12456 J. Agric. Food Chem. 2010, 58, 12456–12461
DOI:10.1021/jf102363a

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Improvement of Fish Freshness Determination Method by the Application of Amorphous Freeze-Dried Enzymes

Paveena Sirirangsan,[†] Naoko Hamada-Sato,*[,‡] Kiyoshi Kawai,[§] Manabu Watanabe,[†] and Toru Suzuki[†]

[†]Department of Food Science and Technology, and [‡]Course of Safety Management in Food Supply Chain, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan, and [§]Department of Biofunctional Science and Technology, Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan

Alkaline phosphatase (ALP), nucleoside phosphorylase (NP), and xanthine oxidase (XOD) were used in a colorimetric method for evaluation of fish freshness based on the K_i value. Two enzyme mixtures, NP-XOD and ALP-NP-XOD, were prepared with a color developing agent, and stabilities of the enzymes were improved by freeze-drying with glass-forming additives, i.e., sucrose and sucrose-gelatin. As a result, a linear relationship was obtained between the K_i values determined by the developed colorimetric method and a conventional high-performance liquid chromatography with a high correlation coefficient of 0.997. All enzyme samples containing the additive(s) were amorphous, and higher enzymes activities were maintained compared to those freeze-dried without an additive. Sucrose-gelatin/enzyme mixtures showed higher glass transition temperature; consequently, the enzymes were better stabilized than the sucrose/enzyme formulations. Using the sucrose-gelatin/enzyme mixture, K_i values of fish meat could be accurately determined even after 6-month storage of the dried enzymes at 40 °C.

KEYWORDS: Fish freshness; *K* value; K_i value; enzyme; sucrose; gelatin; glass transition; freeze-drying

1. INTRODUCTION

Evaluation of freshness is important for quality control of fish and marine products. The freshness-lowering of fish meat generally depends upon storage temperature and time. A variety of physical and chemical methods have been employed for freshness measurement of fish, including sensory analysis, microbiological techniques, assessment methods of total volatile basic nitrogen, trimethylamine, and nucleotide compounds (*1*, *2*). Among these methods, the relative content of nucleotides produced by the adenosine 5′-triphosphate (ATP) degradative pathway, in terms of the freshness index "*K* value", are considered the most reliable and useful indicator (*2*, *3*),

$$\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP} \rightarrow \text{Ino} \rightarrow \text{Hx} \rightarrow \text{uric acid}$$

where ADP, AMP, IMP, Ino, and Hx are adenosine 5′-diphosphate, adenosine 5′-monophosphate, inosine 5′-monophosphate, inosine, and hypoxanthine, respectively. The relative content of these compounds drastically changes during the decomposition. Therefore, the freshness index "*K* value" can be defined by the following equation (*4*):

$$K \text{ value } (\%) = \frac{[\text{Ino}] + [\text{Hx}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}] + [\text{IMP}] + [\text{Ino}] + [\text{Hx}]} \times 100$$

*Corresponding author. Tel: +81-3-5463-0389. Fax: +81-3-5463-0389. E-mail: hsnaoko@kaiyodai.ac.jp.

Because ATP, ADP, and AMP contents decrease rapidly and nearly become nil after 24 h storage at 0 °C, the *K* value can be simplified to the index "K_i value" (*3*, *5*) as follows:

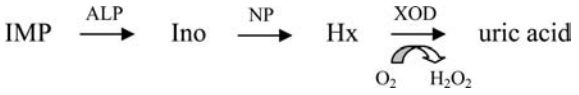
$$K_i \text{ value } (\%) = \frac{[\text{Ino}] + [\text{Hx}]}{[\text{IMP}] + [\text{Ino}] + [\text{Hx}]} \times 100$$

In general, the contents of IMP, Ino, and Hx have been conventionally measured by high-performance liquid chromatography (HPLC) and column chromatography (*6*, *7*). However, these methods require complicated and time-consuming procedures. Therefore, development of a simple and rapid method for routine use in food industries is required. Many efforts have been devoted to developing an easy method for measuring the freshness of fish, marine products, and livestock meat based on the *K* or K_i value. A portion of such research studies has been based on the use of enzymes, e.g., enzymatic methods (*8*), colorimetric methods (*6*, *9*−*13*), and electrode enzyme sensors (*3*, *5*, *14*−*19*). Unfortunately, all of the enzymes used are unstable and lose their activities during processing and storage. Therefore, it is necessary to maintain the activities of the enzymes.

In the present study, a colorimetric method for measurement of the K_i value using alkaline phosphatase (ALP), nucleoside phosphorylase (NP), and xanthine oxidase (XOD) was developed. The principle of this method was as follows. The extracted solution of fish meat is assayed using two freeze-dried enzyme mixtures: NP-XOD and ALP-NP-XOD. The decomposition of IMP and the subsequent accumulation of

 Published on Web 11/08/2010

Ino and Hx then occur through enzymatic reactions as shown below.



