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Rapid Production of Fish Sauce from the Internal Organs of White Sturgeon, *Acipenser transmontanus* Richardson, 1836

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Abstract: The internal organs of white sturgeon in Miyazaki Prefecture are discarded during processing. Therefore, we tried to produce fish sauce using a short-term manufacturing method. The minced internal organs were autolyzed by endogenous proteases at 50 °C. During autolysis, the protein contents of the supernatant and precipitate after centrifugation were analyzed by the Kjeldahl method, and the protein size was monitored by SDS-PAGE. This analysis showed that the extraction rate was about 60% after treatment at 50 °C for 24 h. The major bands at 200 kDa, 43 kDa, and 40 kDa detected before the start of the treatment gradually disappeared over time. Fifteen components were detected as the main volatile components. These components increased sharply and then decreased during incubation at 50 °C for 24 h. The fish sauce produced had a good aroma after incubation at 50 °C for 72 h.

Keywords: fish sauce; *Acipenser transmontanus*; white sturgeon; aroma; protease; fatty acid; histamine; volatile compound

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1. Introduction

Fish sauce is a traditional Japanese fermented food that is produced by aging seafood with a high concentration of salt. As in soy sauce, amino acids produced by the decomposition of proteins are used as seasoning. Soy sauce is formed by the decomposition of soybean proteins with koji enzymes. In addition, several microorganisms, such as koji, yeast, and lactic acid bacteria, cooperate to make the soy sauce.

However, fish sauce is formed by the decomposition of seafood protein with self-digesting enzymes of the seafood itself. Fish sauce is valued for its taste and preservative properties, and improvement in the odor of the strong scent that is peculiar to fish sauce has been studied using koji or yeast [1–9].

The production of microbial fermented fishery seasoning has recently increased. For example, fish sauce with an increased γ-aminobutyric acid (GABA) content is produced by adding lactic acid bacteria isolated from pickles [10].

Sturgeons (*Acipenseridae*) are valued for their nutritious meat and valuable caviar [11]. In recent years, sturgeon aquaculture has developed in many countries. In Miyazaki Prefecture, sturgeon aquaculture is flourishing, and the production of sturgeon eggs is the highest in Japan. However, the internal organs are disposed of as waste during processing. Therefore, we have examined the development of fermented foods using these internal organs.

Flavor is formed by the degradation of proteins and lipids in fish via various metabolic pathways, through the actions of halophilic microorganisms and enzymes [12]. In a previous study, we described two methods for producing fish sauce using soy sauce koji or salt, with the discarded internal organs as raw materials. An investigation of the properties of each fish sauce showed differences in the types and abundance of aromatic components.

In particular, there was a marked increase in 2-furanmethanol in the fish sauce after heating, while furfuryl acetate was only detected in fish sauce made using soy sauce koji. These findings suggest that these components may have an effect on the scent. We also found differences in the types and abundance of aromatic components, with 2-hexenal only being detected in fish sauce made using salt [13]. This ingredient has been reported to have a grassy scent [14]. The internal organs of sturgeon contain a large amount of hexanal, and the components with a grassy odor were reduced by fermentation with the addition of koji. A large amount of isovaleric acid was also detected in fish sauce produced using soy sauce koji, but this did not have an unpleasant odor, which suggests that other ingredients had positive effects.

The extraction rate was about 30% for both methods, while a rate of about 40% has been achieved after fermentation of the internal organs of bluefin tuna for 3 months [15]. Utagawa reported rapid production of fish sauce from the internal organs of mackerel, horse mackerel, herring and ayu [16].

These results show the need to improve manufacturing conditions in order to increase the extraction rate. In this study, we tried to produce fish sauce efficiently from the residue of sturgeon products.

2. Materials and Methods

2.1. Proximate Analysis

Internal organs of white sturgeon, *Acipenser transmontanus* Richardson, 1836, were obtained from an aquafarm in Nichinan, Miyazaki, and stored at −20 °C until analysis. The internal organs were cut into small pieces with a kitchen knife and crushed well with a mixer. The minced internal organs were used for proximate analysis. The protein content was analyzed by the Kjeldahl method. Ash content and moisture were analyzed based on the guidelines published by the Association of Official Analytical Chemists [17].

