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〔資　　料〕

Aging of Dried Japanese Anchovies (*Engraulis Japonicus*) during the Processing Period

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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. The present study was undertaken to provide additional research findings in support of the authors' preceding studies[3, 4)] on aging for short durations (mainly by drying). The objective of this study was to clarify the difference in aging produced by drying fish versus storing them in a refrigerator. Each procedure requires several days' worth 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 processed in three ways as described here (Table 1). The first batch were placed in 10% (w/w) brine for 1, 3, 10 and 30 minutes after being cut into fillets using a binding string of approximately 10 cm in length[3)], and were then stored at 4°C for periods of 11, 22 and 44 hours. The second batch were cut in the same way and placed in 10% (w/w) brine for 1, 3, 6, 10, 20 and 30 minutes, and were then dried on a net cage in a shaded area outdoors around 28°C for periods of 11 and 22 hours. The third batch were boiled whole in 3% (w/w) brine for 4 minutes and then dried on a net cage in a shaded area outdoors around 28°C for 44 hours. The products of these three processing methods were then packed into freezer bags and stored below −40°C until the time of analysis.

Table. 1　Immersion and storage time of fillets

Immersion time in 10% (w/w) brine (minutes)	Storage time (hours)
1, 3, 10, 30	11, 22, 44 (at 4°C)
1, 3, 6, 10, 20, 30	11, 22 (around 28°C)
Boiled in 3% (w/w) brine for 4 minutes	44 (around 28°C)

Determination of volatile basic nitrogen value

The Conway method[5)] was used to detect the volatile basic nitrogen (VBN) value, which enabled evaluation of the extent of deterioration of the preserved fish[3, 4)]. 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

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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 processing, each sample was minced into small particles, and water activity (Aw) was monitored using a Water Activity Meter (SP-W, AS ONE, 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[4, 6]. 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 by reference to the *K*-value as defined by

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

Also, the percentage of IMP (an umami component) was judged according to

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

Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the characteristics of fish protein decomposition via the previously reported procedure[3, 4]. The Laemmli method[7] 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. 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 35 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, *K*-values, and IMP of fillets during the storage period

Figure 1 shows variations in Aw (water activity), VBN (the volatile basic nitrogen value), *K*-value (the index of the freshness of the fish), and the percentage of IMP (inosine 5'-monophosphate, an umami component) in the fillets during each storage period. During the storage period in which they were kept at 4°C (Fig. 1: A, B-1), no significant changes in the Aw or VBN values of the fillets were observed, and it is supposed—judging from the VBN

values, which were below the value taken as representing the initial stage of putrefaction (30 mg%)—that none of the fillets kept at 4°C for 44 or fewer hours decomposed.

By contrast, during the storage period in which the fillets were kept at around 28°C, a clear decrease in Aw values (Fig. 1: A-2) and a clear increase in VBN values (Fig. 1:

