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Food Chemistry 76 (2002) 443–447

Food Chemistry

www.elsevier.com/locate/foodchem

Changes in acid and alkaline phosphatase activities during the spoilage of raw muscle from horse mackerel *Trachurus japonicus* and gurnard *Lepidotriga microptera*

Takashi Kuda*, Chiharu Matsumoto, Toshihiro Yano

Department of Food Science, Ishikawa Agricultural College, Nonoichi, Ishikawa 921-8836, Japan

Received 24 April 2001; received in revised form 17 July 2001; accepted 17 July 2001

Abstract

The aim of this work was to provide information on the role of acid phosphatase (Acp) and alkaline phosphatase (Alp) activities as spoilage indicators of raw fish. Horse mackerel *Trachurus japonicus*, a red-flesh fish, and gurnard *Lepidotriga microptera*, a white-flesh bottom fish, were stored at 4 °C. The aerobic bacteria count (APC) of horse mackerel skin increased from 10^3 to 10^7 CFU/cm^2 in seven days, the Alp activity increased about from 10 to 150 nmol *p*-nitrophenol/min/g tissue with increase of volatile basic nitrogen (VBN), an index of fish spoilage. The APC of gurnard skin also reached 10^7 CFU/cm^2 after 8 days' storage. The Alp activity was twice that of the Acp activity in the fresh gurnard and the activity decreased slightly during the initial 4 days and increased from four days' storage.

Keywords: Phosphatase activity; Spoilage; Horse mackerel *Trachurus japonicus*; Gurnard *Lepidotriga* microptera

1. Introduction

Non-specific acid phosphatase (Acp, EC 3.1.3.2) and alkaline phosphatase, (Alp, EC 3.1.3.1) activities are widely distributed throughout the living world (Fernley, 1971; Pacova & Kocur, 1978). The relative distribution of the activity of Alp has been reported as: intestinal mucosa = placenta > kidney = bone > liver = lung = spleen in mammals (Fernley, 1971). The phosphatases are very important for regulation of various metabolic processes that occur by phosphorylation and dephosphorylation, with kinases (Sparks & Brautigan, 1986). Some phosphatase inhibitors, such as okadaic acid, can cause diarrheic shellfish poisoning (Quilliam, 1999).

In the test for phosphatase activity, a yellow compound *p*-nitrophenol (PN), liberated from *p*-nitrophenylphosphate (PNP) by phosphatases, is quantified colorimetrically. This test can assay tens or hundreds of samples per hour easily and routinely, and has been used extensively in food safety and medical checks (Kissmehi, Treptau, Kottwitz, & Plattner, 1997; Panara, 1988). For example, in the dairy industry, the destruction of enzymes by ordinary pasteurization or sterilization temperatures can be checked to determine whether milk has been adequately pasteurized (Richardson, 1975).

In many studies of marine food, phosphatase activities in fish muscles, particularly ATP, ADP and IMP-degrading enzymatic activities, have been reported to be related to the *K* value, an index of freshness (Gill, 1992), and the umami taste compounds (Tomioka & Endo, 1984). However, the changes of phosphatase activities and bacteria of fish during spoilage are not well clarified. Viable bacterial counts on aerobic agar plates (APC), volatile basic nitrogen (VBN) and trimethylamine (TMA) are regarded as spoilage indices in fish and other marine products which are protein-rich foods (Gram & Huss, 1996; Koutsoumanis & Nychas, 1999). However, these assays need many hours, e.g. 2 or more days for APC (Houk & Hardy, 1987), or complicated techniques. We consider that, if Acp or Alp activity is related to the spoilage, the Acp or Alp assay can be useful as a spoilage indicator.

* Corresponding author. Tel.: +81-76-248-3135; fax: +81-76-248-8402.

E-mail address: kuda@ishikawa-c.ac.jp (T. Kuda).

0308-8146/02/$ - see front matter
PII: S0308-8146(01)00302-8

The aim of this work was to provide information on the role of Acp and Alp activities as indicators of spoilage in raw fish and other marine products stored at low temperature.

