Utilization of ATP and Its Derivatives as an Index of Freshness of Nila (*Oreochromis niloticus*) During Storage

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ABSTRACT

The role of a reliable and reproducible, index of fish freshness, is important. The objective of this research was to examine the utilisation of ATP and its derivatives (expressed as K-value) as an index of fish freshness using Nila (Oreochromis niloticus) as a model of study. K-value is [(Inosine + Hypoxanthine) / (ATP + ADP + AMP + IMP + Inosine + Hypoxanthine)] x 100%. Live fish were killed, packed individually using polyethylene bag and stored at 4 and 28°C. At defined time, some fishes were examined their ATP and its breakdown products using a reverse phase HPLC system. The results showed that ATP, ADP and AMP were degraded rapidly and disappeared within 12 hours. Degradation of IMP in the samples stored at 4°C was slower than those of samples stored at 28°C. Interestingly, the accumulation of Inosine occurs only in the samples stored at 28°C while the accumulation of Hypoxanthine occurs only in the samples stored at 4°C. The data suggested that the activity of IMP-degrading enzyme (5'-nucleotidase) at 4°C was higher than that of Inosine-degrading enzyme, but at 28°C both enzymes have similar activities. Linear regression analysis between K-value and storage time showed that increasing rate of K-value or rate of the lowering freshness of samples stored at 28°C was 4 times higher than that of samples stored at 4°C. Limits of fish acceptability (K-value 60%) of Nila stored at 28 and 4°C were reached at 12 and 72 hours, respectively. The results confirmed that K-value was the best as an index of fish freshness compared with IMP, Inosine and Hypoxanthine and it could be used as an index of freshness of Nila (Oreochromis niloticus) during storage.

INTRODUCTION

Safety and freshness of fishery products are important factors in the international trade. Based on this, it is important to develop a method that could be detected fish freshness having high reproducibility, high reliability and not time consuming.

In 1959, Saito et al. has been proposed a new index for fish freshness using ATP and its breakdown products namely K-value. K-value is [(Inosine + Hypoxanthine)/ (ATP + ADP + AMP + IMP + Inosine + Hypoxanthine)] x 100%. The higher K-value the higher lowering freshness. In 1987, Ehira and Uchiyama have been comprehensively reviewed on the lowering freshness of 104 fish samples using TMA-N, TVB-N, and K-value. They concluded that only K-value that could reflects the real freshness and gave almost the same results as the commercial sensory evaluation based on actual purchases. Based on this, K-value has been widely used in Japan as a standard of fish freshness either commercially or officially.

In 1964, Jones et al. proposed Hipoxanthine as an index of fish freshness. Unfortunately, formation of Inosine and Hypoxanthine in fish muscle during iced storage differs among species. Therefore, it was believed that the freshness of Inosine-forming species could not be estimated by Hypoxanthine. In the previous paper (Marseno et al., 1998), we developed a new rapid method to detect fish freshness using immobilized ADP-ase and 5'-nucleotidase on polyacrylamide gel. The presence of ADP and IMP, that could be detected by ADPase and 5'-nucleotidase, respectively, reflects that the fish examined is fresh. However, the presence of free inorganic phosphate reduced the accuracy and reproducibility of the method.

In the previous paper, we found that K-value is the

best parameters for estimating the freshness of Mas (*Cyprus carpio)* compared with IMP, Inosine or Hypoxanthine (Sudarmanto et al., 1998). The aim of this research is to examine the utilisation of ATP and its derivative (expressed as K-value) as an index of freshness of Nila *Oreochromis niloticus* during storage.

MATERIALS AND METHODS

Chemicals

Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Inosine monophosphate (IMP), Inosine (HxR) and Hypoxanthine (Hx) were purchased from Sigma Chemicals Co. (USA). All other chemicals used were of analytical grade.

Preparation of fish

Live fish Nila *Oreochromis niloticus* was obtained from local market in Yogyakarta. Live fishes were transported to the laboratory using polyethylene tank, and killed immediately to reduce the effect of stress that could affects on the content of ATP and its derivatives. The fish were packed individually using polyethylene bag and stored at 4°C and 28°C (room temperature). At defined time, the fishes were withdrawn, cut into small pieces, mixed and extracted their ATP and its derivatives.