2.2. Fatty Acid Analysis

Total lipids were determined using the method described by Folch et al. [18]. Fatty acid compositions of the lipids were determined using a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) equipped with a capillary column (0.25 mm × 30 m, HR-Thermon-3000B, Shinwa Chemical Industries, Ltd., Kyoto, Japan) after methanolysis with 10% (*w*/*v*) HCl methanol solution. The quantities of individual fatty acids were estimated from the peak areas on the chromatogram using C19:0 fatty acid (nonadecanoic acid) as an internal standard [19].

2.3. Preparation of Crude Enzyme Solution from White Sturgeon

A portion of the minced fish was added to an equal volume of distilled water and mixed well with a vortex mixer. The resulting solution was centrifuged at 5000× *g* for 15 min at 4 °C, and the supernatant was used as a crude enzyme solution for the protease assay.

2.4. Protease Assay

Protease activity was determined by measuring the amount of tyrosine liberated from casein [20]. A crude enzyme solution of 100 µL was mixed with 500 µL of 3.0% casein solution. The mixture was incubated at 25 °C or 50 °C for 10 min, and then 500 µL of 5.0% trichloroacetic acid (*w*/*v*) was added to stop the enzyme activity. The mixture was centrifuged at 5000× *g* for 10 min, and the supernatant was used to determine the amount of tyrosine liberated from casein. For the measurement, 250 µL of supernatant was mixed with 0.55 mol/L sodium carbonate solution and incubated at room temperature for 10 min. Then, 125 µL of 660 mM Folin's phenol reagent was added and the mixture was incubated at 25 °C or 50 °C for 30 min to generate a measurable color, based on the reaction with tyrosine. The absorbance of the supernatant at 660 nm was measured with a UV-vis spectrophotometer (V-530, Shimadzu Co., Kyoto, Japan), and the amount of liberated

tyrosine was calculated using a calibration curve previously made for a standard tyrosine solution. One unit of enzyme activity was defined as the amount of enzyme that could liberate the equivalent of 1 µg of tyrosine from casein per 1 min.

2.5. Preparation of Fish Sauce from Internal Organs of White Sturgeon

To prepare the fish sauce, sturgeons were treated using the procedure for making the crude enzyme solution described above. Temperatures of 25 °C and 50 °C were selected to induce autolysis based on a previous study [13]. A mixture weighing 10 g was placed in a vial vessel and incubated at 25 °C or 50 °C for 96 h after the addition of 10 mL of distilled water. An aliquot of each sample was collected for chemical analysis every 24 h. The mixture was then centrifuged at 5000× *g* for 10 min. The supernatant was collected as sturgeon fish sauce, and the concentrations of histamine and volatile compounds were analyzed. The protein concentration was determined by the Kjeldahl method.

2.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

The protein content of autolyzed fish bodies was adjusted to 1.5 µg/µL and mixed with an equal amount of 2 × SDS-PAGE buffer solution consisting of 0.125 M (*w*/*v*) Tris-HCl buffer (pH 6.8), 4% SDS (*w*/*v*), 8% (*w*/*v*) 2-mercaptoethanol, 40% (*v*/*v*) glycerol, and 0.004% (*w*/*v*) bromophenol blue. The mixture was heated at 100 °C for 3 min. Protein size profiles in the samples were monitored with SDS-PAGE, using the Laemmli method [21]. Samples were loaded onto 12.5% (*w*/*v*) polyacrylamide gels and run at 20 mA. To visualize protein bands, the gels were stained with 0.1% (*w*/*v*) Coomassie Brilliant Blue R250 (CBB) in 30% (*v*/*v*) methanol, 10% (*v*/*v*) acetic acid, and 60% (*v*/*v*) distilled water, and destained with CBB destaining solution consisting of 10% (*v*/*v*) methanol, 10% (*v*/*v*) acetic acid, and 80% (*v*/*v*) distilled water.

