

## 凍結・解凍魚と未凍結魚との鑑別に用いる簡便法と試験紙法の開発

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## A Routine Method and Test Paper Method for the Differentiation Between Frozen-Thawed and Unfrozen Fish

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We developed a simple routine method and test paper method for differentiating frozen-thawed fish from unfrozen one. The suggested procedure consists of two steps; (i) incubation of a drop of fish blood with a fluorescent substrate in a Durham tube for 10 min at room temperature, (ii) termination of the reaction by adding an alkaline buffer and observing on the color change of the blood in Durham tube under a UV-lamp. For unfrozen fish, the blood shows red while it shows blue for frozen-thawed fish. The method requires only 10  $\mu$ l of the blood and about 15 min for the entire procedure. Based on the routine method, a test paper method was developed. The differentiation can be performed by smearing a drop of the blood on the test paper, and after keeping it at room temperature for 10 min, placing a drop of the alkaline buffer on the paper, and then observing the color change under a UV-lamp. Both the routine and test paper methods are applicable for testing the most common edible fish.

In the previous paper,<sup>1)</sup> we described a method for differentiating frozen-thawed fish from unfrozen one by the determination of neutral  $\beta$ -*N*-acetylglucosaminidase activity in the blood. We report here a method developed from our previous study and test paper which makes feasible differentiation of frozen-thawed from unfrozen fish.

### Materials and Methods

#### Fish

Eighty samples of live carp *Cyprinus carpio* and thirty samples of red sea bream *Chrysophrys major*, with average body weights of about 500 g and 800 g respectively, were purchased from fish shops in Fukuoka city and killed in our laboratory by a blow to the head. The carp samples were divided into four equal groups, which were wrapped in polyethylene bags and stored at 4, -1, -20, and -40°C respectively, and the red sea bream samples were into two equal groups, stored at 4 and -20°C respectively. At intervals, two samples of each group were removed and thawed in tap water of about 15°C.

#### Routine Method

The caudal part of the fish was excised and 10  $\mu$ l

of blood was aspirated from the dorsal aorta by an aspirator tube. To 20  $\mu$ l of the isotonic solution (10 mM phosphate buffer, pH 7.0, containing 0.11 M NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 20 mM *N*-acetyl-D-galactosamine) were added 10  $\mu$ l of the blood and 10  $\mu$ l of the substrate solution (0.8 mM 4-methylumbelliferyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside which had been dissolved in 2-methoxyethanol in 10 mM concentration as a stock solution, and diluted with the isotonic solution when used). The above mixture in a Durham tube (6 $\times$ 49 mm) was incubated at room temperature (about 20°C) for 10 min, then 1 ml of the stop solution (50 mM glycine buffer, pH 10.4, with 5 mM EDTA and 0.1 M NaCl) was added. The fluorescence was observed under a UV-lamp (Hirai Science Institute) at 360 nm.

#### Preparation of the Test Paper

A test paper (0.7 $\times$ 6 cm) was made of the Toyo filter paper No. 6 (0.7 $\times$ 1 cm) cohered with Pylontape (0.7 $\times$ 5 cm) on one end. The filter paper was permeated with the isotonic solution containing 0.2 mM substrate. After being dried at room temperature, the test paper was put into a dark bottle and stored at 4°C.

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### Application of the Test Paper

The caudal part of fish was excised and the blood aspirated from the dorsal aorta using a micropipette. A drop of the blood was smeared on the test paper. After 10 min, a drop of the stop solution was put onto the test paper and the color change was immediately observed under a UV-lamp (360 nm).

### Chemicals

All of the chemicals used were the same sources as described elsewhere.<sup>1)</sup>

## Results and Discussion

### Differentiation by the Routine Method

Fig. 1 shows the result of the differentiation by the routine method. Tube A contained blood from the unfrozen carp, and tube B that from the carp stored at  $-40^{\circ}\text{C}$  for one day and then thawed. It was easy to distinguish which was unfrozen or frozen-thawed just by glancing at the color of the blood in the Durham tube, because for the unfrozen fish the blood remained red while for the frozen-thawed fish the blood showed blue (fluorescence). Here the neutral  $\beta$ -*N*-acetylglucosaminidase activity of 0.2 mU/ml was the critical activity lower than that the fluorescence could not be seen with the naked eye, under given conditions. As reported in the preceding paper,<sup>1)</sup> for the carp stored at  $4^{\circ}\text{C}$  and at  $-1^{\circ}\text{C}$ , the activities were lower than the criterion up to the 5th and 12th days, respectively. Similarly, for the red sea bream stored at  $4^{\circ}\text{C}$ , it was lower up to the 3rd day. However, when the carp and red sea bream were frozen at  $-20^{\circ}\text{C}$  or  $-40^{\circ}\text{C}$  even for one day and then thawed, the activities become much higher. This method was valid to most of the common fish listed in our foregoing paper.<sup>1)</sup> The method is considered to be practical, and food inspectors can do on-the-spot inspection. Species such as Ayu *Plecoglossus altivelis* and Striped puffer *Fugu xanthopterus* have a lower activity of neutral  $\beta$ -*N*-acetylglucosaminidase after freezing and thawing. For these species, modifications in the examining conditions have to be designed.

