Effects of long-term frozen storage on the compositions of free amino acids and nucleotide-related compounds of the coconut crab *Birgus latro*

Sato T.[1*]; Ohgami S.[2]; Kaneniwa M.[3]

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Abstract

This study examined the effects of long-term frozen storage (-20°C for 5 months) of free amino acids (FAAs) and nucleotide-related compounds (NRCs) in muscle and hepatopancreas of the coconut crab *Birgus latro*. Although long-term frozen storage had little effect on FAA composition in muscle, the amounts of several FAAs increased in the hepatopancreas that may be the result of protein decomposition during the frozen storage. Long-term storage at -20°C significantly increased the amounts of disodium 5'-inosine monophosphate (IMP) in muscle and hepatopancreas, resulting from the deaminase activity of 5'-adenosine monophosphate (AMP) which deaminates AMP to IMP. Changes in FAAs and NRCs may result in changes in the flavor of muscle and hepatopancreas of frozen stored coconut crabs, both through altered amounts of each substance and synergistic interactions among substances.

Keywords: *Birgus latro*, Free amino acid, Nucleotide-related compound, Fatty acid, Taste active value

1-Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Japan Fisheries Research and Education Agency, 148-446 Fukaiota, Ishigaki, Okinawa 907-0451, Japan.
Present address: Research Center for Marine Invertebrates, National Research Institute of Fisheries and Environment on Inland Sea, Japan Fisheries Research and Education Agency, 1760 Momoshima, Onomichi, Hiroshima 722-0061, Japan.
2- Japan Fisheries Research and Education Agency, 15F Queen's Tower B, 2-3-3 Minato Mirai, Nishi-ku, Yokohama, Kanagawa 220-6115, Japan.
3-Research Center for Biochemistry and Food Technology, National Research Institute for Fisheries Science, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa 236-8648, Japan.
*Corresponding author's email: takusato@affrc.go.jp

Introduction

Since fish and shellfish are highly perishable and quickly lose freshness, they must be stored at low temperatures. Freezing is currently one of the most widespread methods of conserving seafood. Freezing can lengthen the storage life of foods by reducing enzyme activity (Namboodiri and Gopakumar, 1992) and inhibiting microbial growth (Troller, 1980). However, freezing can also result in the deterioration of food quality through processes such as freezer burn, physical damage to tissue by water crystallization, free drip, oxidation of unsaturated fatty acids, and protein denaturation (James, 1996).

The coconut crab, *Birgus latro* also known as the robber crab or palm thief, lives in coastal areas of the tropical Indo-Pacific region and is the largest terrestrial arthropod in the world. Individual coconut crabs can weigh up to 4 kg (Brown and Fielder, 1991) and live up to approximately 50 years (Sato *et al.*, 2013). In Okinawa prefecture of Southwest Japan, coconut crabs have been eaten traditionally by locals and have been recently served to tourists in some restaurants and bars as a special food of Okinawa (Sato *et al*., 2010). Both the muscle and hepatopancreas of coconut crabs are eaten after being steamed or boiled. Adult coconut crabs molt once per year during the winter dry season in holes or crevices (Fletcher *et al.*, 1990; Sato *et al.*, 2013), limiting the period of coconut crab harvest season. However, the demand for coconut crabs as food is constant throughout the year. In addition, because keeping live coconut crabs is difficult due to their quarrelsome habits and cannibalism, raw coconut crabs are generally stored in the freezers of restaurants and bars in Okinawa before being cooked with some crabs frozen for over six months (T. Sato, pers. comm. with local people. 2008). However, it has not yet been determined whether long-term frozen storage influences the taste of coconut crab although free amino acids (FAAs), nucleotide-related compounds (NRCs), fatty acid compositions, and their seasonal variations of coconut crabs have been determined (Sato *et al*. 2015a; 2015b).

Freezing of foods has been reported to affect their compositions of FAAs and NRCs (Castrillón *et al.*, 1996; Yamamoto *et al.*, 2005). The concentrations of FAAs and NRCs have been widely used as indicators of the taste of fish and shellfish. Most FAAs have sweet, bitter, sour and/or umami components, and contribute to the characteristic flavors of foods (Kato *et al.*, 1989). The disodium salts of 5'-nucleotides also have sweet and/or unami components, and many types of FAAs synergize with 5′-nucleotides (Komata, 1990). Thus, a determination of the compositions of FAAs and NRCs may provide information about the effects of frozen storage on the taste of seafood. This study, therefore, analyzed the effects of long-term frozen storage on compositions of FAAs and NRCs of

coconut crabs caught in Okinawa.