ALP is used to convert IMP to Ino. Likewise, Ino is converted to Hx by NP, and subsequently, XOD oxidizes Hx to uric acid. Then, hydrogen peroxide, which is formed during the oxidation of Hx to uric acid, reacts with a color developing agent (WST-8), resulting in the change in the sample solution color from colorless to orange of formazan. The intensity of developed color was measured spectrophotometrically at 454 nm for determination of [Ino + Hx] and [IMP + Ino + Hx] contents, which were used in the calculation of the K_i value.

The enzymes ALP, NP, and XOD are, however, thermally unstable and become almost completely inactive during freeze-drying and subsequent storage. Thus, it is of practical importance to stabilize these enzymes. In our previous study (*20*), the effects of various glass-forming additives, i.e., sucrose, trehalose, bovine serum albumin (BSA), and dextran, on the stabilization of freeze-dried XOD were investigated. The obtained results indicated that sucrose and sucrose−BSA mixtures are relatively good stabilizers. For application in fish freshness measurement, in this study sucrose and sucrose-gelatin were used to improve the stabilities of freeze-dried NP-XOD and ALP-NP-XOD. Gelatin, one of the protein polymers like BSA, was used due to its availability and low cost. Then, the potential of the developed colorimetric method to determine fish freshness was examined. Finally, the stability of the enzyme mixtures was investigated by observing the accuracy of measuring the K_i value after long-term storage of the dried enzymes at different temperatures.

2. MATERIALS AND METHODS

2.1. Reagents. NP (bacterial EC 2.4.2.1) was purchased from Sigma-Aldrich, Co., USA. 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8), was provided by Dojindo Laboratories, Japan. ALP (from calf intestine EC 3.1.3.1), XOD (from butter milk EC 1.1.3.22), inosine 5′-monophosphate disodium salt, inosine, analytical grade sucrose, gelatin, and other reagents were obtained from Wako Pure Chemical Industries, Ltd., Japan.

2.2. Preparation of Freeze-Dried Enzyme Mixtures. ALP, NP, and XOD were separately dialyzed against 20 mM potassium phosphate buffer (pH 7.8) at 4 °C for 48 h to remove stabilizing agents. Two enzyme mixtures, NP-XOD and ALP-NP-XOD, were prepared, for which the activities of NP:XOD and ALP:NP:XOD were determined to be in the proportions of 0.4:0.8 U/mL and 60:0.4:0.8 U/mL, respectively. The solution of each enzyme mixture was prepared with 0.3 mM WST-8 and 200 mM sucrose (SUC) or 200 mM sucrose + 0.5% (w/v) gelatin (SUC-GE) in 20 mM potassium phosphate buffer (pH 7.8). As the control, nonadditive samples of both enzyme mixtures were also prepared. Samples for the measurement of enzyme stability were prepared without WST-8. Aliquots (1 mL) of each solution were placed in 2 mL polypropylene tubes and frozen instantaneously with liquid nitrogen for at least 1 min. The frozen solids were transferred to a precooled freeze-drier. Freeze-drying was performed with a gradual increase in the temperature from −40 to 5 °C at the rate of 5 °C per 3 h, and from 5 to 25 °C at the rate of 10 °C per 3 h. The total time for freeze-drying was 36 h. The chamber pressure was maintained at 3.0×10^{-2} Torr throughout the drying process. After freeze-drying, the residual water in all samples was further removed over phosphorus pentoxide in a vacuum desiccator for 7 days at room temperature. The samples were hermetically sealed in a dry nitrogen-purged glovebox and stored at 5, 25, and 40 °C for a period of up to 172 days. Measurement of the K_i value of horse mackerel (*Trachurus japonicas*) meat with the freeze-dried enzyme mixtures was carried out at appropriate intervals during the storage.

2.3. Moisture Content Analysis. A Metrohm Karl Fisher coulometer (737 KF, Herisau, Switzerland) was used to measure the moisture content of freeze-dried samples.

2.4. Thermal Analysis. The physical properties of freeze-dried samples were examined by differential scanning calorimetry (DSC-50: Shimadzu, Co., Japan). Indium and distilled water were used to calibrate temperature and heat capacity for DSC measurements. Alumina powder was used as a reference material. A sample (approximately 15 mg) was weighed on an aluminum DSC pan in a dry nitrogen-purged glovebox and sealed hermetically. All measurements were performed from 0 to 180 °C at a scan rate of 5 °C/min. The values of the glass transition temperature (T_g) and crystallization temperature (T_c) were determined from the onset temperatures of endothermic shift and exothermic peak, respectively.

2.5. Assay of Enzyme Activity. The activities of NP-XOD and ALP-NP-XOD were separately assayed by the enzymatic conversion of substrate Ino and IMP, respectively. The freeze-dried samples (without WST-8) were rehydrated with distilled water to render the previous concentration, and the sample solution (75 μL) was added to 225 μL of 1.33 mM substrate (Ino for the NP-XOD samples and IMP for the ALP-NP-XOD samples) in 20 mM potassium phosphate buffer (pH 7.6). In this study, the enzymatic activity of each enzyme mixture was evaluated as "total enzymatic activity" for catalyzing the reduction of the substrate to produce uric acid. The time course for absorbance at 292 nm, which is the maximal absorption peak of uric acid, was determined at 20 °C using a UV−vis spectrophotometer (V-630BIO, Jasco, Tokyo, Japan). Enzyme activity was evaluated from the initial reaction rate. Remaining activity was expressed as a percentage of the activity relative to that determined before freeze-drying.

