Effect of chemical cross-talk caused by fusion of the sensors

Oxidizing enzymes were used in the diamine and K-value sensor; when these flow sensors were fused and placed on the same line, chemical cross-talk occurred in which oxygen consumed in the oxidation reaction in the upstream sensor caused a response at the

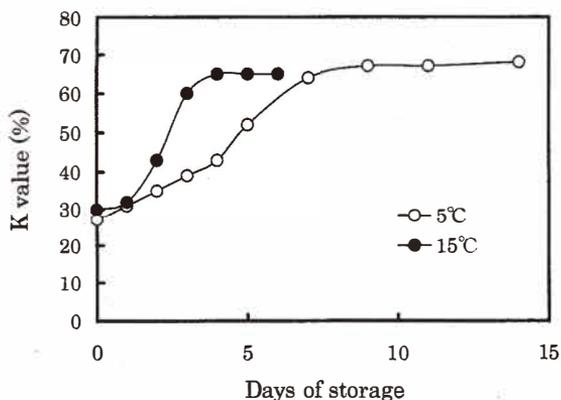


Fig. 3. Changes in K-value of chicken muscle during storage at 5 and 15°C. ○: Chicken extract during storage at 5°C. ●: Chicken extract during storage at 15°C.

downstream sensor. In chicken and fish, almost no diamine is produced in the very fresh range with a K-value of 30% or lower, and the diamine sensor does not respond. Therefore, to reduce chemical cross-talk, the diamine sensor was placed upstream in the construction of the fused system. The fused sensor system is shown in Fig. 4.

Since the optimal pH was 7.5~8.0 for the diamine sensor and 8.0~8.5 for the K-value sensor, Tris-HCl buffer, pH 8.0, was used in the fused sensors and the flow rate was 1.2 ml/min.

To the standard samples at K-values of 15% and 40%, putrescine at a specified concentration was added. Forty microliters of the sample was injected into the sensor system, and the effect of chemical cross-talk on the K-value sensor was investigated. The results are shown in Fig. 5. Deviation of the K-value occurred due to cross-talk at a putrescine concentration of 0.71 mg/100 ml. We attempted to correct the effect of cross-talk. Since the cross-talk increased linearly with putrescine concentration, the output of the K-value sensor was corrected using the slope as the correction coefficient, and the K-value was re-calculated. The results are shown in Fig. 6. By correcting the K-value, sufficient measurement precision with a correlation coefficient of 0.955 was obtained even in a range of high putrescine concentration. One assay could be completed within 5 min. The precision of the analysis was 2% of the coefficient of variation. The immobilized enzymes were stable over 100 days at 4°C.

#### 4. Conclusion

The K-value sensor was used to evaluate early stage freshness, and the diamine sensor could estimate the incipient stage of spoilage by measuring diamines. An enzyme flow reactor system could be constructed by fusing the K-value sensor and a diamine sensor for

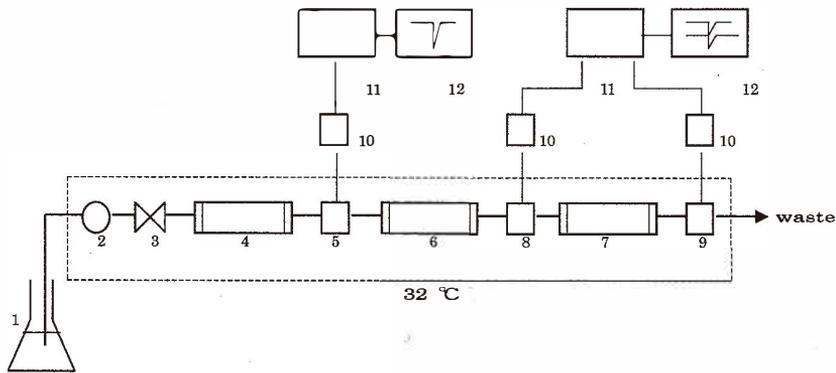


Fig. 4. Construction of the fused biosensor system for determination of freshness. 1. Buffer, 2. peristaltic pump, 3. injection port, 4. column for determination of diamine, 5. hydrogen peroxide electrode, 6. column (A) for determination of K-value, 7. column (B) for determination of K-value, 8. oxygen electrode (EL1), 9. oxygen electrode (EL2), 10. A/D converter, 11. computer, 12. recorder.

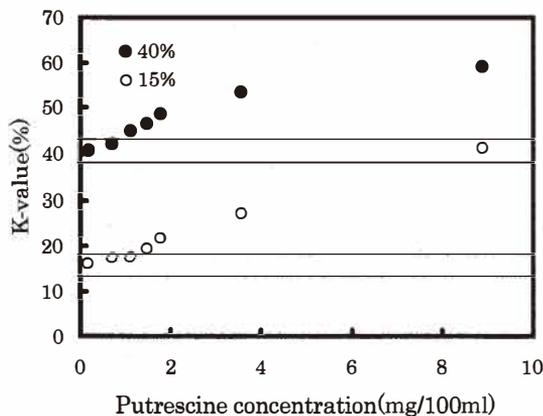


Fig. 5. Effect of chemical cross-talk on K-value. Measurement was carried out in 0.05M Tris-HCl buffer at 1.2ml/min, 32°C and pH 8.0. Forty microliter samples containing putrescine were injected. ○: Standard sample at K-value of 15%, putrescine at a specified concentration. ●: Standard sample at K-value of 40%, putrescine at a specified concentration.

serially detecting freshness up to the incipient stage of spoilage. Sufficient precision in the measurement could be achieved by correcting the effect of chemical cross-talk by fusing these reactors. Therefore, an enzyme reactor system can be developed for the determination of chicken quality over a wide range including both freshness and spoilage.

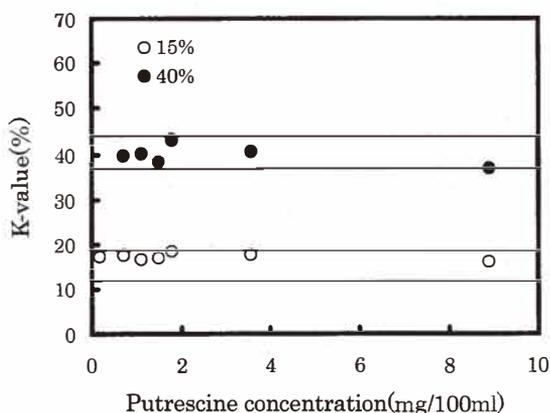


Fig. 6. Correlation between the corrected K-values and putrescine concentration. Measurement was carried out in 0.05 M tris-HCl buffer at 1.2ml/min, 32°C and pH 8.0. Forty microliter samples containing putrescine were injected. ○: Standard sample at K-value of 15%, putrescine at a specified concentration. ●: Standard sample at K-value of 40%, putrescine at a specified concentration.

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