ATP degradation products as freshness indicator of flatfish during storage

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Abstract In this study, the ATP degradation products and microbial growth during storage of flatfish were measured for assessing its freshness. LOD and LOQ of the ATP degradation products including ADP, AMP, IMP, HxR and Hx were 0.01–0.11 and 0.02–0.37 µg/g respectively, and the recovery ranged from 35.8 to 98.8%. The Hx level increased significantly during the storage period, regardless of storage temperature ($p < 0.05$). The initial Hx level was 245.27 µg/g, and this rapidly increased to 2563.72, 6643.69, and 4236.65 µg/g at 4, 10, and 25 °C at 14 days, 8 days, and 12 h, respectively. The correlation coefficient (R^2) between microbial growth and Hx percentage ranged from 0.7709 to 0.8939. Based on the nitroblue tetrazolium colorimetric assay, the optical density of flatfish stored at 4 °C increased from 2.45 to 13.29 at 82 h of storage, which was equivalent to 442% increment.

Keywords Flatfish · ATP degradation products · Hx percentage · NBT solution

Introduction

With a recent focus on health and wellness, the consumption of fish and fishery products is greatly increasing. In 2012, the Korean per capita consumption of marine products was 44.5 kg per year, which was greater than that of meat products (KOSIS, 2012). Among all fish species, flatfish is one of the most consumed fish species in Korea, due to its distinctive flavour and muscle characteristics. A well-accepted, and widely consumed flatfish dish is Korean-style sashimi and sushi, which requires a freshly-caught, and -served fish fillet. Korean-style sashimi is minimally processed, hence, the freshness of the flatfish is crucial for its safe consumption. Recently, a seafood traceability system to predict the freshness of fishery products, was introduced into Korea (MOF, 2015). In the seafood traceability system, all the information regarding the distribution history of the fish, including trade name, caught and shipping date, manufacturer, and distributor is available. This information allows consumers to make a smarter choice in purchasing fishery products. The limitation of this system is that the information only allows a rough estimate of the freshness. Therefore, development of a direct and accurate measurement of fish freshness is necessary.

Many attempts have been made to predict the freshness of fish products. The sensory indices, including flavour, texture and appearance evaluation are well-established (Olafsdottir et al., 1997; Reineccius, 1990). However, person-to-person variability during the evaluation of certain sensory attributes poses a risk of using sensory cues for the prediction of fish freshness. Compared to sensory evaluation, physiochemical analyses provide more objective, documentable and reportable data on fresh freshness, such as colour, water-holding capacity, total volatile bases,

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concentrations of trimethylamine (Natale et al., 2001; Ocaño-Higuera et al., 2011), thiobarbituric and peroxide values, free fatty acid content (Ozogul et al., 2005), adenosine 5'-triphosphate (ATP) catabolites (Ocaño-Higuera et al., 2011; Ryder, 1985) and microbial analyses.

In fish muscle, ATP decreases rapidly within 24 h post-mortem. The ATP is metabolised to adenosine-5'-diphosphate (ADP), when is then degraded sequentially into adenosine-5'-monophosphate (AMP), inosine-5'-monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx) at varying rates (Ehira, 1976; Kuda et al., 2007; Ocaño-Higuera et al., 2011). Relevant to the ATP degradation products, the *K* value, can be calculated from the concentrations of ATP and its degradation products to reveal the relative rate of ATP degradation, which can also be valuable to predict fish freshness (Lougovois et al., 2003; Ocaño-Higuera et al., 2011).

In this study, we did investigate the biochemical analyses of the ATP degradation products and microbial growth during storage of flatfish for assessing its freshness. The association between the accumulation of ATP degradation products and microbial growth was also investigated.