2.6. Preparation of Fish Extract. After being caught, horse mackerel were killed by stabbing on board and then immediately stored in a refrigerator (0 °C) for up to 7 days. Fish extraction was carried out every day during the storage in accordance with the modified method of Ryder (*21*). Two grams of fish meat was homogenized with 10 mL of 10% perchloric acid. The homogenate was centrifuged at 13400*g* for 10 min at 5 °C, and the supernatant was neutralized to pH 7.0 with 8 and 1 N KOH. The neutralized mixture was recentrifuged at 15940*g* for another 2 min at 5 °C. The supernatant was filtrated through a 0.45 μM diameter filter and adjusted to 25 mL with a neutralized solution of 10% perchloric acid, and then filtered prior to storage at −90 °C for subsequent analysis.

2.7. HPLC Method. HPLC was employed as the conventional method. ATP-related compounds were separated using a multimode column (GS-320 HQ, Asahipak, Kanagawa, Japan). The mobile phase of 0.2 M sodium phosphate buffer (pH 3.8) was used at a flow rate of 1 mL/min at 25 °C. The eluent was monitored at 258 nm for each ATP-related compound. The experiment was done in triplicate.

2.8. The Developed Colorimetric Method. The freeze-dried samples of NP-XOD and ALP-NP-XOD were separately rehydrated with distilled water to render the previous concentration. For the preparation of calibration curves, the NP-XOD solution (150 μL) was added into 150 μL each of 0, 5, 10, 20, 30, and 50 μM Ino standard solution prepared in 20 mM potassium phosphate buffer (pH 7.8). Similarly, the ALP-NP-XOD solution (150 μL) was added into 150 μL each of 0, 5, 10, 20, 30, and 50 μM IMP standard solutions prepared in 20 mM potassium phosphate buffer (pH 7.8). For fish freshness measurement, both the NP-XOD and the ALP-NP-XOD solutions (150 μL) were separately added into 20 μL of fish extracts and mixed with 130 μL of 20 mM potassium phosphate buffer (pH 7.8). Then, the mixtures of each sample solution were incubated at 37 °C for 20 min. Then, absorbance at 454 nm, which is the maximal absorption peak of the produced formazan, was measured at 25 °C using a UV−vis spectrophotometer (V-630BIO, Jasco, Tokyo, Japan). A calibration curve was plotted for each of the enzyme mixtures. The absorbance results of the fish extracts were converted into the concentrations of [Ino + Hx] and [IMP + Ino + Hx] using the calibration curves, and then converted to the K_i value. To confirm the reliability of the results obtained from the fish extracts, the freeze-dried enzyme mixtures were also assayed using Ino and IMP standard solutions at 5, 20, and 50 μM. All experiments were done in triplicate.

2.9. Statistical Analysis. Analysis of variance (ANOVA) was used to search for significant differences between mean values of the different results (at $p < 0.05$). The parallels $n = 3$ were used for all analyses. Significant differences between groups in the changes of [Ino + Hx], [IMP + Ino + Hx] contents, and K_i (%) as measured by colorimetric method and

Table 1. Sample Abbreviations, T_g, T_c, and the Moisture Content (%) of Freeze-Dried Samples

enzyme	formulation	abbreviation	T_g[a] (°C)	T_c[a] (°C)	% moisture content[b]
NP-XOD	200 mM sucrose	SUC	54	105	2.64 ± 0.76
	200 mM sucrose + 0.5% gelatin	SUC-GE	75	139	0.34 ± 0.05
ALP-NP-XOD	200 mM sucrose	SUC	53	103	2.82 ± 0.13
	200 mM sucrose + 0.5% gelatin	SUC-GE	74	134	0.73 ± 0.20

[a] The values are expressed as mean (n = 2). [b] The values are expressed as mean ± SD (n = 3).