2.7. Bacteria Plate Count and Histamine Analysis

Each fish sauce sample was boiled at 90 °C for 10 min. The bacteria and coliform bacteria counts of each sample were then obtained using Nissui Standard Method Agar and Desoxycholate Agar Medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), according to the standard plate count method. The histamine concentration was measured with a Check-Color Histamine kit (Kikkoman Co., Tokyo, Japan).

2.8. Volatile Compound Analysis

Solid-phase microextraction (SPME) was used to quantify compounds in the sturgeon fish sauce. Volatile compounds in the samples were extracted using a 2 cm, 23-gauge SPME fiber coated with 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). Then, 0.2 mL of fish sauce was placed in a 20 mL glass vial and the SPME fiber was exposed to the headspace at 50 °C for 20 min. The SPME fiber was then injected into a gas chromatograph (7890 B, Agilent Technologies Inc., Santa Clara, CA, USA) and analyzed by GC using a DB-WAX column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, Wien, Austria) coupled with MS (5977 AMSD, Agilent Technologies). The oven temperature was initially held at 50 °C for 5 min, then increased to 250 °C at a rate of 3 °C/min, and then held at 250 °C for 10 min. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The compounds were identified based on their retention times in the GC column. The relative amounts of compounds in each processing step were determined using an extracted ion chromatogram from the MS analysis. The results for volatile compounds are quantitative and are expressed as mg/L, relative to an internal standard (cyclohexanol). All analyses were performed in triplicate.

2.9. Statistical Analysis

Differences between data were evaluated by Student *t*-test (Microsoft Excel, 2013), with $p < 0.05$ considered to be significant.

3. Results

3.1. Proximate Analysis of Minced Internal Organs

The protein content and the pH in the minced internal organs of white sturgeon were 14.1% and 6.08, respectively. The contents of moisture, lipid, and ash in the minced internal organs were 80.16%, 2.83%, and 1.01%, respectively. The lipid content was lower than that in the internal organs of other freshwater fishes, such as carp and ayu [22]. The major fatty acid was oleic acid (C18:1), which accounted for 30.6% of the total fatty acids, while linoleic acid was the third most abundant (Figures 1 and 2). Polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA, C22:5n-3) and eicosapentaenoic acid (EPA, C20:5n-3), were also present, although only in small amounts.

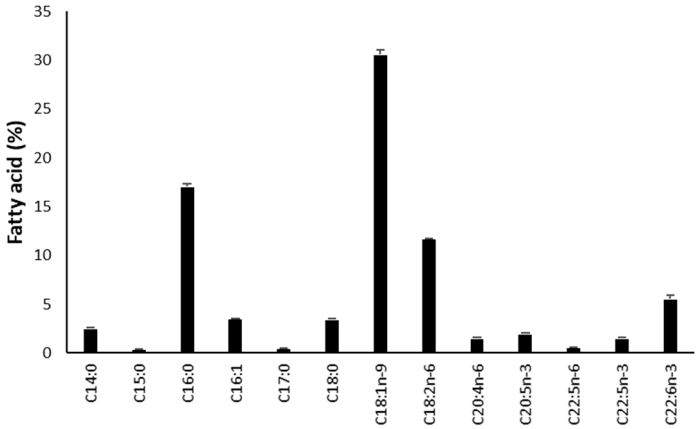

Figure 1. The percentage of each fatty acid compared with the total fatty acid. Values are mean (n = 3).

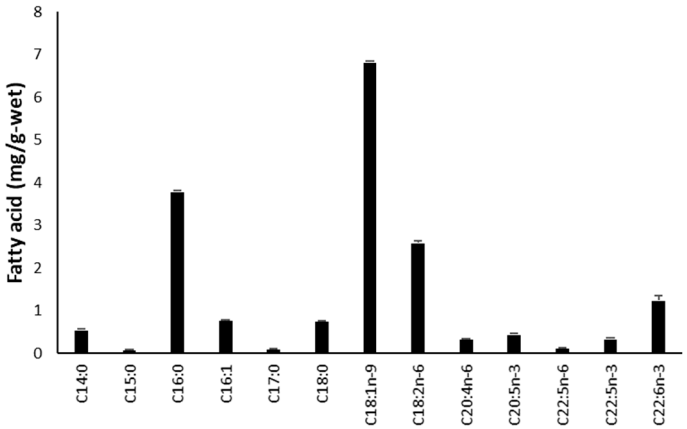

Figure 2. Fatty acid composition (mg/g wet weight) of internal organs of white sturgeon. Values are mean (n = 3).

