Environment-friendly Utilization of Squid Pen with Water: Production of β-chitin Nanofibers and Peptides for Lowering Blood Pressure

Mitsumasa Osada[a,], Mizuki Nishiwaki[a], and Takashi Watanabe[b]*

[a]Department of Chemistry and Materials, Faculty of Textile Science and Technology, Shinshu University, 3-15-1, Tokida, Ueda, Nagano 386-8567, Japan

[b]Division of Chemical Engineering and Biotechnology, National Institute of Technology, Ichinoseki College, Ichinoseki, Iwate 021-8511, Japan

1. Introduction

Biomass resources, such as carbohydrates and proteins, should ideally be used as a whole. Chitin is normally present combined with proteins and inorganics, so to obtain a specific part of chitin, the other part of the protein must be removed. Because proteins are traditionally removed by alkaline aqueous solution treatment, the protein changes to an alkaline mixture, which is discarded. From a perspective of the sustainable utilization of biomass, using only a specific part and discarding the other is not desirable. However, the purification of other parts, such as proteins from the mixture solution, is not cost- or energy-effective. Additionally, it is difficult to treat wastewater after separation. Therefore, it is important to develop a method for the complete utilization of biomass components. High-temperature water treatment is a promising technique for the entire use of biomass.[1]

To obtain α-chitin from crab shells using traditional methods, the protein needs to be removed by alkaline aqueous treatment, and the ash is then removed by acid aqueous treatment.[2–4] There are two types of chitin, α-chitin and β-chitin, which have different crystalline structures[5,6] and it is difficult to use, except for α-chitin components such as protein and ash. However, various studies have promoted the application of high-temperature water treatment for the hydrolysis of proteins without acid and alkaline conditions.[7–9] In a previous study, we demonstrated that the protein in the crab shell could be removed by high-temperature water treatment at 300 °C within 30 min because it can be hydrolyzed into water-soluble peptides.[1] We obtained a solid residue containing α-chitin and ash, namely calcium carbonate, from the crab shell and confirmed that the chemical structure of α-chitin was relatively stable after treatment at 300 °C. It is important to note that the decomposition conditions (i.e., temperature and time) are

different for α-chitin and protein.[10] However, residues of ash from crab shells remain mixed with α-chitin.

The squid pen contains 30%–50% β-chitin, 50%–70% protein, and less than 1% ash.[11] Therefore, pure β-chitin may be obtained from the squid pen as a solid residue and the protein converted to a water-soluble peptide by controlling the temperature and treatment time. In general, to obtain β-chitin from a squid pen, the protein is removed by alkaline aqueous treatment.[11] In contrast, for the high-temperature water treatment, the water-soluble protein can be obtained using only water. However, there are no reports on applying high-temperature water treatment for the separation of β-chitin and protein to date. In addition, the properties of products such as β-chitin and protein obtained from the high-temperature water are not clear in comparison with those obtained by traditional methods.

In this work, the properties of β-chitin obtained by high-temperature water treatments as its nanofibers (NFs) are also evaluated. We uses wet pulverization using a water jet to convert it into β-chitin NFs (ChNFs). This system uses only water with no additional acids and achieves the environment-friendly production of ChNFs. β-Chitin comprises parallel molecular chains without significant hydrogen bonding between intermolecular sheets[12–14]; therefore, β-chitin powder can be easily disintegrated into NFs.[15,16] Therefore, the physicochemical properties, such as transmittance and viscosity, of β-ChNF dispersions can vary as much as those of α-ChNF dispersions.[17–20] ChNFs are characterized by a high modulus,[21] high relative surface area (i.e., exposure of many hydrophilic functional groups),[22] and an excellent biocompatibility.[23]

In this study, the function of the peptide for lowering blood pressure, namely the inhibition of Angiotensin-1 converting enzyme (ACE) is evaluated. ACE inhibition is a promising application of peptides.[24,25] If the peptide obtained from the squid pen by high-temperature water treatment has the activity of ACE inhibition, the protein component in the squid pen could also be converted as a functional food instead of waste.

Therefore, a new method of squid pen into solid β-chitin and water-soluble peptides using only water at high temperatures is proposed. The physicochemical properties of β-chitin when converted into NFs using only water were studied. Because this method uses only water, the protein in the squid pen could also be a functional peptide for lowering blood pressure, namely the inhibition of ACE.

2. Experimental Section

Materials. Squid pen (*Todarodes pacificus*) was obtained from Marutatsu Kamasui Co., Ltd., (Iwate, Japan) and stored as never-dried chitins at 4 °C before use. Pure β-chitin was obtained from Seikagaku Corporation (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Angiotensin-1 converting enzyme (ACE) from bovine lungs was obtained from Life Laboratory (Yamagata, Japan).

High-temperature water treatment. Treatment of squid pen in high-temperature water was conducted in a stainless steel 316 (SS-316) tube reactor with an inner volume of 6 cm^3.[26,27]

Squid pen (0.2 g) and water (3 g) were loaded into the reactor, and submerged in a molten-salt bath (KNO_3–$NaNO_3$) at reaction temperatures of 150, 200, 250, and 300 °C. The internal reactor temperature was measured by inserting a thermocouple directly into the reactor. Approximately 4 min was required to heat the reactor to the reaction temperature, which was added to treatment times. The saturated vapor pressures in the reactor at150, 200, 250, and 300 °C are calculated to be 0.48, 1.6, 4.0, and 8.6 MPa, respectively [28]. After a pre-defined treatment time, the reactor was removed from the bath and rapidly quenched in a water bath to cool to 25 °C. After cooling, the products were collected from the reactor and divided into water-soluble and solid fractions using a 0.20 μm membrane filter, after which the latter was dried at 90 °C for 24 h.

Amino acid analysis. Squid pen (5 mg) was hydrolyzed with 1 mL of 6 mol/L hydrochloric acid in hydrolysis vials at 105 °C for 16 h. The sample was diluted with a 0.2 mol/L sodium citrate loading buffer (pH 2.2) to a final volume of 2 mL with water and filtered through a 0.45 μm membrane filter. The amino acid content of the reaction products was determined using a Shimadzu analyzer (Shimadzu Corp., Kyoto, Japan), equipped with an ion-exclusion column (ShimPack Amino-Na, Shimadzu Corp., Kyoto, Japan), and post-column labeling methods using a spectrophotometer (RF-10A, Shimadzu Corp., Kyoto, Japan).

Weight change. We evaluated the weight change of the squid pen after high-temperature water treatment using equation 1:

$$Weight\ change\ [\%] = \frac{Weight\ of\ squid\ pen\ recovered\ [g]}{Weight\ of\ squid\ pen\ loaded\ (0.2\ [g])} \times 100 \quad (1)$$

X-ray diffraction (XRD). Equatorial diffraction profiles were obtained with Cu-Kα radiation from a powder X-ray generator (Japan Electronic Organization Co. Ltd., JDX-3530) operating at 30 kV and 30 mA. The crystallinity index was calculated from normalized diffractograms using the intensities of the peaks at the [1–10] lattice ($I_{1–10}$, at 2θ = 20 corresponding to the maximum intensity of chitin) and I_{am} at 2θ = 16 (amorphous diffraction), used to calculate the crystallinity index (2):[29]

$$Crystallinity\ index\ [\%] = \frac{I_{1-10} - I_{am}}{I_{1-10}} \times 100 \quad (2)$$

The *d*-spacing of the peaks at the [1–10] lattice was calculated using Bragg's equation:

$$2d \sin\theta = \lambda \quad (3)$$

where *d* is the spacing between the planes in the lattice, θ is the Bragg angle, and λ is the X-ray wavelength.