2. Materials and methods

2.1. Phosphatase activity in edible part of fishes

The marine foods used in this study, as fresh as possible, were purchased in Ishikawa, Japan. The dorsal muscle were minced with 9 volumes of 1% Triton X-100 and centrifuged (2220 ***g*** at 4 °C for 10 min). The supernatant was used as a crude enzyme solution. Phosphatase activities, at pH ranging from 4 to 11 in the crude enzyme solutions, were then measured.

2.2. Storage of horse mackerel (Trachurus japonicus) at 0 and 10 °C

Three fresh horse mackerel (red-flesh fish) were each packed with polyethylene film and stored at 0 or 10 °C for 7 or 15 days, respectively. The changes of colony forming units (CFU) on aerobic plate count agar (APC) in dorsal skin, pH value of dorsal muscle and the phosphatase activities in the dorsal muscle of the fishes were measured at 2-day intervals and at 10 °C and 3-day intervals at 0°C.

2.3. Storage of horse mackerel and gurnard (Lepidotriga microptera) at 4 °C

Three fresh samples of horse mackerel or gurnard (white-flesh and bottom fish) were each packed with polyethylene film and stored at 4 °C for 9–13 days. The changes of APC, pH, phosphatase activities and volatile basic nitrogen (VBN) of the fish were measured. In this experiment, protein concentration in the 1% Triton X-100 solutions was measured, using the DC protein assay kit (Bio-Rad, CA). Samples were analyzed at intervals.

2.4. Assay of phosphatase activity and measurement pH and APC and VBN

To assay phosphatase activities, *p*-nitrophenyl phosphate disodium salt hexahydrate (PNP, Wako Pure Chemical Industries, Ltd, Osaka) was used (Berberian & Beauge, 1992; Houk & Hardy, 1987). 0.15 ml of working solution, containing the 15 mmol/l PNP, 1 mmol/l $MgCl_2$ and buffer solution, were put into a 96 micro-well plate. The buffer solution was adjusted to pH 4.0, 5.0 and 6.0 by 0.1 mol/l CH_3COOH-CH_3COONa, to pH 7.0, 8.0 and 9.0 by 0.1 mol/l Tris-HCl and to pH 10.0 and 11.0 by 0.05 mol/l $NaHCO_3^-$ 0.1 mol/l NaOH. Then 0.025 ml of crude enzyme solutions were added. After mixing, the micro-well plate was incubated at 37 °C for 30 min. To stop the reaction, 0.05 ml of 0.5 mol/l NaOH were added. Absorbance at 405 nm was measured using the microplate reader (Model 550, Bio-Rad, CA).

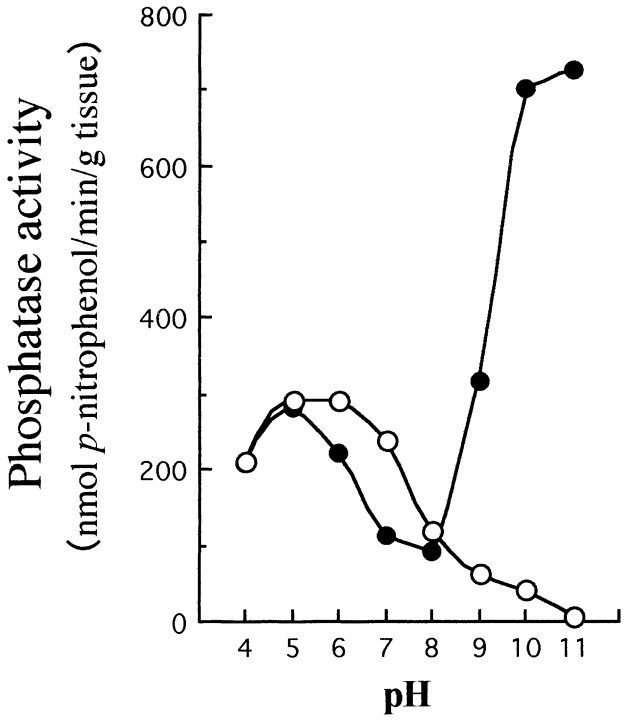

Fig. 1. Phosphatase activity in dorsal muscle of marine horse mackerel *Trachurus japonicus* (open circle) and gurnard *Lepidotrigla microptera* (closed circle).