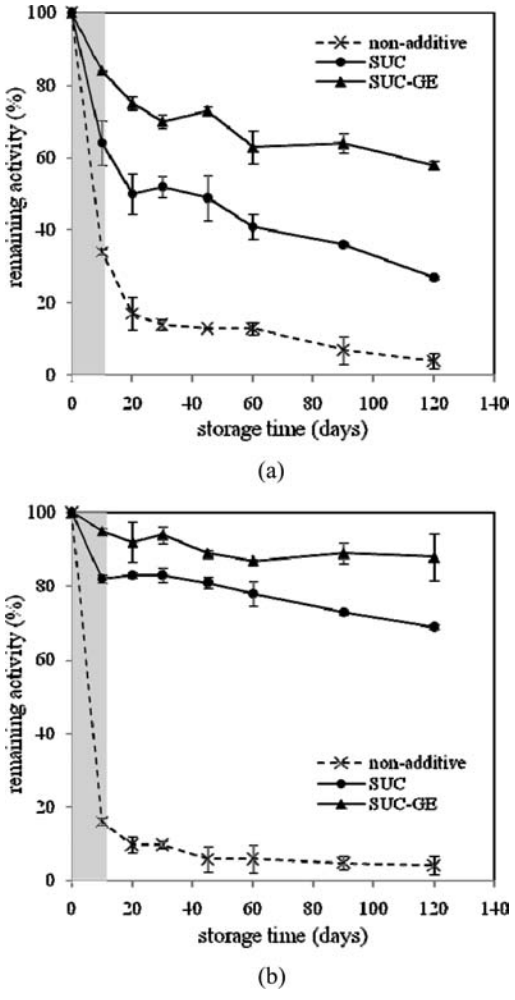


Figure 1. Remaining activities (%) of (**a**) NP-XOD and (**b**) ALP-NP-XOD after freeze-drying and storage at 25 °C. The shaded areas mean the time for 36 h freeze-drying and subsequent 7-day further dehydration. The values are mean ± SD (n = 3).

HPLC were determined by the Student's t test using XLSTAT (Addinsoft Inc., France). The significant level was $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Stability of Enzyme Mixtures. The stabilities of ALP, NP, and XOD are very important for the performance of the colorimetric method in the fish freshness measurement. The stabilities of the two enzyme mixtures, NP-XOD and ALP-NP-XOD, were therefore initially investigated. **Table 1** shows a list of the abbreviations of all samples and the T_g, T_c and moisture content of the freeze-dried samples. The moisture content of SUC was slightly higher than those of SUC-GE samples. SUC-GE showed a higher T_g than SUC. This is because a high molecular weight gelatin has a higher T_g and a greater resistance to a decrease in T_g, induced by the plasticizing effect of water, than sucrose. In addition, T_c values of SUC-GE were higher than those of SUC samples. This indicates

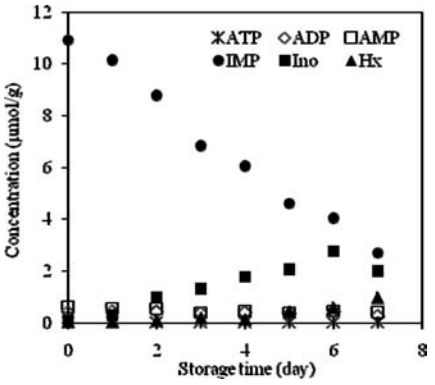


Figure 2. Changes in concentrations of ATP-related compounds in horse mackerel meat stored at 0 °C as measured by HPLC.

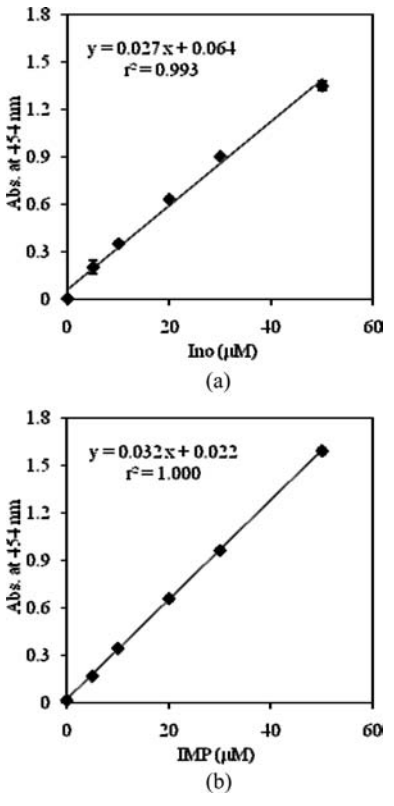


Figure 3. Calibration curves for (**a**) Ino and (**b**) IMP assayed using NP-XOD and ALP-NP-XOD, respectively. The values are mean ± SD (n = 3).

that the physical stability of the dried samples is improved by the addition of gelatin. In the comparison between NP-XOD and ALP-NP-XOD mixtures, it was noted that the presence of ALP showed little or no effect on the T_g and T_c of the samples.

The remaining activities of NP-XOD and ALP-NP-XOD during storage at 25 °C for up to 120 days are shown in **Figure 1**. The activities decreased markedly during freeze-drying and subsequent further dehydration, and then gradually decreased during storage.

Nonadditive samples of either NP-XOD or ALP-NP-XOD showed a drastic decrease in enzyme activity to approximately 4% after 120 days of storage. SUC, on the other hand, maintained the activity to a certain extent; about 30 and 70% remained in NP-XOD and ALP-NP-XOD, respectively, after 120 days of storage. Furthermore, SUC-GE protected the enzyme mixtures most effectively, that is, approximately 60% activity of NP-XOD and 90% activity of ALP-NP-XOD were preserved. SUC and SUC-GE formed high-viscous glassy matrices, embedding the enzymes, and the rate of degradation is consequently decreased owing to the restrictive molecular mobility (*20*, *22*−*24*). In addition, the higher the T_g, the lower the molecular mobility at storage temperature (*25*). Based on this finding, SUC-GE showed a higher T_g than SUC; therefore, SUC-GE prevented enzyme degradation to a greater degree than SUC because of its lower molecular mobility.