3.2. Protease Activity

The protease activity in the internal organs of white sturgeon is shown in Figure 3. This activity at 50 °C was about twice as high as that at 25 °C, but without a significant difference.

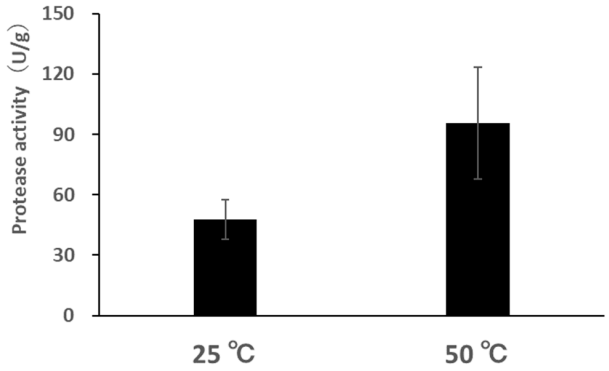

Figure 3. Specific protease activity in internal organs of white sturgeon at different temperatures. Values are mean (n = 3).

3.3. Time Course of Autolysis

3.3.1. Changes in Extraction Rate

The extraction rates at 25 °C for 96 h and at 50 °C for 24 h are shown in Figure 4. The extraction rate at 50 °C was higher than that at 25 °C. The sample incubated at 25 °C for 96 h turned black and had a putrid odor.

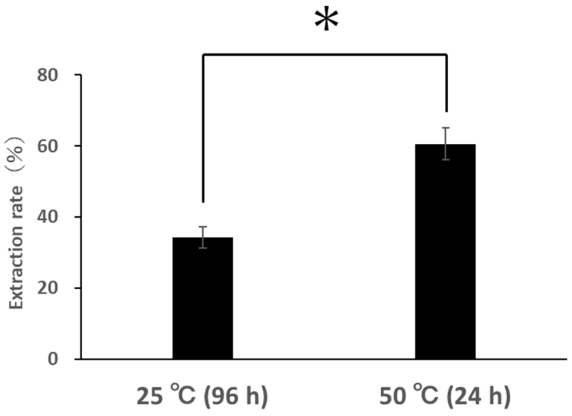

Figure 4. Extraction rate from internal organs of white sturgeon at different temperatures and times. Values are mean (n = 3). Asterisks mean significant difference ($p < 0.05$).

3.3.2. Changes in Protein Concentration

The changes in protein levels over 96 h are shown in Figure 5. At 50 °C, the protein level ranged from 6.6% to 7.3%. In all groups, the protein level did not change significantly over the course of 96 h.

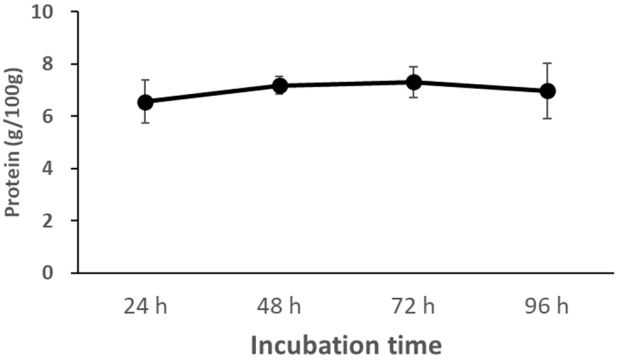

Figure 5. Changes in protein concentrations in mixtures of internal organs of white sturgeon reacted at 50 °C for 96 h. Values are mean (n = 3).

3.3.3. Distribution of Protein Molecular Size

The results of the SDS-PAGE analysis are shown in Figure 6. While incubated at 50 °C, the major bands at 200 kDa, 43 kDa, and 40 kDa detected at 0 h gradually disappeared over the incubation time. Other protein bands also tended to fade over time.