Extraction of ATP and its derivatives

The extraction procedure of Tsuchimoto et al. (1985) and Ryder (1985) was adopted with slight modification as described previously (Kayama et al., 1988; Suwetja, 1988; Marseno, 1993; Sudarmanto et al., 1998). One gram of muscle was homogenized with 2 ml of cold 10% perchloric acid (PCA). The homogenate was centrifuged at 1,900xg (3000 rpm) for 10 min. at 4°C, and the supernatant was kept in ice and the precipitate was mixed with 2 ml of cold 5 % PCA, and then centrifuged at 1900 x g for 3 min. The resulted supernatants were collected and immediately neutralized with KOH to pH 6.4 - 6.8. After standing for 30 min. in ice, it was centrifuged at 1,900 x g for 3 min. at 4°C. The resulted supernatant was collected and diluted to 10 ml by neutralized cold 5% PCA of pH 6.4. The extract was kept at -80°C until the time of analysis.

HPLC Analysis

The reversed-phase HPLC was performed according to the method of Suwetja (1988) with slight modification as described previously (Sudarmanto et al., 1998) using a Beckman 116 HPLC system equipped with an injector, a program panel gradient, a unit panel of elution holder (IBM-System Gold), a chromatocorder (Crompack-Cr 3A) and a UV-detector (Beckman 166-UV). The column used was a reverse phase type ODS stainless steel column Cosmosil 5C-18 (4,6 mm I.D x 25 cm, Nakalai Chemicals, Japan) equipped with a pre-column type 10C-18 (4.6 mm I.D x 5 cm, Nakalai Chemicals, Japan).

The operation condition of HPLC was adopted from Suwetja (1988) and Sudarmanto et al., 1998. Two eluents were used, namely, eluent A (0.05 M Potassium phosphate buffer pH 6.4) and eluent B (eluent A containing 37.5 % methanol). Both eluents were used at a flow rate of 0.7 ml/min., operation pressure was about 1000 psia and absorbency was at 254 nm. All of the solutions used were filtered using 0.45 µm Toyo filter before injected into the column. Injected volume was 20 µl. The areas of peaks were automatically calculated by the chromatocorder, and the quantity of ATP and its derivatives were identified by comparing with standards.

Rate of the lowering freshness

In 1988, Suwetja proposed that rate of the lowering freshness (Kf) in fish = slope x 2.303. Log (100 - KV) = Log (100 - Ko) - $\frac{Kf}{2.303}t$

If the formula was simplified that Log (100-KV) as Y and t as X, so the slope = Kf/2.303 or Kf = slope x 2.303. In this case (100-KV) is remaining amount of total nucleotide at defined time t; and (100-Ko) is amount of total nucleotide at the beginning of storage time; t is storage time. Log (100-KV) will give a linear regression against storage time.

RESULTS AND DISCUSSIONS

Changes of ATP and its derivatives

The changes of ATP and its derivatives of Nila *Oreochromis niloticus* during storage at 28°C and 4°C·

are shown in Table 1 and 2. The data shows that either stored at 28 or 4°C ATP could not be detected at the beginning of storage, while ADP disappeared within 12 hours. Decreasing rate of IMP in the samples stored at 28°C was faster than that of the samples stored at 4°C. Accumulation of IMP in the samples stored at 4°C reached a highest concentration at 12 hours storage, then gradually decreased. On the other hand, IMP content in the samples stored at 28°C disappeared within 24 hours.

Table 1. **Changes of ATP and its derivatives of Nila *Oreochromis niloticus* during storage at 28°C.**

torage time hours)	µmole/g muscle						K-value (%)
	ATP	ADP	AMP	IMP	HxR	Hx	
0	–	1,38	0,18	4,41	1,69	0,40	25,93
4	–	1,20	0,15	4,96	2,17	0,92	32,87
8	–	0,58	0,09	5,22	3,73	1,01	44,59
12	–	0,46	0,04	4,07	3,28	2,92	57,57
24	–	–	–	–	3,39	5,66	100
36	–	–	–	–	3,01	6,83	100
48	–	–	–	–	1,30	3,42	100