APC was measured by a general method (Helrich, 1990). Briefly, samples (0.1 ml) of serial dilutions of fish homogenates were spread on the surface of aerobic plate count agar (Standard Method agar, Nissui, Tokyo) and incubated at 30 °C for 72 h. The bacterial data were shown as mean and standard error (SE) of log CFU/cm^2 skin.

The pH was measured using a pH meter (F-12, Horiba, Kyoto) after being diluted with four times the volume of deionized water.

VBN was measured using Conway's microdiffusion method (Conway, 1950) after being extracted with four volumes of 10% trichloroacetic acid.

3. Results and discussion

3.1. Phosphatase activities in edible part of various fishes

Phosphatase activities, at pH ranging from 4 to 11 in the edible part of 39 fresh fishes and seven fresh mollusks, were measured. Fig. 1 shows the activities in dorsal muscle of horse mackerel and gurnard.

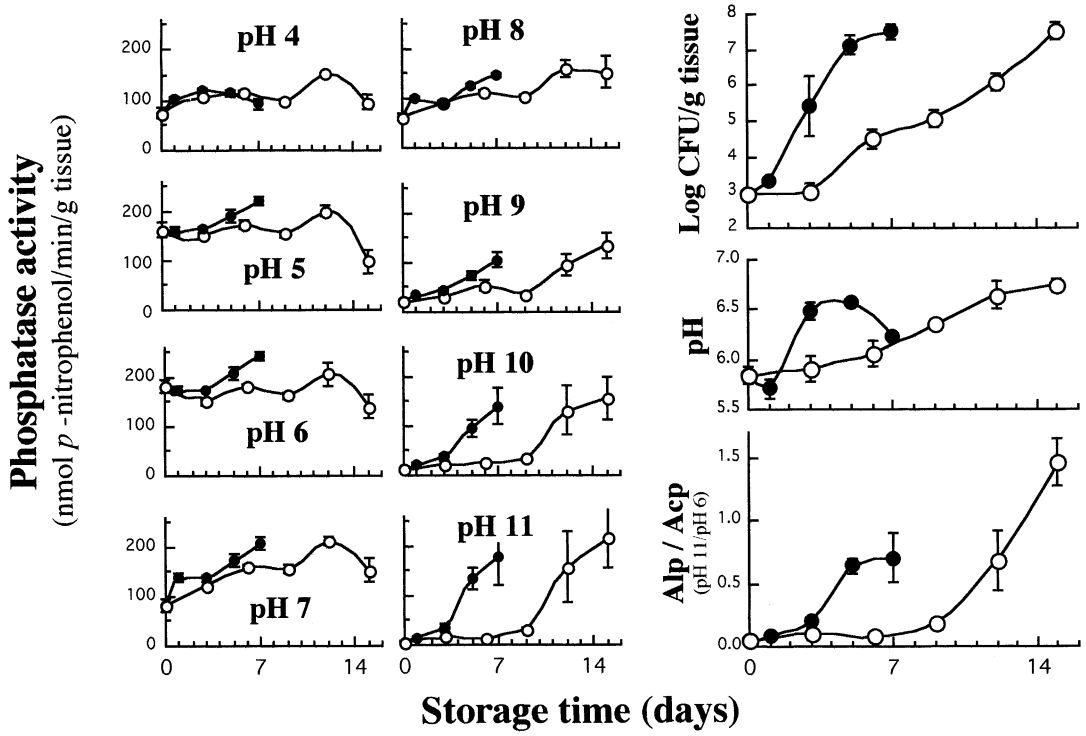

Fig. 2. Aerobic plate counts and phosphatase activity in horse mackerel *Trachurus japnonicus* stored at 0 °C (open circle) and 10 °C (closed circle). Values are mean±S.E. ($n=3$).

