Influence of Free Sugars by Glycolysis on the Formation of the Characteristic Flavor in the Brew of Cooked Clam

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Changes in the glycogen, free sugars, and amino acids contained in the clam body during storage for 24 h at 4 °C were investigated. The relationship was also studied between the changes in these constituents and the formation of the main cooked odor compounds, 3-hydroxy-2-methylpyran-4-one (maltol) and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF). Glycogen was decreased to 68% of the initial value and, in contrast, glucose, glucose-6-phosphate (G6P), fructose-1,6-diphosphate (F1,6P), and maltose were increased when clam bodies were stored for 24 h. In particular, glucose increased markedly from 11.1 mg/100 g to 104.9 mg/100 g. However, the amino acids in the clam body were unchanged during storage. The results of an addition test confirmed that free sugars played an important role in the formation of maltol and DMHF which were mainly generated as the flavor impact compounds of cooked clam. It is concluded that the increase in free sugars, especially glucose, that were degraded from glycogen in the muscle part during storage promoted the formation of maltol and DMHF.

Keywords: *Clam; flavor; maltol; DMHF; glycolysis; free sugars*

INTRODUCTION

When clam (*Meretrix lusoria*) is heated, a characteristically pleasant odor is generated, which affects the cooked clam flavor as well as its taste. We have previously reported that four compounds, 3-hydroxy-2-methylpyran-4-one (maltol), 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), 2-acetyl-2-thiazoline, and 2-acetylthiazole, were organoleptically and quantitatively present as potent odor compounds in cooked clam soup (Sekiwa et al., 1997). In particular, maltol and DMHF possess a sweet odor and have been identified in many heated foods (Ito, 1972). We have also reported that the raw clam body stored for 24 h presented a more preferable cooked odor than that just after being unshelled and furthermore, that the concentrations of both maltol and DMHF were markedly increased with storage time (Sekiwa et al., 1997). However, it remained unclear which factors enhanced their formation during storage of the clam body. In the present work, the precursors generating these odor compounds in the clam were investigated.

It is generally known that bivalves contain much more glycogen than other seafoods (Yamanaka, 1988). Watanabe et al. (1990) have also reported that glycogen affected the mild taste of scallop. Glycogen in organisms is usually decomposed to free sugars and sugar phosphates by glycolysis under anaerobic conditions. Therefore, during storage of the clam body after unshelling, it is assumed that the free or phosphate sugars are derived from glycogen. Yamanaka (1974) has reported that the increase in sugar phosphates by glycolysis was related to the browning of fish meat after heating, and Kawashima and Yamanaka (1995) have also recognized the same browning mechanism in the scallop muscle. It was expected that the change in the amount of those free sugars in the clam body would have an effect on

the formation of the characteristic odor compounds that are thermally generated from sugars and amino acids. In this study, the increase in the amounts of nonvolatile constituents such as free sugars and free amino acids during storage of the clam body was measured to correlate this change with the formation of the main odor compounds, especially of maltol and DMHF.

MATERIALS AND METHODS

Materials. Live clams (*M. lusoria*:; Mie coast, Japan) were purchased at Tokyo central market during the period from October 1995 to January 1996. After being prepared for 24 h in water with 1% NaCl, the body was separated from the shell by hand-shucking and then measured. The muscle part of each clam body was cut off and divided into four portions, each being stored at 4 °C for 30 min, 2 h, 12 h, or 24 h before being assayed.

Chemicals. Most of the chemicals, including standard sugars, amino acids and enzymes for the assays, were commercially available. 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone was a gift from the T. Hasegawa Co. Ltd.

Quantification of Glycogen Content. The glycogen in each sample was extracted by following the procedure of Watanabe et al. (1992). A 3 mL portion of 30% KOH was added to 0.2 g of a sample, and the resulting suspension was heated for 30 min in a boiling-water bath, before the liberated glycogen was precipitated by 3 mL of ethanol. The glycogen was separated by centrifugation (1000 rpm for 15 min) and made up to a 5 mL solution with purified water. An aliquot (1 mL) of this solution was hydrolyzed to glucose with 1 M H_2SO_4. After neutralization, the resulting glucose was quantified by the enzymatic method (Wako glucose CII−test, Wako Pure Chemical Industries Ltd.). The actual quantity of glycogen was calculated from the quantity of glucose.