3.2. Changes in Concentrations of ATP-Related Compounds in Horse Mackerel. The contents of ATP-related compounds in horse mackerel meat during storage in a refrigerator (0 °C) for up to 7 days were determined by the conventional HPLC method, and the results are shown in **Figure 2**. ATP and ADP concentrations rapidly decreased immediately after death. AMP concentration also drastically decreased and became less than 0.50 μmol/g. Thus, instead of the *K* value the freshness index can be defined as the K_i value. IMP concentration decreased almost linearly from 10.91 to 2.70 μmol/g during storage, whereas Ino concentration increased from 0.12 to 2.76 μmol/g in the early period of storage

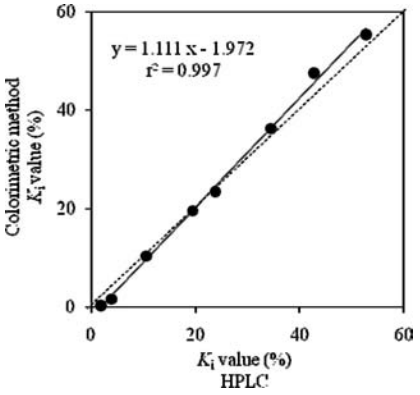


Figure 4. Comparison of K_i values determined by colorimetric method and HPLC. The dashed line indicates the expected linear relation when the slope is 1.

Table 2. Changes in [Ino + Hx] and [IMP + Ino + Hx] Contents and K_i (%) in Horse Mackerel Meat during Storage at 0 °C as Measured by Colorimetric Method and HPLC[a]

	HPLC			colorimetric method		
storage time (days)	Ino + Hx (μmol/g)	IMP + Ino + Hx (μmol/g)	K_i (%)	Ino + Hx (μmol/g)	IMP + Ino + Hx (μmol/g)	K_i (%)
0	0.19	11.10	1.7	0.04	11.71	0.3
1	0.39	10.22	3.8	0.19	12.02	1.6
2	1.05	9.98	10.5	1.19	11.51	10.3
3	1.63	8.38	19.4	1.63	8.32	19.6
4	1.95	8.21	23.7	2.13	9.12	23.3
5	2.54	7.35	34.6	2.76	7.63	36.2
6	3.17	7.42	42.7	3.41	7.18	47.5
7	3.01	5.71	52.7	2.83	5.10	55.5

[a] The values shown in this table are mean of results obtained from three experiments. There were no significant differences ($p > 0.05$) in the values obtained from HPLC and colorimetric method.

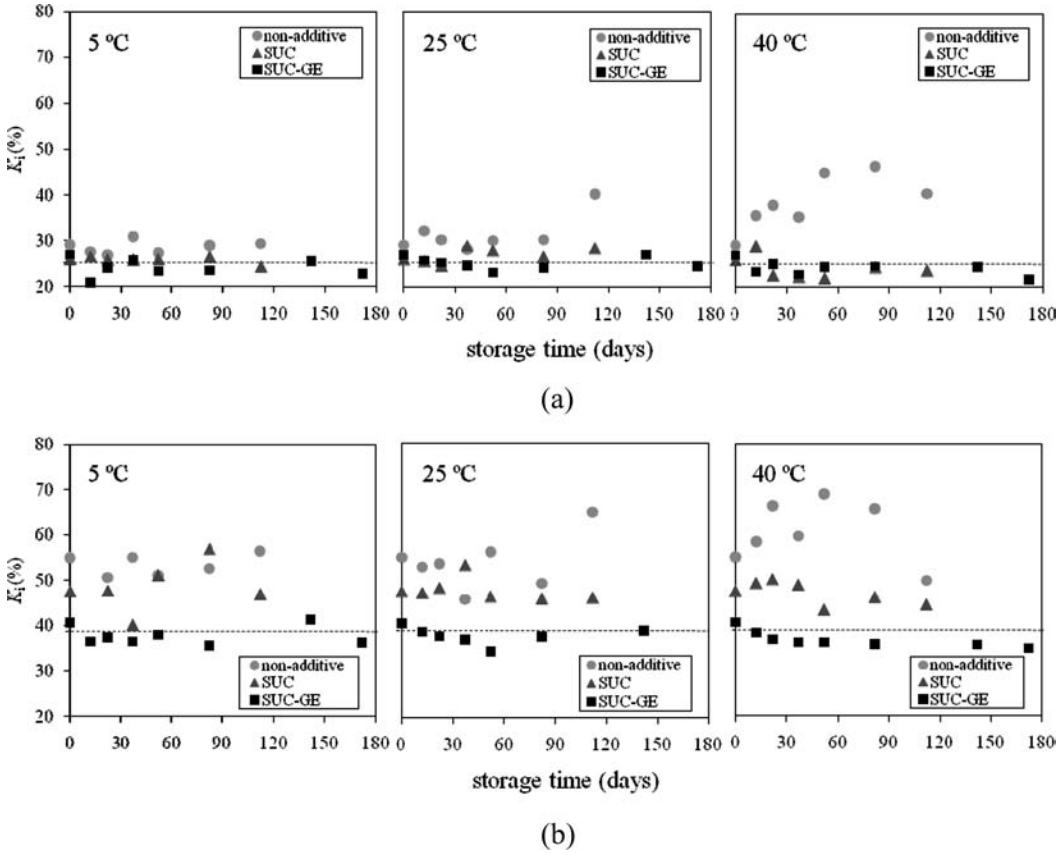


Figure 5. K_i values of (**a**) 4-day-old and (**b**) 6-day-old fish extracts measured with the stored enzyme mixtures at 5, 25, and 40 °C. Dashed lines represent the original K_i values.