All samples of 11 red-flesh fish and 14 samples among 23 white-flesh fish indicated maximum activity at pH 5 or 6 as horse mackerel. The Acp activity of red-flesh fishes ranged from 200 to 450 nmol *p*-nitrophenol (PN)/min/g tissue, and Alp activity at pH 10 and 11 of the fish was very low compared with the Acp activity. The Acp activity of white-flesh fish ranged from 100 to 350 nmol PN/min/g tissues.

These results agree with some reports that indicate that the IMP-degrading enzyme activity in dark muscle is far higher than that in ordinary muscle and, for the enzyme in dark muscle, the optimal pH is about 6 (Nakagawa & Nagayama, 1994). Additionally, it is well known that the tissue pH values of most red-flesh fish and white-flesh fish are 5.6–5.8 and 6.2–6.3, respectively (Nishioka, 1984).

On the other hand, both of Acp and Alp activities in the muscle of gurnard, a white-flesh bottom fish, were significant. Interestingly, the Alp activity (700 nmol PN/min/g tissue at pH 10 and 11) was far higher than the Acp activity (290 nmol PN/min/g tissue at pH 5).

Three shellfishes hard clam, clam and cockle showed very high phosphatase activity (500–1000 nmol PN/min/g tissue) at pH 5, 6 and 11, respectively. The edible parts of these shellfishes include digestive organs. The phosphatase activities of scallop ligament muscle were not as high as the same activities in ordinary muscle of octopus and squids (<200 nmol PN/min/g tissue).

3.2. Changes of the phosphatase activities in fish during storage

Fig. 2 shows the changes of phosphatase activities, APC and pH in dorsal muscle of horse mackerel during storage. After 5 days at 10 °C and 15 days at 0 °C, the APC reached 7.0 log CFU/cm^2 skin, which indicates spoilage (Roberts & Weese, 1998). The phosphatase activities at pH 10 and 11 increased with the APC in both samples stored at 0 or 10 °C. While the APC increased from 0 day storage to a level of 7.0 log CFU/cm^2 skin, the Alp activity at pH 10 and 11 increased from <10 to about 150 nmol/PN/g tissue.

Changes of APC, pH, phosphatase activities and VBN in horse mackerel and gurnard stored at 4 °C are shown in Fig. 3. For red-flesh fish, horse mackerel, the APC reached 7.0 log CFU/cm^2 skin in 7 days. The VBN was higher than 50 mg/100g tissue after 9 days' storage. Changes of phosphatase activities (per g protein) were similar to data for fish stored at 0 and 10 °C.

The APC of gurnard, a white-flesh fish, also reached 7.0 log CFU/cm^2 skin after 7 days' storage. The VBN was 36 mg/100g tissue after 8 days storage. The Alp activity was twice higher than the Acp activity in the fresh gurnard. The Alp activity decreased slightly in the initial 4 days' and increased from 4 days' storage. The standard errors of Alp activity were large in the samples of spoiled horse mackerel and gurnard after 6 days' storage.