Materials and methods

Sample collection and preparation

Live crabs were captured at night by hand without bait during May 2013 at Hatoma Island (24º28'N, 123º49'E) located southwest of Okinawa. Only male crabs were examined to exclude any effects of sex on FAAs and NRCs (Sato *et al*., 2015a). Sex was determined by the presence of pleopods on the left ventral surface (Fletcher, 1993). Thoracic length (hereafter, ThL) was measured using Vernier calipers (Mitutoyo Corporation, CD-20PM). A total of 6 males, of mean±SD ThL 40.9±0.8 mm, were collected.

The crabs were transported live to the Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute (24º34'N, 123º16'E) soaked in iced water and euthanized by freezing. Three of the collected crabs were steamed for 30 min and their muscle and hepatopancreas were separated manually. Collected samples were stored individually at -80 °C until chemical analyses. The other three crabs were sealed individually in plastic bags and stored for 5 months at -20°C. These crabs were subsequently steamed without thawing and their muscle and hepatopancreas were separated and stored as above. All chemical analyses were performed by a commercial service, the Japan Food Research Laboratories (Tokyo, Japan). The means ±SD ThL of crabs steamed immediately and those frozen and steamed were similar (41.1±1.0 mm vs. 40.8±0.7 mm; Welch's *t* test, p=0.70).

Amino acid analysis

To determine the amino acid composition of muscle, a 6 g muscle sample of each individual crab was added to 25 mL of 10% sulfosalicylic acid solution and shaken for 20 min. The pH of the sample was adjusted to pH 2.2 with 3 mol/L sodium hydroxide, its volume was made up to 50 mL with sodium citrate buffer solution (pH 2.2), and it was filtered through filter paper (No. 1, Advantec Toyo Kaisha Ltd., Tokyo, Japan).

To analyze FAAs other than tryptophan, the filtrate was diluted 5-40 fold with sodium citrate buffer (pH 2.2) and filtered through a 0.45 μm membrane (GL Science Inc., Tokyo, Japan). FAAs were analyzed using an automatic (JCL-500/V, Jeol Ltd., Tokyo, Japan) or a high-speed (L-8800, Hitachi Ltd., Tokyo, Japan) amino acid analyzer. To determine tryptophan content, 2 mL of the filtrate was adjusted to pH 10.0 with 3 mol/L sodium hydroxide and made up to 10 mL with pure water. After filtration through a 0.45 μm membrane (GL Science Inc.), the filtrate was analyzed by high performance liquid chromatography (HPLC) fitted with a CAPCELL PAK C18 AQ column (250 mm×4.6 mm inside diameter, Shiseido Ltd., Tokyo, Japan) and a fluorometer (fluorescence excitation spectrum, 305 nm). The mobile phase consisted of eluents A (20 mmol/L perchloric acid) and B (eluent A:methanol=8:2, v/v). The flow rate was 0.7 mL/min, and the

column temperature was 40 °C. The effluent was monitored at 348 nm.

To determine the amino acid composition of hepatopancreas, a 6 g sample from each individual crab was added to 25 ml of 10% sulfosalicylic acid and shaken for 20 min and its pH was adjusted to pH 2.2 with 3 mol/L sodium hydroxide. To each was added 10 mL diethyl ether and the samples were mixed and allowed to stand for 10 min. The diethyl ether layer was removed and the aqueous layer was made up to 50 ml with sodium citrate buffer (pH 2.2) and filtered through filter paper (No. 1, Advantec Toyo Kaisha Ltd.). To analyze FAAs other than tryptophan, each filtrate was diluted 8-25 fold with sodium citrate buffer (pH 2.2) and filtered through a 0.45 μm membrane, (GL Science Inc.). FAAs, including tryptophan, were analyzed as above.

Amino acids classified as sweet included alanine (Ala), glycine (Gly), proline (Pro), serine (Ser), and threonine (Thr); those classified as bitter included arginine (Arg), lysine (Lys), histidine (His), phenylalanine (Phe), tyrosine (Tyr), leucine (Leu), isoleucine (Ile), methionine (Met), and valine (Val); and those classified as umami included glutamic acid (Glu) and aspartic acid (Asp) (Funatsu *et al.*, 2004).

Nucleotide analysis

Since 5'-nucleotides (disodium 5'-inosine monophosphate (IMP), disodium 5'-adenosine monophosphate (AMP), and disodium 5'-guanosine monophosphate (GMP)) provide umami, these were extracted and analyzed. A 4 g muscle or hepatopancreas sample from each individual was added to 25 mL cold perchloric acid, shaken for 10 min and made up to 50 mL with pure water. After sonication for 10 min, the sample was filtered through filter paper (No. 1, Advantec Toyo Kaisha Ltd.). A 5 ml aliquot of filtrate was shaken after adding 0.4 mL of 3 mol/L sodium hydroxide. After radiational cooling and filtering (0.45 μm membrane, GL Science Inc., Tokyo, Japan), the filtrate was analyzed by HPLC with a MCI GEL CDR-10 column (250 mm × 4.6 mm inside diameter, Mitsubishi Chemical, Tokyo, Japan). The mobile phase consisted of acetate buffer (pH 3.3). The flow rate was 1.5 mL/min, the column temperature was 60 °C, and detection was monitored at 260 nm. AMP was analyzed after 2-5-fold dilution with pure water.