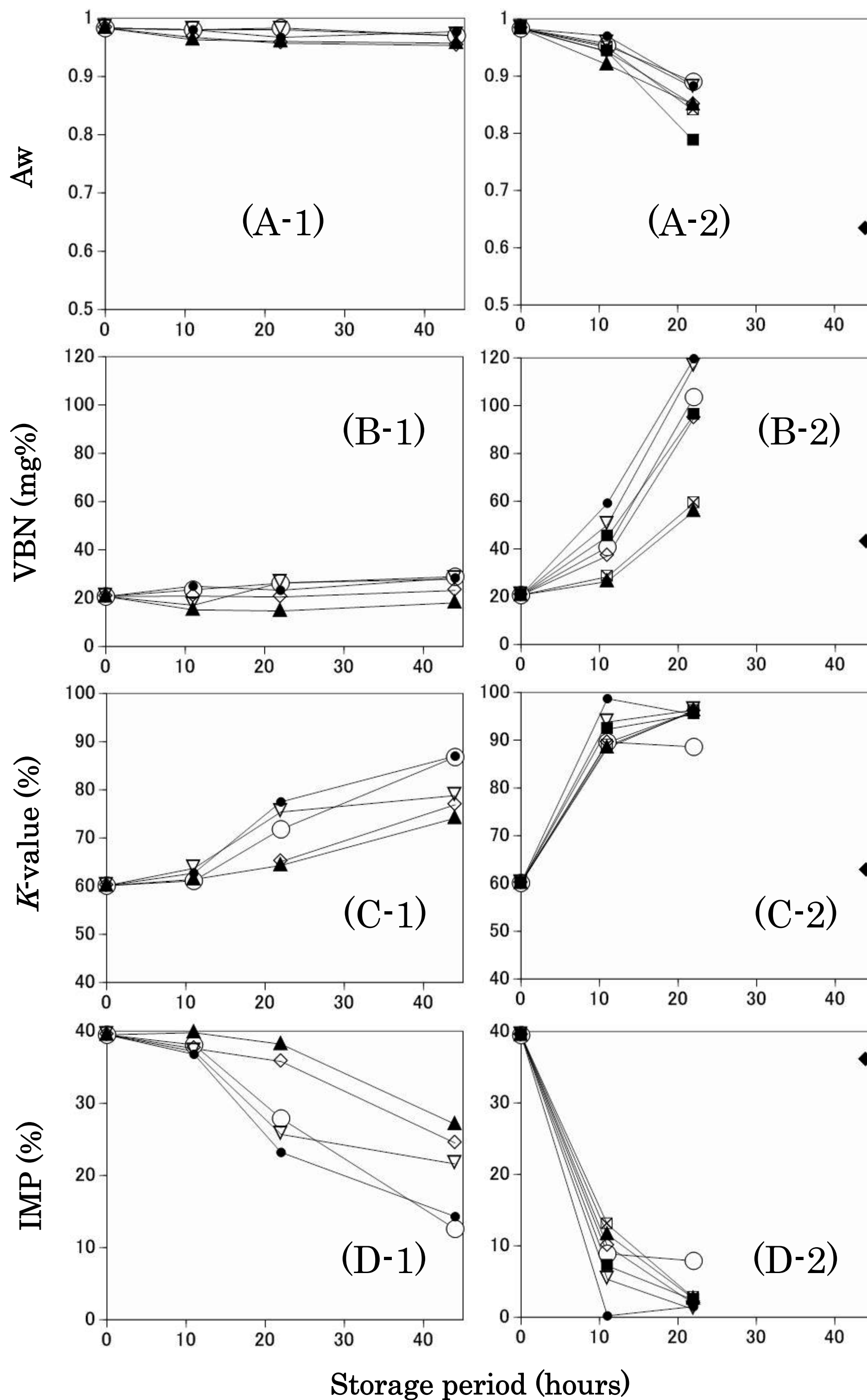

Fig. 1 Variations in Aw (A-1,2), VBN (B-1,2), *K*-value (C-1,2) and IMP (%) (D-1,2) in the fillets during the storage period. Fillets were dipped into 10% brine for ○ 0, ● 1, ▽ 3, ■ 6, ◇ 10, ☒ 20 and ▲ 30 minutes, and stored (A,B,C,D-1: 4°C; A,B,C,D-2: around 28°C) for each period indicated. The values for the fish dried at around 28°C for 44 hours after being boiled in 3% brine for 4 minutes are shown as ◆. The symbols in the figure indicate the means for three to five samples.

B-2) were observed. Longer periods of immersion in brine before drying appeared to reduce the degradation of dried fish more in terms of volatile base production. Based on VBN values (which were over 30 mg% after the 22-hour drying process), the flavor component derived from protein decomposition increased. Here, the dried fillets were considered to have matured but not spoiled. Aw values were under 0.90, and the propagation of most putrefaction-causing bacteria are considered to have been prevented at this stage.

Meanwhile, the Aw value for boiled and dried fish (Fig. 1: A-2) at 44 hours was the lowest because of drying, and the related VBN value (Fig. 1: B-2) was lower than that of dried fish at 22 hours, possibly due to the cessation of autolytic enzyme activity associated with the boiling process before drying.