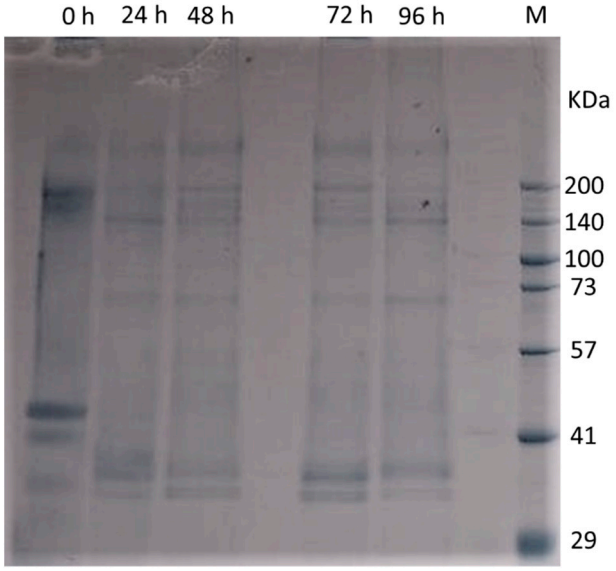

Figure 6. SDS-PAGE analysis of mixtures of internal organs of white sturgeon reacted at 50 °C for 96 h. M, protein molecular weight standards.

3.3.4. Bacterial Counts and Histamine Concentration

In all groups, the total viable count of the fish sauce was less than 300 CFU/mL, and the coliform bacteria count was negative (data not shown). In addition, the histamine concentration was ≤60 ppm throughout the incubation for 96 h, with no significant changes during this time (Figure 7). This was clearly lower than the standard value of 400 ppm specified by CODEX [23].

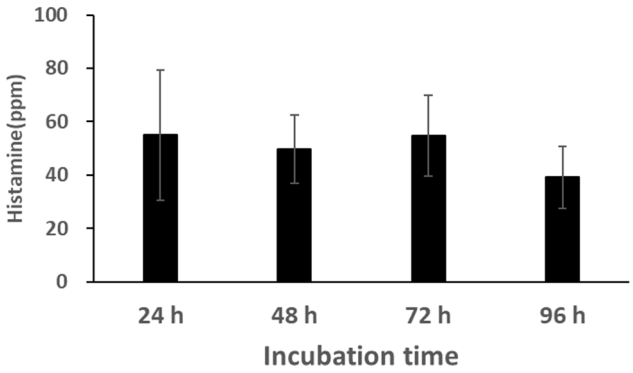

Figure 7. Histamine concentrations in mixtures of internal organs of white sturgeon reacted at 50 °C for 96 h. Values are mean (n = 3).

3.3.5. Volatile Compound Analysis

More than 200 components were detected by SPME-GC/MS in all samples, of which 15 were identified with high abundance (Table 1). These components tended to increase over time. Components other than acetic acid increased significantly after incubation at 50 °C for 24 h.

Table 1. Volatile components in the fish sauce, with concentration of each component based on the internal standard (2.439 ppm) (n = 3).

