



HOKKAIDO UNIVERSITY

Title	Production and antioxidant activity of peptides from sturgeon head
Author(s)	Islam, Md Rashidul; Li, Wen; Ogata, Yumi et al.
Citation	Sustainable Chemistry and Pharmacy, 31 https://doi.org/10.1016/j.scp.2022.100944
Issue Date	2022-12-20
Doc URL	https://hdl.handle.net/2115/93915
Type	journal article
File Information	SUSCP_accepted manuscript_Antioxidant_Rashidul.pdf



1 *Research paper*

2

3 **Production and antioxidant activity of peptides from sturgeon head**

4

5 Md. Rashidul Islam^{a, b}, Wen Li^a, Yumi Ogata^c, Takeya Yoshioka^c, Kazuhiro Ura^d, Yasuaki
6 Takagi^d

7 ^aGraduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-Cho, Hakodate,
8 Hokkaido 041-8611, Japan

9 ^bDepartment of Fisheries Biology and Genetics, Hajee Mohammad Danesh Science and
10 Technology University, Dinajpur-5200, Bangladesh

11 ^cHokkaido Industrial Technology Center, 379 Kikyo-Cho, Hakodate, Hokkaido 041-0801,
12 Japan

13 ^dFaculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-Cho, Hakodate, Hokkaido
14 041-8611, Japan

15

16 Corresponding author: Md. Rashidul Islam

17 Email: mrislam_fbg@hstu.ac.bd

18 Tel/Fax: +88 0531 51734/+88 0531 61311

19

20 Declaration of Interest: None

21 **Abbreviations**

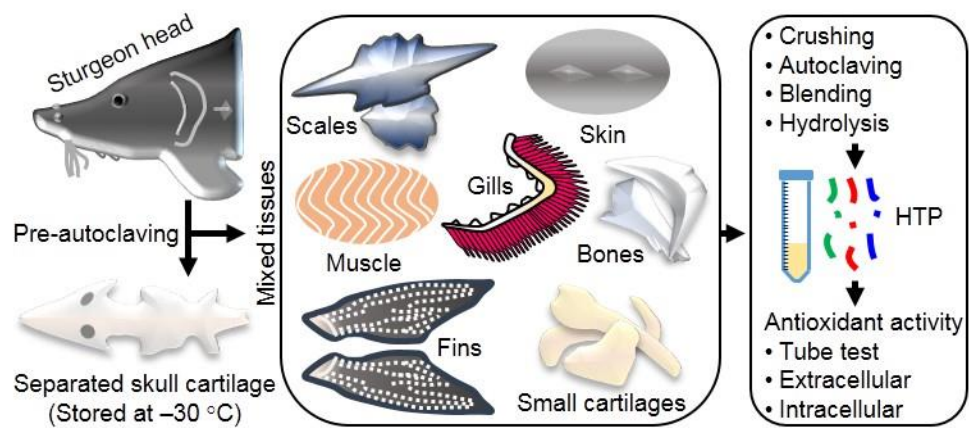
22

23	18S rRNA	18S ribosomal RNA
24	ABTS [•]	2, 2-Azino- <i>bis</i> (3-ethylbenzothiazoline-6-sulfonic acid)
25	CCK-8	Cell counting kit-8
26	DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
27	DW	Distilled water
28	DW-P	Autoclaved in DW and hydrolyzed with papain
29	DW-P+B	Autoclaved in DW and hydrolyzed with papain and bromelain
30	EFW	Endotoxin-free water
31	EU	Endotoxin unit
32	GPX1	Glutathione peroxidase-1
33	HBSS	Hanks' balanced salt solution
34	HO1	Heme oxygenase-1
35	HTP	Head tissue peptide
36	LMM	Low molecular mass
37	LPS	Lipopolysaccharide
38	OH [•]	Hydroxyl radical
39	ROS	Reactive oxygen species
40	SCP	Skin collagen peptide
41	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
42	SE	Standard error
43	STP	Skin tissue peptide
44	WDW-P	Autoclaved without DW and hydrolyzed with papain
45	WDW-P+B	Autoclaved without DW and hydrolyzed with papain and bromelain

Highlights

- We succeeded in producing peptides from unutilized sturgeon head.
- Peptides with <500 Da are high in head tissue peptide (HTP).
- In the tube test, HTP showed high ABTS[•] scavenging activity.
- HTP significantly protected extracellular and intracellular oxidative stress.

Graphical Abstract



Abstract

The head is a significant, but typically unused, fish by-product. Here, we developed an industrially applicable new method to make head tissue peptides (HTPs) by autoclaving, blending and enzymatic hydrolysis of sturgeon head tissue. Four conditions were tested: head tissue autoclaved in distilled water (DW) and hydrolyzed with papain (DW-P) or papain and bromelain (DW-P+B), or head tissue autoclaved without DW and hydrolyzed with papain (WDW-P) or papain and bromelain (WDW-P+B). Among these conditions, DW-P+B resulted in the highest peptide yield, more peptides of <500 Da, and peptides with the highest antioxidant activity. Next, the same method produced sturgeon skin tissue peptide (STP) and skin collagen peptide (SCP) and the bioactivities were compared. The gross yield of HTP per fish was higher than that of STP and SCP because the sturgeon's head is ~3 times heavier than its skin. The HTP contained more reactive oxygen species (ROS)-scavenging amino acids (Glx, His, Leu, Lys, Met, Phe, Tyr, Val) and showed significantly higher ABTS[•] scavenging activity than STP and SCP. The extracellular cell-protection and intracellular anti-ROS activities of HTP on the application of oxidative stress were similar to those of STP and SCP. Moreover, HTP showed high transparency and high safety (a low endotoxin level). All these features suggest that HTP is suitable for percutaneous applications. Extracellular oxidative stress promotes human skin aging; thus, HTP may be a novel, low-cost ingredient for antiaging cosmetics. The present study will help to exploit zero industrial discards, resulting in blue transformation of by-products.

Keywords: sturgeon's by-product, hydrolysate, production, antioxidant

1. Introduction

According to FAO (2022), fishery by-products are increasing because of growing global fish production and processing. The developing Asian aquaculture practices lead the trend. Fish by-products have been globally diverted to produce low-cost fishmeal (27%) and fish oil (48%) or discarded as waste, resulting in economic losses and environmental deterioration (FAO, 2022; Le Gouic et al., 2019). Due to the global crisis of energy and food as well as climate change, it is a time to search for new resources and increase their usage (Chen et al., 2020). In addition, production technologies must consider zero discards. Therefore, FAO listed the "repurposing, reuse and recycling, and the valorization of waste products" as one of the nine blue transformations of Asian aquaculture.

Fish by-products are a good source of peptides (Henriques et al., 2021; Le Gouic et al., 2019), which can be natural antioxidants. They are considered sustainable alternatives to peptides from livestock by-products, which have risks of infectious diseases and religious objections (Al Khawli et al., 2020; León-López et al., 2019). Antioxidants support healthy aging, scavenging free radicals to prevent disorders (Loboda et al., 2016; Tan et al., 2018). For example, antioxidants donate electrons to free radicals, balance redox reactions, decrease oxidative stress (Lobo et al., 2010; Sharma and Kim, 2021), and keep human skin healthy as anti-aging compounds (Liu et al., 2022). Several natural or synthetic antioxidants, such as vitamin C, vitamin E, and butylated hydroxytoluene, are commercially available; however, prolonged use is toxic or contributes to allergic contact dermatitis (Ham et al., 2020; Kosari et al., 2010). On the other hand, collagen and protein hydrolysates are natural antioxidants widely accepted for their low antigenicity, biocompatibility, and biodegradability (Aguirre-Cruz et al., 2020; Henriques et al., 2021; León-López et al., 2019). Moreover, they have

bioactivities to activate fibroblasts (Chan et al., 2020; Chen et al., 2020; Edgar et al., 2018; Li et al., 2021b), which metabolize skin dermis to keep skin healthy.

The major fish by-products used for peptide production are skin (Abuine et al., 2019; Park and Jo, 2019) and viscera (FAO, 2022). However, fish heads remain unutilized, though they usually account for 9–12% of total fish weight (FAO, 2022) and, in some species like sturgeon, occupy the dominant weight of the fish body (17% of total body weight; Islam et al., 2020; 2021). Many heads with other body parts are discarded as waste from the sturgeon aquaculture because its primary marketable product is only caviar. Therefore, developing effective methods to transform fish heads into highly value-added products is a matter of emergency need. Although, in the case of sturgeon, the use of skin as a source of collagen, gelatin, and collagen peptides has been studied (Atef et al., 2021; Meng et al., 2019; Nikoo et al., 2014; Zhang et al., 2014, 2019, 2020), the head is left unused except in our previous studies on gelatin (Islam et al., 2020, 2021).

Therefore, in this study, we report a trial to produce antioxidant peptides from sturgeon heads, a complex mixture of collagen-rich tissues (e.g., skin or scales) and collagen-poor tissues (e.g., muscle or gills). The difficulty lies in the hardness of the head. Consequently, the sturgeon heads were first autoclaved to soften them and then blended. Then, considering the large-scale productivity, the relatively low-cost proteolytic enzymes such as papain and bromelain (Table 1) were adopted for the industrial production of the peptides. The adopted procedures produced smaller peptides with higher bioactivities, e.g., antioxidant activity, than other proteolytic enzymes (Li et al., 2021a; Slizyte et al., 2016, 2018). Thus, finally, the antioxidant activities of the peptides produced from the sturgeon head were compared with those produced from the skin.