During the period of storage at 4°C, *K*-values (Fig. 1: C-1) gradually increased and VBN values (Fig. 1: D-1) gradually decreased, particularly in fillets immersed in brine for longer. This suggests that longer immersion resulted in better preservation.

By contrast, during the period of storage at around 28°C, *K*-values (Fig. 1: C-2) rapidly increased and IMP values (Fig. 1: D-2) rapidly decreased. This suggests an absence of effectiveness in keeping freshness and IMP content of fish in drying process.

The *K*-values of boiled and dried fish (Fig. 1: C-2) at 44 hours were as low as those of fresh fillets at 0 hours, and IMP values (Fig. 1: D-2) were as high. This suggests that ATP-related compounds in fresh fillets were preserved by stopping autolytic enzyme activity via boiling before drying.

These results indicate a clear difference in IMP content between dried fish and boiled/dried fish, with the latter being similar to that of fresh fish.

Fillet protein decomposition during storage

SDS electrophoretic patterns and related quantitative imagery for fillets stored at 4°C ((A) to (J)) and around 28°C ((L) to (T)) are shown in Figures 2 and 3. The top image shows the results of electrophoresis, with fish proteins in the fillets separated in gel from top to bottom in line with molecular weight. The lower graphs show quantitative images for each lane from left to right in line with molecular weight. In the lanes of these figures, protein decomposition is observed during the storage period in line with time elapsed, and actual decomposition is seen in the quantitative images in the lower graphs. In Figure 2 (for fillets stored at 4°C), protein decomposition is observed from fresh (A) to 11 h-stored (B, E, H) fillets, but no clear temporal change from 11 h-stored (B, E, H) to 44 h-stored (D, G, J) fillets is seen. In Figure 3 (for fillets stored at around 28°C), the lanes from 11 h-stored (L, N, P, R) to 22 h-stored (M, O, Q, S) fillets show that protein was decomposed and concentrated via the process of drying, and indicate ordered molecular weight distribution from big to small (M, O, Q, S). The molecular weight distribution imagery for boiled and dried fish (T) was similar to that for fresh fish (K), while protein became