Materials and methods

Chemicals

ADP sodium salt ($\geq 95\%$ purity), disodium salts of IMP ($\geq 98\%$ purity) and AMP ($\geq 99.0\%$ purity), ATP disodium salt hydrate ($\geq 95\%$ purity), Hx ($\geq 99\%$ purity), inosine ($\geq 99\%$ purity) and nitroblue tetrazolium (NBT, 10 mg) were purchased from Aldrich Chemical Co (Germany). Triethylamine (99% purity) and perchloric acid (70% purity) were obtained from Samchun Chemical Co (Korea). Water and methanol (HPLC grade) were purchased from JT Baker (Phillipsburg, NJ, USA).

Sample preparation and storage conditions

Freshly-caught flatfish was purchased on the day of its catch at a fish market located in Seoul (Karak market, Seoul, Korea). After the catch, the fish was kept on ice during transportation to the market, and was purged in fresh water at the fish market. Therefore, the fish was alive at the time of purchase. Upon purchasing, the flatfish were de-headed and tailed and kept on ice until reaching the laboratory, where they were de-skinned, and the internal organs carefully removed. All muscle parts were homogenised and then sub-divided into 50-ml falcon tubes for storage study. The storage durations were set at 4 and 10 °C, for 8 days and 14 days, respectively, and at 25 °C for 12 h. The storage temperature (4 and 10 °C) was chosen by common fish storage at home and market. High

temperature (25 °C) was chosen as one of the extreme temperature environment for fish storage. The NBT colour measurements were determined in flatfish samples stored at 4 and 10 °C for 82 h, respectively, and at 25 °C for 44 h.

Microbiological analysis

For the total aerobe count (expressed as colony forming units, CFU), 10 g of homogenised flatfish muscle was mixed with 90 ml of 0.1% sterile peptone water in a sterile bag. The mixture was blended for 1 min using a stomacher (Bag mixer, Interscience, France). After, 1 ml aliquots were serial-diluted with sterile buffered peptone water (0.1%) and plated on a Petrifilm (3 M, St Paul, MN, USA) that was then incubated at 37 °C for 48 h.

Calibration and quantification of ATP degradation products

A stock solution of ATP degradation products was prepared at 0.01% in high performance liquid chromatography (HPLC) grade water and stored at 4 °C. The working solutions were prepared at 1, 5, 10, 20, 50, 100, 200, 500 and 1000 µg/L by dilution with HPLC grade water. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as $3 \times \sigma/S$ and $10 \times \sigma/S$, respectively. These values were based on the y-intercept ($\sigma_n = 7$) and the average slope ($S_n = 7$). The method detection limit (MDL) and method detection quantification (MDQ) values were calculated by dividing the LOD and LOQ, respectively, by sample weight.

Extraction of ATP degradation products and analysis of Hx percentage

The methods of Ryder (1985) were used to extract the ATP degradation products and determine the Hx percentage, respectively. Briefly, 5 g of de-skinned flatfish muscle was homogenised with 25 ml of cold 0.6 M perchloric acid using a macro homogeniser (Omni International, USA) at 0 °C for 1 min. The homogenate was centrifuged (Combi-514R, Hanil, Seoul, Korea) at $6500 \times g$ at 0 °C for 20 min, and 10 ml of the supernatant filtered through filter paper. The filtrate was immediately neutralised to pH 6.5–6.8 with 1 M KOH (5.5 ml). The neutralised mixture was allowed to stand for 20 min in an ice bath and then centrifuged at $6500 \times g$ at 0 °C for 10 min to precipitate the potassium perchlorate. A 5 ml aliquot of the supernatant was made up to 10 ml with 0.01 M potassium phosphate buffer (pH 6.5). An aliquot (2 ml) of the diluent was mixed with 2 ml of dichloromethane to remove impurities and left to stand for 10 min. The supernatant was filtered using a syringe filter (SmartPor GHP, 0.2 µm, Fisher, Seoul,

Table 1 Validation specification of ATP degradation products including LOD, LOQ and recoveries