2. Materials and methods

2.1. Production of peptides

Heads of bester sturgeon (355.0 ± 15.6 g, mean $\pm$ SE) and skin samples were collected from a fish processing plant (Bifuka Shinko Kosha, Bifuka, Japan) and stored at -20 °C. Heads were thawed at 4 °C for overnight, washed, and weighed. Each head was placed in a stainless-steel bucket (200 mm diameter $\times$ 220 mm height; Tsubame Bussan, Niigata, Japan) and pre-autoclaved (121 °C, 100 kPa) for 10 min. The skull cartilage, which accounts for approximately $9.6 \pm 0.3\%$ of the head weight, was manually separated from the head after the autoclaving (Supplementary Fig. 1) and stored at -30 °C for later use as a source of type II gelatin or chondroitin sulfate. The remaining head tissues ($89.7 \pm 0.3\%$), defined as mixed tissue, were ground into a paste and used to produce head tissue peptides (HTPs). In our previous study, the wet weight ratio of each tissue in the mixed tissue to head weight was 6.7% bones, 6.3% pectoral fins, 11.4% gills, 26.2% muscle, 5.6% scales, 7.7% skin, and 6.8% small cartilages from kalamtra sturgeon (Islam et al., 2020). Four conditions were applied for HTP production (Supplementary Fig. 1). 1) DW-P: samples were autoclaved for 15 min, defatted in ethanol for 24 h, autoclaved in distilled water (DW) again for 15 min, and hydrolyzed with papain. 2) DW-P+B: samples were pretreated as DW-P and hydrolyzed with papain and bromelain. 3) WDW-P: samples were autoclaved without DW for 30 min, defatted in ethanol for 24 h, and hydrolyzed with papain. 4) WDW-P + B: samples were pretreated as WDW-P and hydrolyzed with papain and bromelain. The final concentration of each enzyme was 0.625% (dry weight enzyme per wet weight tissue), and the hydrolysis condition was 50 °C, 4 h at pH 7.0. The concentration and hydrolysis condition of papain were considered following Ji et al. (2013), Li et al. (2021a), and Noman et al. (2018). A

mixture of papain and bromelain was applied following Slizyte et al. (2016, 2018) with minor modifications.

Skin tissue peptide (STP) and skin collagen peptide (SCP), the latter prepared from purified skin collagen, were produced using the same conditions for DW-P+B-treated head samples. Skin samples were a mixture of bester (*Huso × Acipenser ruthenus*) and kalamtra (*Huso dauricus × Acipenser schrenckii × Acipenser transmontanus*) sturgeon. The skin collagen was purified as described by Islam et al. (2021).

2.2. Molecular mass distribution of peptides

The molecular mass distribution of peptide samples was determined by size exclusion chromatography using a TSKgel G2500PW_{XL} column (7.5 mm × 300 mm; Tosoh Bioscience, Tokyo, Japan), following the method reported by Fekete et al. (2014) and Li et al. (2021a). The mobile phase was 35% acetonitrile with 0.05% trifluoroacetic acid (pH 2.1) at a flow rate of 0.8 mL/min (~25 °C). The elution peaks were detected at 214 nm. Insulin (5807 Da), vitamin B₁₂ (1355 Da), and glutathione (307 Da) were used as standards. A 30-μL aliquot of sample or standard was injected into a high-performance liquid chromatography system (10AVP, Shimadzu Corporation, Tokyo, Japan). The amount of each molecular mass fraction was calculated as:

$$\text{Molecular mass fraction (\%)} = (S_{AF}/S_{AC}) \times 100$$

where S_{AF} is the area of the fraction (<500 Da, 500 Da–1 kDa, 1–3 kDa, 3–5 kDa, or >5 kDa) in the chromatogram and S_{AC} is the total area of the chromatogram.

2.3. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

Tricine-SDS-PAGE was performed following the method of Schägger (2006) and Islam et al. (2021).

2.4. Amino acid composition and collagen-derived peptide content in the total protein fraction

The amino acid composition in the total protein fraction and the collagen-derived peptide content in the total protein fraction of peptides were quantified as described in our previous study (Islam et al., 2021). Briefly, three replicates of each sample were mixed and hydrolyzed in 6 M HCl at 110 °C for 24 h. Next, the hydrolysates were dried by evaporation and dissolved in a citric acid buffer solution, and amino acid concentrations were quantified using an automated amino acid analyzer (L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan). Tryptophan was not quantified because it cannot be detected using this method. Then, the collagen-derived peptide content in the total protein (CCp; %) was calculated as Hyp in the sample per Hyp in the purified collagen, where Hyp is the number of hydroxyproline residues per 1000 amino acid residues; Hyp is an amino acid specifically carried in collagen molecules. Purified collagen was used as the standard, defined as containing 100% collagenous protein.

2.5. Proximate composition

The proximate composition of samples was measured according to Islam et al. (2021). Briefly, the collagen-derived peptide content (CCs) and non-collagenous protein-derived peptide content (NCCs) in samples were calculated using the following formulae:

$$\text{CCs (\%)} = [(\text{Hyp of the sample})/(\text{Hyp of the purified collagen})] \times 100$$

$$\text{NCCs (\%)} = [100 (\text{CCs/CCp}) - \text{CCs}]$$

Lipid and ash contents were measured following the method of previous reports (AOAC 2000, # 920.153; Bligh and Dyer, 1959; Islam et al., 2021). Finally, the remaining non-proteinaceous substances, such as carbohydrates, were determined as the remainder after summing the collagen, non-collagenous protein, lipid, and ash and subtracting their content from the total mass of a sample.

2.6. Transparency of peptide solutions

The absorbance of sample solutions (w/v 1%) was measured using a spectrophotometer at 600 nm (UH5300, Hitachi, Tokyo, Japan), following Islam et al. (2021). The formula for calculating transparency (%) was as follows:

$$\text{Transparency (\%)} = 10^{-(\text{Absorbance at 600 nm})} \times 100$$

2.7. Endotoxin levels

The endotoxin levels in peptide samples were measured using a Limulus Amebocyte Lysate assay (Endospecy[®] ES-24S Kit, Seikagaku Corporation, Tokyo, Japan), in line with the instructions of the manufacturer.

2.8. In vitro antioxidant activities of peptides

2.8.1. Test tube-based antioxidant activities

In test tube-based assays, antioxidant activities of one electron and hydrogen-atom sharing free radicals—2, 2-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS[•]) and hydroxyl radical (OH[•]), respectively—were applied.

ABTS[•] scavenging activity was measured following Chi et al. (2015a). Briefly, ABTS stock solution (7 mM) was mixed with K₂S₂O₈ (2.45 mM) at a ratio of 2:1 (v/v) and kept in the dark for 16 h at room temperature (~25 °C). After diluting the solution with 5 mM phosphate-buffered saline (pH 7.4), the absorbance at 734 nm was adjusted to 0.700 ± 0.02. Next, the solution was mixed with peptide samples dissolved in DW at a ratio of 1:1 (v/v) and incubated for 10 min in the dark. The absorbance was measured at 734 nm using a spectrophotometer (UH5300, Hitachi), and the scavenging activity was calculated as:

$$\text{ABTS}^{\bullet} \text{ scavenging activity} = [(S_A - N_A)/(N_A)] \times 100 (\%)$$

where S_A and N_A are the absorbances of the treated sample and a blank control (DW), respectively.

The OH[•] scavenging activity was measured as reported by Chi et al. (2015a). Peptide samples in DW were mixed with 1, 10-phenanthroline (1.865 mM), FeSO₄·7H₂O (1.865 mM), and H₂O₂ (0.03% v/v) at a ratio of 2:1:1:1 (v/v), and incubated for 60 min at 37 °C. Then, the absorbance at 536 nm was measured, and the scavenging activity was calculated as:

$$\text{OH}^{\bullet} \text{ scavenging activity} = [S_A - N_A]/[B_A - N_A] \times 100 (\%)$$

where S_A, N_A, and B_A are the absorbances of the treated sample with H₂O₂, a blank control (DW), and a sample without H₂O₂ (a negative control), respectively.

2.8.2. Extracellular antioxidant activities

Mouse fibroblast L929 cells (RCB1451, RIKEN, Tsukuba, Japan) were seeded at 1.2×10⁴ cells/well in 96-well culture plates and incubated in a humidified atmosphere at

37 °C with 5% CO₂. The cell culture medium was the Minimum Essential Medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Peptide samples were dissolved in endotoxin-free water (EFW) and sterilized by 0.2-µm syringe filtration.

The assay was conducted following the work of Li et al. (2021b) with minor modifications. First, cells were incubated until confluence. Then, the culture medium was changed to a fresh medium containing peptide samples, and the cells were further incubated for 24 h. After that, a freshly prepared 0.25 mM H₂O₂ solution was added to each well, and the cells were incubated for 24 h. In the negative control group, EFW was added instead of the peptide sample solution.

Finally, cell viability was assessed with a Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). After washing cells with Hanks' balanced salt solution (HBSS), the HBSS was replaced with 10% (v/v) CCK-8 solution in the culture medium and incubated for 30 min. Then, the absorbance was measured at 450 nm using a microplate reader (Infinite F50, Tecan Japan, Kawasaki, Japan), and the relative cell viability (%) normalized to the control group, which was not subjected to oxidative stress (i.e., H₂O₂ treatment), was obtained.

2.8.3. Intracellular antioxidant activities

2.8.3.1. Intracellular reactive oxygen species (ROS) levels

The assay was conducted following Li et al. (2021b) with minor modifications. First, L929 cells were seeded in black/transparent microtiter 96-well plates (Falcon; Corning Incorporated, Corning, NY, USA) and incubated until confluence as described above. Then, cells were treated with samples or EFW in the culture medium for 24 h. After washing with

HBSS, cells were treated in the dark for 20 min at 37 °C with 20 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St Louis, MO, USA) in HBSS. DCFH-DA diffuses through the cell membrane and is hydrolyzed into nonfluorescent dichlorofluorescein (DCFH) by intracellular esterase.

Next, cells were washed twice with HBSS, and freshly prepared 0.25 mM H₂O₂ in HBSS was added. Intracellular DCFH reacts with H₂O₂ and is oxidized to DCF, a green fluorescent dye. Thus, DCF reveals positively correlated with the intracellular H₂O₂ levels. After incubating the cells at 37 °C for 15 min in the dark, DCF was detected with excitation/emission at 485/535 nm using a microplate reader (WallAc ARVO 1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA). Then, the intracellular ROS level is expressed as the relative fluorescence intensity normalized to the control group, which was not subjected to oxidative stress.

2.8.3.2. Expression of antioxidant genes

L929 cells were seeded in 24-well culture plates at 1.2×10^4 cells/well and incubated until confluence. Cells were treated with peptide samples for 24 h, as described in section 2.8.2. To prevent instability of housekeeping gene expression, no oxidative stress was applied. Total RNA was extracted using ISOGEN II (Nippon Gene Co., Ltd., Tokyo, Japan) and quantified with a spectrophotometer (ND-1000, Thermo Fisher Scientific). Next, cDNA was synthesized from 50 ng of total RNA using a reverse transcription kit (ReverTra Ace® qPCR RT Master Mix with gDNA remover, Toyobo, Osaka, Japan). The expression of antioxidant genes [glutathione peroxidase-1 (*GPX1*) and heme oxygenase-1 (*HO1*)] was quantified with the synthesized cDNA diluted fivefold as the template. The assay was carried out using SYBR green-based THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Osaka, Japan)

and a Roche LightCycler® Nano Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). The amplification conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, each annealing temperature (58.5 °C for *GPX1*, 55 °C for *HO1*, and 58.5 °C for *18S rRNA*) for 10–15 s, and extension at 72 °C for 15 s. A housekeeping gene, *18S rRNA*, was used to normalize the expression of target genes. The $2^{-\Delta\Delta CT}$ method was used to determine gene expression (Livak and Schmittgen, 2001). The specificity of each primer was checked by TA cloning using the pGEM-T Easy vector (Promega, Madison, USA) and DNA sequencing (data not shown). Primer sequences are given in Supplementary Table 1.

