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Effect of Processing and Storage on the Fate of Vitamins B_1, B_2, and B_6 and Nicotinamide of Sea Urchin Gonads

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Changes in the B′ complex vitamin content in salted sea urchin gonads during processing (by adding sodium chloride and ethyl alcohol) and 180 days of storage for maturation were investigated. Thiamin comprised more than 90% of total vitamin B_1 (thiamin + TMP + TDP + TTP) in the raw gonads and was degraded appreciably due to the gonadal enzymes during processing and storage. FAD, the major compound among B_2 vitamers (RF, FMN, FAD) in the raw gonads, was hydrolyzed into RF enzymatically during the initial 15 days of storage. RF in the matured–salted gonads was relatively stable. B_6 vitamers existed mainly as PLP in the raw gonads and were degraded appreciably by the gonadal enzymes during processing and the initial storage period of the salted gonads. The residual B_6 vitamer was PM. Nicotinamide was stable in the salted gonads.

INTRODUCTION

Sea urchin gonads are generally eaten raw with lemon juice, soy sauce, and other condiments. In addition to the raw gonad preparation, there is a Japanese salted preparation of sea urchin gonads in which sodium chloride and ethyl alcohol are added as a preservative. The salted gonads are manufactured in Japan, with a production over 1700 tons/year. The typical storage period is several months. Protein, carbohydrates, nucleotides, and lipids in the salted gonads were hydrolyzed by enzyme reactions existing in the cells of the gonads during storage (Ohshima *et al.*, 1986; Shimada and Okajima, 1989; Shimada, 1989; Shimada and Ogura, 1990). It has been suggested that the low molecular weight components, resulting from the autolysis, contribute to the tasty salted gonads (Shimada *et al.*, 1989). In addition to other nutrients, the salted gonads may be a good source of many vitamins, especially B′ complex vitamins, in the Japanese diet, because the sea urchin gonads contain a large amount of B′ complex vitamins (Higashi *et al.*, 1965). However, the B′ complex vitamin content of the salted gonads has yet to be further elucidated. The B′ complex vitamins in salted gonads may be degraded enzymatically or nonenzymatically during processing and storage for maturation.

Numerous studies have shown that the processing and storage of various types of food cause a loss of B′ complex vitamins in food. Ranhotra *et al.* (1985) found that half to two-thirds of four B vitamins (thiamin, riboflavin, niacin, and vitamin B_6) in pasta-type products still existed after cooking. Takahashi and Khan (1987) reported that the thiamin and riboflavin content in salmon steaks decreased to 87.2 and 92.6%, respectively, by treatment in an infrared oven. The loss of thiamin and riboflavin was mainly attributed to leaching during the canning process of faba beans, and very little thermal destruction was observed (Lu *et al.*, 1984). Mean losses of vitamin B_6 in canned fish, seafood, meats, and poultry were reported to range from 42.6 to 48.9% (Schroeder, 1971). Thermal loss of vitamin B_6 in chicken liver and muscle amounted to 22.2 and 11.7%, respectively (Gregory *et al.*, 1986). The variation in stability of B′ complex vitamins observed among these types of food is indicative of the important effects of food composition on degradation of B vitamins

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during food processing. However, loss of B′ complex vitamins in the salted gonads during processing and storage for maturation has still not been clarified.

The objectives of this study were to examine the stability of vitamins B_1, B_2, and B_6 and nicotinamide in sea urchin gonads during processing and storage and to determine the conversion of these vitamins. Furthermore, to clarify the participation of enzymes in the cells of the gonads, changes in the B′ complex vitamins in steamed and salted gonads, which are not commonly eaten, were investigated.

MATERIALS AND METHODS

Preparation of Salted Sea Urchin Gonads. Sea urchin gonads were excised from sea urchins (*Hemicentrotus pulcherrimus*) collected from the coast of Kitaura (Yamaguchi Prefecture, Japan). The sea urchin gonads were slightly dehydrated for several hours at 4 °C by being left to stand in a basket with small holes (raw gonads). Half of the dehydrated gonads were steamed for 20 min to inactivate the enzymes (steamed gonads). The raw gonads were processed and stored as follows. The raw gonads were gently mixed with sodium chloride (7% w/w). Ethyl alcohol (10% w/w) was added to the mixture in a glass bottle. The bottle was tightly sealed with a cap and then stored at room temperature for 0–180 days in total darkness (salted gonads). The steamed gonads were mixed with sodium chloride and ethyl alcohol as described above, and the steamed and salted gonads were stored at room temperature for 0–180 days (steamed and salted gonads).