### Differentiation by the Test Paper

The test paper developed on the basis of the routine method makes the procedure of the differentiation easy and simple. The results obtained with the test paper are shown in Fig. 2 and Table 1. The test paper A smeared with the blood from the unfrozen carp did not change in color

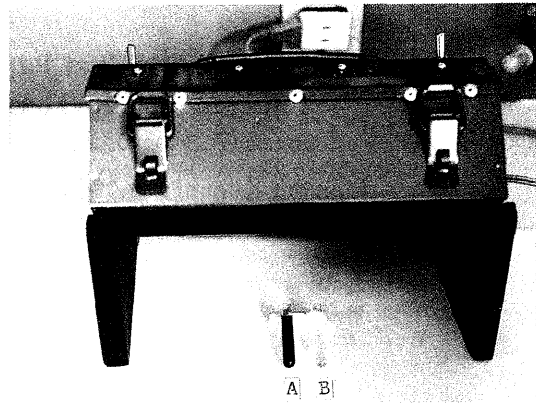


Fig. 1. A routine method for differentiating frozen-thawed from unfrozen fish. Tube A contained unfrozen carp blood and tube B frozen-thawed carp blood. After incubation at  $20^{\circ}\text{C}$  for 10 min, the tubes were observed under a UV-lamp at 360 nm.

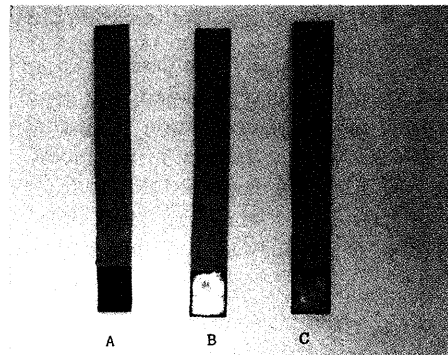


Fig. 2. Test paper method for differentiating frozen-thawed from unfrozen fish. Test paper A (red) was smeared with unfrozen carp blood, paper B (blue) was with the frozen-thawed carp blood with the activity of  $>1.1$  mU/ml, and paper C (blue but dim) was with the frozen-thawed carp blood diluted to the activity of about 0.3 mU/ml with isotonic solution. After being kept for 10 min at room temperature, the test papers were observed under a UV-lamp at 360 nm.

while the test paper B smeared with the blood from frozen-thawed fish with an activity over 1.1 mU/ml and the paper C smeared with a frozen-thawed blood with an activity of about 0.3 mU/ml turned blue, and fluorescence was visible. These results are the same as for the routine method, that is, for carp stored at 4 and  $-1^{\circ}\text{C}$ , the test papers did not change color until the 5th day and more than a week respectively, and until the 3rd day for red sea bream stored at  $4^{\circ}\text{C}$ , while for the frozen-thawed fish, both carp and red sea bream

**Table 1.** Results of the differentiation between frozen-thawed and unfrozen fish using the test paper

Storage temperature	Storage period (day)							
	1	2	3	4	5	6	7	8
	Carp							
4°C	—	—	—	—	—	+	++	+++
-1°C	—	—	—	—	—	—	—	—*1
-20°C	+++	+++	+++	+++	+++	+++	+++	+++
-40°C	+++	+++	+++	+++	+++	+++	+++	+++
	Red sea bream							
4°C	—	—	—	++	+++	+++*2		
-20°C	+++	+++	+++	+++	+++	+++		

\*1 "—" means no fluorescence.

\*2 "+, ++, +++" mean the degree of the fluorescence. +, slight; ++, middle; +++, strong.

stored at -20 or -40°C even for one day, the test paper gave a strong fluorescence and changed from red to blue. These results suggest that the test paper is also useful to the differentiation. Compared with other methods reported so far,<sup>2-6)</sup> our method is reliable and practical.

When the routine method or the test paper method was applied, the blood should not be mixed with body fluids because the result might be obscured. Fish gills are usually congested, and sampling of the blood can be done by inserting the test paper into the gills. It should be confirmed that the blood on the gills has not been subjected to hemolysis by washing water.

The test paper is stable at least for 6 months, when stored at 4°C.

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