2.9. Data analysis

All experiments were repeated two or three times. Three replicate samples were mixed for amino acid, Hyp content, and Tricine-SDS-PAGE analyses. When necessary, data are presented as the mean $\pm$ SE. One-way analysis of variance and the Tukey-Kramer test were performed using Microsoft Excel add-in statistical software (version 2.12, Social Survey Research Information Co., Ltd., Tokyo, Japan). The level of statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Determination of peptide production conditions

This study developed a green, industrially applicable method to produce peptides from sturgeon heads for their blue transformation. The most significant hurdle—the hardness

of the sturgeon head—was overcome by applying autoclaving, which softens the head and makes blending possible.

The enzymatic hydrolysis conditions affected the peptides' yield and molecular mass distribution. Double-enzymatic treatment (using papain and bromelain) resulted in significantly higher yields and smaller peptides than treatment with papain alone (Table 2), probably because of the different cleavage specificities of the two enzymes. Smaller peptides are better antioxidants than larger ones because of their higher accessibility to free radicals (Park and Jo, 2019; Sila and Bougatef, 2016; Tejpal et al., 2017). The autoclaving conditions did not significantly affect the yield or molecular mass of the peptides (Table 2); however, the DW-P+B treatment resulted in a product significantly rich in smaller peptides. This result was supported by data from 15.5% Tricine SDS-PAGE (Fig. 1A); the low staining intensity observed for the DW-P+B-treated sample (lane 2 in Fig. 1A) suggested a higher proportion of peptides with molecular mass <1.7 kDa in this sample than in other samples.

The P+B-treatments resulted in slightly but significantly higher ABTS[•] and OH[•] scavenging activities than the P-treatments in either DW or without DW (WDW) (Supplementary Fig. 2).

Based on these results, the DW-P+B condition was employed to produce HTP, STP, and SCP.

3.2. Yields of HTP, STP, and SCP

The STP yield was highest, followed by SCP and HTP (Table 3); the collagen purification step significantly decreased the SCP yield compared with STP, while bones and scales in the head, which were hard for the enzymes to hydrolyze, decreased the yield of HTP compared with STP and SCP. The yield of HTP on the wet tissue basis was $12.1 \pm 0.3\%$,

which was higher than that of *Sardinella* head and viscera peptides (4.3%, Souissi et al., 2007), close to that of codfish and tilapia skin peptides (11%–14%, Chen et al., 2017; Wang et al., 2013), but slightly lower than that of Chinese sturgeon muscle peptides (17.5%, Noman et al., 2018). Moreover, a higher amount of sturgeon head (17.1% of the body weight) than skin (6.1%) (Islam et al., 2020) makes the expected gross yield (dry weight) of HTP higher than STP and SCP (from a 2-kg commercial-sized fish for meat, the gross yield of HTP is 127.9 g, whereas that of STP and SCP are 94.1 g and 67.6 g, respectively). Therefore, the sturgeon head is a potential source of peptides for industrialization, having a good yield.

3.3. Molecular mass distribution of peptides

The HTP contained a significantly higher percentage of peptides with a molecular mass <500 Da than SCP and STP (Table 3). Similarly, HTP had the lowest staining intensity in 15.5% Tricine-SDS-PAGE (lane 4 in Fig. 1B, compared with lanes 2 and 3), suggesting a higher amount of peptides with molecular mass <1.7 kDa. Smaller peptides have greater functionality (Halim et al., 2016). For example, human skin prevents the penetration of large molecules; peptides of <500 Da more easily penetrate intact skin and show functionalities in cosmetics or skin therapeutics (Chaulagain et al., 2018; Schagen, 2017). Besides, the dominant low-molecular-mass (LMM) fragments were <500 Da in HTP (Table 3). The earlier reports also showed that seabass skin collagen peptides (the dominant LMM fragments: 1–1.3 kDa) and salmon skin collagen peptides (the dominant LMM fragments: 100–860 Da) were useful in skin care (Benjakul et al., 2018; Wang et al., 2015). Therefore, HTP may have greater potential for percutaneous applications such as cosmetics.

3.4. Amino acid composition and proximate composition

364

365 The amino acid composition and the proximate composition of peptide-containing
366 samples are shown in Tables 4 and 5. The levels of amino acid residues that show ROS
367 scavenging activity, such as glutamine/glutamic acid (Glx), histidine (His), leucine (Leu),
368 lysine (Lys), methionine (Met), phenylalanine (Phe), tyrosine (Tyr), and valine (Val) (Cojocar
369 et al., 2014; You et al. 2010; Zhao et al., 2018), were higher in HTP than in STP or SCP (Table
370 4). The collagen-derived peptide contents, both in the total protein fraction and sample weight,
371 were highest in SCP, followed by STP and HTP (Tables 4 and 5). Therefore, more amino acid
372 residues that show ROS scavenging activity were contained in the non-collagenous proteins of
373 HTP. Because the sturgeon head contains collagen-poor tissues such as muscle, it is reasonable
374 that more non-collagenous protein derivative was found in the HTP.

375 This is the first report to estimate the contents of collagen-derived and non-collagenous
376 protein-derived peptides. According to Table 5, the total protein content (collagen + non-
377 collagenous proteins) was lower in HTP (76.2%) than in STP (98.4%) and SCP (which is
378 defined as 100% pure collagenous peptide). The head also contains scales, bones, and small
379 cartilages; thus, more non-proteinaceous substances, such as ash and sugar (polysaccharides),
380 were found in the HTP (Table 5). In addition, HTP contained significantly less lipid than STP
381 (Table 5), suggesting that the defatting process was more efficient in the head tissue than in the
382 skin.

383 The total protein content of HTP (76.2%) was higher than that in sturgeon viscera
384 hydrolysates (65.8%, Ovissipour et al., 2009) and sardine, horse mackerel, and axillary
385 seabream protein hydrolysates (60.2%–74.9%, García-Moreno et al., 2014), close to that in
386 bogue protein hydrolysates (75.7%–76.4%, García-Moreno et al., 2014), but lower than that in
387 small-spotted catshark protein hydrolysates (87.0%–89.5%, García-Moreno et al., 2014). Also,
388 the lipid content of the HTP (0.5%) was lower than those of several fish protein hydrolysates

(4.6%–25.3%, García-Moreno et al., 2014) but higher than that of sturgeon viscera hydrolysate (0.2%, Ovissipour et al., 2009). In summary, HTP is a protein-rich and lipid-poor peptide than most fish peptides previously reported.

3.5. Transparency

The transparencies of SCP, STP, and HTP were $96.9 \pm 0.1\%$, $98.6 \pm 0.1\%$, and $98.8 \pm 0.1\%$, respectively, showing excellent clarity as 1% solutions. HTP contained more non-collagenous proteins and non-proteinaceous substances (Table 5) than the others, but these substances did not affect its transparency. Therefore, all the samples would be suitable for industrial applications; particularly, high transparency is essential for cosmetics.

3.6. Endotoxin levels

Endotoxins are fever-generating lipopolysaccharides (LPS) from certain Gram-negative bacteria, having multiple injurious biological effects such as inflammation (Miyamoto et al., 2009). The endotoxin levels in HTP, SCP, and STP were 0.4 ± 0.1 , 0.2 ± 0.0 , and 0.3 ± 0.0 endotoxin unit (EU)/mg, respectively. The maximum limit of endotoxin in a sample depends on its applications. For example, the usable limit of endotoxin is 0.5 EU/mL or 20 EU/device for a medical device directly or indirectly contacting the cardiovascular or lymphatic system (Cavaillon, 2018; FDA, 2012). LPS at <1 ng/mL (equivalent to 8 EU/mg in the present measurement system) did not induce human monocytes to secrete inflammatory cytokines such as interleukin-6 or tumor necrosis factor- α (Cavaillon, 2018; Morris et al., 2014). Therefore, the endotoxin levels of the present peptides were below the applicable limits in the following cell culture experiments (the maximum concentration of

peptides in the cell culture experiments was 1 mg/mL, which equaled 0.4 EU/mL in the case of HTP). Keeping fish by-products clean during fish processing is essential to reduce the endotoxin level. For example, the immediate freezing of by-products may greatly contribute to the production of low-endotoxin peptides as fish by-products spoil quickly. Presently, endotoxin levels of fish peptides were quantified only in the articles reported by our colleagues (Li et al., 2019; Li et al., 2021a).

3.7. In vitro antioxidant activities of peptides

3.7.1. Test tube-based antioxidant activities

The antioxidant activity of collagen peptides prepared from fish skin is well known (Nurilmala et al., 2019; Park and Jo, 2019), but the comparison of the activity with that of peptides prepared from other tissues, such as the head, is scarce. In the present study, the highest ABTS[•] scavenging activity was found in HTP, followed by STP and SCP (Fig. 2). These data corresponded well to the numbers of ROS-scavenging amino acid residues (Glx, His, Leu, Lys, Met, Phe, Tyr, and Val), which are plentiful in non-collagenous proteins, in the samples (Table 4). Thus, antioxidant activity correlated with the amount of non-collagenous proteins in peptides (Fig. 2, Table 5). These data suggest that the by-products with lower collagen content are more suitable for producing antioxidant peptides. In addition, pretreatments of tissues to remove non-collagenous proteins, such as the NaOH treatment, are not recommended. The higher ABTS[•] scavenging activity may also relate to the higher abundance of smaller peptides in HTP (Fig. 2, Table 3), as several reports showed that smaller peptides have higher antioxidant activity (Chi et al., 2015b; Park and Jo, 2019).

3.7.2. Extracellular antioxidant activities

The addition of 0.25 mM H₂O₂ significantly reduced cell viability to approximately 60% of the level for control cells (i.e., cells without H₂O₂ treatment) (Fig. 3A, B). The extracellular application of peptide samples at a dose of 500 µg/mL increased the viability to 77% of the control level (Fig. 3A), suggesting that the samples protected the cells from H₂O₂ oxidative stress. When a sample dose of 1000 µg/mL was applied, cell viability increased to approximately 84%–90% of the control level (Fig. 3B). At this concentration, differences in viability between control cells and HTP- and STP-treated cells were statistically insignificant. However, the difference between control cells and SCP-treated cells was significant. Thus, the antioxidative potency was lower in SCP, showing a similar tendency to that observed in the ABTS[•] scavenging assay.