The gonad samples (the salted gonads and the steamed and salted gonads) were collected from separate bottles at various intervals during 180 days and stored at −40 °C until analyzed.

Vitamins and Reagents. Thiamin hydrochloride, riboflavin (RF), and flavin adenine dinucleotide disodium salt (FAD) were obtained from E. Merck (Darmstadt, Germany). Thiamin monophosphate chloride (TMP) was purchased from Sigma Chemical Co. (St. Louis, MO). Thiamin diphosphate chloride (TDP), pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), pyridoxal 5′-phosphate (PLP), and pyridoxamine 5′-phosphate (PMP) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Thiamin triphosphate (TTP) was provided from Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Flavin mononucleotide sodium salt dihydrate (FMN) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Nicotinamide and isonicotinamide were obtained from Wako Pure Chemical Ltd. (Osaka, Japan). Pyridoxine 5′-phosphate (PNP) was prepared by reduction of PLP with sodium borohydride. All other reagents were of analytical grade.

Determination of Moisture and Sodium Chloride Contents. Moisture was determined by drying samples at 110 °C until their weight became constant. Thus, the moisture content determined for the salted gonads and the steamed and salted gonads included ethyl alcohol as well as water. Sodium chloride content was determined with a sodium ion-selective electrode (Horiba SH-7) according to the method of Shimada *et al.* (1989).

Vitamin B_1 Analysis. The gonads (approximately 0.4 g) were homogenized in 4 mL of 10% trichloroacetic acid for 2 min at 4 °C with a laboratory disperser (Ystral-Mitamura Riken Kogyo, Germany). The homogenates were centrifuged at 22000*g* for 15 min at 5 °C. Trichloroacetic acid in the supernatants obtained was removed by extraction five times with the same volume of diethyl ether. The water layers were filtered through a 0.65-μm pore size membrane. Thiamin and its phosphate esters in the filtrates were converted to corresponding thiochromes according to the procedure of Ishii *et al.* (1979a). The filtrate (0.4 mL) was mixed with 0.05 mL of 0.3 M BrCN. The mixture was vigorously shakened for 1 min, followed by an addition of 0.05 mL of 1 N NaOH. A control sample was prepared by sequential additions of 0.05 mL of 1 N NaOH and 0.05 mL of 0.3 M BrCN to 0.4 mL of the extract. The control sample was analyzed to correct the baseline of the elution profile. Standard solutions were prepared from solutions of thiamin and its phosphate derivatives in exactly the same manner as that used for the gonad extract. An aliquot (1–5 μL) of the oxidized sample was analyzed using a Hiber LiChrosorb Si 60 column (7-μm particle size, 250 × 4 mm, Merck Co.) by HPLC system (Hitachi, liquid chromatograph 638-30, fluorescence spectrophotometer F-1050, chromatoprocesser 833-0001) according to a modified method of Ishii *et al.* (1979b). The spectrofluorometer was operated with the excitation wavelength set at 365 nm and the fluorescence wavelength at 430 nm. The solvent system was acetonitrile/50 mM potassium phosphate buffer (pH 8.4) at the ratio of 60:40 (v/v). The solvent flow rate was 0.8 mL/min.

Table I. Moisture and Sodium Chloride Contents of Samples

sample	moisture, %	sodium chloride, %
raw gonads	60.91	0.30
steamed gonads	60.62	0.30
salted gonads[a]	58.01[b]	5.82
steamed and salted gonads[a]	58.13[b]	6.06

[a] Sample at 0 day. [b] Moisture + ethyl alcohol.