Taste Activity Value (TAV) and Equivalent Umami Concentration (EUC)

TAV and EUC were calculated as described (Chen and Zhang, 2007; Sato *et al.*, 2015a; 2015b). TAV is the ratio of the concentration of taste compounds and its threshold value and is generally measured in water or in a simple matrix. Generally speaking, a TAV higher than 1 is considered active in food taste. Taste threshold parameters of FAAs and NRCs in water were in

accordance with those previously reported (Yamaguchi *et al.*, 1971; Kato *et al.*, 1989; Fuke and Ueda, 1996).

EUC (g monosodium glutamate (MSG)/100 g) has been defined as the concentration of MSG equivalent to the umami intensity provided by a mixture of MSG-like amino acids and 5′-nucleotides and is represented by the following equation (Yamaguchi *et al.*, 1971):

$$Y = \sum a_i b_i + 1218\left(\sum a_i b_i\right)\left(\sum a_j b_j\right) \quad (1)$$

where Y is the EUC of the mixture in g MSG/100 g; a_i is the concentration (g/100 g) of each umami amino acid (Glu and Asp); b_i is the relative umami concentration (RUC) for each umami acid to MSG (Glu, 1; Asp, 0.077); a_j is the concentration (g/100 g) of each umami 5′-nucleotide (IMP, AMP, GMP); b_j is the RUC for each umami 5'-nucleotide relative to IMP (IMP, 1; and AMP, 0.18; GMP, 2.3) and 1218 is a synergistic constant based on the concentration (g/100g wet weight sample) used. The EUC of each group was calculated using the mean concentrations of MSG-like amino acids and 5'-nucleotides.

Statistical analysis

Each sample was analyzed twice and the results were averaged. The FAA and NRC compositions of edible parts were compared using Welch's *t* test (Welch, 1947).

Results

Amino acid analysis

The amounts of each FAA in muscle except for Val were similar in crabs steamed immediately and those frozen and steamed (Table 1). The amount of Val was significantly greater in the muscle of crabs steamed immediately, but the difference was insignificant.

In hepatopancreas, however, the concentrations of Tyr, Val, Ala, Glu, Thr, and Asp were significantly greater in crabs frozen and steamed than in those steamed immediately, with the former group having higher levels of total, bitter, and umami FAAs (Table 2).

Nucleotide analysis

The amounts of IMP and GMP were significantly greater in the muscles of crabs frozen and steamed than in those steamed immediately (Table 1), but the difference in GMP was very small. The amount of IMP was also significantly greater in the hepatopancreas of crabs frozen and steamed than in those steamed immediately (Table 2). However, the total amounts of NRCs were similar in the muscle and hepatopancreas of the two groups of crabs (Tables 1, 2).

TAV and EUC

Taste active contents of FAAs in muscle were similar in the two groups of crabs with Arg, His, Met, Ala, Gly, and Glu considered active in taste (TAVs >1) in both groups (Table 3). Of the NRCs tested in muscle, only AMP had a TAV >1 and was considered an

active ingredient in both groups.

In contrast, the composition of taste active contents in the hepatopancreas differed between crabs steamed immediately and those of frozen and steamed. Arg, Lys, and His were active contents in both groups, whereas Met, Val, Ala, and Glu were active contents only in crabs frozen and steamed (Table 3).

The EUC value of muscles was similar in the two groups, but the EUC value of hepatopancreas was significantly higher in crabs frozen and steamed than in those steamed immediately (Table 3).

Discussion

Little is known about the compositions of FAAs and NRCs of seafood, especially shellfish, after long-term frozen storage. These data are important because of the widespread use of freezing to preserve seafood and the increase in the number of such items frozen. Studies assessing the effects of long-term frozen storage on fish muscle reported that the amounts of FAAs decreased slightly (Beklevik *et al*., 2005; Tokur *et al*., 2006) or were nearly unchanged (Wesselinova, 2000). Similarly, we found that long-term frozen storage had little effect on FAA composition in coconut crab muscle with only Val differing significantly in the muscles of crabs steamed immediately and those steamed after long-term frozen storage.