The crystallite size of chitin in the [1–10] lattice was calculated using the Scherrer equation:

$$L = \frac{0.9\lambda}{H \cos\theta} \quad (4)$$

where *L* is the crystallite size perpendicular to the plane, and *H* is the full width at half maximum in radians.

Fourier transform infrared (FTIR) spectroscopy. The FTIR spectra of the squid pen were measured using a Nicolet iS10 spectrometer (Thermo Fisher Scientific Inc.).

Preparation of chitin nanofibers (ChNFs). The solid residue of the squid pen after high-temperature water treatment was used to prepare the ChNF dispersions. After suspension of the solid residue in distilled water, disintegration into NFs was performed using a Star Burst system (Star Burst Mini, Sugino Machine Co., Ltd., Uozu, Japan). The solid residue can be converted to NFs by the Star Burst system, and the details of the disintegration process were described in our previous study.[11] The suspension was pressurized at approximately 235 MPa and the number of collisions with the ceramic ball was set to 10 times (i.e., 10 passes).

Field-emission scanning electron microscopy (FE-SEM). To prepare the FE-SEM samples, the squid pen dispersion after high-temperature water treatment was diluted with *tert*-butyl alcohol and precipitated by centrifugation at 20,000 ×*g* at 25 °C for 5 min. After repeating this process several times, the water supernatant was replaced with tert-butyl alcohol. The precipitated sample was frozen in a glass bottle and dried under vacuum conditions (0.02 MPa) for 3 h. The dried samples were coated with an osmium layer (~2 nm thick) using an osmium coater (Neoc-STP, Meiwafosis Co., Ltd., Tokyo, Japan). The sample was observed using a FE-SEM (S-5000, Hitachi Co., Ltd., Tokyo, Japan) operated at 5.0 kV. A histogram of the ChNF widths was constructed from several FE-SEM images using ImageJ software (NIH, Bethesda, MD, USA).

Optical transmittance. The ChNF dispersions (1 wt%) were loaded into quartz cuvettes. The transmittance was measured in the range of 200–700 nm at 25 °C using a spectrophotometer (V530, Jasco Co., Ltd., Tokyo, Japan) with distilled water as the blank. Transmittance at 600 nm was used to compare the different conditions.

Protein measurement. The protein content of the hydrolysate was analyzed according to the Lowry assay using BSA as the standard.[30]

Molecular weight (MW) distribution of proteins. The MW distribution of the protein obtained was measured using a size-exclusion high-performance liquid chromatography (SEC-HPLC), which was equipped with a UV detector (SPD-20AV, Shimadzu Corp., Kyoto, Japan) at a wavelength of 215 nm, and a size-exclusion column (Superdex Peptide10/300GL, GE Healthcare, IL, USA). The standards used were Ala (98 Da), D-Ala-Gly-Gly (203 Da), aprotinin (6,500 Da), ribonuclease A (13,700 Da), and carbonic anhydrase (29,000 Da). The mobile phase was 0.05 mol/L phosphate buffer, 0.15 mol/L sodium chloride, pH 6.8 at a flow rate of 0.7 mL/min. A calibration curve was plotted for the elution time vs. the absolute MW of the standards, from which the relative mean MW of the protein samples was estimated.

Measurement of ACE inhibitory activity. ACE inhibitory activity was assessed according to the method described by Cushman and Cheung with some modifications.[31] A sample solution (25 µL) was mixed with 25 µL of ACE (50 U/L), BSA (0.2 g/L) and sodium borate buffer (100 mmol/L), and then pre-incubated for 5 min at 37 °C. Separately, 8.3 mmol/L of *N*-Hippuryl-His-

Leu tetrahydrate (HHL) (Sigma-Aldrich, MO, USA) solution was prepared by dissolving 0.1 mol /L sodium borate buffer, pH 8.3, containing 0.5 mol/L sodium chloride. The reaction was initiated by adding 75 μL of HHL solution to 50 μL of the sample and ACE solution, which was incubated for 60 min at 37 °C. The reaction was stopped by adding of 250 μL of captopril solution 10 mg/L in a mixture of acetonitrile-methanol- acetic acid (5:5:1). The sample was separated by centrifugation (10,000 ×*g*, 5 min, 4 °C). The hippuric acid liberated by the ACE reaction in the supernatant was analyzed by HPLC on a J'sphere-ODS-M80 (4.6 mm × 250 mm) column (YMC, Kyoto, Japan). The flow rate was 1 mL/min with a mobile phase of 20% methanol and 80% ammonium phosphate buffer (20 mmol/L, pH 3.2) and monitored by UV at 228 nm. ACE inhibition was calculated using the following equation:

$$ACE\ inhibition\ (\%) = \frac{\left(Abs_{no\ sample} - Abs_{sample}\right)}{Abs_{no\ sample}} \times 100 \quad (5)$$

where $Abs_{no\ sample}$ is the absorbance of the enzyme-substrate mixture in the absence of squid pen proteins, whereas Abs_{sample} is the absorbance of the enzyme-substrate mixture in the presence of proteins. The protein concentration effecting a 50% reduction of ACE activity was defined as the 50% inhibitory concentration (IC_{50}) value.

3. Results and Discussion

Composition of squid pen. The inorganic component of the squid pen was determined by weight difference after burning, which was less than 1%. The protein composition of the squid pen is 61 wt% (Table 1). High ratio amino acids are alanine, glycine, proline, and histidine. Furthermore, the protein composition was also evaluated from the remaining weight after deproteinization, in

which the squid pen (2 g) was soaked in an aqueous sodium hydroxide solution (1 mol/L) for 2 h at 70 °C with stirring and we obtained 61 wt% of the protein composition. Therefore, the β-chitin composition was evaluated as 39 wt% by subtracting the weight ratio of the protein from the squid pen.

Table 1. Amino acid composition of squid pen.