Quantification of Free Sugars and Free Amino Acids. A portion (10 g) of the muscle part of the clam body stored for each period was deproteinized by homogenization in 7% cold perchloric acid (30 mL). The supernatant was separated by centrifugation (3500 rpm at 4 °C for 15 min), and the resulting precipitate was homogenized with 7% cold perchloric acid (10 mL) before its solution was centrifuged. The combined supernatant was neutralized to pH 7.0 with KOH. The generated white precipitate was separated by centrifugation, and

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Table 1. Change in the Amounts of Free Sugars and Glycogen during the Storage of Clam at 4 °C

sugar	n	concentration of free sugar[a] (mean ± SE; mg/100 g of body weight)				
		0 h	0.5 h	2 h	12 h	24 h
glucose	3	11.1 ± 1.3a	16.4 ± 1.5a	28.2 ± 3.0a	53.8 ± 2.9b	104.9 ± 13.9c
G6P	3	3.2 ± 1.0a	5.2 ± 0.5ab	6.0 ± 0.9ab	8.7 ± 1.4b	12.1 ± 1.1c
F6P	3	0.5 ± 0.3a	1.0 ± 0.5a	0.9 ± 0.3a	0.6 ± 0.3a	0.5 ± 0.3a
F1,6P	3	1.5 ± 0.8a	4.7 ± 1.2ab	7.0 ± 0.8b	12.1 ± 2.4c	12.5 ± 2.6c
maltose	3	3.5 ± 0.5a	5.1 ± 0.8a	4.7 ± 0.7a	19.2 ± 1.8b	36.1 ± 1.2b
glycogen[b]	8	5.3 ± 0.4a	4.5 ± 0.7a	3.6 ± 0.3b	3.3 ± 0.6b	2.9 ± 0.4b

[a] Means followed by different letters in each row are significantly different ($p \leq 0.05$). [b] Concentrations are presented in g/100 g clam body weight.

the resulting supernatant was subjected to assays for free sugars and free amino acids.

The free sugars, glucose, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (F1,6P), and maltose in the body were quantified by the enzymatic methods of Bergmeyer (1963), Racker (1963), and Beutler (1984), while free amino acids were quantified by a Hitachi 835 amino acid analyzer. Each quantification was performed three times.

Addition of Sugars to the Sample and Separation of the Volatile Compounds Formed by Cooking. The effect of free sugars on the formation of the main odor compounds was investigated. A 0.67 mmol amount of D-glucose, G6P, F6P, or F1,6P, 0.33 mol of maltose, or 1.0 g of glycogen was added to unshelled clam bodies (100 g) that had been stored at 4 °C for 30 min. A control sample without additional sugar was also prepared. The sample and hot water (100 mL) in a glass flask were boiled for 30 min by an electric mantle heater. The odor concentrate was prepared by the column adsorption method, using Tenax TA as described in our previous report (Sekiwa et al., 1997). After filtration through cotton gauze and centrifugation for 10 min at 3000 rpm, the obtained liquid was passed through a 2 cm × 15 cm bed-height column packed with preconditioned Tenax TA resin. The volatile compounds adsorbed to the resin were eluted with diethyl ether (150 mL). After being dried with anhydrous sodium sulfate, the ethereal extract was concentrated at 39 °C. The flavors in each sample were then isolated by duplication.

Gas Chromatography (GC) and Gas Chromatography−Mass Spectrometry−Selected Ion Monitoring (GC−MS−SIM). A Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionization detector (FID) was used for analysis with a DB-WAX (60 m × 0.25 mm i.d., J&W) fused silica capillary column. The oven temperature was held initially at 60 °C for 4 min and then raised to 180 °C at 2 °C/min. The injection temperature was set at 200 °C, the detection temperature was set at 210 °C, and helium at 1.0 mL/min was used as the carrier gas. A Hewlett Packard 5972 mass-selective detector was interfaced with a Hewlett Packard 5890 series II gas chromatograph for the GC−MS analysis, the GC conditions for GC−MS being the same as those for the GC analysis. The concentrations of maltol and DMHF in each volatile concentrate were analyzed by the selected ion monitoring (SIM) method. The selected ions for each compound were as follows: m/z 126, 71, 55, and 97 for maltol and m/z 128, 43, 57, and 85 for DMHF. A calibration curve for each compound was established with standard solutions containing four defined amounts of each compound. The concentration range of each compound was then determined to obtain good linearity.