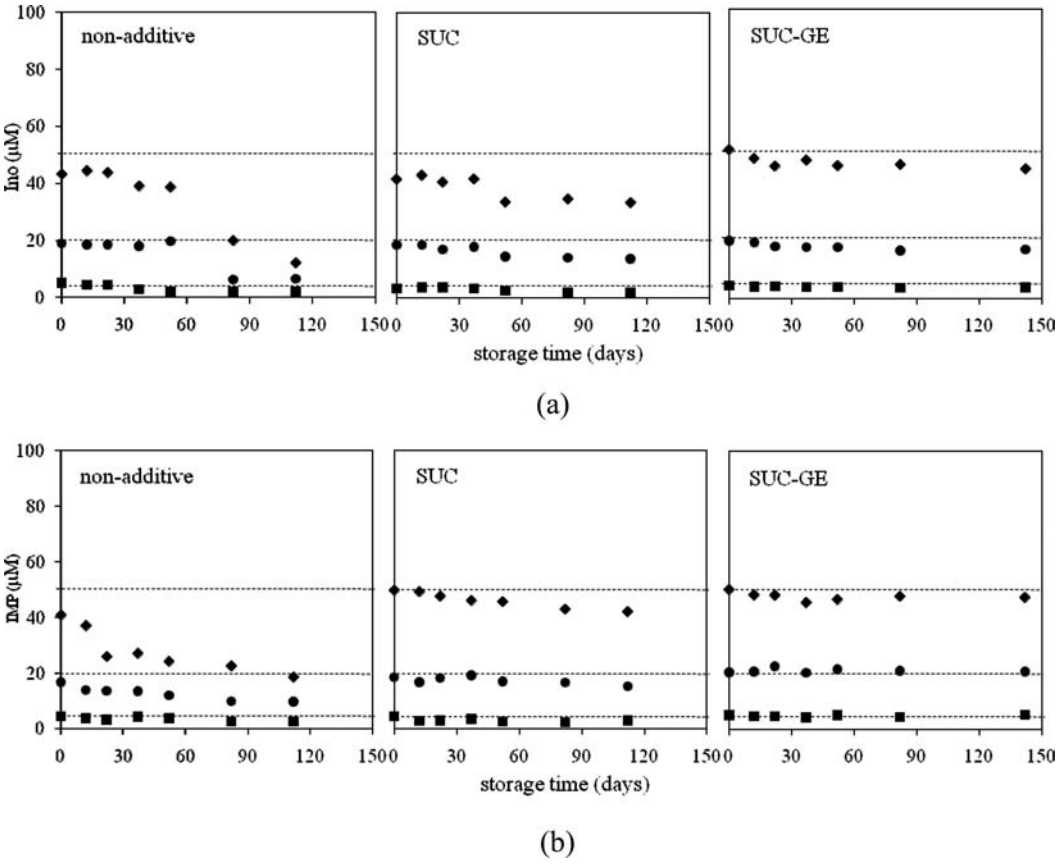


Figure 6. Precision of (**a**) Ino and (**b**) IMP concentrations at (■) 5, (●) 20, and (◆) 50 μM measured with the stored enzyme mixtures at 40 °C. Dashed lines represent the actual concentration.

and then inversely decreased to 2.01 μmol/g. Hx concentration increased from 0.07 to 1.00 μmol/g over the storage period.

3.3. Calibration Curves. The calibration curves for Ino and IMP are shown in **Figure 3**. A linear relationship between the concentrations of Ino (and IMP) and the absorbance of the compound was observed. The correlation coefficients were 0.993 and 1.000, and the regression equations were $y = 0.027x + 0.064$ and $y = 0.032x + 0.022$ for the curves of Ino and IMP, respectively. Because the total concentration of IMP, Ino, and Hx in the fish extract was adjusted to 50 μM or less, the evaluation of K_i was considered to be possible using these calibration curves.

3.4. Comparison of K_i Values Determined by Colorimetric Method and HPLC. The extracts of horse mackerel stored at 0 °C for up to 7 days were assayed using the two enzyme mixtures, NP-XOD and ALP-NP-XOD. Then, the contents of [Ino + Hx] and [IMP + Ino + Hx] were obtained from the calibration curves, and K_i values of the fish samples were consequently calculated from the relative content of these compounds, as shown in **Table 2**. The [Ino + Hx] and [IMP + Ino + Hx] contents and K_i values determined using the enzyme mixtures (the colorimetric method) were comparable to those determined by HPLC. There were no significant differences ($p > 0.05$) in the results obtained from both methods. A good correlation between K_i values determined by both methods was observed, as shown in **Figure 4**. A linear relationship was obtained with a high correlation coefficient of 0.997, and the regression equation was $y = 1.111x - 1.972$. From these results, the proposed colorimetric method was applicable for the determination of the K_i value for evaluation of fish freshness.