	Total amino acids	
	mg/g	mol%
Asp	27.6	4.5
Thr	17.6	2.9
Ser	17.5	2.9
Glu	28.3	4.6
Pro	76.2	12.5
Gly	82.3	13.5
Ala	89.4	14.6
Cys	4.1	0.7
Val	39.9	6.5
Met	6.8	1.1
Ileu	14.5	2.4
Leu	38.6	6.3
Tyr	59.8	9.8
Phe	19.3	3.2
His	63.3	10.4
Lys	22.1	3.6

Try	ND	ND
Arg	3.5	0.6
Total	610.8	100

Weight change of squid pen. Figure 1 shows the weight change of the high-temperature water-treated squid pen at (a) 150 °C, (b) 200 °C, (c) 250 °C, and (d) 300 °C. At 150ºC, the weight change of the squid pen decreases slowly. At 200 °C, the weight change of the squid pen decreases gradually, and plateaus at approximately around 30%–40%. At 250 °C, the weight change of the squid pen decreases to 30%–40% at 30 min, which was faster than that at 150 and 200 °C. Whereas at 300 °C, the weight change of the squid pen decreases drastically and reaches 0% at 30 min. The weight change of squid pen is due to the progress of protein hydrolysis as described later. The rate of weight change increases with increasing treatment temperature. Additionally, the final values of weight change after treatment with high-temperature water at 200 and 250 °C were approximately 30%–40%.

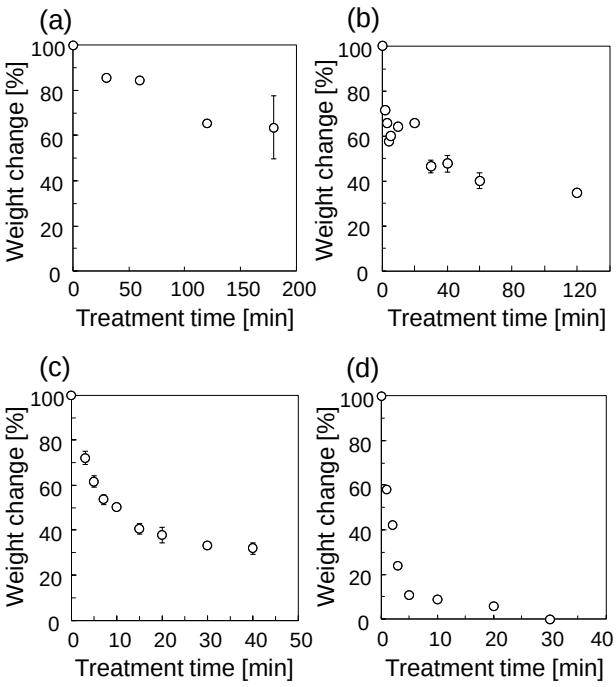


Figure 1. Effect of high-temperature water treatment on the weight of squid pen at (a) 150 ºC, (b) 200 ºC, (c) 250 ºC, and (d) 300 ºC.

Crystallographic parameters of the solid residue. Figure 2 shows the XRD patterns of untreated and 250 ℃ water treated squid pen. The crystallographic parameters of the squid pen are listed in Table 2. For the untreated squid pen, we observed a peak derived from β-chitin which has two diffraction peaks at 8.5 and 19.7° corresponding to the [010] and [1–10] mixed planes, respectively.[12] The crystallinity index (CI) and the crystal size (CS) of the squid pen increase with high-temperature water treatments, although the *d*-spacing remains unchanged. Previously, we reported the effect of high-temperature water treatment on the crystallographic parameters of pure β-chitin and observed the same tendency of squid pen in this work.[27]

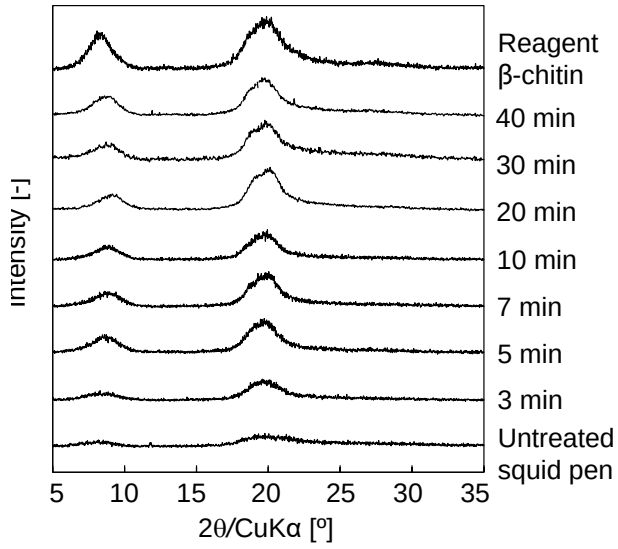


Figure 2. Effect of high-temperature water treatment at 250 ºC on the X-ray diffraction pattern of squid pen.

Table 2. Crystallinity index (CI), *d*-spacing, and crystal size (CS) values determined for squid pen before and after the high-temperature water treatment.

Heating temperature [°C]	Heating time [min]	CI [%]	*d*-spacing [Å]		CS [nm]
		[1–10]	[010]	[1–10]	[1–10]
No treatment		76	10.8	4.5	5.7
150	30	79	10.1	4.5	6.2
	60	79	10.1	4.5	6.0
	120	81	10.0	4.5	6.2
	180	84	10.3	4.4	6.5
200	10	85	11.3	4.6	9.4
	20	89	10.6	4.6	7.2
	40	83	10.1	4.5	7.2
	60	89	10.5	4.4	7.0
250	3	87	10.7	4.5	6.6
	5	88	10.5	4.5	7.4
	7	91	10.3	4.4	7.5
	10	89	9.9	4.7	8.1
	20	86	9.7	4.4	7.0
	30	78	10.1	4.5	6.6
	40	82	10.3	4.5	6.6
Reagent β-chitin		85	10.8	4.5	5.8

Chemical structure of the solid residue. Figure 3 shows the FTIR spectra of untreated and at 250 °C high-temperature water treated squid pen. The peaks of the untreated squid pen are like those of reagent β-chitin. The peaks at 3400 and 3300 cm^{-1} are derived from O−H and N−H

stretching, the peaks at 1655 and 1630 cm^{-1} are amide I bands and the peak at 1550 cm^{-1} is an amide II band.[32] Even after the high-temperature water treatment, the peaks did not change notably. These results indicate that the chemical structure and the intra- and inter-molecular hydrogen bonds of β-chitin are relatively stable for up to 40 min below 250 °C.

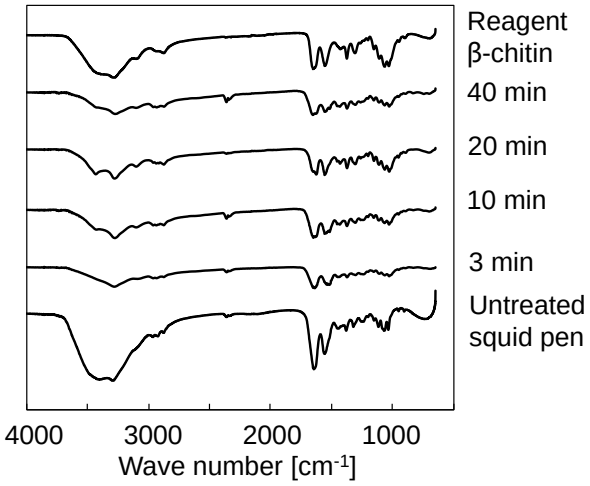


Figure 3. Effect of high-temperature water treatment at 250 ºC on the Fourier transformed infrared spectra of squid pen.