Vitamin B_2 Analysis. The gonads (0.4 g) were homogenized in 4 mL of distilled water for 2 min at room temperature with a laboratory disperser. The homogenate was heated for 15 min at 80 °C as described by Yagi and Sato (1981). After cooling, the homogenate was centrifuged at 22000*g* for 15 min at 10–15 °C and filtered through a 0.65-μm membrane filter. The filtrate was subjected to HPLC with a Hiber LiChrosorb RP-18 column (5 μm, 250 × 4 mm, Merck Co.). The system of HPLC was the same as that used for the vitamin B_1 analysis (Ohkawa *et al.*, 1982). The filtrate was eluted at a flow rate of 0.8 mL/min with 35% methanol containing 10 mM NaH_2PO_4 (pH 5.5) at room temperature. The column effluents were monitored with the spectrofluorometer. Excitation and emission wavelengths were set at 440 and 530 nm, respectively.

Vitamin B_6 Analysis. Vitamin B_6 compounds were determined according to the reversed-phase isocratic HPLC method described by Edwards *et al.* (1989) with some modifications. All manipulations were carried out under the light-shielded condition. The gonads (about 1.0 g) were homogenized in 30 mL of 10 mM potassium phosphate buffer (pH 7.0) for 1 min at 4 °C with a Polytron homogenizer. To 1 mL of the homogenate was added 27 μL of 9 N $HClO_4$. After a vigorous mixing and standing for 10 min at room temperature, the mixture was centrifuged at 7000*g* for 5 min at room temperature. To the resulting supernatant (300 μL) were added 1.2 mL of water and 32 μL of $HClO_4$, and then the mixture was filtered through a 0.2-μm membrane filter. The filtrate (100 μL) was applied to an HPLC apparatus. The HPLC system was described previously (Yagi *et al.*, 1991). To determine the recovery of vitamin B_6 compounds from the gonads, 30 μL of a standard mixture of the compounds (10 μM each) was added to 1 mL of the homogenate, and then the sample was passed through the assay procedure.

Nicotinamide Analysis. Nicotinamide was extracted from sea urchin gonads by the method of Shibata (1988). The sample (0.6 g) was homogenized in 3 mL of distilled water containing isonicotinamide (used as the internal standard). A screw-capped vial containing 1 mL of the homogenate and 4 mL of distilled water was autoclaved for 10 min at 121 °C. After cooling, the sample was centrifuged at 10000*g* for 10 min. The precipitate was again extracted with 5 mL of distilled water. The pooled supernatant (1.2 mL) was added to 0.07 mL of 70% perchloric acid, and the mixture was centrifuged at 10000*g* for 3 min. Nicotinamide was extracted from the supernatant with diethyl ether for analysis by HPLC according to the method described by Shibata *et al.* (1987). The HPLC system consisted of an LC-4A liquid chromatograph (Shimadzu, Kyoto, Japan), an SPD-2AS UV detector (Shimadzu), a Chromatopac C-R3A (Shimadzu), and a TSK-GEL ODS 80TM column (250 × 4.6 mm, Toyo Soda Co.). The eluent was 10 mM KH_2PO_4 [pH 3.0, adjusted by addition of concentrated phosphoric acid/acetonitrile in the ratio 96:4 (v/v)], the flow rate 0.7 mL/min, the detection wavelength 260 nm, and the column temperature 25 °C (Shibata *et al.*, 1988).

RESULTS AND DISCUSSION

Moisture and Sodium Chloride Contents. The moisture contents of the gonads ranged from 58.01 to 60.91% (Table I). The moisture contents in the salted gonads and in the steamed and salted gonads included

Table II. Vitamin B Contents of Raw Gonads and Steamed Gonads[a]

vitamin B	concn, μmol/100 g raw gonads	steamed gonads
vitamin B_1		
thiamin	0.84 ± 0.02	0.68 ± 0.03
TMP	0.01 ± 0	0.01 ± 0.01
TDP	0.03 ± 0	0.02 ± 0
TTP	0.01 ± 0	0.01 ± 0
total	0.89 ± 0.02	0.72 ± 0.04
vitamin B_2		
RF	0.04 ± 0	0.02 ± 0
FMN	0.04 ± 0	0.13 ± 0.01
FAD	1.41 ± 0.19	0.98 ± 0.02
total	1.49 ± 0.19	1.13 ± 0.03
vitamin B_6		
PN	0	0
PL	0	0
PM	0.07 ± 0.01	0.06 ± 0.01
PNP	0	0
PLP	0.95 ± 0.17	0.18 ± 0.03
PMP	0	1.00 ± 0.18
total	1.02 ± 0.17	1.24 ± 0.20
niacin		
nicotinamide	2.60 ± 0.15	9.30 ± 0.13