3.5. Accuracy of Measuring the K_i Value by Colorimetric Method after Long-Term Storage of Dried Enzymes. The operational storage stability of freeze-dried NP-XOD and ALP-NP-XOD in measuring the K_i value of horse mackerel was investigated. Freeze-dried enzyme mixtures stored at 5, 25, and 40 °C for a period of up to 172 days were used. K_i values were determined using these enzyme mixtures to assay 4- and 6-day-old fish extracts (approximately 26 and 40% K_i, respectively, evaluated by HPLC) that were stored at −90 °C to prevent decomposition of ATP-related compounds until the analyses. The results are shown in **Figure 5**. K_i values measured with enzyme mixtures freeze-dried without protective additives significantly deviated ($p < 0.05$) from the original measurements made before enzyme storage for both the fish extracts, and these deviations increased as storage temperature increased. The enzyme mixtures freeze-dried in SUC formulation accurately measured K_i of the 4-day-old fish extract regardless of storage temperature and time with no significant changes in the K_i values ($p > 0.05$); however, for the 6-day-old fish extract, the obtained value significantly deviated ($p < 0.05$) from its original value at all given storage temperatures. Okuma and Watanabe (*16*) proposed that the main factor for the decrease in measuring accuracy of the enzyme reactor in their study is probably inactivation of XOD. A reduction in the activity of XOD would result in lower uric acid content being measured (by color) than there would have been if complete conversion had occurred. This would tend to underestimate both the numerator and denominator of the K_i equation. But the denominator underestimation would be worse since it should have more Hx after both IMP and Ino are converted, this would get incompletely converted to uric acid, and the [IMP + Ino + Hx] absorbance would be really low. With a low denominator, the K_i value would then be a falsely higher value. In addition, it was noted that the K_i values of the 6-day-old fish extract measured with the stored enzymes of nonadditive and SUC formulations were overestimated worse than those of the 4-day-old fish extract. This can be understood by considering that the high numerator of the K_i value results in the great falsely

higher value of K_i. On the other hand, the enzyme mixtures freeze-dried with SUC-GE accurately determined the K_i of either 4- or 6-day-old fish extracts even after 172-day storage of the enzymes at all given temperatures. There were no significant changes ($p > 0.05$) in the K_i values obtained during the storage. Only a slight decrease of the K_i values was found at 40 °C because of a decrease in reaction sensitivity of WST-8 at high temperature.

To confirm the reliability of the results obtained from the fish extracts, standard solutions of Ino and IMP at 5, 20, and 50 μM were also assayed using the freeze-dried enzyme mixtures, which were stored at 40 °C prior to the analysis. The results are shown in **Figure 6**. Similar to the results of fish extracts, nonadditive mixtures of NP-XOD and ALP-NP-XOD respectively failed to evaluate concentrations of Ino and IMP ($p < 0.05$), particularly at high concentrations of the solutions. The enzyme mixtures freeze-dried with SUC enabled the determination of the concentrations of the standard solutions accurately in the early period of storage; however, with increasing storage time the observed concentrations of Ino and IMP were considerably and slightly lower ($p < 0.05$) than those of the actual concentrations, respectively. On the other hand, the dried enzyme mixtures in the SUC-GE formulation enabled the accurate determination of the concentrations of both standard solutions for all given concentrations with no significant differences ($p > 0.05$).

3.6. Conclusions. Changes in the concentrations of ATP-related compounds of horse mackerel meat were examined during storage at 0 °C. The proposed enzymatic method was capable of measuring the K_i value accurately, and was therefore useful for assessing the freshness of fish meat. NP-XOD and ALP-NP-XOD freeze-dried with sucrose-gelatin showed sufficiently high stabilities for determining K_i efficiently even after storage at 40 °C for up to 172 days. This colorimetric method enables an economical and reliable measurement for fish freshness with simple and rapid operation. For further study, this developed method should be modified to facilitate users in more practical approaches rather than using a spectrophotometer.

LITERATURE CITED

(1) Ehira, S.; Uchiyama, H. Determination of fish freshness using the *K* value and comments on some other biochemical changes in relation to freshness. In *Seafood Quality Determination*; Kramer, D. E., Liston, J., Eds.; Elsevier Science Publisher B. V.: Amsterdam, 1986; pp 185−207.

(2) Hanna, J.; Rapid microbial methods and fresh fish quality assessment. In *Fish Processing Technology*; Hall, G. M., Ed.; Blackie Academian & Professional: Glasgow, 1992; pp 275−305.

(3) Karube, I.; Matsuoka, H.; Suzuki, S.; Watanabe, E.; Toyama, K. Determination of fish freshness with an enzyme sensor system. *J. Agric. Food Chem.* **1984**, *32*, 314−319.

(4) Saito, T.; Arai, A.; Matsuyoshi, M. A new method for estimating the freshness of fish. *Bull. Jpn. Soc. Sci. Fish.* **1959**, *24*, 749−750.