Properties of β-chitin nanofibers (β-ChNFs). The solid residue obtained by high-temperature water treatment was disintegrated into NF by wet pulverization with 10 passes. FE-SEM images of the squid pen after high-temperature water treatment and wet pulverization are shown in Figure 4. NF structures observed for the squid pen treated with high-temperature water. However, lumps are observed even after 10 passes (Figure 4 (h)), indicating that the untreated squid pen is not converted to NFs. Therefore, it may be difficult to disintegrate into NFs from protein and β-chitin structures.

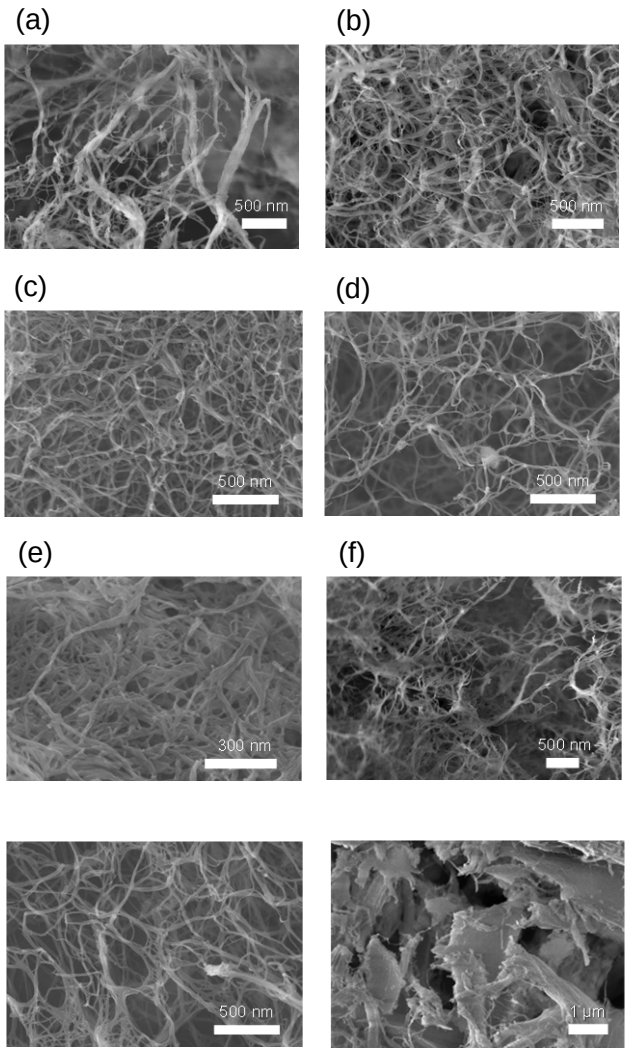


Figure 4. Field-emission scanning electron microscopy micrographs of the β-chitin nanofibers at (a-b) 150 ºC for 120 and 180 min, (c-d) 200 ºC for 60 and 120 min, (e-f) 250 ºC for 10 and 30 min, respectively; (g) Reagent β-chitin; and (h) squid pen (untreated).

The width distributions of the β-ChNFs are shown in Figure 5. The histogram of the reagent β-ChNFs after 10 passes shows a narrow distribution with a 22.2 nm average. Squid pens treated at 150 °C for 120 min and 180 min show wide distributions, and thick fibers above 80 nm in width are often observed. For the squid pen treated at 200 °C, the distribution becomes narrower with

increasing treatment time from 60 to 120 min. Distributing the squid pen treated at 250 ℃ for 10 min is the narrowest, and the distribution is like the reagent β-ChNFs. However, for the squid pen treated at 250 ℃ for 30 min, the distribution becomes wider again.

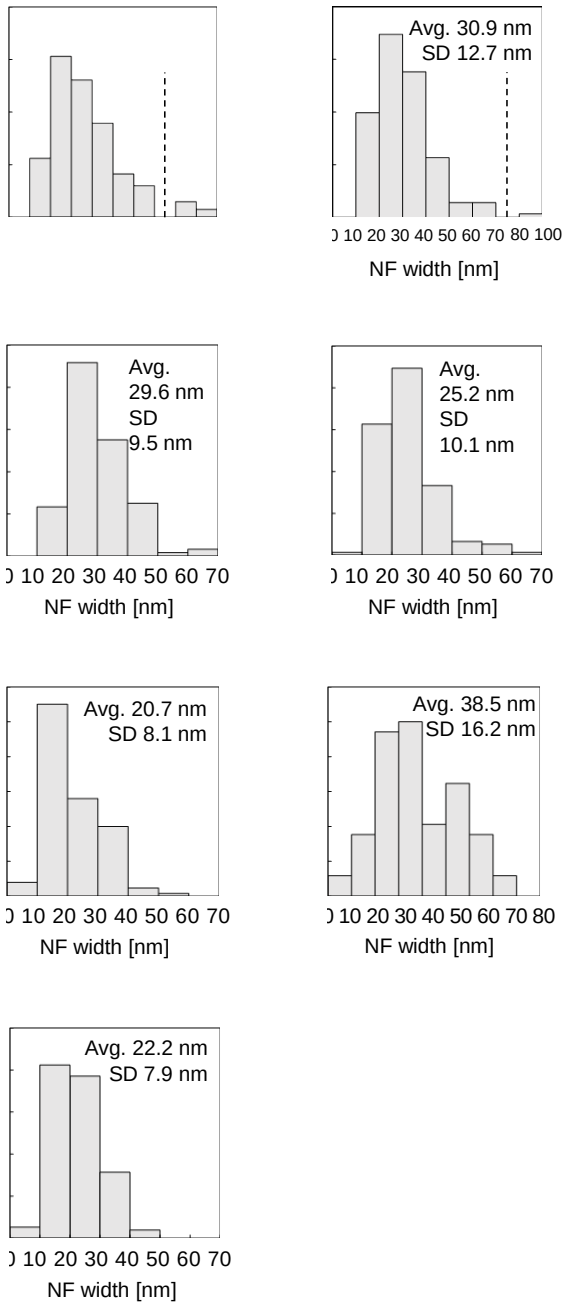


Figure 5. Width distributions of the β-chitin nanofibers at (a-b) 150 ºC for 120 and 180 min, (c-d) 200 ºC for 60 and 120 min, (e-f) 250 ºC for 10 and 30 min, respectively, and with (g) β-chitin reagent.

Photographs of the squid pen and β-ChNFs (1 w/v%) after 10 passes and the transmittance are shown in Figure 6. The transmittance of the samples separated into two phases was measured during one temporary phase after shaking. The reagent β-ChNFs are clear and homogeneous. β-ChNFs obtained at 150 °C for 120 and 180 min become turbid, and two phases can be observed. The β-ChNFs obtained at 200 °C for 60 min also show two phases. The β-ChNFs from the 200 °C treatment for 120 min become clear and one phase. The β-ChNFs obtained at 250 °C for 60 and 120 min separate the two phases again, and the color changes to brown. The transmittance at 200 °C for 120 min is like the reagent β-ChNFs and the appearance of both samples did not change for a week.

