広島女学院大学人間生活学部紀要　第６号
Journal of the Faculty of Human Life Studies
Hiroshima Jogakuin University ***6****: 81–84, Mar. 2019*
〔資　　料〕

Storage Properties of Japanese Anchovies (*Engraulis Japonicus*) during the Salting Period

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（2018年12月９日　受理）

1. Introduction

The Japanese anchovy—a small marine fish—is an everyday staple in Japan. Some anchovies are consumed fresh, but most are boiled or processed in a variety of ways. A major problem with anchovy storage is the rapid autolytic degradation of their abdominal tissue—a phenomenon often referred to as belly bursting[1)]—which may be mainly caused by the high proteolytic activity of intestinal and pyloric caeca[1, 2)]. Myofibrils in the fish also readily deteriorate as a result of autolysis. However, these properties can be advantageously used in the aging of fish. The present study was undertaken to provide additional research findings in support of the authors' preceding studies[3)] in terms of differences in storage temperatures during salting regarding measurement items in the study, with new additional results for ATP-related compounds and *K*-values. The objective of this study was to enable reporting on advantages and disadvantages relating to the progress of aging during storage and the state of non-related fish putrefaction.

2. Materials and Methods

Fish samples

The fish used for this study were Japanese anchovies (*Engraulis Japonicus*) bought from an ordinary market in Hiroshima Prefecture. Each weighed between 8.4 and 16.2 g, with lengths ranging from 9.8 to 11.6 cm. The fish were cut into fillets with a binding string of approximately 10 cm in length and packed in plastic containers filled with salt for storage at 4, 20 and 35°C in incubators for 1, 2, 4, 8, 12 and 25 weeks (Table 1). Before analysis, salt was removed from the fillets by shaking them in a basket until no more crystals were observed on their surfaces.

Table. 1　Storage temperature and period of fillets

Storage temperature (°C)	Salting period (weeks)
4	1, 2, 4, 8, 12, 25
20	
35	

Determination of volatile basic nitrogen value

The Conway method[4)] was used to detect the volatile basic nitrogen (VBN) value, which enabled evaluation of the extent of putrefaction of the preserved fish[3)]. First, each of the storage fillets was minced into small particles, and 3 g of the particles were homogenized at 25,000 rpm for 1 minute with 24 mL of distilled water. Then 3 mL of 20 w/v% trichloroacetic acid was added, and the mixture was stirred in a mixer and then left for 10 minutes. Finally the mixture was centrifuged at 3,000 rpm for 10 minutes. The filtrate of the supernatant was used as a sample solution. The inner section of a Conway microdiffusion unit (Shibata, Japan) was filled with 1 mL of boric acid solution in which 3.3 μg of bromocresol green, 3.3 μg of methyl red, 210 μL of ethanol, and 10 mg of boric acid were dissolved. The outer section was filled with 1 mL of the sample solution mixed with 1 mL of 33 w/w% potassium carbonate. The unit with its contents was incubated at 37°C for 80 minutes. After the incubation period, the boric acid solution containing the VBN compounds was titrated with 0.02 mol/L of hydrochloric acid. The VBN value was calculated using the following equation, where "V" is the consumed volume of 0.02 mol/L hydrochloric acid, "f" is a titration factor of 0.02 mol/L hydrochloric acid, and "B" is the consumed volume of a blank test:

$$\text{VBN value (mg\%)} = 14 \times 0.02 \times (V-B) \times f \times 1000$$

Monitoring of water activity

After the salt crystals were removed from the samples

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(by shaking them in a basket), each sample was minced into small particles, and water activity (Aw) was monitored using a Pawkit Water Activity Meter (Ainex, Tokyo, Japan).

Determination of ATP-related compounds via HPLC

After each storage period the fillets were minced and analyzed to determine their ATP-related compounds via the previously reported method[5]. Briefly, a 1-gram portion of each sample was homogenized with 2 mL of iced 10% PCA, followed by centrifugation at 3,000 rpm for 3 minutes. The precipitate was washed with 2 mL of 5% PCA, followed by centrifugation at 3,000 rpm for 3 minutes, and the wash liquid was added to the supernatant. This process was carried out twice, and the mixture of the supernatant and the wash liquid was neutralized to pH 6.5 with 10 M potassium hydroxide and submitted as a test solution, which was filtered through a 0.45 μm membrane filter (Nacalai Tesque, Kyoto, Japan) before injection. A 10 μL portion of the test solution was injected into a Mightysil RP-18GP column (4.6 × 250 mm, Kanto Chemical, Tokyo, Japan) eluted with a mixture of 20 mM citric acid monophosphate, 20 mM acetic acid, and 40 mM triethylamine (pH 4.8). The flow rate of the elute was 1.0 mL/min, and the column was at room temperature. The elute was monitored with UV absorption at 260 nm, and the ATP-related compounds were analyzed by comparing the retention times of HPLC peaks between samples and authentic compounds. The freshness of the muscle was judged from the *K*-value as defined by

$$K\text{-value}(\%) = (\mathrm{HxR} + \mathrm{Hx})/(\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP} + \mathrm{IMP} + \mathrm{HxR} + \mathrm{Hx}) \times 100$$

Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the characteristics of fish protein decomposition via the previously reported method[3]. The Laemmli method[6] was used, and 5 – 20% polyacrylamide gels (e-PAGEL HR, Atto, Japan) at room temperature were employed. Approximately 20 μg (wet weight) of white muscle was dissolved in the sample solution (EzApply, Atto, Japan) for each lane to enable comparison of the differing states of the stored fish[3]. The correlation of molecular weight to relative mobility was determined with Phosphorylase B (molecular weight: 97,200), albumin (66,400), ovalbumin (45,000), carbonic anhydrase (29,000), trypsin inhibitor (21,100) and α-lactalbumin (14,300) as standard proteins (EzStandard, Atto, Japan). After being run in an electrode buffer (EzRun MOPS, Atto, Japan) for around 30 minutes, the gels were stained with Coomassie brilliant blue (EzStain AQua, Atto, Japan). The JustTLC program (Ver. 4.5, Sweday, Sweden) was subsequently used to determine the distribution characteristics of the various molecular weights of the proteins in the stained gels.

Chemicals

The analytical-grade trichloroacetic acid, bromocresol green, methyl red, ethanol, boric acid, potassium carbonate, perchloric acid (PCA), phosphoric acid, potassium hydroxide, potassium carbonate anhydrous, sodium dihydrogenorthophosphate, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx) and 0.02 and 0.1 mol/L hydrochloric acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

3. Results and Discussion

Aw, VBN and *K*-values of fillets during the salting period

Variations of Aw, VBN and K values in the fillets during the salting period are shown in Figure 1. Aw values immediately decreased and subsequently remained at under 0.80 – a state in which the growth and propagation of most putrefaction-causing bacteria are prevented – until the end of the period (Fig. 1 (A)). As seen in Fig. 1 (B), VBN values also immediately decreased just after the commencement of salting, and then slightly rose with increased salting periods and storage temperatures. Values were less than half of those considered to represent the initial stage of putrefaction (30 mg%). These results suggest that the ratio of free water in the fillets decreased in the early stage through dehydration (which resulted from high osmotic pressure) from the influence of salt and hydration with ions. The growth and propagation of putrefactious bacteria appear to have been prevented by the salt throughout the salting period.

Meanwhile, *K*-values (*K*-value (%)=(HxR + Hx)/(ATP + ADP + AMP + IMP + HxR + Hx) ×100), which indicate the freshness of meat and the percentages of HxR and Hx in all ATP-related compounds (ATP, ADP, AMP, IMP, HxR and Hx), increased to over 80% at every storage temperature (4, 20 and 35°C) after a week's storage (Fig. 1 (C)). The

percentages of IMP (an umami component) in all ATP-related compounds decreased to less than 1%, and *K*/ IMP values remained stable until the end of the storage period. These results suggest that IMP does not remain even at lower temperatures (4°C) in salted fillets during aging.

Decomposition of fillet protein during salting periods

SDS electrophoretic patterns and related quantitative images of fresh and salted fillets ((A) to (I)) are shown in Figure 2. The upper photograph shows the results of electrophoresis, with fish proteins in the fillets separated in the gel from top to bottom in line with molecular weight. The lower graphs show quantitative images of each lane from left to right in line with molecular weight. In the lanes, protein decomposition is observed during the salting period in line with time elapsed (fresh: 0 weeks; (A): 2, (B): 12, (C): 25 weeks at 4°C; (D): 2, (E): 12, (F): 25 weeks at 20°C; (G): 2, (H): 12, (I): 25 weeks at 35°C) and actual decomposition is seen in the quantitative images in the lower graphs from (A) to (C), (D) to (F) and (G) to (I). In