Vitamin C is a potent antioxidant; however, it showed a significant toxic effect on L929 cells when its concentration was >50 µg/mL (data not shown). An earlier report stated that overuse of vitamin C or E resulted in reductive stress that is toxic to cells and favorable for cancer (Henkel et al., 2019). However, the present peptide samples showed no cytotoxicity at 1000 µg/mL. The papain-hydrolyzed collagen peptide from skate cartilage protected the L929 cells from oxidative stress (Li et al., 2021b) similar to the present finding. Besides, Nile tilapia and monkfish muscle peptides protected HepG2 cells from extracellular H₂O₂ (Hu et al., 2020; Yarnpakdee et al., 2015). The present study is the first to compare the cell-protective activities of peptides prepared from different tissues in the same fish species.

3.7.3. Intracellular antioxidant activities

3.7.3.1. Intracellular ROS levels

L929 cells treated with 0.25 mM H₂O₂ showed a 10-fold increase in ROS compared with those treated with endotoxin-free water (EFW) (Fig. 4A, B). Pretreatment with peptide samples at a dose of 500 µg/mL did not change the endogenous ROS levels of H₂O₂-treated cells (Fig. 4A), while that with 1000 µg/mL significantly reduced the ROS levels (Fig. 4B). No differences were evident in the potency of HTP, SCP and STP (Fig. 4B). In this experiment, the peptide samples were removed before H₂O₂ was added. Thus, possibly, peptides were transported into fibroblasts and worked as antioxidants to decrease the ROS induced by H₂O₂. The proton-coupled transporters of the solute carrier 15 (SLC15) family are integral membrane proteins responsible for absorbing pharmacological compounds such as oligopeptides (Viennois et al., 2018). For example, PEPT1 and PEPT2 are involved in the cellular uptake of peptides in epidermal keratinocytes (Kudo et al., 2016). Peptides from Nile tilapia skin gelatin failed to suppress ROS production under H₂O₂-induced oxidative stress in PEPT1 knockdown porcine intestinal enterocytes (Zheng et al., 2018). These studies strongly suggest that the antioxidative effects of peptides depend on a transporter-related mechanism.

3.7.3.2. *Expression of antioxidant genes*

The endogenous antioxidant system is complex and depends on enzymatic and non-enzymatic molecules that collectively act against free radicals or ROS in four ways: (1) they suppress or prevent the formation of ROS; (2) they neutralize or scavenge ROS; (3) they repair biomolecules affected by ROS; or (4) they use signals essential for ROS production and react, transporting antioxidants to where they are needed (Ighodaro and Akinloye, 2018). Enzymes such as glutathione peroxidase (GPX), catalase, and superoxide dismutase are the first-line defensive antioxidants that detoxify the ROS (Kuciel-Lewandowska et al., 2020; Kurahashi and Fujii, 2015). Heme oxygenase-1 (HO1) degrades heme into biliverdin, which

is reduced to bilirubin by biliverdin reductase as a cytoprotective antioxidant (Kurahashi and Fujii, 2015). In this study, the effects of peptide samples on the mRNA levels of *GPX1* and *HO1* were tested using L929 fibroblasts without external oxidative stress. The peptide samples did not significantly stimulate the mRNA levels of either antioxidant gene (Supplementary Fig. 3A, B). These genes may be expressed more in stressful conditions (Yamamoto et al., 2018); thus, the samples should be tested with external oxidative stress. However, in such conditions, the housekeeping (internal reference) genes for qPCR may also be affected; thus, the qPCR results would become hard to interpret. Therefore, it is necessary to develop ways to be able to apply oxidative stress in this type of gene expression assay.

4. Conclusions

Head and skin are major by-products of commercial sturgeon, but the head remains typically unused because of its hardness. Furthermore, the head is a complex mixture of multiple tissues; thus, this may also prevent its usage. In this study, aiming at the blue transformation of the sturgeon head, we applied a green, industrially applicable method involving autoclaving, blending, and enzymatic hydrolysis and successfully produced HTP for the first time. Autoclaving effectively softened the head and was essential to crushing its hard, complex tissues. The present study also revealed that HTP contains more ROS-scavenging amino acid residues and shows significantly higher ABTS[•] scavenging activity than STP or SCP. A new finding is that non-collagenous tissues contain more antioxidant amino acids; thus, the hydrolysis results in peptides with higher antioxidant activity. Moreover, HTP showed protective activity against extracellular oxidative stress and decreased intracellular ROS levels; both potencies were comparable to SCP and STP. HTP was also rich in low-molecular-mass peptides and showed a low endotoxin level. All these

features suggest that HTP is more suitable than SCP and STP for percutaneous applications against skin aiming. The present study proceeds with proper usage of the sturgeon head and a blue transformation of sturgeon aquaculture to establish a bio-circular economy in the future.

Acknowledgments

This study was partly supported by a Grant-in-Aid for Scientific Research (B) 18H02273 from the Japan Society for the Promotion of Science. We thank the Edanz group (<https://jp.edanz.com/ac>) for the English language editing of this manuscript.

References

- Abuine, R., Rathnayake, A.U., Byun, H.G., 2019. Biological activity of peptides purified from fish skin hydrolysates. *Fish. Aquat. Sci.* 22, 10. <https://doi.org/10.1186/s41240-019-0125-4>
- Aguirre-Cruz, G., León-López, A., Cruz-Gómez, V., Jiménez-Alvarado, R., Aguirre-Álvarez, G., 2020. Collagen hydrolysates for skin protection: Oral administration and topical formulation. *Antioxidants*. 9, 181. <https://doi.org/10.3390/antiox9020181>
- Al Khawli, F., Martí-Quijal, F.J., Ferrer, E., Ruiz, M.J., Berrada, H., Gavahian, M., Barba, F.J., de la Fuente, B., 2020. Aquaculture and its by-products as a source of nutrients and bioactive compounds, in: Lorenzo, J.M., Barba, F.J. (Eds.), *Advances in Food and Nutrition Research*, Academic Press, Cambridge, MA, USA. 92, 1–33. <https://doi.org/10.1016/bs.afnr.2020.01.001>
- AOAC, 2000. *Official Methods of Analysis*, seventeen ed. The Association of Official Analytical Chemists, Gaithersburg, MD, USA.

538 Atef, M., Ait Chait, Y., Ojagh, S.M., Latifi, A.M., Esmaeili, M., Hammami, R., Udenigwe,
 539 C.C., 2021. Anti-salmonella activity and peptidomic profiling of peptide fractions
 540 produced from sturgeon fish skin collagen (*Huso huso*) using commercial enzymes.
 541 Nutrients. 13, 2657. <https://doi.org/10.3390/nu13082657>
 542 Benjakul, S., Karnjanapratum, S., Visessanguan, W., 2018. Hydrolysed collagen from *Lates*
 543 *calcarifer* skin: its acute toxicity and impact on cell proliferation and collagen
 544 production of fibroblasts. Int. J. Food Sci. Technol. 53, 1871–1879.
 545 <https://doi.org/10.1111/ijfs.13772>
 546 Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can.
 547 J. Biochem. Physiol. 37, 911–917. <https://doi.org/10.1139/y59-099>
 548 Cavaillon, J.M., 2018. Exotoxins and endotoxins: Inducers of inflammatory cytokines.
 549 Toxicon. 149, 45–53. <https://doi.org/10.1016/j.toxicon.2017.10.016>
 550 Chan, P.T., Matanjun, P., Budiman, C., Shapawi, R., Lee, J.S., 2020. ACE-inhibitory and
 551 antioxidant activities of hydrolysates from the by-products of hybrid grouper
 552 (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*). Sains Malays. 49, 261–270.
 553 <https://doi.org/10.17576/jsm-2020-4902-04>
 554 Chaulagain, B., Jain, A., Tiwari, A., Verma, A., Jain, S.K., 2018. Passive delivery of protein
 555 drugs through transdermal route. Artif. Cells, Nanomedicine Biotechnol. 472–487.
 556 <https://doi.org/10.1080/21691401.2018.1430695>
 557 Chen, M., Li, Y., Huang, G., 2020. Potential health functions of collagen bioactive peptides:
 558 A review. Am. J. Biochem. Biotechnol. 16, 507–519.
 559 <https://doi.org/10.3844/ajbbbsp.2020.507.519>
 560 Chen, X.L., Peng, M., Li, J., Tang, B.L., Shao, X., Zhao, F., Liu, C., Zhang, X.Y., Li, P.Y.,
 561 Shi, M., Zhang, Y.Z., Song, X.Y., 2017. Preparation and functional evaluation of
 562 collagen oligopeptide-rich hydrolysate from fish skin with the serine collagenolytic

protease from *Pseudoalteromonas* sp. SM9913. *Sci. Rep.* 7, 15716.
<https://doi.org/10.1038/s41598-017-15971-9>

Chi, C.F., Hu, F.Y., Wang, B., Li, T., Ding, G.F., 2015a. Antioxidant and anticancer peptides from the protein hydrolysate of blood clam (*Tegillarca granosa*) muscle. *J. Funct. Foods* 15, 301–313. <https://doi.org/10.1016/j.jff.2015.03.045>

Chi, C.F., Wang, B., Wang, Y.M., Zhang, B., Deng, S.G., 2015b. Isolation and characterization of three antioxidant peptides from protein hydrolysate of bluefin leatherjacket (*Navodon septentrionalis*) heads. *J. Funct. Foods* 12, 1–10.
<https://doi.org/10.1016/j.jff.2014.10.027>

Cojocaru, E., Filip, N., Ungureanu, C., Filip, C., Danciu, M., 2014. Effects of valine and leucine on some antioxidant enzymes in hypercholesterolemic rats. *Health* 6, 2313–2321. <https://doi.org/10.4236/health.2014.617266>

Edgar, S., Hopley, B., Genovese, L., Sibilla, S., Laight, D., Shute, J., 2018. Effects of collagen-derived bioactive peptides and natural antioxidant compounds on proliferation and matrix protein synthesis by cultured normal human dermal fibroblasts. *Sci. Rep.* 8, 10474. <https://doi.org/10.1038/s41598-018-28492-w>

FAO, 2022. The state of world fisheries and aquaculture 2022: Towards blue transformation. Rome, FAO. pp. 236. <https://doi.org/10.4060/cc0461en>

FDA, U.S., 2012. Guidance for industry pyrogen and endotoxins testing: Questions and Answers. <https://www.fda.gov/media/83477/download> (accessed 25 November 2021).