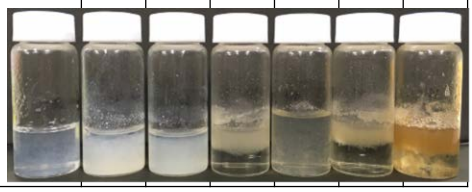
Samples	Reagent β-chitin	Squid pen after high-temperature water treatment					
Temp. [ºC]	-	150	150	200	200	250	250
Time [min]	-	120	180	60	120	10	30
Transparent [%]	82	16	26	21	69	19	8
Photographs							

Figure 6. Transmittance and photographs of the β-chitin nanofiber dispersions.

Protein measurements in the liquid product. Figure 7 shows the effect of high-temperature water treatment on the protein yield in the aqueous liquid phase. At 150 °C, the protein yield slowly increases and plateaus at approximately 60%. At 200 °C and 250 °C, the protein yield gradually increases to 50%–60% at 40 min and 15 min, respectively, and subsequently decreases. At 300 °C, the protein yield quickly increases to 50% within 5 min and then decreases gradually. Therefore, the rate of protein yield increases with increasing temperature. Additionally, the maximum value

of the protein yields after high-temperature water treatments is approximately 60%, which is like the protein composition of the raw squid pen.

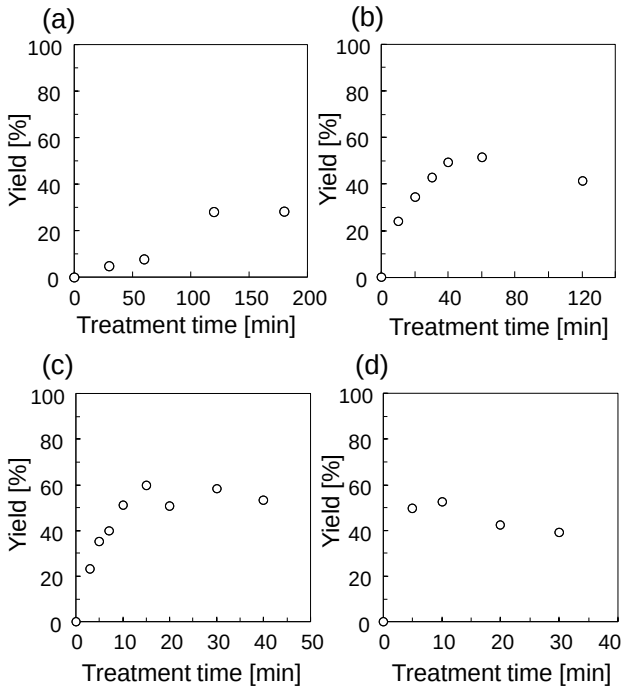


Figure 7. Effect of high-temperature water treatment on the protein yield of squid pen at (a) 150 ºC, (b) 200 ºC, (c) 250 ºC, and (d) 300 ºC.

Molecular weight (MW) of the liquid product. Figure 8 shows the effect of high-temperature water treatment on the MW distribution of the liquid products. Table 3 shows the area ratio of each MW presented in Figure 8. For the 200 °C treatment for 10 min, the main MW is in the range of 1×10^3 ~ 5×10^3. The MW decreases with increasing treatment time, and the main MW is in the range of 2×10^2 ~ 5×10^2 at 200 °C for 60 min. At 250 °C for 3 min, the MW distribution is like that at 200 °C for 10 min. The main MW decreases to 2×10^2 ~ 5×10^2 with the 250 °C treatment for 15

min. Therefore, the rate of MW change increases with increasing treatment temperature. Further, the MW of the liquid products can be controlled by changing the treatment temperature and time.

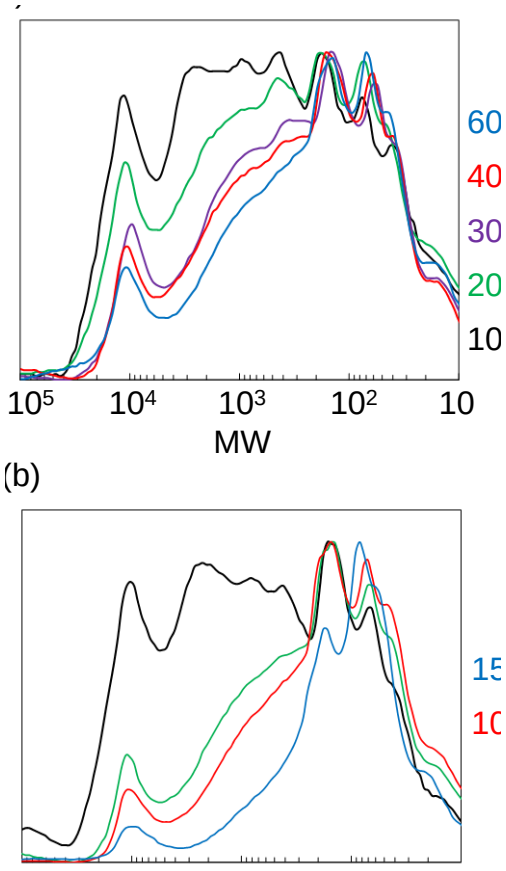


Figure 8. Molecular weight (MW) distribution of the liquid products from squid pen after high-temperature water treatment at (a) 200 ºC and (b) 250 ºC.

Table 3. Molecular weight (MW) distribution of the liquid products from squid pen after high-temperature water treatment.

Temperature [°C]		200					250			
Treatment time [min]		10	20	30	40	60	3	7	10	15
MW	$5\times10^4 \sim 1\times10^5$	0.2	0.4	0.2	0.6	0.4	1.4	0.4	0.1	0.6
	$1\times10^4 \sim 5\times10^4$	13.3	11.2	6.5	6.5	7.9	12.9	5.9	4.2	4.3
	$5\times10^3 \sim 1\times10^4$	9.8	8.7	8.1	6.9	6.4	11.2	6.0	5.2	4.6
	$1\times10^3 \sim 5\times10^3$	31.1	27.5	24.5	24.4	20.6	30.9	22.0	17.8	10.2
	$5\times10^2 \sim 1\times10^3$	13.9	14.6	15.8	15.4	15.0	13.2	15.6	15.0	11.5
	$2\times10^2 \sim 5\times10^2$	18.4	21.4	24.1	23.9	25.2	16.5	24.9	27.8	30.8
	$1\times10^2 \sim 2\times10^2$	13.2	16.2	20.8	22.2	24.6	14.1	25.1	29.8	38.0

ACE inhibitory activity of the liquid product. Figure 9 shows the effect of high-temperature water treatment on the ACE inhibitory activity of the liquid products. At 150 °C, the ACE inhibitory activity remains at approximately 40% regardless of treatment time. At 200 °C, the ACE inhibitory activity peaks at approximately 40 min. At 250 °C, the ACE inhibitory activity is higher at shorter treatment times, and it gradually decreases to approximately 10% at 40 min.