	Retention time	LOD ^a (µg/g)	LOQ ^b (µg/g)	MDL (µg/g)	MDQ (µg/g)	Recovery (%)
IMP	8.481 ± 0.025	0.01	0.02	0.02	0.08	35.8
ATP	9.829 ± 0.242	0.04	0.12	0.11	0.38	37.9
ADP	12.837 ± 0.188	0.05	0.17	0.16	0.53	55.5
AMP	14.495 ± 0.026	0.11	0.37	0.35	1.18	86.6
Hx	18.368 ± 0.066	0.03	0.10	0.09	0.31	98.8
HxR	17.120 ± 0.035	0.04	0.12	0.12	0.39	83.2

Recovery (%) (Volume of each degradation product in spiked standard solution – volume of each degradation product in standard solution) * 100/volume of spiked each degradation product

^aLOD = 3 × SD/slope (µg/g)

^bLOQ = 10 × SD/slope (µg/g)

Korea). The individual ATP degradation compounds were calculated as a percentage of Hx. Thus, the Hx percentage was calculated as follows:

$$\text{Hx}(\%) = \text{Hx} / (\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx}) \times 100.$$

HPLC analysis of ATP degradation products

The ATP degradation products were analysed using an Agilent 1200 series HPLC equipped with a UV detector (Agilent Technologies, Palo Alto, CA) and Zorbax SB-Aq column (StableBond analytical 4.6 × 250 mm, 5 µm). An 0.01 M potassium phosphate buffer (pH 6.5) and 0.1% of 50 mM triethylamine was used as mobile phase A and methanol as mobile phase B. The buffer solution was prepared daily and filtered (0.45 µm) prior to injection. The flow rate was 0.5 ml/min, detection wavelength set at 254 nm, and the column temperature at 30 °C. The elution program was as follows: 0 min 100% A, 3 min 85% A and 15% B, 5 min 85% A and 15% B.

Colour measurement of flatfish with NBT solution

NBT was diluted at 250 ppm and mixed with Tris–HCl buffer (pH 7.6) and 1 M NaOH at 2:1:1 ratio. The NBT solution was mixed with the liquid extract collected before dilution with dichloromethane for HPLC analysis (Sect. 2.5), and left to stand for 5 min. The optical density (OD) was then measured at 575 nm.

Statistical analysis

All result was the means of triplicate treatments and the data are expressed as mean ± standard deviation. Differences between groups were analyzed using the Duncan test and *t* test. Statistical analysis were performed by IBM SPSS Statistics 21 (IBM, Chicago, USA).

Results and discussion

ATP degradation products analysis

Table 1 shows the retention time, LOD, LOQ, MDL, MDQ and recovery of the ATP degradation products. Six ATP degradation products in flatfish were sufficiently resolved in this method. The LOD and LOQ of the ATP degradation products ranged from 0.01–0.11 and 0.02–0.37 µg/g, respectively. The recovery ranged from 35.8 to 98.77%.

The concentrations of the ATP degradation products found in flatfish stored at 4, 10 and 25 °C (Table 2) revealed noticeable changes in IMP and Hx. The initial (day 0) concentration of IMP and Hx were 1844 and 245 µg/g, respectively. The concentration of IMP was almost unchanged at 14 days of storage at 4 °C, showing a final concentration of 1901 µg/g. In contrast, the concentration of Hx increased dramatically during storage with 2563 µg/g detected at 4 °C at 14 days, equivalent to about 946% increment the initial level. The level of Hx is regarded as one of the most important biochemical changes in fish associated with ATP degradation (Suh et al., 2017). Hence, Hx has been previously used to evaluate the shelf-life of fish (Vázquez-Ortiz et al., 1997).