Retention Time (min)		0 h	24 h	48 h	72 h	96 h
9.0	Ethanol	0.002 ± 0.004	15.001 ± 24.061	1.914 ± 0.557	3.665 ± 1.160	2.788 ± 1.914
12.4	1-Penten-3-one	0.056 ± 0.062	0.352 ± 0.139	0.114 ± 0.009	0.102 ± 0.026	0.053 ± 0.010
15.6	Hexanal	2.352 ± 2.681	9.877 ± 11.198	0.667 ± 0.588	0.439 ± 0.002	0.858 ± 0.874
20.8	1-Penten-3-ol	2.275 ± 0.441	7.135 ± 9.662	1.237 ± 0.259	1.041 ± 0.180	0.934 ± 0.353
26.7	1-Pentanol	0.332 ± 0.162	6.439 ± 6.923	2.466 ± 0.240	3.557 ± 0.041	3.660 ± 0.232
35.5	3-Hexen-1-ol	0.005 ± 0.009	7.308 ± 12.658	-	-	0.013 ± 0.011
39.5	Methional	0.005 ± 0.003	6.257 ± 10.648	0.641 ± 1.068	0.008 ± 0.0004	0.043 ± 0.058
39.8	1-Octen-3-ol	0.716 ± 0.491	3.023 ± 2.305	0.959 ± 1.653	3.068 ± 0.742	2.471 ± 0.088
39.9	Acetic acid	-	-	0.083 ± 0.144	0.223 ± 0.315	-
40.0	Furfural	-	0.133 ± 0.201	0.033 ± 0.057	0.044 ± 0.027	-
50.2	Butanoic acid	-	4.944 ± 8.564	0.235 ± 0.407	0.683 ± 0.967	0.424 ± 0.734
57.0	Pentanoic acid	0.001 ± 0.002	0.887 ± 0.244	0.002 ± 0.003	0.471 ± 0.616	0.070 ± 0.122
64.0	Hexanoic acid	-	0.922 ± 1.213	0.002 ± 0.004	0.852 ± 1.205	0.102 ± 0.169
74.6	Octanoic acid	-	0.341 ± 0.202	0.005 ± 0.008	0.002 ± 0.002	0.011 ± 0.019
79.7	Nonanoic acid	-	0.472 ± 0.635	0.005 ± 0.004	0.006 ± 0.009	0.022 ± 0.038

The volatile compounds in fish sauce samples were identified by selected ion monitoring (SIM), with comparisons made to the NIST database. These compounds comprised acids, alcohols, ketones, aldehydes, and sulfur-containing molecules. The concentrations of the 15 main volatile components are shown in Table 1. The alcohols included ethanol, 1-penten-3-ol, 1-pentanol, 3-hexen-1-ol, and 1-octen-3-ol. The acids included acetic acid, butanoic acid, pentanoic acid, hexanoic acid, octanoic acid, and nonanoic acid. Autolysis decreased the levels of hexanal and 1-penten-3-ol, but increased those of 1-pentanol and 1-octen-3-ol.

4. Discussion

Fish contains saturated and unsaturated fatty acids, with the latter being beneficial for human health (for example, to help to protect the body against heart disease [24]). However, it has also been reported that these unsaturated fatty acids are easily oxidized, and their decomposition products are the causative components of the fishy off-flavor of fisheries products [25].

The fatty acid analysis showed that the internal organs of white sturgeon contained oleic acid (C18:1) and linoleic acid (C18:2), which are presumed to be causative components of the odor of fish sauce (Figures 1 and 2). The protease activity in crude enzyme solutions from the minced internal organs of white sturgeon was clearly higher at 50 °C than at 25 °C (Figure 3). Treatment at 50 °C improved the extraction rate (Figure 4), but further measurement of the amount of free amino acids is needed in a future study. The protein concentration measured every 24 h after treatment at 50 °C did not change significantly for 96 h. This suggests that after 24 h, the activity of endogenous proteases had reached a certain level (Figure 5). SDS-PAGE showed the degradation of proteins to low-molecular-weight substances, which indicates that indigenous proteases of white sturgeon are active at higher temperatures (Figure 6). The results of the study show that proteins in the internal organs of white sturgeon were significantly degraded when incubated at 50 °C for 1 day or longer. Similarly, Furutani and Satomi monitored protein degradation by SDS-PAGE during fish sauce preparation with *Glossanodon semifasciatus*, showing disappearance of the band at 45–66 kDa due to autolysis of proteins [26]. Moreover, Tang et al. reported that the main proteins in sturgeon surimi gels are myosin heavy chain (MHC, 200 kDa) and actin (AC, 43 kDa) [27,28]. After the sturgeon surimi paste was incubated at 40 °C for 1 h, the MHC band on SDS-PAGE disappeared, along with the appearance of lower-molecular-weight products that probably originated from MHC. This loss of protein bands in SDS-PAGE is suggestive of proteolytic activity. Tang et al. speculated that the modori (gel degradation)

phenomenon in sturgeon surimi gels occurs at 40 °C, and that the mechanism is related in part to the degradation of myofibrillar proteins by cathepsin L. In that study, actin degradation was not observed, which may be due to the difference in treatment time.