(5) Nanjyo, Y.; Yao, T. Rapid measurement of fish freshness indices by an amperometric flow-injection system with a 16-way switching valve and immobilized enzyme reactors. *Anal. Chim. Acta* **2002**, *470*, 175−183.

(6) Kaminishi, Y.; Nakaniwa, K.; Kunimoto, M.; Miki, H. Determination of *K*-value using freshness testing paper and freshness prediction of the finfishes stored at some different temperatures by the kinetic parameters. *Fish. Sci.* **2000**, *66*, 161−165.

(7) Valle, M.; Malle, P.; Bouquelet, S. Evaluation of fish decomposition by liquid chromatographic assay of ATP degradation product. *J. AOAC Int.* **1998**, *81*, 571−575.

(8) Ehira, S.; Uchiyama, H. Rapid estimation of freshness of fish by nucleoside phosphorylase and xanthine oxidase. *Bull. Jpn. Soc. Sci. Fish.* **1969**, *35*, 1080−1085 (in Japanese).

(9) Ehira, S.; Saito, K.; Uchiyama, H. Accuracy of measuring *K* value, an index for estimating freshness of fish, by freshness testing paper. *Bull. Tokai Reg. Fish. Res. Lab.* **1986**, *120*, 73−82 (in Japanese).

(10) Negishi, S.; Karube, I. An enzymatic assay method for IMP determination using IMP dehydrogenase and an application of the principle to a test paper method. *Nippon Suisan Gakkaishi* **1989**, *55*, 1591−1597 (in Japanese).

(11) Negishi, S.; Watanabe, E. Colorimetric determination of freshness index *K*-value with thiazolyl blue tetrazolium bromide (MTT). *Bull. Jpn. Soc. Sci. Fish.* **1986**, *52*, 1695 (in Japanese).

(12) Uda, F.; Hayashi, E.; Uchiyama, H.; Kakuda, K. Colorimetric method for measuring *K* value, an index for evaluating freshness of fish. *Bull. Tokai Reg. Fish. Res. Lab.* **1983**, *111*, 55−62.

(13) Watanabe, E.; Tamada, Y.; Hamada-Sato, N. Development of quality evaluation sensor for fish freshness control based on K_i value. *Biosens. Bioelectron.* **2005**, *21*, 534−538.

(14) Okuma, H.; Watanabe, E. Flow system for fish freshness determination based on double multi-enzyme reactor electrodes. *Biosens. Bioelectron.* **2002**, *17*, 367−372.

(15) Park, I. S.; Cho, Y. J.; Kim, N. Characterization and meat freshness application of a serial three-enzyme reactor system measuring ATP-degradative compounds. *Anal. Chim. Acta* **2000**, *404*, 75−81.

(16) Shin, S. J.; Yamanaka, H.; Endo, H.; Watanabe, E. Development of ornithine biosensor and application to estimation of prawn freshness. *Anal. Chim. Acta* **1998**, *364*, 159−164.

(17) Watanabe, E.; Ando, K.; Karube, I.; Matsuoka, H.; Suzuki, S. Determination of hypoxanthine in fish meat with an enzyme sensor. *J. Food Sci.* **1983**, *48*, 496−500.

(18) Watanabe, E.; Toyama, K.; Karube, I.; Matsuoka, H.; Suzuki, S. Determination of inosine 5′-monophosphate in fish tissue with an enzyme sensor. *J. Food Sci.* **1984**, *49*, 114−116.

(19) Yano, Y.; Kataho, N.; Watanabe, M.; Nakamura, T.; Asano, Y. Evaluation of beef aging by determination of hypoxanthine and xanthine contents: application of a xanthine sensor. *Food Chem.* **1995**, *52*, 439−445.

(20) Srirangsan, P.; Kawai, K.; Hamada-Sato, N.; Watanabe, M.; Suzuki, T. Improvement in the remaining activity of freeze-dried xanthine oxidase with the addition of a disaccharide-polymer mixture. *Food Chem.* **2010**, *119*, 209−213.

(21) Ryder, J. M. Determination of adenosine triphosphate and its breakdown products in fish muscle by high performance liquid chromatography. *J. Agric. Food Chem.* **1985**, *33*, 678−680.

(22) Anchordoquy, T. J.; Izutsu, K.; Randolph, T. W.; Carpenter, J. F. Maintenance of quaternary structure in the frozen state stabilizes lactate dehydrogenase during freeze-drying. *Arch. Biochem. Biophys.* **2001**, *390*, 35−41.

(23) Franks, F. Solid aqueous solutions. *Pure Appl. Chem.* **1993**, *65*, 2527−2537.

(24) Wang, W. Lyophilization and development of solid protein pharmaceuticals. *Int. J. Pharm.* **2000**, *203*, 1−60.

(25) Roos, Y. H. Prediction of the physical state. In *Phase Transitions in Food*; Roos, Y. H., Ed.; Academic Press: San Diego, 1995; pp 157−188.

Received for review June 19, 2010. Revised manuscript received October 11, 2010. Accepted October 14, 2010.