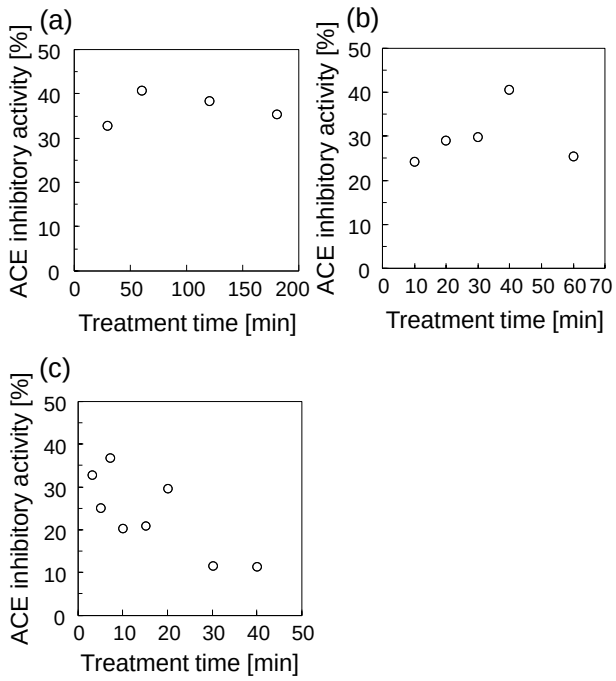


Figure 9. Effect of high-temperature water treatment on the angiotensin 1-converting enzyme (ACE) inhibitory activity of liquid product from squid pen at (a) 150 ºC, (b) 200 ºC, and (c) 250 ºC.

Figure 10 shows the ACE inhibitory activity of the squid pen treated at 200 °C for 40 min and at 250 °C for 3 min in a dose-dependent manner, with half maximal inhibitory concentration (IC_{50}) values of 0.15 mg/mL and 0.2 mg/mL, respectively. These IC_{50} values are comparable with those of reported peptides from marine sources derived from enzymatic hydrolysis such as cobia head and dwarf gulper shark,[24] sea bream scales,[33] and Wakame (*Undaria pinnatifida*).[34] However, unlike these marine peptides, we did not use an expensive enzyme, which is an advantage of this method.

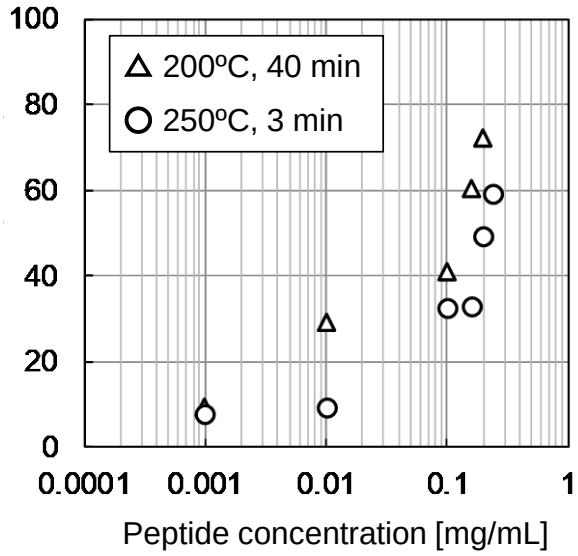


Figure 10. Half maximal inhibitory concentration (IC_{50}) of the liquid product from squid pen.

Conversion of squid pen into solid β-chitin and water-soluble peptides. The final weight change after treatment with high-temperature water below 250 °C was at approximately 30%–40%, which corresponds to the composition of β-chitin in the squid pen. The protein yields in aqueous medium after treatment with high-temperature water were approximately 50%–60%, which is close to the protein composition in the squid pen. From the XRD and IR analyses (Fig. 2, 3, and Table 2), the solid residue is mainly β-chitin. The chemical structure of β-chitin is relatively stable up to 40 min at 250 °C. These results indicate that after high-temperature water treatment, β-chitin and protein can be obtained as a solid residue and liquid-based solution, respectively.

Relationship between β-ChNFs properties and the weight change and protein yield. The weight change of the squid pen and amount of remaining protein were strongly related to the

physicochemical properties of β-ChNFs such as their distribution and transmittance. At 150 °C for 120 and 180 min, the weight change (Fig. 1 (a)) and the protein yield (Fig. 7 (a)) are 60% and 30%, respectively, indicating that 30%–40% of protein remained in the solid residue. The average NF width at 150 °C for 120 and 180 min is greater than reagent β-ChNF (Fig. 5), which is probably because of the remaining protein. At 200 °C for 60 min, the weight change (Fig. 1 (b)) and the protein yield (Fig. 7 (b)) were 40% and 50%, respectively, indicating that 0%–10% of the protein remains in the solid residue. The NF width at 200 °C for 60 min is slightly greater than reagent β-ChNF (Fig. 5), which is probably related to the low amount of remaining protein. At 200 °C for 120 min, the weight change is 40% with a 40% protein yield, which is observed after the peak of protein yield. In high-temperature water, the protein is hydrolyzed to amino acids, and thereafter, the amino acid is decomposed.[7–9] In this work, the protein yield was estimated by the Lowry method, and the amino acid and decomposition products could not be detected. Therefore, the protein yield showed a peak relative to the treatment time. Thus, there is no remaining protein was after treatment at 200 °C for 120 min. The width of the correspondent NF is like that of reagent β-ChNF (Fig. 7), which is probably due to the complete removal of the protein. The weight change (Fig. 1 (c)) and protein yield (Fig. 7 (c)) at 250 °C for 10 min are both 50%, indicating that 10% of the protein remains in the solid residue. However, the average NF width at 250 °C for 10 min is smaller than reagent β-ChNF (Fig. 5), which is probably due to the partial decomposition of β-chitin.[35–37] At 250 °C for 30 min, the weight change and protein yield are 30% and 60%, respectively, indicating that 0% of the protein remains in the solid residue. However, distributing the NF width at 250 °C for 30 min differs from that of reagent β-ChNF (Fig. 5), which is probably due to the partial decomposition of β-chitin. From the XRD and IR data, the crystallite structure

and chemical bonds do not change significantly; however, they are slightly decomposed, and the properties of β-ChNF are changed at 250 °C for 30 min.

Relationship between ACE inhibitory activity and molecular weight (MW) of protein. The MW of the protein decreased through hydrolysis in high-temperature water; however, it occurred at lower temperatures than β-chitin. Proteins with a high MW may change a peptide with a low MW. The function of peptides, such as ACE inhibitory activity, is affected by the MW.[24] High-temperature water treatment can achieve not only extraction of peptides but also control the MW by treatment temperature and time.

As shown in Figure 9, the ACE inhibitory activities peak at 200 °C for 40 min and at 250 °C for 3 min. The MW distribution at 200 °C for 40 min was between 30 min and 60 min. Peptides with a higher MW within 30 min and those with a lower MW over 60 min are not effective for ACE inhibitory activities. At 250 °C, the MW distribution at 3 min was higher than that at 7 min, and the maximum ACE inhibitory activity was also observed at 3 min. These results indicate that there is an appropriate MW for the ACE inhibitory activity, and higher and lower MW are not effective. However, the MW distributions at 200 °C for 40 min and at 250 °C for 3 min were different. Previous studies have demonstrated how peptides with a specific sequence of amino acid are effective for ACE inhibitory activity.[24,25] The high ACE inhibitory activity at 200 °C for 40 min and at 250 °C for 3 min is probably related to the amount of the specific peptide; however, it is not clear which peptide was effective in this work.