FDA, U.S., 2018. Enzyme preparations used in food (partial list).
<https://www.fda.gov/food/generally-recognized-safe-gras/enzyme-preparations-used-food-partial-list> (accessed 18 November 2022).

586 Feketea, S., Beck, A., Veuthey, J., Guillarme, D., 2014. Theory and practice of size exclusion
587 chromatography for the analysis of protein aggregates. *J. Pharm. Biomed. Anal.* 101,
588 161–173. <http://dx.doi.org/10.1016/j.jpba.2014.04.011>

589 García-Moreno, P.J., Batista, I., Pires, C., Bandarra, N.M., Espejo-Carpio, F.J., Guadix, A.,
590 Guadix, E.M., 2014. Antioxidant activity of protein hydrolysates obtained from
591 discarded Mediterranean fish species. *Food Res. Int.* 65, 469–476.
592 <https://doi.org/10.1016/j.foodres.2014.03.061>

593 Halim, N.R.A., Yusof, H.M., Sarbon, N.M., 2016. Functional and bioactive properties of fish
594 protein hydolysates and peptides: A comprehensive review. *Trends Food Sci.*
595 *Technol.* 51, 24–33. <https://doi.org/10.1016/j.tifs.2016.02.007>

596 Ham, J., Lim, W., Whang, K.Y., Song, G., 2020. Butylated hydroxytoluene induces
597 dysregulation of calcium homeostasis and endoplasmic reticulum stress resulting in
598 mouse Leydig cell death. *Environ. Pollut.* 256, 113421.
599 <https://doi.org/10.1016/j.envpol.2019.113421>

600 Henkel, R., Sandhu, I.S., Agarwal, A., 2019. The excessive use of antioxidant therapy: A
601 possible cause of male infertility? *Andrologia.* 51, e13162.
602 <https://doi.org/10.1111/and.13162>

603 Henriques, A., Vázquez, J.A., Valcarcel, J., Mendes, R., Bandarra, N.M., Pires, C., 2021.
604 Characterization of protein hydrolysates from fish discards and by-products from the
605 north-west spain fishing fleet as potential sources of bioactive peptides. *Mar. Drugs.*
606 19, 338. <https://doi.org/10.3390/md19060338>

607 Hu, X.M., Wang, Y.M., Zhao, Y.Q., Chi, C.F., Wang, B., 2020. Antioxidant peptides from
608 the protein hydrolysate of monkfish (*Lophius litulon*) muscle: Purification,
609 identification, and cytoprotective function on HepG2 cells damage by H₂O₂. *Mar.*
610 *Drugs.* 18, 153. <https://doi.org/10.3390/md18030153>

611 Ighodaro, O.M., Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase
612 (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in
613 the entire antioxidant defence grid. Alexandria J. Med. 54, 287–293.
614 <https://doi.org/10.1016/j.ajme.2017.09.001>

615 Islam, M.R., Yuhi, T., Meng, D., Yoshioka, T., Ogata, Y., Ura, K., Takagi, Y., 2021. Purity
616 and properties of gelatins extracted from the head tissue of the hybrid kalamtra
617 sturgeon. LWT - Food Sci. Technol. 142, 110944.
618 <https://doi.org/10.1016/j.lwt.2021.110944>

619 Islam, M.R., Yuhi, T., Ura, K., Takagi, Y., 2020. Optimization of extraction of gelatin from
620 the head of kalamtra sturgeon (*Huso dauricus* × *Acipenser schrenckii* × *Acipenser*
621 *transmontanus*). Appl. Sci. 10, 6660. <https://doi.org/10.3390/app10196660>

622 Ji, Y., Zhang, G., Li, X., Zhao, B., Zhou, S., 2013. Enzymatic hydrolysis of protein from
623 small yellow croaker (*Pseudosciaena polyactis*) and evaluation of its antioxidant
624 activity. J. Food Biochem. 37, 278–285. [https://doi.org/10.1111/j.1745-](https://doi.org/10.1111/j.1745-4514.2011.00640.x)
625 [4514.2011.00640.x](https://doi.org/10.1111/j.1745-4514.2011.00640.x)

626 Kosari, P., Alikhan, A., Sockolov, M., Feldman, S.R., 2010. Vitamin E and allergic contact
627 dermatitis. Dermatitis. 21, 48–153. <https://doi.org/10.2310/6620.2010.09083>

628 Kuciel-Lewandowska, J., Kasperczak, M., Bogut, B., Heider, R., Laber, W.T., Laber, W.,
629 Paprocka-Borowicz, M., 2020. The impact of health resort treatment on the
630 nonenzymatic endogenous antioxidant system. Oxid. Med. Cell. Longev. 2020,
631 8423105. <https://doi.org/10.1155/2020/8423105>

632 Kudo, M., Katayoshi, T., Kobayashi-Nakamura, K., Akagawa, M., Tsuji-Naito, K., 2016.
633 H⁺/peptide transporter (PEPT2) is expressed in human epidermal keratinocytes and is
634 involved in skin oligopeptide transport. Biochem. Biophys. Res. Commun. 475, 335–
635 341. <https://doi.org/10.1016/j.bbrc.2016.05.093>

636 Kurahashi, T., Fujii, J., 2015. Roles of antioxidative enzymes in wound healing. *J. Dev. Biol.*
 637 3, 57–70. <https://doi.org/10.3390/jdb3020057>
 638 Le Gouic, A. V., Harnedy, P.A., FitzGerald, R.J., 2019. Bioactive peptides from fish protein
 639 by-products, in: Mérillon, J.M., Ramawat, K. (Eds.), *Bioactive molecules in food.*
 640 *Reference series in phytochemistry*, Springer, Cham. pp. 355–388.
 641 https://doi.org/10.1007/978-3-319-78030-6_29
 642 León-López, A., Morales-Peñaloza, A., Martínez-Juárez, V.M., Vargas-Torres, A., Zeugolis,
 643 D.I., Aguirre-Álvarez, G., 2019. Hydrolyzed collagen-sources and applications.
 644 *Molecules*. 24, 4031. <https://doi.org/10.3390/molecules24224031>
 645 Li, W., Kobayashi, T., Meng, D., Miyamoto, N., Tsutsumi, N., Ura, K., Takagi, Y., 2021a.
 646 Free radical scavenging activity of type II collagen peptides and chondroitin sulfate
 647 oligosaccharides from by-products of mottled skate processing. *Food Biosci.* 41,
 648 100991. <https://doi.org/10.1016/j.fbio.2021.100991>
 649 Li, W., Kobayashi, T., Moroi, S., Kotake, H., Ikoma, T., Saeki, H., Ura, K., Takagi, Y., 2019.
 650 Anti-obesity effects of chondroitin sulfate oligosaccharides from the skate *Raja*
 651 *pulchra*. *Carbohydr. Polym.* 214, 303–310.
 652 <https://doi.org/10.1016/j.carbpol.2019.03.025>
 653 Li, W., Terauchi, N., Meng, D., Miyamoto, N., Tsutsumi, N., Ura, K., Takagi, Y., 2021b.
 654 Antioxidant and fibroblast-activating activities of the by-product of skate chondroitin
 655 extractive production. *Sustain. Chem. Pharm.* 23, 100499.
 656 <https://doi.org/10.1016/j.scp.2021.100499>
 657 Liu, M., Chen, S., Zhang, Z., Li, H., Sun, G., Yin, N., Wen, J., 2022. Anti-ageing peptides
 658 and proteins for topical applications: a review. *Pharm. Dev. Technol.* 27, 108–125.
 659 <https://doi.org/10.1080/10837450.2021.2023569>

660 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
 661 quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. 25, 402–408.
 662 <https://doi.org/10.1006/meth.2001.1262>

663 Lobo, V., Patil, A., Phatak, A., Chandra, N., 2010. Free radicals, antioxidants and functional
 664 foods: Impact on human health. *Pharmacogn. Rev.* 4, 118–126.
 665 <https://doi.org/10.4103/0973-7847.70902>

666 Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., Dulak, J., 2016. Role of Nrf2/HO-1
 667 system in development, oxidative stress response and diseases: an evolutionarily
 668 conserved mechanism. *Cell. Mol. Life Sci.* 73, 3221–3247.
 669 <https://doi.org/10.1007/s00018-016-2223-0>

670 Meng, D., Tanaka, H., Kobayashi, T., Hatayama, H., Zhang, X., Ura, K., Yunoki, S., Takagi,
 671 Y., 2019. The effect of alkaline pretreatment on the biochemical characteristics and
 672 fibril-forming abilities of types I and II collagen extracted from bester sturgeon by-
 673 products. *Int. J. Biol. Macromol.* 131, 572–580.
 674 <https://doi.org/10.1016/j.ijbiomac.2019.03.091>

675 Miyamoto, T., Okano, S., Kasai, N., 2009. Inactivation of *Escherichia coli* endotoxin by soft
 676 hydrothermal processing. *Appl. Environ. Microbiol.* 75, 5058–5063.
 677 <https://doi.org/10.1128/AEM.00122-09>

678 Morris, M.C., Gilliam, E.A., Button, J., Li, L., 2014. Dynamic modulation of innate immune
 679 response by varying dosages of Lipopolysaccharide (LPS) in human monocytic cells.
 680 *J. Biol. Chem.* 289, 21584–21590. <https://doi.org/10.1074/jbc.M114.583518>

681 Nikoo, M., Benjakul, S., Bashari, M., Alekhorshied, M., Cissouma, A.I., Yang, N., Xu, X.,
 682 2014. Physicochemical properties of skin gelatin from farmed Amur sturgeon
 683 (*Acipenser schrenckii*) as influenced by acid pretreatment. *Food Biosci.* 5, 19–26.
 684 <https://doi.org/10.1016/j.fbio.2013.10.004>

685 Noman, A., Xu, Y., AL-Bukhaiti, W.Q., Abed, S.M., Ali, A.H., Ramadhan, A.H., Xia, W.,
686 2018. Influence of enzymatic hydrolysis conditions on the degree of hydrolysis and
687 functional properties of protein hydrolysate obtained from Chinese sturgeon
688 (*Acipenser sinensis*) by using papain enzyme. *Process Biochem.* 67, 19–28.
689 <https://doi.org/10.1016/j.procbio.2018.01.009>