Compared to the levels of IMP and Hx detected in the flatfish stored at 4 °C, the concentrations of IMP and particularly, Hx, were dramatically increased during storage at 10 °C. IMP was 0.08 µg/g at 10 °C at 8 days of storage, and Hx was 6643 µg/g, corresponding to a 560% increase since day 0. For the flatfish stored at 25 °C, the concentration of IMP and Hx at 12 h was 201 and 4236 µg/g, respectively. Typically, IMP is responsible for the sweetness of fresh fish, while Hx is associated with an intense bitter taste in fish (Howgate, 2006). In these results, the flatfish stored at 10 °C displayed the biggest changes in the concentrations of the ATP degradation products up to 560% among the three storage conditions. Also, the concentration of IMP was inversely proportional to the Hx concentration.

Table 2 Concentrations of ATP degradation products during the storage at 4 °C, 10 °C and 25 °C

Storage temperature	ATP degradation products	Storage (days)												
		0	2	4	6	8	10	12	14					
4 °C	IMP	1844.02 ± 11.02 ^g	2685.38 ± 13.11 ^a	2129.95 ± 9.09 ^b	2115.88 ± 0.06 ^c	2095.20 ± 0.09 ^d	2001.36 ± 0.01 ^e	1990.68 ± 0.07 ^e	1901.09 ± 0.04 ^f					
	ATP	66.20 ± 1.44 ^c	157.41 ± 2.47 ^a	72.33 ± 35.25 ^c	54.50 ± 0.07 ^c	105.00 ± 0.08 ^b	11.78 ± 0.02 ^d	20.36 ± 1.06 ^d	20.67 ± 0.03 ^d					
	ADP	24.63 ± 0.21 ^c	9.98 ± 0.71 ^d	87.84 ± 16.85 ^a	16.65 ± 0.02 ^{cd}	39.00 ± 0.14 ^b	27.11 ± 0.02 ^c	38.97 ± 0.51 ^b	23.82 ± 0.09 ^c					
	AMP	0.66 ± 0.29 ^c	0.27 ± 0.42 ^c	7.09 ± 0.150 ^a	0.27 ± 0.01 ^c	4.60 ± 0.01 ^b	5.13 ± 0.04 ^b	0.20 ± 0.98 ^c	0.25 ± 0.13 ^c					
	Hx	245.27 ± 10.50 ^g	83.54 ± 2.15 ^h	771.34 ± 3.52 ^e	660.44 ± 9.75 ^f	941.54 ± 4.03 ^d	1104.39 ± 3.75 ^c	2084.23 ± 8.67 ^b	2563.72 ± 6.15 ^a					
	HxR	4.31 ± 0.30 ^b	5.08 ± 0.20 ^a	4.39 ± 0.44 ^b	2.72 ± 0.12 ^c	4.71 ± 0.04 ^{ab}	2.38 ± 0.03 ^c	0.96 ± 0.82 ^d	0.11 ± 0.23 ^e					
10 °C	IMP	2050.68 ± 15.05 ^a	2009.18 ± 6.35 ^b	1304.03 ± 1.04 ^c	0.08 ± 5.45 ^d	0.08 ± 7.02 ^d	0.08 ± 9.47 ^d	0.08 ± 5.76 ^d	0.08 ± 1.45 ^d					
	ATP	23.98 ± 6.02 ^b	15.87 ± 0.22 ^{cd}	4.22 ± 0.24 ^e	17.35 ± 0.03 ^c	12.26 ± 0.05 ^d	1.51 ± 0.35 ^e	4.39 ± 0.72 ^e	159.66 ± 0.08 ^a					
	ADP	13.20 ± 8.03 ^c	23.52 ± 0.12 ^b	27.43 ± 0.35 ^b	34.72 ± 0.05 ^a	17.31 ± 0.03 ^c	4.24 ± 0.14 ^d	15.37 ± 0.51 ^d	3.81 ± 0.05 ^d					
	AMP	0.27 ± 7.01 ^b	0.41 ± 0.02 ^b	5.51 ± 0.87 ^a	0.76 ± 0.07 ^b	0.25 ± 0.26 ^b	7.03 ± 0.03 ^a	0.25 ± 0.81 ^b	0.25 ± 0.47 ^b					
	Hx	1005.11 ± 8.03 ^h	1734.43 ± 0.08 ^g	2772.88 ± 22.75 ^f	8367.04 ± 5.01 ^a	7903.78 ± 9.38 ^b	7043.05 ± 13.05 ^c	7014.34 ± 12.04 ^d	6643.69 ± 6.89 ^e					
	HxR	5.09 ± 12.02 ^a	2.15 ± 0.09 ^a	0.11 ± 0.02 ^a	0.11 ± 0.28 ^a	1.67 ± 0.64 ^a	0.11 ± 0.03 ^a	0.11 ± 0.57 ^a	0.19 ± 0.41 ^a					
25 °C	Storage (hours)													
	0	2	4	6	8	10	12							
	IMP	1844.02 ± 11.02 ^b	2512.53 ± 7.81 ^a	1712.90 ± 3.91 ^c	30.92 ± 3.37 ^g	1047.44 ± 4.25 ^d	184.99 ± 5.26 ^f	201.76 ± 3.99 ^e						
	ATP	66.20 ± 1.44 ^e	120.33 ± 0.26 ^c	64.06 ± -0.06 ^f	55.38 ± 0.03 ^g	150.56 ± 0.02 ^b	151.72 ± 0.01 ^a	105.61 ± 0.49 ^d						
	ADP	24.63 ± 0.21 ^b	4.87 ± 0.01 ^e	5.70 ± 0.85 ^d	38.79 ± 0.06 ^a	7.65 ± 0.72 ^c	7.76 ± 0.08 ^c	0.20 ± 0.42 ^f						
	AMP	0.66 ± 0.29 ^c	0.26 ± 0.07 ^c	9.46 ± 0.61 ^a	1.94 ± 0.08 ^b	0.27 ± 0.70 ^c	0.23 ± 0.02 ^c	0.28 ± 0.23 ^c						
Hx	245.27 ± 12.50 ^g	1438.47 ± 15.08 ^f	3055.52 ± 5.52 ^e	3481.36 ± 3.02 ^d	5285.70 ± 17.67 ^b	7570.04 ± 2.81 ^a	4236.65 ± 7.20 ^c							
HxR	4.31 ± 0.30 ^b	2.89 ± 0.36 ^c	1.96 ± 0.04 ^d	93.67 ± 0.09 ^a	0.11 ± 0.81 ^e	0.10 ± 0.08 ^e	0.11 ± 0.56 ^e							