High-temperature water treatment for complete utilization of squid pen. The non-catalytic hydrolysis of the protein in high-temperature water is due to the higher concentration of OH^- ions at 150–250 °C than that at ambient temperature. The concentration of OH^- ion at 25 °C is 1.0×10^{-7} mol/kg, which increases with temperatures up to approximately 250 °C. For example, the concentrations of OH^- ions at saturated vapor pressures of 150 °C, 200 °C, and 250 °C are 1.8×10^{-6} mol/kg, 2.3×10^{-6} mol/kg, and 2.5×10^{-6} mol/kg, respectively. The dissociation of water molecules into H^+ and OH^- ions is an endothermic process; therefore, the equilibrium constant for this process increases with temperature. The combined effects of high OH^- concentrations and temperatures are responsible for the non-catalytic hydrolysis of proteins observed in the absence of added alkali.

An alkaline aqueous solution is needed for the hydrolysis of proteins, which yields a mixture of protein and an alkaline solution. To use this protein, the alkaline must be neutralized and removed. Therefore, the utilization of the protein obtained from the β-chitin production was not achieved commercially. The process should be optimized to use most of this natural resource, as retaining a limited portion while discarding the residue is not sustainable. A solution to this problem is the use of high-temperature water treatment. The complete utilization of squid pen (i.e., β-chitin and protein) can be achieved using only water.

4. Conclusions

(1) The squid pen was treated with high-temperature water (150–250 °C, 30–120 min), obtaining β-chitin as a solid residue and protein as a water-soluble peptide.

(2) The properties of the β-chitin obtained were like those of the reagent β-chitin.

(3) The physicochemical properties of the β-ChNFs obtained were almost the same as those of the reagent β-ChNFs. β-ChNFs can apply for medical supplies, cosmetics, and food.

(4) The molecular weight of the water-soluble peptide could be controlled by changing the treatment conditions, such as temperature and reaction time.

(5) The water-soluble peptide can be used for lowering blood pressure, which may augment circulation levels.

(6) These results indicate that high-temperature water treatment is an effective method for the complete utilization of squid pen.

(7) In the future, high-temperature water treatment will be used for not only squid pen but also various biomass resources containing carbohydrates and proteins.

AUTHOR INFORMATION

Corresponding Author

*E-mail: osadam@shinshu-u.ac.jp; Tel.: +81-268-21-5458; Fax: +81-268-21-5391

*ORCID: 0000-0003-3232-5857

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was supported by JSPS KAKENHI (grant number 17H04893), Japan.

ACKNOWLEDGMENT

We thank Dr. Nobuhide Takahashi, Dr. Hiroshi Fukunaga, Dr. Iori Shimada, and Dr. Kazuhide Totani, for their substantial intellectual contributions to the conception of the project.

References

[1] M. Osada, C. Miura, Y.S. Nakagawa, M. Kaihara, M. Nikaido, K. Totani, Effect of sub- and supercritical water treatments on the physicochemical properties of crab shell chitin and its enzymatic degradation, Carbohydr. Polym. 134 (2015) 718–725. https://doi.org/10.1016/j.carbpol.2015.08.066.

[2] J.N. Bemiller, R.L. Whistler, Alkaline Degradation of Amino Sugars, J. Org. Chem. 27 (1962) 1161–1164. https://doi.org/10.1021/jo01051a009

[3] K. Gopalan Nair, A. Dufresne, Crab shell chitin whisker reinforced natural rubber nanocomposites. 1. Processing and swelling behavior, Biomacromolecules. 4 (2003) 657–665. https://doi.org/10.1021/bm020127b.

[4] S. Ifuku, H. Saimoto, Chitin nanofibers: Preparations, modifications, and applications, Nanoscale. 4 (2012) 3308–3318. https://doi.org/10.1039/c2nr30383c.

[5] P. Sikorski, R. Hori, M. Wada, Revisit of α-chitin crystal structure using high resolution X-ray diffraction data, Biomacromolecules. 10 (2009) 1100–1105. https://doi.org/10.1021/bm801251e.

[6] F.C. Yang, R.D. Peters, H. Dies, M.C. Rheinstädter, Hierarchical, self-similar structure in native squid pen, Soft Matter. 10 (2014) 5541–5549. https://doi.org/10.1039/c4sm00301b.

[7] A.T. Quitain, H. Daimon, K. Fujie, S. Katoh, T. Moriyoshi, Microwave-assisted hydrothermal degradation of silk protein to amino acids, Ind. Eng. Chem. Res. 45 (2006) 4471–4474. https://doi.org/10.1021/ie0580699.

[8] T. Powell, S. Bowra, H.J. Cooper, Subcritical Water Processing of Proteins: An Alternative to Enzymatic Digestion?, Anal. Chem. 88 (2016) 6425–6432. https://doi.org/10.1021/acs.analchem.6b01013.

[9] T.M. Aida, M. Oshima, R.L. Smith, Controlled Conversion of Proteins into High-Molecular-Weight Peptides without Additives with High-Temperature Water and Fast Heating Rates, ACS Sustain. Chem. Eng. 5 (2017) 7709–7715. https://doi.org/10.1021/acssuschemeng.7b01146.

[10] M. Osada, H. Kobayashi, T. Miyazawa, S. Suenaga, M. Ogata, Non-catalytic conversion of chitin into Chromogen I in high-temperature water, Int. J. Biol. Macromol. 136 (2019) 994–999. https://doi.org/10.1016/j.ijbiomac.2019.06.123.

[11] S. Suenaga, N. Nikaido, K. Totani, K. Kawasaki, Y. Ito, K. Yamashita, M. Osada, Effect of purification method of β-chitin from squid pen on the properties of β-chitin nanofibers, Int. J. Biol. Macromol. 91 (2016) 987–993. https://doi.org/10.1016/j.ijbiomac.2016.06.060.

[12] Y. Nishiyama, Y. Noishiki, M. Wada, X-ray structure of anhydrous β-chitin at 1 Å resolution, Macromolecules. 44 (2011) 950–957. https://doi.org/10.1021/ma102240r.

[13] D. Sawada, Y. Nishiyama, P. Langan, V.T. Forsyth, S. Kimura, M. Wada, Direct determination of the hydrogen bonding arrangement in anhydrous β-chitin by neutron fiber diffraction, Biomacromolecules. 13 (2012) 288–291. https://doi.org/10.1021/bm201512t.

[14] D. Sawada, Y. Nishiyama, P. Langan, V.T. Forsyth, S. Kimura, M. Wada, Water in crystalline fibers of dihydrate β-chitin results in unexpected absence of intramolecular hydrogen bonding, PLoS One. 7 (2012) 4–11. https://doi.org/10.1371/journal.pone.0039376.