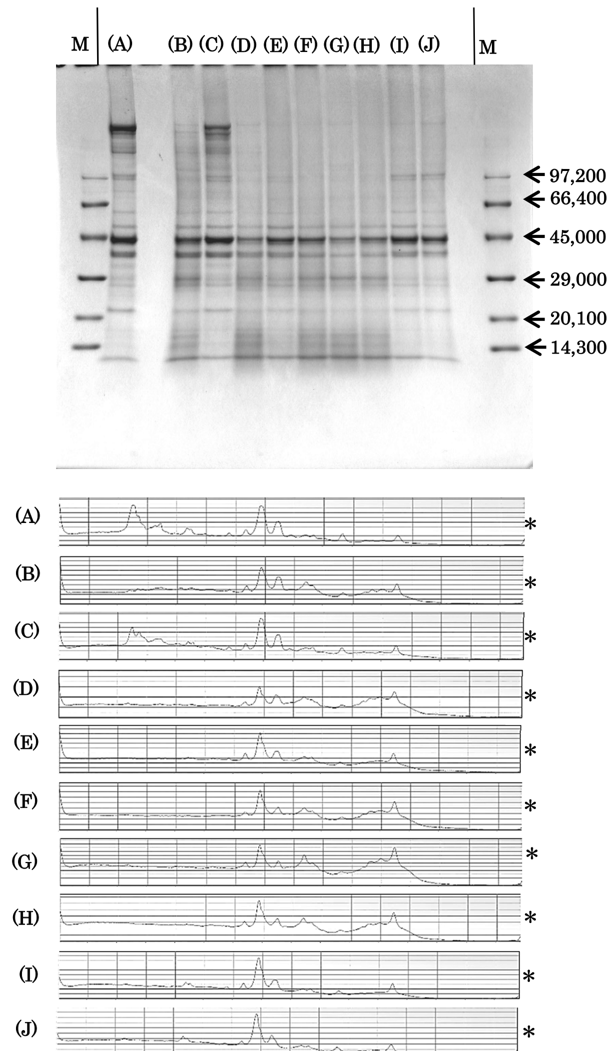

Fig. 2 SDS electrophoretic patterns and related quantitative images (JustTLC) of stored fillets (A: fresh; B: washed-11 h; C: washed-22 h; D: washed-44 h; E: 10 min-11 h; F: 10 min-22 h;G: 10 min-44 h; H: 30 min-11 h; I: 30 min-22 h; J: 30 min-44 h). Marker proteins: M is shown on each side of the gel as reference. Asterisks indicate measured values of 100 on the vertical axis with JustTLC.

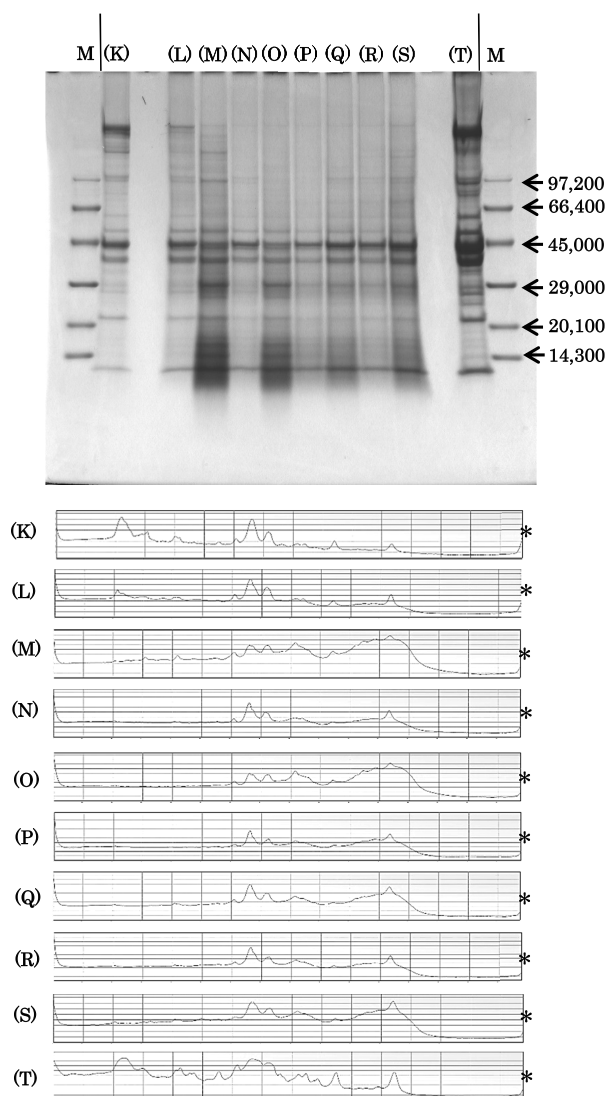

Fig. 3 SDS electrophoretic patterns and related quantitative images (JustTLC) of dried fillets (K: fresh; L: washed-11 h; M: washed-22 h; N: 3 min-11 h; O: 3 min-22 h; P: 10 min-11 h; Q: 10 min-22 h; R: 30 min-11 h; S: 30 min-22 h; T: boiled and dried). Marker proteins: M is shown on each side of the gel as reference. Asterisks indicate measured values of 100 on the vertical axis with JustTLC.

concentrated via the drying process.

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[8], Spanish mackerel meat cured in sake lees[9] or miso[10], sardine meat cured in rice bran[11], and sablefish meat cured in miso or sake lees[12]). In sardine meat cured in rice bran in particular, free amino acid content generally increased in line with the length of the aging period. It may be inferred that the variety and content of low-molecular-weight compounds contributes more to aging taste of dried fish than the IMP, although the latter is clearly a major factor in fresh or boiled and dried fish.

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