Values in the same column with different letters are significantly different ($p < 0.05$)

Fig. 1 Relationship between microbial growth and concentration of hypoxanthine in flatfish during storage (A) at 4 °C, (B) 10 °C and (C) 25 °C

Association between microbial growth and Hx concentration

Figure 1 shows the association between microbial growth and the concentration of Hx in flatfish during storage at 4, 10 and 25 °C. The total aerobe count of flatfish was initially 4.03 CFU/g. During storage, the count reached 7.65 CFU/g at 4 °C at 14 days, 8.61 CFU/g at 10 °C at 8 days and 8.02 CFU/g at 25 °C at 12 h. While the aerobe count gradually increased during storage at all temperatures studied, it occurred fastest in the flatfish stored at 10 and 25 °C.

The concentration of Hx increased as the aerobe count gradually increased. The concentrations of Hx in flatfish stored at 10 and 25 °C were dramatically increased from 4 days and 2 h, respectively. As Hx can be oxidized to xanthine, which in turn, is oxidized to uric acid, its content can reach a maximum during storage and then start to decline (Barat et al., 2008). Upon comparing the microbial growth and the concentration of Hx in flatfish stored at the three different storage temperature, a linear correlation was observed between the two variables. This result supported previous studies, showing the concentration of Hx can be a

valid indicator of fish freshness (Burns and Kee, 1985; Jacober and Rand, 1982; Zhang and Lee, 1977).