Not Detected

Table 2. **Changes of ATP and its derivatives of Nila *Oreochromis niloticus* during storage at 4°C.**

orage time ours)	µmole/g muscle						K-value (%)
	ATP	ADP	AMP	IMP	HxR	Hx	
0	–	1,56	tr	4,21	0,97	tr	14,39
12	–	tr	tr	7,57	1,25	0,14	15,51
24	–	–	–	5,15	1,27	0,16	21,73
36	–	–	–	5,01	3,49	0,20	42,41
48	–	–	–	4,84	4,15	0,22	47,39
72	–	–	–	3,44	4,26	0,23	56,62
96	–	–	–	2,94	5,64	0,3	66,97
20	–	–	–	1,99	6,66	0,36	77,91
44	–	–	–	1,65	6,56	0,58	81,23
68	–	–	–	–	4,03	0,72	100

Not Detected
trace

At the fresh stage (K-value below 60%), formation nosine (HxR) in the samples stored at 28°C was faster n those of samples stored at 4°C. Interestingly, in the eriorate stage (K-value greater than 60%), accumu- on of HxR occurred only in the samples stored at 4°C accumulation of Hipoxanthine (Hx) occurred only ne samples stored at 28°C. Moreover, Table 3 shows the samples stored at 28°C have the same ratio of ' : HxR and HxR : Hx, but for samples stored at 4°C e a higher ratio for HxR:Hx than that of IMP : HxR. data suggested that IMP degrading enzyme (5'-nucleotidase) has a higher activity than that of HxR degrading enzyme at 4°C, but at 28°C both enzymes have the same activity.

Table 3. **Ratio of IMP : HxR and HxR : Hx of Nila *Oreochromis niloticus* during storage at 28°C (A) and 4°C (B)**

(A)

Storage time (hours)	K-value (%)	IMP:HxR	HxR:Hx
0	25.93	2.60 : 1	4.23 : 1
4	32.87	2.29 : 1	2.36 : 1
8	44.59	1.40 : 1	3.69 : 1
12	57.57	1.24 : 1	1.12 : 1

(B)

Storage time (hours)	K-value (%)	IMP:HxR	HxR:Hx
0	14.39	4.34 : 1	–
12	15.51	6.06 : 1	8.93 : 1
24	21.73	4.05 : 1	7.94 : 1
36	42.41	1.44 : 1	17.45 : 1
48	47.39	1.17 : 1	18.86 : 1
72	56.62	0.81 : 1	18.52 : 1

Changes of K-value

The changes of K-value of Nila *Oreochromis niloticus* during storage were expressed in Figure 1.

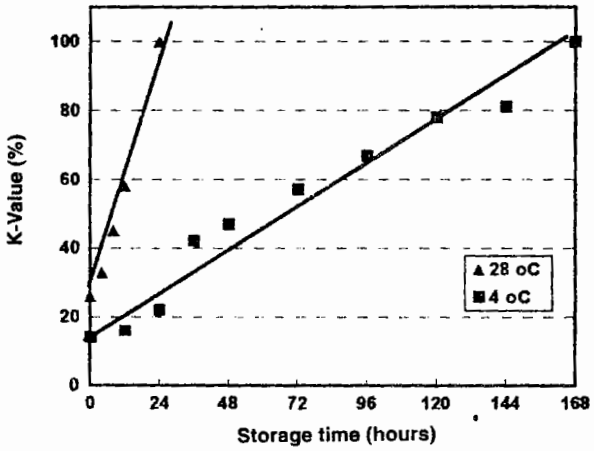

Figure 1. **Changes of K-value as an index of freshness of Nila *Oreochromis niloticus* during storage.**

K-value was calculated from the beginning of storage until reached a K-value of 100 %. The data shows that K-values of the samples stored at 28°C increased rapidly and reached 60% within 12 hours of storage, and

the same K-value level for samples stored at 4°C reached within 72 hours of storage. Based on the regression linear analysis, it could be calculated that the increasing rate of K-value of the samples stored at 28°C (Y= 24.244 + 2.666; r = 0.992 X) was 4 times higher than those of samples stored at 4°C (Y= 11.597 + 0.669 X; r = 0.960). In other word, it could be said that the rate of lowering freshness of the samples stored at 28°C was 4 times faster than those of samples stored at 4°C.