[a] Mean ± SD, $n = 3$.

ethyl alcohol as well as water. The sodium chloride content (0.3%) of the steamed gonads was the same as that of the raw gonads. The steamed and salted gonads contained almost the same amount of moisture (about 6%) as the salted gonads. Thus, individual differences in moisture and sodium chloride contents were slight between the raw gonads and the steamed gonads and between the salted gonads and the steamed and salted gonads. The B' complex vitamin contents, which are expressed as micromoles per 100 g of sample in the following experiments, were not corrected for moisture and sodium chloride contents.

Vitamin B Contents of the Gonad Preparations. Concentrations of thiamin and its phosphate esters in the raw gonads and in the steamed gonads were determined as shown in Table II. The two gonad preparations resembled plant seeds (Steyn-Parvé and Monfoort, 1963) in the composition of vitamin B_1. Thiamin consisted of 94.4% of the total thiamin compounds in both gonads. The most abundant thiamin compound in rat tissues has been reported to be TDP, which represented an average of 85–90% of the total thiamin compounds present (Rindi and De Giuseppe, 1961; Ishii *et al.*, 1979a). On the contrary, thiamin is the main component of vitamin B_1 in plant seeds (Steyn-Parve and Monfoort, 1963). It may be possible that TDP and other phosphate esters were hydrolyzed into thiamin by phosphatases in the raw gonads during the dehydration of several hours. However, the possibility did not seem to be plausible because the dehydration step did not affect the phosphoester compound of pyridoxal, as shown in Table II. When the raw gonads were steamed, 19% of the total thiamin was lost, indicative of moderate stability of thiamin (Table II). Processing of the gonad preparations with sodium chloride and ethyl alcohol decreased the thiamin contents as shown in Figure 1 (data on 0 day): thiamin contents of the salted gonads and the steamed and salted gonads were 0.49 (loss after processing calculated from dry weights except sodium chloride; 37.8%) and 0.57 μmol/100 g (loss after processing; 8.6%), respectively. Thiamin consisted of more than 90% of the total thiamin compounds in the two kinds of salted gonads throughout 180 days of storage (data not shown). Thiamin contents of the salted gonads and the steamed and salted gonads gradually decreased (Figure 1). The

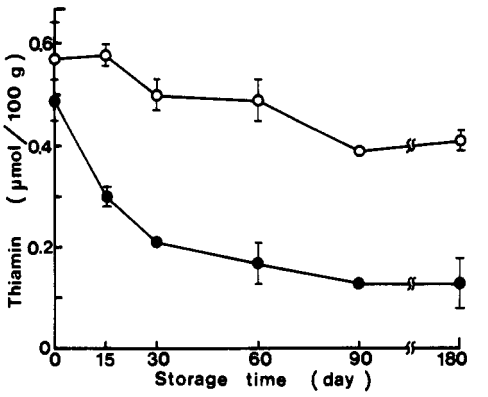

Figure 1. Changes in thiamin contents of the salted gonads (●) and the steamed and salted gonads (○) during storage. The bars show the standard deviation of the three replicate determinations.