[15] Y. Fan, T. Saito, A. Isogai, Preparation of chitin nanofibers from squid Pen β-chitin by simple mechanical treatment under acid conditions, Biomacromolecules. 9 (2008) 1919–1923. https://doi.org/10.1021/bm800178b.

[16] S. Suenaga, K. Totani, Y. Nomura, K. Yamashita, I. Shimada, H. Fukunaga, N. Takahashi, M. Osada, Effect of acidity on the physicochemical properties of α- and β-chitin nanofibers, Int. J. Biol. Macromol. 102 (2017) 358–366. https://doi.org/10.1016/j.ijbiomac.2017.04.011.

[17] S. Suenaga, M. Osada, Self-Sustaining Cellulose Nanofiber Hydrogel Produced by Hydrothermal Gelation without Additives, ACS Biomater. Sci. Eng. 4 (2018) 1536–1545. https://doi.org/10.1021/acsbiomaterials.8b00026.

[18] S. Suenaga, M. Osada, Parameters of Hydrothermal Gelation of Chitin Nanofibers Determined Using a Severity Factor, Cellulose. 25 (2018) 6873–6885. https://doi.org/10.1007/s10570-018-2053-3.

[19] S. Suenaga, M. Osada, Preparation of β-chitin nanofiber aerogels by lyophilization, Int. J. Biol. Macromol. 126 (2019) 1145–1149. https://doi.org/10.1016/j.ijbiomac.2019.01.006.

[20] J. Machida, S. Suenaga, M. Osada, Effect of the Degree of Acetylation on the Physicochemical Properties of α-Chitin Nanofibers, Int. J. Biol. Macromol. 155 (2020) 350–357. https://doi.org/10.1016/j.ijbiomac.2020.03.213.

[21] Y. Ogawa, R. Hori, U.J. Kim, M. Wada, Elastic Modulus in the Crystalline Region and the Thermal Expansion Coefficients of α-Chitin Determined using Synchrotron Radiated X-ray Diffraction, Carbohydr. Polym. 83 (2011) 1213–1217. https://doi.org/10.1016/j.carbpol.2010.09.025.

[22] L. Liu, R. Wang, J. Yu, J. Jiang, K. Zheng, L. Hu, Z. Wang, Y. Fan, Robust Self-Standing Chitin Nanofiber/Nanowhisker Hydrogels with Designed Surface Charges and Ultralow Mass Content via Gas Phase Coagulation, Biomacromolecules. 17 (2016) 3773–3781. https://doi.org/10.1021/acs.biomac.6b01278.

[23] R. Jayakumar, V. V. Divya Rani, K.T. Shalumon, P.T.S. Kumar, S. V. Nair, T. Furuike, H. Tamura, Bioactive and Osteoblast Cell Attachment Studies of Novel α- and β-Chitin Membranes for Tissue-Engineering Applications, Int. J. Biol. Macromol. 45 (2009) 260–264. https://doi.org/10.1016/j.ijbiomac.2009.06.002.

[24] A. Kaur, B.A. Kehinde, P. Sharma, D. Sharma, S. Kaur, Recently Isolated Food-derived Antihypertensive Hydrolysates and Peptides: A Review, Food Chem. 346 (2021) 128719. https://doi.org/10.1016/j.foodchem.2020.128719.

[25] L. Xue, R. Yin, K. Howell, P. Zhang, Activity and Bioavailability of Food Protein-derived Angiotensin-I-converting Enzyme–inhibitory Peptides, Compr. Rev. Food Sci. Food Saf. 20 (2021) 1150–1187. https://doi.org/10.1111/1541-4337.12711.

[26] M. Osada, C. Miura, Y.S. Nakagawa, M. Kaihara, M. Nikaido, K. Totani, Effect of sub- and supercritical water pretreatment on enzymatic degradation of chitin, Carbohydr. Polym. 88 (2012) 308–312. https://doi.org/10.1016/j.carbpol.2011.12.007.

[27] M. Osada, C. Miura, Y.S. Nakagawa, M. Kaihara, M. Nikaido, K. Totani, Effects of supercritical water and mechanochemical grinding treatments on physicochemical properties of chitin, Carbohydr. Polym. 92 (2013) 1573–1578. https://doi.org/10.1016/j.carbpol.2012.10.068.

[28] W. Wagner, A. Pruß, The IAPWS formulation 1995 for the thermodynamic properties of ordinary water substance for general and scientific use, J. Phys. Chem. Ref. Data. 31 (2002) 387–535. https://doi.org/10.1063/1.1461829.

[29] R.L. Lavall, O.B.G. Assis, S.P. Campana-Filho, β-Chitin from the Pens of Loligo sp.: Extraction and Characterization, Bioresour. Technol. 98 (2007) 2465–2472. https://doi.org/10.1016/j.biortech.2006.09.002.

[30] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent., J. Biol. Chem. 193 (1951) 265–275. https://doi.org/10.1016/s0021-9258(19)52451-6.

[31] D.W. Cushman, H.S. Cheung, Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung, Biochem. Pharmacol. 20 (1971) 1637–1648. https://doi.org/10.1016/0006-2952(71)90292-9.

[32] J. Brugnerotto, J. Lizardi, F.M. Goycoolea, W. Argüelles-Monal, J. Desbrières, M. Rinaudo, An infrared investigation in relation with chitin and chitosan characterization, Polymer. 42 (2001) 3569–3580. https://doi.org/10.1016/S0032-3861(00)00713-8.

[33] A. Fahmi, S. Morimura, H.C. Guo, T. Shigematsu, K. Kida, Y. Uemura, Production of Angiotensin I Converting Enzyme Inhibitory Peptides from Sea Bream Scales, Process Biochem. 39 (2004) 1195–1200. https://doi.org/10.1016/S0032-9592(03)00223-1.

[34] M. Sato, T. Hosokawa, T. Yamaguchi, T. Nakano, K. Muramoto, T. Kahara, K. Funayama, A. Kobayashi, T. Nakano, Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Wakame (Undaria pinnatifida) and Their Antihypertensive Effect in Spontaneously Hypertensive Rats, J. Agric. Food Chem. 50 (2002) 6245–6252. https://doi.org/10.1021/jf020482t.

[35] M. Osada, K. Kikuta, K. Yoshida, K. Totani, M. Ogata, T. Usui, Non-catalytic synthesis of Chromogen i and III from N-acetyl-d-glucosamine in high-temperature water, Green Chem. 15 (2013) 2960–2966. https://doi.org/10.1039/c3gc41161c.

[36] M. Osada, K. Kikuta, K. Yoshida, K. Totani, M. Ogata, T. Usui, Non-catalytic dehydration of N,N′-diacetylchitobiose in high-temperature water, RSC Adv. 4 (2014) 33651–33657. https://doi.org/10.1039/c4ra06319h.

[37] M. Osada, S. Shoji, S. Suenaga, M. Ogata, Conversion of N-acetyl-D-glucosamine to nitrogen-containing chemicals in high-temperature water, Fuel Process. Technol. 195 (2019) 106154. https://doi.org/10.1016/j.fuproc.2019.106154.

TOC/Abstract Graphic.