Histamine poisoning frequently occurs in fishery products such as red meat fish, especially Scombridae, which contains a high level of free histidine (the precursor of histamine) of 700–1800 mg/100 g [29,30]. Histamine poisoning can be caused by the ingestion of foods containing 22–370 mg of histamine [31], whereas the histamine concentration in fish sauce produced at 50 °C was ≤60 ppm (Figure 7). This result indicates that high-temperature autolysis of sturgeon organs without salt did not cause microbial or histamine contamination.

Color, taste, smell, and texture are factors that determine the taste of food; among these, smell is particularly important. There may be as many as 400,000 types of substances that have an odor, but only about 300 types have been detected above threshold concentrations [32]. There are also five basic tastes, but these are difficult to quantify because there are no standardized odors. Marine products have a strong odor, and they emit a fishy odor as their freshness decreases with time after landing. The odor of marine products also differs depending on the habitat, and has been studied for more than 40 years [33,34].

The production of volatile compounds during the manufacturing of fish sauce has an effect on the flavor of the final product. In this study, few Maillard-derived products were detected, due to the short-term treatment. The content of acids such as acetic acid, which is mainly produced by microbial metabolites [35], was the highest among the volatile compounds, but made a lesser contribution to the overall aroma profile of the fish sauce, due to the high odor thresholds. In contrast, alcohols contribute to the characteristic aroma of fish sauce, due to their low odor thresholds. For example, 1-octen-3-ol, which has a mushroom-like odor and has been detected in fish products [36], was found in the current study. Aldehydes were the largest contributor to the odor of the fish sauce, due to their low odor thresholds, and mainly comprised hexanal, which has a grassy scent, and furfural. Sulfur-containing molecules were also key compounds in fish sauce, despite their relatively low content among all volatile compounds. For example, methional has various odors depending on its concentration, and is an important aroma component of soy sauce [37].

Aroma substances are markedly influenced by the lipid contents in raw materials [38]. The fatty acid analysis showed that the internal organs of sturgeon contained the third highest amount of linolenic acid among the detected fatty acids. Large amounts of hexanal and 1-octen-3-ol, which are produced by the oxidation of fatty acids, were also detected in the internal organs. In soybean, linoleic acid is converted to linoleic acid 13-hydroperoxide by lipoxygenase and to n-hexanal by hydroperoxide lyase [39], and in mushroom, linoleic acid is converted to linoleic acid 10-hydroperoxide by dioxygenase and to 1-octen-3-ol by hydroperoxide lyase [40–42]. Ding et al. suggested that similar auto-oxidation pathways of linoleic acid occur in fish sauce [43], and these results may be due to the effect of lipoxygenase, which is present in the internal organs of white sturgeon [44].

In this study, volatile components increased sharply during incubation at 50 °C for 48 h, and then decreased (Table 1). The abundancy of these components was reduced by incubation at 50 °C and the aroma improved after 72 h. However, at 96 h, an unpleasant odor still remained. This suggests that the mixture contains components with different odor thresholds, for which a more detailed study is required.

All of the above results suggest that efficient production of fish sauce from internal organs of sturgeon can be achieved, although there are still problems in terms of flavor. Incubation at 50 °C for 72 h was considered to be the most suitable.

5. Conclusions

The aim of this study was to produce fish sauce rapidly from the internal organs of white sturgeon cultivated in Miyazaki Prefecture, using a short-term and high-temperature manufacturing method. The results indicated avoidance of microbiological contamination, and the fish sauce had characteristic aroma components.

Fifteen components were detected as the main volatile components. These components increased sharply and then decreased when incubated at 50 °C for 24 h. The fish sauce had a good aroma when incubated at 50 °C for 72 h. Using this method, fish sauce can be quickly produced from the internal organs of white sturgeon. We plan to perform further studies on the production of fish sauce using this method and evaluate the aroma of the resulting sauce.

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Institutional Review Board Statement: This study was not involving humans or animals.

Informed Consent Statement: This study was not involving humans or animals.

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Conflicts of Interest: The authors declare no conflict of interest.

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