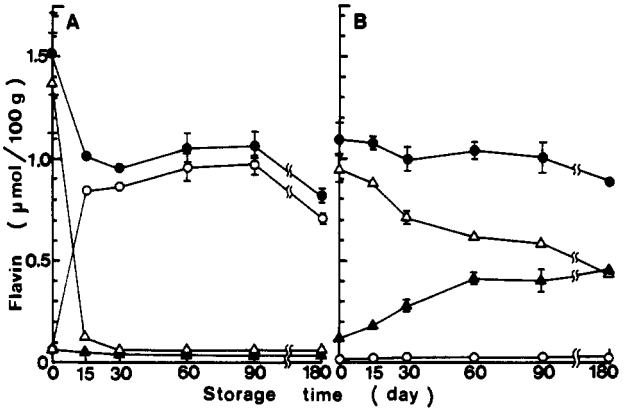

Figure 2. Changes in flavin contents of the salted gonads (A) and steamed and salted gonads (B) during storage: (●) total flavin; (○) RF; (▲) FMN; (△) FAD. The bars show the standard deviation of the three replicate determinations.

decrease in the thiamin content was higher in the salted gonads than in the steamed and salted gonads during the storage. Because of the addition of ethyl alcohol, the viable cell (bacteria) adhering to the salted gonads was less than 1×10^2/g of salted gonads throughout 180 days of storage, and it was suggested that the component changes in the salted gonads occurred independently of the reactions catalyzed by bacterial enzymes during the storage (Shimada and Okajima, 1989). Thus, the rapid decrease in thiamin, especially at the initial storage period (0–30 days), in the salted gonads may be attributable to the degradation by enzymes such as thiaminase present in the gonadal cells. The loss of thiamin in the salted gonads during the storage of 180 days was about 74% of the value at 0 day. A small but significant decrease in the thiamin content was observed in the storage of the steamed and salted gonads. Thus, thiamin is liable to be degraded enzymatically and nonenzymatically in the salted gonads.

The contents of RF, FMN, and FAD in the raw gonads and the steamed gonads are shown in Table II. FAD was the major compound, which comprised 94.6% of total flavin in the raw gonads and 86.7% in the steamed gonads. The total flavin content decreased from 1.49 ± 0.19 to 1.13 ± 0.03 μmol/100 g (loss; 24.2%) by the heat treatment. Neither the salted gonads nor the steamed and salted gonads were changed in their total and individual flavin contents by the processing with sodium chloride and ethyl alcohol (Figure 2). However, the storage did affect the flavin content in the gonad preparations. The total flavin content decreased greatly during the initial 15 days of storage (loss; 34.4%) and then became nearly constant in the salted gonads, while the total flavin content remained

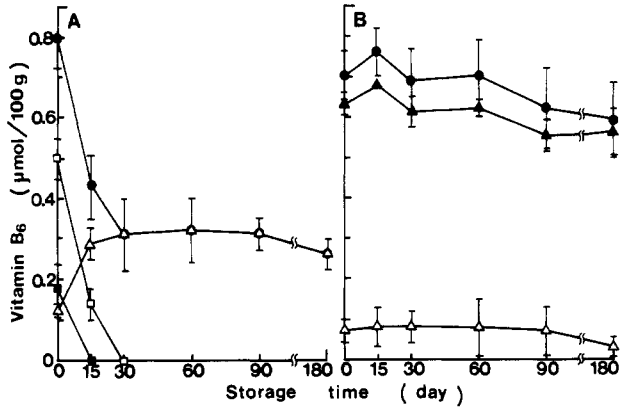

Figure 3. Changes in vitamin B_6 content of the salted gonads (A) and the steamed and salted gonad (B) during storage: (●) total vitamin B_6; (△) PM; (▲) PMP; (□) PL; (■) PLP. The bars show the standard deviation of the three replicate determinations.

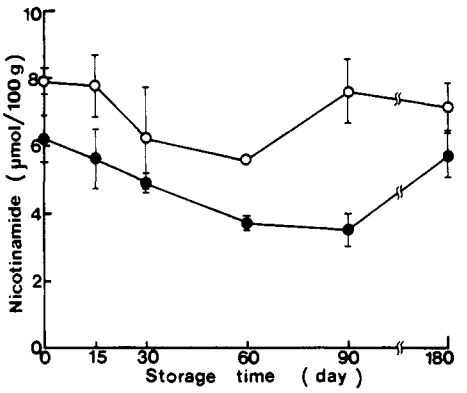

Figure 4. Changes in nicotinamide contents of the salted gonads (●) and the steamed and salted gonads (○) during storage. The bars show the standard deviation of the three replicate determinations.