Association between microbial growth and percentage of Hx

Figure 2 shows the correlation between microbial growth and percentage of Hx during storage at 4, 10 and 25 °C. The Hx percentage, or *K* value, was calculated from the percentage ratio of Hx to the total ATP degradation products. The *K* value has been used previously to show the relative rate of ATP degradation (Lougovois et al., 2003). As the microbial count increased, the Hx percentage increased. The initial Hx percentage of flatfish was 3.3%. As the concentration of Hx increased, the *K* value increased, expectantly. At 4 °C and 14 days of storage, the Hx was 56.9%. It is considered that a *K* value less than 20% indicates the fish is fresh, while it should be rejected if the value is above 65% (Ehira, 1976). When the *K* value reaches 60%, the fish is considered to be in the initial decomposition step (Kuda et al., 2007). According to the results, the flatfish stored at 4 °C is not considered sashimi-grade at 2 days. The Hx was 67.4% at 3 days storage at

Fig. 2 Relationship of microbial growth and percent of Hx value during storage of flat fish (A) at 4 °C, (B) 10 °C and (C) 25 °C

Fig. 3 Absorbance of xanthine oxidase and NBT solution during storage of flat fish (A) at 4 °C, (B) 10 °C and (C) 25 °C

10 °C and then the level dramatically increased to 99.4% at 4 days. Compared to the value obtained from 4 °C storage, the flatfish decomposed rapidly when stored at 10 °C. Likewise, the Hx reached 63.1% at 6 h and then increased to 96.6% at 25 °C at 10 h. The correlation coefficient (R^2) between microbial growth and Hx percentage during storage was 0.7709, 0.9055 and 0.7353 at 4, 10 and 25 °C, respectively. Based on the correlation coefficients, the flatfish stored at 10 °C showed the highest correlation between microbial growth and Hx percentage.

Colour change of NBT solution by xanthine oxidase

Figure 3 shows the colour change of the NBT solution by xanthine oxidase in flatfish during storage at 4, 10 and 25 °C. The NBT OD of flatfish stored at 4 °C increased from 2.45 to 13.29 after 82 h of storage, equivalent to 442% increment from time 0. The NBT solution changes from yellow to purple during storage. This distinctive purple colour may have been derived from the formation of formazan, which is formed from the interaction between the NBT solution and superoxides produced during the transformation of Hx to xanthine by xanthine oxidase (Agarwal and Banerjee, 2009). Therefore, the colour measurement using NBT solution can be used as an indicator of fish freshness. In comparison to previous assays (Woolfolk and Downard, 1987), this colour assay is a simple and rapid method to detect xanthine oxidase activity, and an efficient screening method for xanthine oxidase inhibitors. The purple intensity is proportional to the concentration of xanthine oxidase. The NBT absorbance of the flatfish stored at 10 °C increased from 4.94 to 14.54 at 3 and 82 h, respectively. In a similar behaviour, the NBT absorbance value of flatfish stored at 25 °C increased from 7.58 to 20.78 from 3 to 44 h of storage. From these results, the NBT solution can be used as a freshness indicator to evaluate fish quality.

In this research, the ATP degradation products, including ADP, AMP, IMP, HxR and Hx were analysed as potential indicators of flatfish freshness. The level of Hx

increased from 245.27 to 6643.69 µg/g during storage. The correlation coefficient between microbial growth and Hx percentage ranged between 0.7709 and 0.8939. The NBT colour change provided a simple and rapid marker of the flatfish freshness. Thus, measurement of the ATP degradation products, microbial growth, Hx percentage and NBT colour change are potential indicators of flatfish freshness.

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Author contributions J-HH carried out this experiment and YK did prepared Fig. 1. HC did wrote introduction part and prepared Tables 1 and 2. K-GL did plan and prepared whole this manuscript.

Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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