690 Nurilmala, M., Pertiwi, R.M., Nurhayati, T., Fauzi, S., Batubara, I., Ochiai, Y., 2019.
691 Characterization of collagen and its hydrolysate from yellowfin tuna *Thunnus*
692 *albacares* skin and their potencies as antioxidant and antiglycation agents. *Fish. Sci.*
693 85, 591–599. <https://doi.org/10.1007/s12562-019-01303-5>

694 Ovissipour, M., Abedian, A., Motamedzadegan, A., Rasco, B., Safari, R., Shahiri, H., 2009.
695 The effect of enzymatic hydrolysis time and temperature on the properties of protein
696 hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera. *Food Chem.* 115,
697 238–242. <https://doi.org/10.1016/j.foodchem.2008.12.013>

698 Park, S.H., Jo, Y.J., 2019. Static hydrothermal processing and fractionation for production of
699 a collagen peptide with anti-oxidative and anti-aging properties. *Process Biochem.* 83,
700 176–182. <https://doi.org/10.1016/j.procbio.2019.05.015>

701 Ramos, O.S., Malcata, F.X., 2017. Food-Grade Enzymes, in: Moo-Young, M. (Ed.),
702 *Comprehensive biotechnology*, Third Edition, Pergamon. pp. 587–603.
703 <https://doi.org/10.1016/B978-0-12-809633-8.09173-1>

704 Schagen, S.K., 2017. Topical peptide treatments with effective anti-aging results. *Cosmetics.*
705 4, 16. <https://doi.org/10.3390/cosmetics4020016>

706 Schagger, H., 2006. Tricine-SDS-PAGE. *Nat. Protoc.* 1, 16–22.
707 <https://doi.org/10.1038/nprot.2006.4>

708 Sharma, C., Kim, S.R., 2021. Linking oxidative stress and proteinopathy in alzheimer’s
709 disease. *Antioxidants.* 10, 1231. <https://doi.org/10.3390/antiox10081231>

710 Sila, A., Bougatef, A., 2016. Antioxidant peptides from marine by-products: Isolation,
 711 identification and application in food systems. A review. J. Funct. Foods. 21, 10–26.
 712 <https://doi.org/10.1016/j.jff.2015.11.007>

713 Slizyte, R., Mozuraityte, R., Remman, T., Rustad, T., 2018. Two-stage processing of salmon
 714 backbones to obtain high-quality oil and proteins. Int. J. Food Sci. Technol. 53, 2378–
 715 2385. <https://doi.org/10.1111/ijfs.13830>

716 Slizyte, R., Rommi, K., Mozuraityte, R., Eck, P., Five, K., Rustad, T., 2016. Bioactivities of
 717 fish protein hydrolysates from defatted salmon backbones. Biotechnol. Reports. 11,
 718 99–109. <https://doi.org/10.1016/j.btre.2016.08.003>

719 Souissi, N., Bougatef, A., Triki-Ellouz, Y., Nasri, M., 2007. Biochemical and functional
 720 properties of sardinella (*Sardinetta aurita*) by-product hydrolysates. Food Technol.
 721 Biotechnol. 45, 187–194.

722 Tacias-Pascacio, V.G., Castañeda-Valbuena, D., Morellon-Sterling, R., Tavano, O.,
 723 Berenguer-Murcia, Á., Vela-Gutiérrez, G., Rather, I.A., Fernandez-Lafuente, R.,
 724 2021. Bioactive peptides from fisheries residues: A review of use of papain in
 725 proteolysis reactions. Int. J. Biol. Macromol. 184, 415–428.
 726 <https://doi.org/10.1016/j.ijbiomac.2021.06.076>

727 Tan, B.L., Norhaizan, M.E., Liew, W.P.P., Rahman, H.S., 2018. Antioxidant and oxidative
 728 stress: A mutual interplay in age-related diseases. Front. Pharmacol. 9, 1162.
 729 <https://doi.org/10.3389/fphar.2018.01162>

730 Tejpal, C.S., Vijayagopal, P., Elavarasan, K., Linga Prabu, D., Lekshmi, R.G.K., Asha, K.K.,
 731 Anandan, R., Chatterjee, N.S. Mathew, S., 2017. Antioxidant, functional properties
 732 and amino acid composition of pepsin-derived protein hydrolysates from whole tilapia
 733 waste as influenced by pre-processing ice storage. J. Food Sci. Technol. 54, 4257–
 734 4267. <https://doi.org/10.1007/s13197-017-2897-9>

735 Viennois, E., Pujada, A., Zen, J., Merlin, D., 2018. Function, regulation, and
 736 pathophysiological relevance of the POT superfamily, specifically PepT1 in
 737 inflammatory bowel disease. *Compr. Physiol.* 8, 731–760.
 738 <https://doi.org/10.1002/cphy.c170032>

739 Wang, J., Xu, M., Liang, R., Zhao, M., Zhang, Z., Li, Y., 2015. Oral administration of marine
 740 collagen peptides prepared from chum salmon (*Oncorhynchus keta*) improves wound
 741 healing following cesarean section in rats. *Food Nutr. Res.* 59, 26411.
 742 <https://doi.org/10.3402/fnr.v59.26411>

743 Wang, W., Li, Z., Liu, J., Wang, Y., Liu, S., Sun, M., 2013. Comparison between thermal
 744 hydrolysis and enzymatic proteolysis processes for the preparation of tilapia skin.
 745 *Czech J. Food Sci.* 31, 1–4. <https://doi.org/10.17221/49/2012-CJFS>

746 Yamamoto, M., Kensler, T.W., Motohashi, H., 2018. The KEAP1-NRF2 system: A thiol-
 747 based sensor-effector apparatus for maintaining redox homeostasis. *Physiol. Rev.* 98,
 748 1169–1203. <https://doi.org/10.1152/physrev.00023.2017>

749 Yarnpakdee, S., Benjakul, S., Kristinsson, H.G., Bakken, H.E., 2015. Preventive effect of
 750 Nile tilapia hydrolysate against oxidative damage of HepG2 cells and DNA mediated
 751 by H₂O₂ and AAPH. *J. Food Sci. Technol.* 52, 6194–6205.
 752 <https://doi.org/10.1007/s13197-014-1672-4>

753 You, L., Zhao, M., Regenstein, J.M., Ren, J., 2010. Purification and identification of
 754 antioxidative peptides from loach (*Misgurnus anguillicaudatus*) protein hydrolysate
 755 by consecutive chromatography and electrospray ionization-mass spectrometry. *Food*
 756 *Res Int.* 43, 1167–1173. <https://doi.org/10.1016/j.foodres.2010.02.009>

757 Zhang, X., Adachi, S., Ura, K., Takagi, Y., 2019. Properties of collagen extracted from Amur
 758 sturgeon *Acipenser schrenckii* and assessment of collagen fibrils *in vitro*. *Int. J. Biol.*
 759 *Macromol.* 137, 809–820. <https://doi.org/10.1016/j.ijbiomac.2019.07.021>

760 Zhang, X., Ookawa, M., Tan, Y., Ura, K., Adachi, S., Takagi, Y., 2014. Biochemical
 761 characterisation and assessment of fibril-forming ability of collagens extracted from
 762 bester sturgeon *Huso huso* × *Acipenser ruthenus*. Food Chem. 160, 305–312.
 763 <https://doi.org/10.1016/j.foodchem.2014.03.075>
 764 Zhang, X., Zhang, H., Toriumi, S., Ura, K., Takagi, Y., 2020. Feasibility of collagens
 765 obtained from bester sturgeon *Huso huso* × *Acipenser ruthenus* for industrial use.
 766 Aquaculture. 529, 735641. <https://doi.org/10.1016/j.aquaculture.2020.735641>
 767 Zhao, W.H., Luo, Q. Bin, Pan, X., Chi, C.F., Sun, K.L., Wang, B., 2018. Preparation,
 768 identification, and activity evaluation of ten antioxidant peptides from protein
 769 hydrolysate of swim bladders of miiuy croaker (*Miichthys miiuy*). J. Funct. Foods. 47,
 770 503–511. <https://doi.org/10.1016/j.jff.2018.06.014>
 771 Zheng, L., Wei, H., Yu, H., Xing, Q., Zou, Y., Zhou, Y., Peng, J., 2018. Fish skin gelatin
 772 hydrolysate production by ginger powder induces glutathione synthesis to prevent
 773 hydrogen peroxide induced intestinal oxidative stress via the Pept1-p62-Nrf2 cascade.
 774 J. Agric. Food Chem. 66, 11601–11611. <https://doi.org/10.1021/acs.jafc.8b02840>

Figure Captions

Fig. 1. (A) Tricine-SDS PAGE patterns of sturgeon head tissue peptides. M, molecular mass markers; DW-P, head tissue was autoclaved in distilled water (DW) and hydrolyzed with papain; DW-P+B, head tissue was autoclaved in DW and hydrolyzed with papain and bromelain; WDW-P, head tissue was autoclaved without DW and hydrolyzed with papain; WDW-P+B, head tissue was autoclaved without DW and hydrolyzed with papain and bromelain. All samples were loaded at 25 µg sample/lane. **(B)** Tricine-SDS PAGE patterns of skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon. M, molecular mass markers. All samples were loaded at 25 µg/lane.

Fig. 2. ABTS[•] scavenging activities of skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon. Columns and bars indicate the mean value ± standard error ($n = 3$). Different letters indicate significant differences ($p < 0.05$).

Fig. 3. Protective effects of skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon on L929 fibroblast cell viability following 0.25 mM H₂O₂-induced oxidative stress. EFW, endotoxin-free water. Columns and bars indicate the mean value ± standard error ($n = 10$). Different letters indicate significant differences ($p < 0.05$).

Fig. 4. Effects of skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon on H₂O₂-induced intracellular ROS production in L929 fibroblast cells. Oxidative stress was induced with 0.25 mM H₂O₂. EFW, endotoxin-

799 free water. Columns and bars indicate the mean value $\pm$ standard error ($n = 10$). Different
800 letters indicate significant differences ($p < 0.05$).