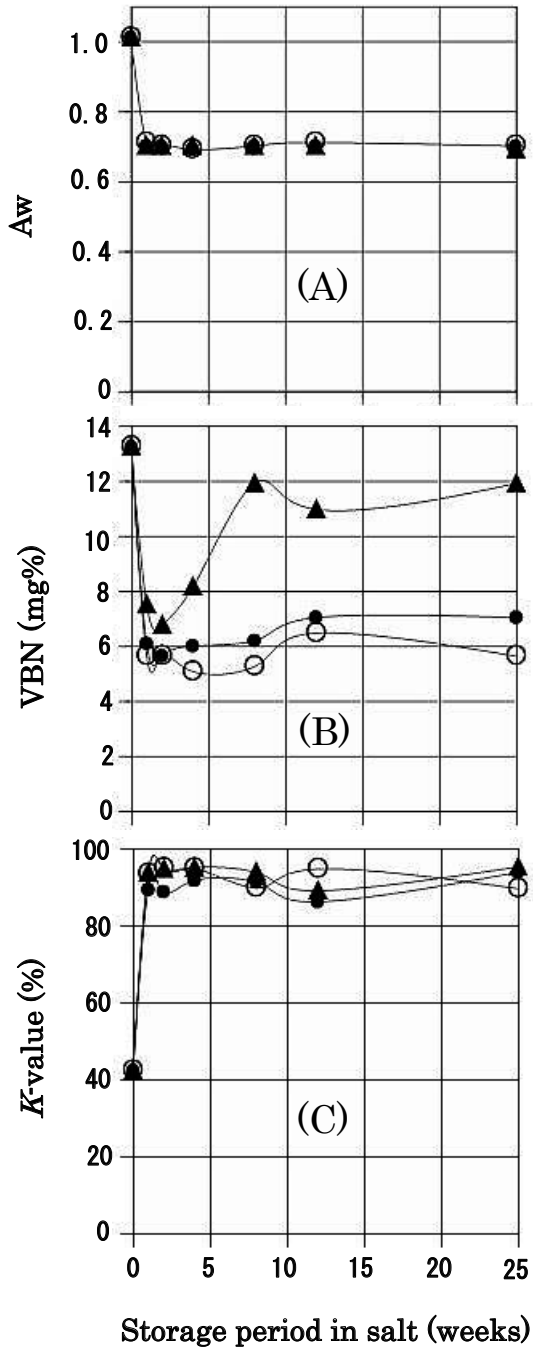

Fig. 1 Variations in (A) Aw, (B) VBN and (C) *K*-values in the fillets during the salting period. Fillets were salted at ○ 4°C, ● 20°C and ▲ 35°C for each duration. Symbols in the figure indicate means for three samples, and are connected via a spline curve.

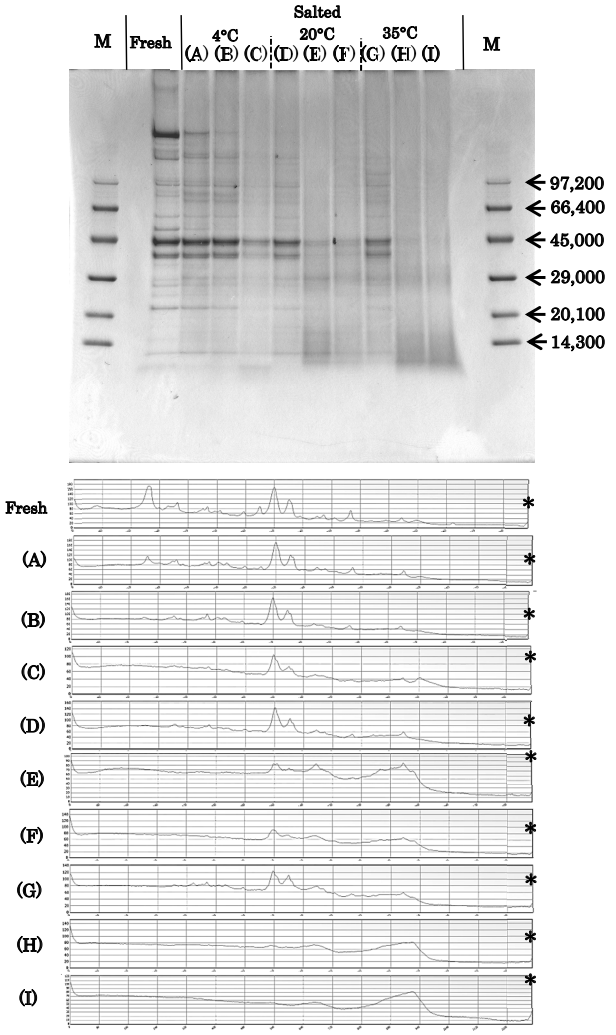

Fig. 2 SDS electrophoretic patterns and related quantitative images (JustTLC) of fresh and salted fillets over periods of (A) 2 weeks, (B) 12 weeks, (C) 25 weeks at 4°C; (D) 2 weeks, (E) 12 weeks, (F) 25 weeks at 20°C; (G) 2 weeks, (H) 12 weeks, (I) 25 weeks at 35°C. Marker proteins: M is shown for each side of the gel as reference. Asterisks indicate measured values of 100 on the vertical axis with JustTLC.

the lanes of (E), (F) and (H), (I) the protein was decomposed, and demonstrated consecutive molecular weight distribution from big to small. Meanwhile, the fillet VBN values were below 15 mg% (Fig. 1(B)), and it was inferred that protein decomposition progressed more in the fillets salted at higher temperatures and for longer periods without putrefaction-causing bacteria activity during the salting period.

Some researchers have reported decomposition of fish protein from high- to low-molecular-weight compounds during the aging period (wahoo meat cured in sake lees[7], Spanish mackerel meat cured in sake lees[8] or miso[9], sardine meat cured in rice bran[10], and sablefish meat cured in miso or sake lees[11]). In sardine meat cured in rice bran in particular, free amino acid content mostly increased in line with the length of the aging period. It may be inferred that a variety of low-molecular-weight compounds such as bioactive peptides might accumulate over time with small amounts of IMP and high *K*-values during the salting period of Japanese anchovy fillets without putrefaction.

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