constant in the steamed and salted gonads during 90 days of storage (Figure 2). The total flavin contents decreased somewhat in the salted gonads and the steamed and salted gonads during 90–180 days of storage. During the initial 15 days of storage, FAD decreased radically in the salted gonads concomitant with an increase in RF. FMN remained constant. The increase in RF content may have resulted from the enzymatic hydrolysis of FAD. In the steamed and salted gonads, FMN increased gradually throughout 180 days of storage concomitant with the decrease in FAD, suggesting that chemical hydrolysis but enzymatic reaction occurred. The increase in RF was not detected during the storage of the steamed and salted gonads. The salted gonads retained significant amounts of vitamin B_2 (total flavin 0.81 ± 0.03 μmol/100 g) even after 180 days of storage.

The raw gonads contained mainly PLP among six vitamin B_6 compounds (Table II). On the contrary, PMP was the major component in the steamed gonads, showing that a large part of PLP converted to PMP when the raw gonads were heated. The same thermal interconversion of the B_6 vitamers has been indicated in chicken muscles by Gregory *et al.* (1986). The heat treatment of the raw gonads also caused the increase in total vitamin B_6 content by 21.6%. The free B_6 vitamer(s) may be liberated from the bound form(s) with unknown component(s) by heat treatment. A rapid conversion of a large part of PLP to PL and PM occurred when sodium chloride and ethyl alcohol were added to the raw gonads, as shown in Figure 3A (0 day). The conversion continued for up to 15 days of storage. The result showed the presence of a rapid phosphatase reaction, followed by a slow transamination reaction, which converts PL to PM. No vitamin B_6 compounds other than PM were found in the salted gonads after 30 days. The loss of total vitamin B_6 during 0–180 days of storage was 67.5% in the salted gonads, indicating the lability of B_6 vitamers. The processing decreased PMP and PLP contents of the steamed gonads by 37.5 and 100%, respectively. PMP and PM contents of the steamed and salted gonads decreased by 11.3 was 55.8%, respectively, after 180 days of storage. These results indicated that amine forms of B_6 vitamers are exclusive compounds found in the matured–salted gonad preparations.

The nicotinamide contents of the raw gonads and the steamed gonads were 2.6 ± 0.2 and 9.3 ± 0.1 μmol/100 g, respectively (Table II). After the processing of the raw gonads (the salted gonads at 0 day), the increase in nicotinamide content (from 2.60 to 6.20 μmol/100 g) was observed (Figure 4). It may be possible that a nicotinamide derivative such as N^1-methylnicotinamide was converted into nicotinamide enzymatically during the rise of temperature on heat treatment and the processing with sodium chloride and ethyl alcohol. However, it was not actually clear why nicotinamide increased with heat treatment and processing. This is a future problem. The contents of nicotinamide decreased until 90 days for the salted gonads and until 60 days of storage for the steamed and salted gonads and, subsequently, increased to near the level at 0 day, respectively (Figure 4). It is likely that the variations of nicotinamide contents during 180 days of storage reflect the sum of the chemical losses and the liberation of nicotinamide from the bound form(s). Generally, it is known that nicotinamide is stable during cooking (boiling, broiling, frying, and heating with a microwave oven) except for leaching into the cooking water (Dexter *et al.*, 1982; Taguchi *et al.*, 1986). Nicotinamide was stable in the salted gonads for 180 days of storage.

Conclusion. This paper presents the stabilities of vitamins B_1, B_2, and B_6 and nicotinamide in salted sea urchin gonads during processing and storage. Vitamin B_1 existed mainly as thiamin in the raw gonads and decreased rapidly during processing and storage. FAD was the major compound among B_2 vitamers in the raw gonads. The loss of vitamin B_2 in the salted gonads was observed during the initial 15 days of storage, and RF resulting from the hydrolysis of FAD was stable for storage. Vitamin B_6 existed mainly as PLP in the raw gonads. Total vitamin B_6 in the salted gonads decreased greatly after 180 days of storage. B_6 vitamer found in the stored–salted gonads was PM, and other B_6 derivatives were not detected. A noticeable loss of nicotinamide was not observed for 180 days of storage.

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