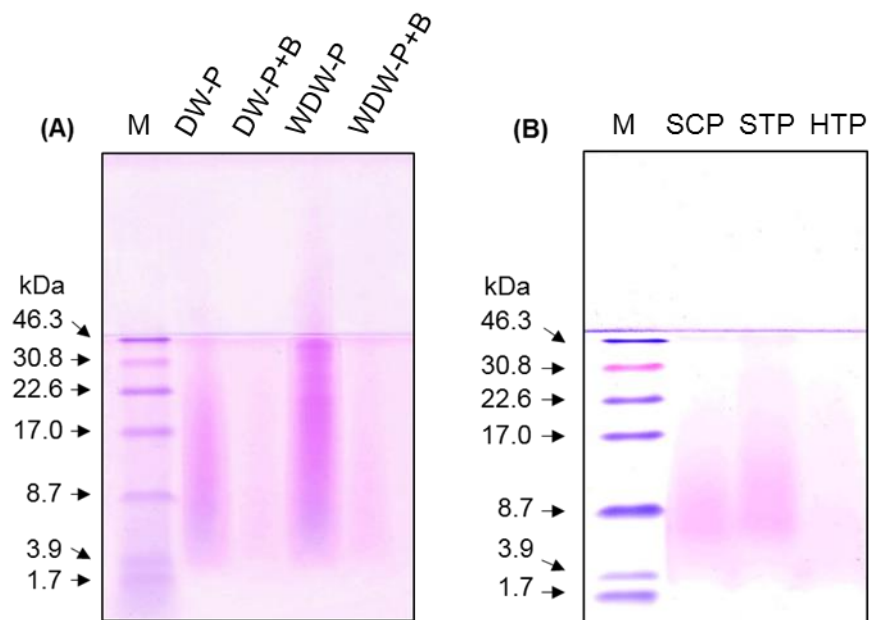
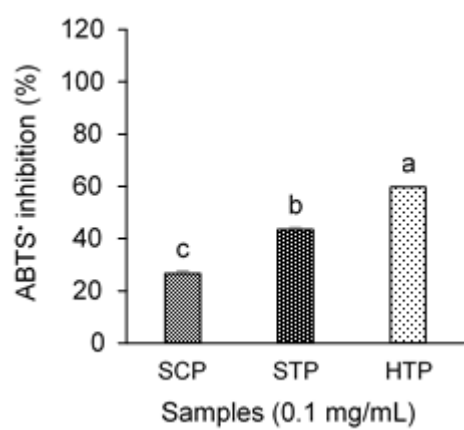


Fig. 1.



803

804 **Fig. 2.**

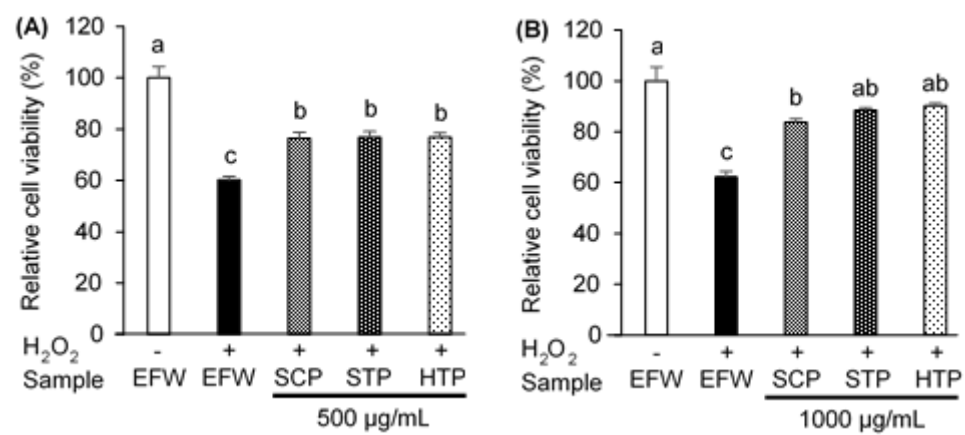


Fig. 3.

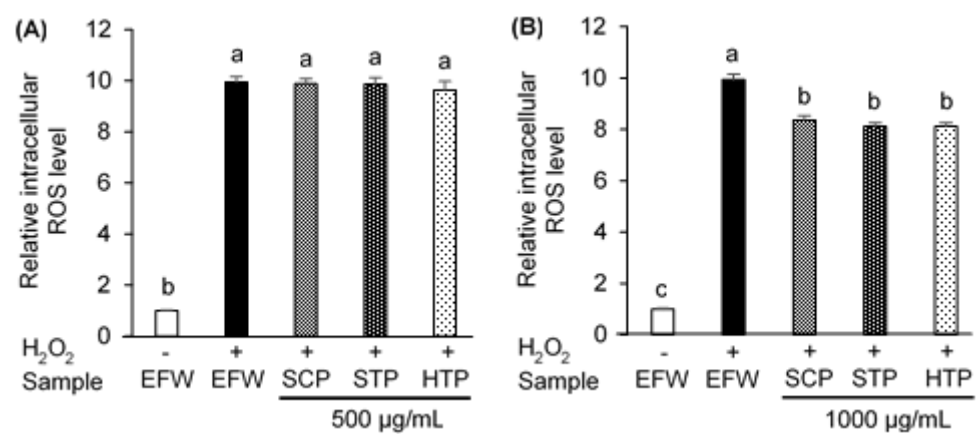


Fig. 4.

809 **Table 1**

810 Major characteristics of enzymes used to produce peptides from sturgeon head.

Enzymes	Main sources	Characteristics	Optimum condition	FDA status
Papain	<i>Carica papaya</i> L.	Cysteine	45–60 °C	Safe to use
Bromelain	<i>Ananas comosus</i> <i>Ananas bracteatus</i>	endoprotease with broad specificity	at pH 4–8	

811 FDA, U.S. Food and Drug Administration

812 From FDA (2018), Ramos and Malcata (2017), Slizyte et al. (2016), and Tacias-Pascacio et

813 al. (2021)

Table 2

Yields (% dry weight sample/dry weight tissue) and molecular mass distribution of peptides produced from sturgeon head tissue in different conditions.

HTPs	Yields	Molecular mass distribution (%)				
		<500 Da	500 Da–1 kDa	1–3 kDa	3–5 kDa	>5 kDa
DW-P	35.3±0.9 ^b	32.1±0.4 ^c	22.9±0.3 ^a	30.4±0.4 ^a	9.1±0.2 ^b	5.6±0.5 ^b
DW-P+B	40.6±0.9 ^a	52.5±1.2 ^a	20.5±0.5 ^{bc}	22.5±0.6 ^c	3.6±0.2 ^d	0.9±0.1 ^d
WDW-P	34.7±1.0 ^b	24.1±0.4 ^d	19.3±0.3 ^c	31.5±0.5 ^a	11.3±0.1 ^a	13.8±0.4 ^a
WDW-P+B	38.9±0.8 ^a	45.9±0.3 ^b	21.7±0.2 ^b	25.2±0.3 ^b	5.2±0.3 ^c	2.0±0.1 ^c

HTPs, head tissue peptides; DW-P, head tissues were autoclaved in distilled water (DW) and hydrolyzed with papain; DW-P+B, head tissues were autoclaved in DW and hydrolyzed with papain and bromelain; WDW-P, head tissues were autoclaved without DW and hydrolyzed with papain; WDW-P+B, head tissues were autoclaved without DW and hydrolyzed with papain and bromelain. All values are the mean ± standard error of three replicates. Different letters in each column indicate a significant difference ($p < 0.05$).

Table 3

Yields (% dry weight sample/dry weight tissue) and molecular mass distribution of skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon.

Peptides	Yields	Molecular mass distribution (%)				
		<500 Da	500 Da–1 kDa	1–3 kDa	3–5 kDa	>5 kDa
SCP	55.4±1.4 ^b	34.3±0.7 ^b	22.2±0.2 ^a	29.7±0.5 ^a	9.8±0.1 ^b	3.8±0.3 ^b
STP	77.1±1.0 ^a	28.9±1.3 ^c	21.9±0.4 ^a	31.2±0.8 ^a	11.7±0.6 ^a	6.3±0.3 ^a
HTP	41.3±0.9 ^c	47.0±0.3 ^a	20.3±0.3 ^b	25.7±0.1 ^b	5.3±0.2 ^c	1.7±0.1 ^c

All values are the mean ± standard error of three replicates. Different letters in each column indicate a significant difference ($p < 0.05$).

Table 4

Amino acid composition (residues/1000 residues) and collagen-derived peptide content (%) in the total protein fraction of the skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon.

Amino acids	Amino acid composition (residues/1000 residues)			
	Purified skin collagen	SCP	STP	HTP
Alanine	115	115	113	101
Arginine	50	52	52	48
Asparagine/aspartic acid	50	50	53	68
Cysteine	0	1	1	1
Glutamine/glutamic acid	71	70	74	98
Glycine	346	344	332	253
Histidine	5	5	6	11
Hydroxyproline	65	64	58	50
Hydroxylysine	9	11	8	7
Isoleucine	13	13	13	23
Leucine	19	19	22	42
Lysine	24	25	28	45
Methionine	10	10	11	16
Phenylalanine	13	13	15	19
Proline	116	117	117	93
Serine	51	48	47	49
Threonine	24	24	25	34
Tyrosine	2	3	6	12
Valine	16	17	20	31
Imino acid	181	181	176	142
Collagen-derived peptide content*	100.0	98.5	89.9	76.9

*Collagen-derived peptide content (%) in the total protein fraction was calculated from the hydroxyproline content. Values for each sample were obtained from mixed samples of three replicates.

Table 5

Proximate composition of skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon.

Contents	Proximate composition (%)		
	SCP	STP	HTP
Collagen [†]	99.1	87.8	58.6
Non-collagenous proteins [†]	0.9	10.6	17.6
Lipid content	-	0.9±0.1*	0.5±0.0
Ash content	-	ND	4.3±0.1
Others (e.g., sugar)	-	0.7*	19.0
Total	100.0	100.0	100.0

All values are expressed as a percentage of the sample weight. -, the purified collagen was assumed to be 100% collagenous protein and thus the non-proteinaceous content to be zero; ND, not detectable. [†]Values were calculated from the hydroxyproline content, which was measured from mixed samples of three replicates. Lipid contents are the mean ± standard error of three replicates. *Significantly different from the value in HTP ($p < 0.05$).