Comparison of the Formation of Flavor Compounds between the Muscle and Internal Parts of the Clam. The internal parts (80 g) and muscle parts (110 g) were prepared from clam bodies (200 g) at 30 min after being unshelled. Both parts were separately boiled for 30 min, and each odor concentrate was obtained in the same way as that already described. The amounts of maltol and DMHF were calculated by comparing with the peak area of an internal standard (methyl decanoate, 3.0×10^{-4} mg per raw clam body) by GC. All measurements were performed in duplicate.

Statistical Analysis. The quantities of free sugars and amino acids were statistically analyzed. The analysis of variance and the statistical difference with Bonferroni's mul-

tiple range tests were performed with SPSS softwere. Significance was defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

Change in the Composition of Some Nonvolatile Components during Storage. The amounts of glycogen and free sugars in the clam body after being unshelled were measured after storing for 0 min, 30 min, 2 h, 12 h, and 24 h at 4 °C (Table 1). The glycogen content in the body just after being unshelled was 5.3% of the clam body weight, and this decreased with increasing storage time. In particular, the glycogen content significantly decreased to 3.6% of the clam body weight in the initial 2 h of storage, corresponding to 68% of its original content. Takagi and Shimidu (1962) have reported that almost all shellfish contain more than 1% glycogen in their body weight. On the other hand, it is known that glycogen is enzymatically degraded by phospholysis or hydrolysis under anaerobic conditions. Glycogen degradation is thus presumed to have occurred during storage of the unshelled clam specimens.

Free sugars, including glucose, G6P, F1,6P, and maltose, that were liberated by glycolysis increased with increasing storage time. Among them, the increase in glucose was the highest, from 11.1 mg/100 g to 104.9 mg/100 g in 24 h. Although F1,6P also increased 10 times during 24 h of storage, the resulting amount was relatively small due to its original amount being very low and its molecular weight being about 1.5 times higher than that of glucose. Nakagawa et al. (1996) have clarified the existence of such hydrolytic enzymes as α-amylase and α-glucosidase in the muscle of fish, and these enzymes are also involved in the degradation of glycogen. On the other hand, Kawashima and Yamanaka (1995) have reported that G6P in the muscle of scallops increased markedly during storage for 3 days at 5 °C. In the case of the clam body, the much greater increase in glucose than G6P means that enzymatic hydrolysis was predominant in its phospholysis.

The change in free amino acids during storage is shown in Table 2. Total content of free amino acids was 22−25 mmol per 100 g of the clam body, the major ones being alanine, taurine and glycine. The total amount of these three amino acids constituted more than 80% of the total amount of free amino acids in the clam body. Since the concentration of these three amino acids scarcely changed, the content of total amino acids also remained unchanged. Therefore, the increase in the concentration of maltol and DMHF is presumed to only have been associated with the increase in free sugars during storage.

Effect of Sugars on the Cooked Clam Odor by the Addition Test. The relationship between the sugars liberated by glycolysis and the cooked flavor of clam was investigated. Glucose, G6P, F6P, or F1,6P (0.67 mmol), maltose (0.33 mmol), or glycogen (1.0 g)

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Table 2. Change in Amino Acid Contents during the Storage of Clam at 4 °C

	concentration of amino acids (mean ± SE; mmol/100 g of clam body weight)[a]				
	0 h	0.5 h	2 h	12 h	24 h
alanine	13.58 ± 0.09	11.85 ± 0.81	13.02 ± 1.34	12.05 ± 0.37	12.42 ± 0.21
taurine	5.14 ± 0.12	5.18 ± 0.32	5.91 ± 0.36	5.44 ± 0.22	5.82 ± 0.28
glycine	1.16 ± 0.15	1.03 ± 0.08	0.89 ± 0.15	0.97 ± 0.19	1.11 ± 0.40
others	4.55 ± 0.27	4.06 ± 0.10	4.10 ± 0.26	4.99 ± 0.38	4.56 ± 0.01
total	24.41 ± 0.07	22.12 ± 1.16	23.91 ± 1.10	23.19 ± 0.40	23.91 ± 0.01

[a] Each value is the mean of triplicate measurements.