However, long-term frozen storage affected FAA composition of the hepatopancreas of coconut crabs with freezing increasing the amounts of several FAAs. These increases may be due to protein decomposition during frozen storage. In another study, we found no noticeable change in FAA composition of the hepatopancreas of coconut crabs steamed immediately and crabs steamed immediately and then stored at -20°C (Sato T, unpubl. data, 2013). Steaming before frozen storage may denature thermally digestive enzymes that are sensitive to heat, thus preventing protein decomposition during frozen storage and the resultant increases in FAA amounts. Therefore, the increases in FAAs of the hepatopancreas observed in crabs that were frozen and steamed was likely due to the activity of enzymes that are sensitive to heat.

Increased amounts of several FAAs were observed in the hepatopancreas, but not in muscle of crabs frozen for 5 months prior to steaming. So, the concentrations of heat-sensitive digestive enzymes differ in different organs of coconut crabs with the hepatopancreas likely having higher concentrations of these enzymes than muscle. The hepatopancreas is known as organ secreting digestive enzymes in shellfish. Although freezing is intended to reduce enzyme activity, thus lengthening the storage life of food (Namboodiri and Gopakumar, 1992), some digestive enzymes of coconut crabs seem to be active at -20°C. The increases in FAAs in the hepatopancreas following long-term

frozen storage resulted in increases in total, bitter tasting, and umami tasting FAAs, as well as the number of FAAs considered active components in taste. These differences may alter the taste of frozen hepatopancreas from that in crabs steamed immediately.

Nucleotide degradation pathways have been analyzed in crustaceans (Cheuk *et al.*, 1979). The observed increases in IMP in both muscle and hepatopancreas of crabs frozen for 5 months, likely results from continued AMP deaminase activity, that is, deamination of AMP to IMP. In contrast, the amounts of AMP in muscle and hepatopancreas of frozen crabs decreased slightly, although the decreases were not significant. The major pathway of adenine nucleotide degradation was found to result in the accumulation of IMP in Alaskan shrimp species, *Pandalus borealis*, *P. pltyceros* and *Pandalopsis dispar* (Stone, 1971). In addition, we previously clarified that the amounts of IMP in muscle and hepatopancreas were unchanged in coconut crabs that were first steamed before being stored frozen at -20°C (Sato T, unpubl. data, 2013), suggesting that steaming thermally denatured AMP deaminase before freezing. Therefore, the increases in IMP in muscle and hepatopancreas during long-term frozen storage may be due to AMP deaminase activity. However, AMP deaminases have been reported unstable in crustaceans (Flick and Lovell, 1972; Cheuk *et al.*, 1979). Further studies focusing on the stability and activity of AMP deaminase in coconut crabs and the mechanism underlying the increased IMP amount during long-term frozen storage are needed.

IMP is an intense enhancer of umami flavor, much stronger than MSG. Although freezing significantly increased the amounts of IMP in both edible parts of coconut crabs, the TAV of IMP in both organs were less than 1, indicating that they were not active in taste. Although the TAV is very useful in evaluating the taste impact of individual compounds in the food matrix, the TAV method cannot consider the effects of masking, enhancing or the additive effects of simultaneously present compounds in the food matrix. The slight increases in IMP induced by freezing may therefore play important roles in the flavors of coconut crab muscle and hepatopancreas. Although the taste intensity of AMP is not as strong as that of IMP, AMP is also an important umami substance (Fuke and Ueda, 1996). The contribution of AMP to taste depends on its concentration with AMP contributing sweetness, but not umami taste at a low concentration (≤100 mg/100 mL). However, AMP and IMP interact synergistically in eliciting umami taste. When a very low concentration of IMP (4 mg/100mL extract) was present simultaneously, umami and complexity tastes were elicited even at the low AMP concentration, and sweetness was further increased (Fuke and Ueda, 1996). In addition, the synergistic effects of flavor 5'-nucleotides with MSG-like components such as Glu and

Asp have been found to greatly increase umami taste (Yamaguchi *et al.*, 1971) and many types of FAAs also synergize with 5'-nucleotides (Komata, 1990). The slight increase in IMP due to long-term frozen storage may impart a superior taste to muscle and hepatopancreas. EUC values also suggested that umami intensity was stronger in crabs that were frozen and steamed than in crabs that were steamed immediately, especially in the hepatopancreas.

This study assessed the effects of long-term frozen storage on the compositions of FAAs and NRCs in coconut crab muscle and hepatopancreas. Long-term frozen storage may impart a superior taste to coconut crab. However, we did not investigate several negative aspects of frozen storage on food quality, including freezer burn, physical damage to tissue by water crystallization, free drip, oxidation of unsaturated fatty acids, and protein denaturation. Although the quality of whole blue crabs *Callinectes sapidus* has been reported unchanged after frozen storage at -18 °C for at least 10 months (Yerlıkaya and Gökoğlu, 2004), further studies are required to clarify the influence of long-term frozen storage on the palatability of coconut crabs.

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