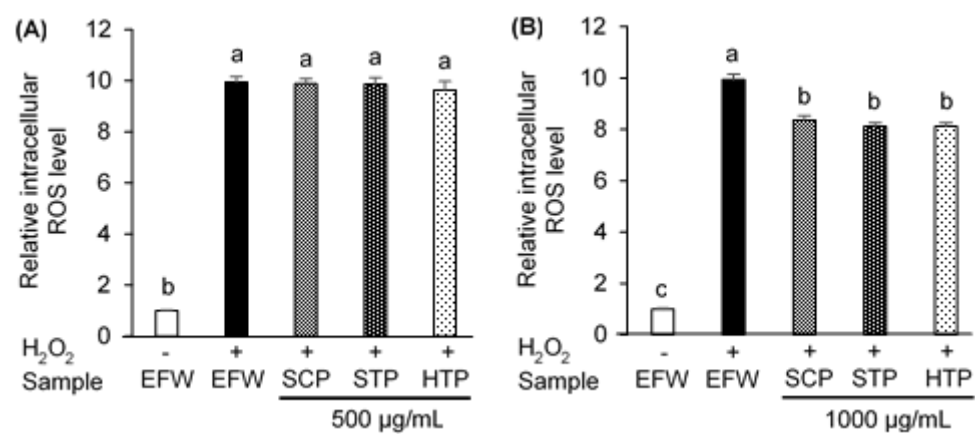
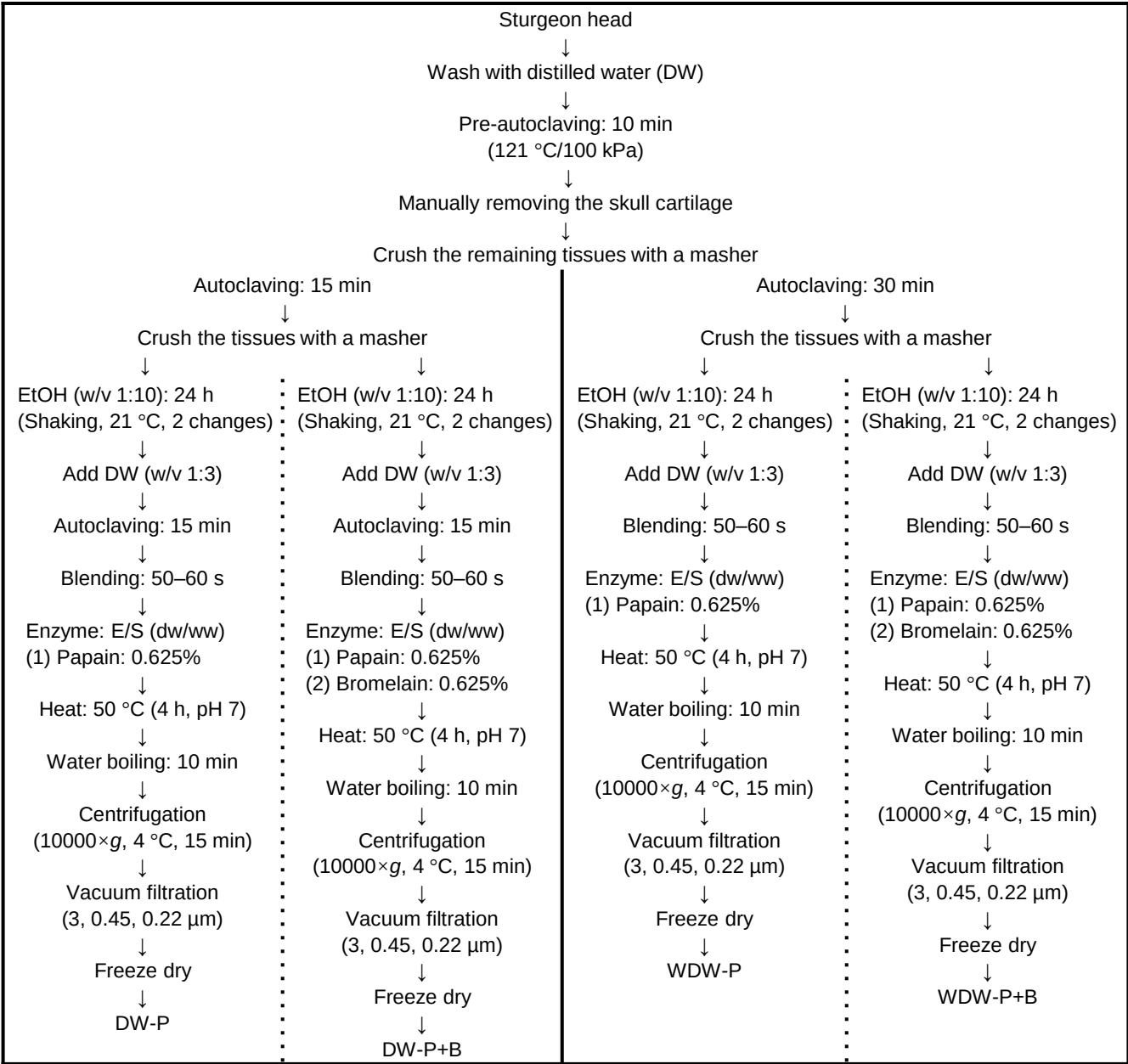
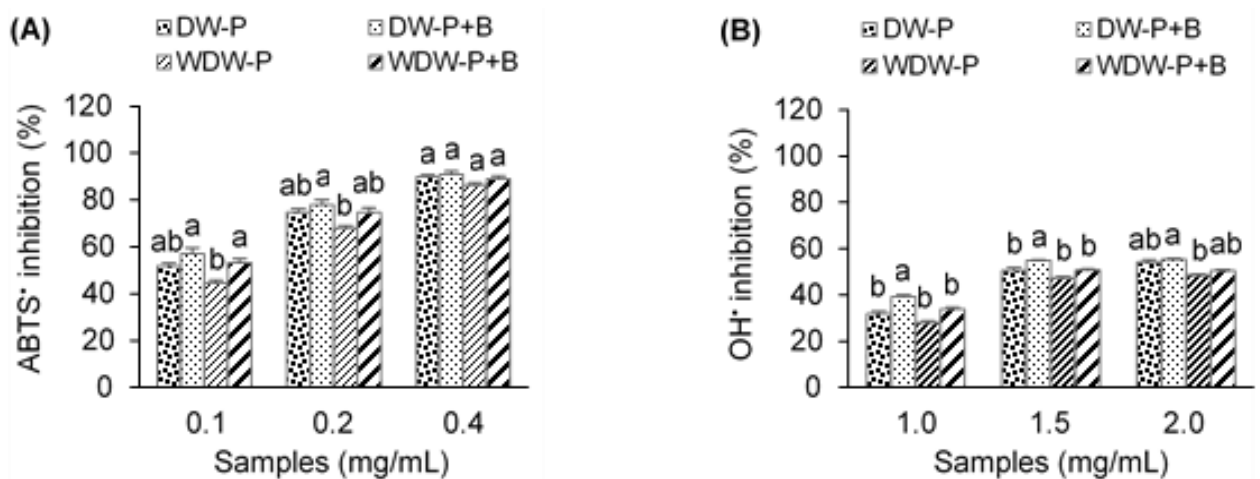


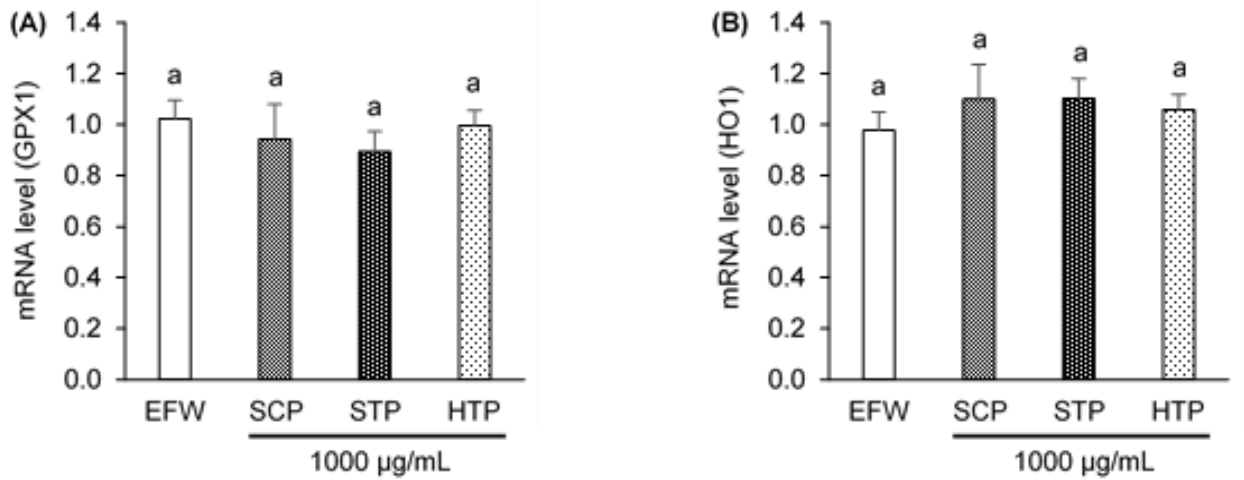
Fig. 4.



Supplementary Fig. 1. Four production methods of sturgeon head tissue peptides (HTPs). EtOH, ethanol; E/S, enzyme per substrate; w/v, wet weight/volume; and dw/ww, dry weight/wet weight.



Supplementary Fig. 2. ABTS• (A) and OH• (B) scavenging activities of peptides produced from the sturgeon head with different treatments. DW-P, head tissues were autoclaved in DW and hydrolyzed with papain; DW-P+B, head tissues were autoclaved in DW and hydrolyzed with papain and bromelain; WDW-P, head tissues were autoclaved without DW and hydrolyzed with papain; WDW-P+B, head tissues were autoclaved without DW and hydrolyzed with papain and bromelain. Columns and bars indicate the mean value $\pm$ standard error ($n = 3$). Different letters in each concentration among samples indicate significant differences ($p < 0.05$).



Supplementary Fig. 3. Effects of skin collagen peptide (SCP), skin tissue peptide (STP), and head tissue peptide (HTP) produced from sturgeon on the mRNA levels of glutathione peroxidase-1 (*GPX1*, **A**) and heme oxygenase-1 (*HO1*, **B**). EFW, endotoxin-free water. Columns and bars indicate the mean value $\pm$ standard error (n = 6). No significant effects of the samples were observed.

Supplementary Table 1

Sequences of the primers for RT-qPCR for antioxidant genes.

Genes	Forward	Reverse	Accession no.
<i>GPX1</i>	5'-TTCGGACACCAGGAGAATGG-3'	5'-AAGGTAAAGAGCGGGTGAGC-3'	NM_008160.6
<i>HO1</i>	5'-ACATTGAGCTGTTTGAGGAG-3'	5'-TACATGGCATAAATTCCCACTG-3'	NM_010442.2
<i>18S rRNA</i>	5'-CTTAGAGGGACAAGTGGCG-3'	5'-ACGCTGAGCCAGTCAGTGTA-3'	NR_003278.3

GPX1, glutathione peroxidase-1; *HO1*, heme oxygenase-1; *18S rRNA*, 18S ribosomal RNA.