Table 3. Effect of Sugars by Glycolysis on the Formation of DMHF[a] and Maltol in Cooked Clam

	generated amount (mean ± SE; ng/g)[b]	
added sugar	maltol	DMHF
control[c]	201.3 ± 10.2	41.0 ± 2.3
glucose[d]	326.7 ± 16.3	106.9 ± 6.5
G6P[d]	256.8 ± 8.5	280.9 ± 3.5
F6P[d]	294.8 ± 8.6	358.6 ± 16.2
F1,6P[d]	262.9 ± 5.7	707.3 ± 18.6
maltose[e]	305.1 ± 13.2	389.2 ± 3.0
glycogen[f]	172.4 ± 12.4	49.5 ± 4.6

[a] DMHF, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone. [b] Each value is the mean of duplicate measurements. [c] Without additional sugar. [d] 0.67 mmol of each sugar was added. [e] 0.33 mmol of maltose was added. [f] 1.0 g of glycogen was added.

Table 4. Yields of Maltol and DMHF by Cooking Different Parts of Clam

	peak area ratio to IS[a]	
cooked part	maltol	DMHF
whole	11.69 ± 0.88	0.97 ± 0.16
muscle	6.29 ± 0.47	0.41 ± 0.02
internals	3.23 ± 0.32	tr[b]

[a] The GC peak area ratio of each component to the internal standard (methyl decanoate, 3.0×10^{-4} mg/100 g of whole raw clam bodies) is presented. Each value is mean ± standard error of duplicate measurements. [b] Detected peak area ratio is trace (<0.01).

was added to a clam sample in water before cooking. The added quantity was calculated from the increase in glucose during 24 h of storage for the sugars and was about half the decrease in glycogen. Each mixture was heated, and the volatiles were collected. The absolute quantities of maltol and DMHF produced were analyzed by the SIM method and are shown in Table 3. Maltol was increased by the addition of every sugar. Many researchers have noted that 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one, instead of maltol, was formed by heating glucose and amino acids or a primary amine (Nishibori and Kawakishi, 1990; Knerr et al., 1993; Hodge et al., 1972). On the other hand, Kim and Baltes (1996) have reported that 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one could be dehydrated to maltol by heating in an aqueous condition of weak basic medium. The pH value of each clam soup with every tested additive was between 7.2 and 7.6 during cooking, which would have caused the formation of maltol from 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one. Therefore, it is considered that the increase in free sugars was directly related to the formation of maltol.

DMHF was also increased by adding almost every type of sugar, except for maltose. Among them, such phosphate sugars as G6P, F6P, and F1,6P accelerated the formation of DMHF. Schieberle (1992) has reported that the phosphate ion promoted the formation of DMHF in wheat bread crust, and our results from the addition test corroborated the promotive effect of phosphate. However, since each clam body contained only 1–8% of the amount of glucose as each phosphate sugar, the increase in glucose during storage seems to have taken the most important role in the formation of DMHF as a precursor in the clam. On the other hand, glycogen did not have any effect on the formation of maltol or of DMHF.

Part of the Body Responsible for Producing the Main Odor. The part of the clam body which was mainly responsible for producing maltol and DMHF during cooking was investigated. The amounts of maltol and DMHF in each part formed by cooking were compared with the ratio of the GC peak area to that of the internal standard (Table 4). Both maltol and DMHF

were produced in much greater quantities in the muscles than in the internal parts, with DMHF being detected in only a trace amount in the internal parts. Since glycogen is generally contained in the muscle parts of animals, glycolysis also mainly occurs in the muscle. However, the results of this study show that the total amount of maltol and DMHF in the muscle and internal parts was less than that of the whole body. This can be explained by assuming that the degradation of sugars in the muscle would be accelerated by amino acids or other factors in the internal parts, and the formation of maltol and DMHF would be promoted secondarily.

We have previously reported that maltol and DMHF predominantly contributed to the odor of cooked clam and that both were also produced in greater quantity with increasing storage time (Sekiwa et al., 1997). In addition, this present work shows that the formation of maltol and DMHF was influenced by free sugars from decomposed glycogen. From these results, it can be concluded that the decomposition of glycogen during clam storage would be related to the formation of a good flavor in cooked clam soup.

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