Relationship between some freshness parameters and storage time

Some metabolites have been used as indices of fish freshness such as Hypoxanthine (Jones, et al., 1964), and IMP and Hypoxanthine (Fatima et al., 1981), Inosine and Hypoxanthine (Ehira and Uchiyama, 1987). The data in Table 4 shows that K-value is the best indicator as a freshness parameter or index of freshness compared with IMP, HxR and Hx because according to the linear regression analysis, the coefficient correlation (r) of K-value against storage time was the highest compared with those of other metabolites. Coefficient correlation (r) between K-value and storage time of the samples stored at 4°C and 28°C were 0.960 and 0.992, respectively. The data suggested that relationship between K-value and the storage time was not affected by temperature of storage, but the increasing rate of K-value during storage was highly depended on the temperature of storage.

Table 4. **Relationship between some freshness parameters and storage time**

Freshness Parameters	Relationship	
	at 28°C	at 4°C
K-value	Y = 24.244 + 2.666X; r = 0.992	Y = 11.597 + 0.669X; r = 0.960
IMP	Y = 4.779 - 0.019X; r = -0.188	Y = 5.915 - 0.027X; r = -0.511
HxR	Y = 1.678 + 0.158X; r = 0.862	Y = 0.807 + 0.055X; r = 0.912
Hx	Y = 0.165 + 0.191X; r = 0.894	Y = 0.068 + 0.003X; r = 0.857

Values in the table were calculated in the range of K-value 0 to 60%.

Rate of the lowering freshness

When the data in Figure 1 was calculated as the rate of lowering freshness using the method of Suwetja (1988) and presented in Figure 2, the results shows that the rate of lowering freshness of the samples stored at 28°C (Y= 1.888 - 0.020 X; r = - 0.980) was 4 times faster than those of samples stored at 4°C (Y = 1.967 - 0.005 X; r = 0.993). This could be explained as follows. The samples stored at 28°C has slope of 0.020, thus it has a rate of lowering freshness of 0.020 x 2.303 = 0.0461/ hours. On the other hand, the samples stored at 4°C has slope of 0.005, thus it has a rate of lowering freshness of 0.005 x 2.303 = 0.011/hours. Based on this, the rate of lowering freshness of samples stored at 28°C is 4 times higher than those of samples stored at 4°C. This data is in concomitant with the increase of K-value in which the increase of K-value of the samples stored at 28°C was 4 times higher than those of samples stored at 4°C (Figure 1).

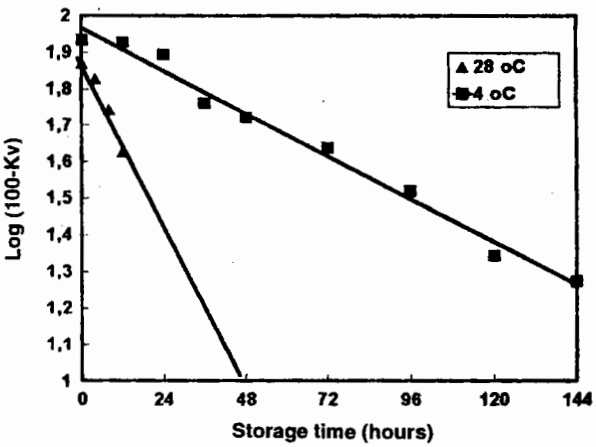

Figure 2. **Rate of lowering freshness of Nila *Oreochromis niloticus* during storage based on K-value.**

CONCLUSION

ATP, ADP and AMP were degraded rapidly and disappeared within 12 hours. Degradation of IMP in the samples stored at 4°C was slower than those of samples stored at 28°C. Interestingly, the accumulation of Inosine occurs only in the samples stored at 28°C while the accumulation of Hypoxanthine occurs only in the samples stored at 4°C. The data suggested that the activity of IMP-degrading enzyme (5'-nucleotidase) at 4°C was higher than that of Inosine-degrading enzyme, but at 28°C both enzymes have same activities. Linear regression analysis between K-value and storage time show that increasing rate of K-value or rate of the lowering freshness of samples stored at 28°C was 4 times higher than that of samples stored at 4°C. Limits of fish acceptability (K-value 60%) of Nila stored at 28 and 4°C were reached at 12 and 72 hours, respectively. The

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