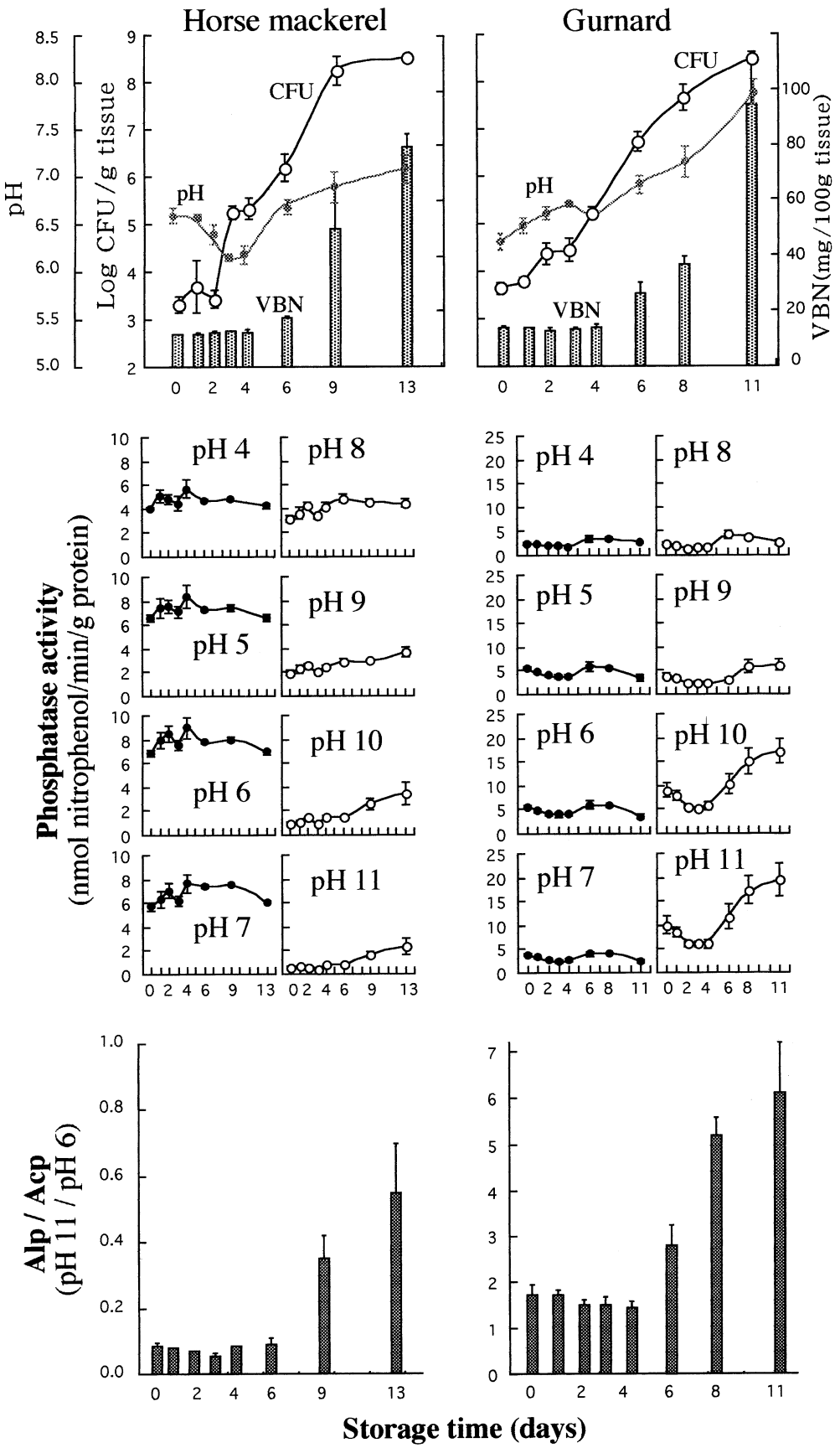

Fig. 3. Spoilage and phosphatase activity of horse mackerel *Trachurus japnonicus* and gurnard *Lepidotrigla microptera* stored at 4 °C. Values are mean±S.E. ($n=3$).

These results suggest that Alp of dorsal muscle can be used as a spoilage index in most red-flesh fishes, the same as VBN. For horse mackerel investigated in this study, an Alp activity of 100 nmol PN/min/g tissue is regarded as initial spoilage. We consider that there are two reasons for the increase of Alp activity. One of the reasons is the Alp, leaching from internal organs and other tissues having large Alp activity, into the dorsal muscle. Another reason is considered to be the increase in microorganisms having Alp activity. The activities of other various enzymes are thought to correlate with spoilage and microflora (Barrett & Kwan, 1985; Brikeland, Stepaniak, & Sorhaug, 1985; Sadovski & Levin, 1969; Stead, 1986). The phosphatase assay, using *p*-nitrophenylphosphate, may be easier, more stable and cheaper than most of the other enzyme assays. We believe that the Alp activity will be more useful as a spoilage index not only in red-flesh fishes, but also in white-flesh fishes and other marine foods, by clarifying changes in the microflora and isozymes of Alp during storage.

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