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Research Article

High Hydrostatic Pressure-Assisted Extraction of High-Molecular-Weight Melanoidins from Black Garlic: Composition, Structure, and Bioactive Properties

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Melanoidin is one of the most important ingredients in black garlic due to the high nutritional values and biological functions. High hydrostatic pressures from 200 to 500 MPa were employed to extract the melanoidins from black garlic for improving the extraction efficiency and enhancing the activities. The results indicated that total phenolics, flavonoids, and sugar yields were all increased when pressure was applied and the antioxidant and overall reducing power was maximized. The bioactive properties of protein tyrosine phosphatase 1B, angiotensin-converting enzyme, and trypsin inhibitory activities were also enhanced compared with the control. Moreover, FT-IR spectroscopy indicated high pressure altered the melanoidin structures to different degrees. It was found that an application of 300 MPa for 5 min was the optimal treatment protocol under all operating conditions.

1. Introduction

Black garlic (*Allium sativum*) is processed by aging whole fresh garlic bulbs at elevated temperature under controlled humidity. This results in a color change from white to dark brown due to the enzymatic browning and Maillard reaction which is a result of a condensation between a reducing sugar carbonyl and an amino group [1]. The primary end products of this reaction are melanoidins that contribute to flavor and color and add nutritional value. These compounds are also responsible for the biological effects that include antitumor, antioxidant, and antihypertensive functions as well as blood sugar modulation [2, 3]. Although black garlic is rich in melanoidins, there are few studies on the positive bioactive properties and applications of melanoidins in black garlic, and most researchers have focused on its polyphenol, flavonoid, and S-allyl cysteine content [4, 5].

Melanoidins are heterogeneous polymers, and their purification is problematic [6]. Melanoidin extraction has been accomplished using water extraction, ethanol precipitation, macroporous resin adsorption, and enzyme treatment [7]. However, these extraction methods have the disadvantages of long reaction times, low product yields, and do not purify away all organic contaminants. High hydrostatic pressure (HHP) is an emerging nonthermal and environmentally friendly food processing technology. Materials to be processed are exposed to 400–600 MPa (58–87,000 psi) conditions for certain amount of time at room temperature [8, 9]. This increases the mass transfer rate, efficiently damages cell membranes, enhances solvent permeability, and improves production efficiency. The processing times are reduced, and overall operating conditions are improved [10]. HHP has been successfully used for bioactive compound extraction from both plant and animal tissues to enhance yields while improving biological activities of target compounds [11–15]. However, there are few studies on the application of HHP treatment to the extraction of melanoidins from black garlic. Therefore, the objective of this study was to apply HHP to extract melanoidins from black garlic and to investigate the effect of

HHP on the compositional, structural, and biological activity properties of melanoidins.

2. Materials and Methods

2.1. Materials and Processing. Black garlic was purchased from Dali Pinhong Plateau Agricultural Science and Technology Development (Yunnan Province, China). Dichloromethane, potassium persulfate, potassium ferricyanide, trichloroacetic acid, ferric chloride, sodium dihydrogen phosphate, and disodium phosphate were purchased from Sinopharm Chemical Reagent (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-*α*-benzoyl-dl-arginine-*p*-nitroaniline (BApNA) was obtained from Macklin Biochemical (Shanghai, China). All chemicals and reagents used were of analytical grade.

Peeled garlic was mixed with distilled water (1 : 3, w/w) and ground for 5 min in a colloid mill. Samples (~150 mL) were packed in thermosealed bags and processed using an S-IL-100-350-09-W HP food processor (Stansted Fluid Power, Harlow, UK) at 25°C and 200 MPa for 5, 15, and 25 min and for 5 min at 300, 400, and 500 MPa. Samples not exposed to high pressure conditions served as controls. Melanoidins were extracted from the processed black garlic samples as previously described with some modifications [16]. In brief, samples were removed from the bags and centrifuged at 7000 × *g* for 5 min. 300 mL of the supernatant was defatted with 200 mL dichloromethane twice, and the aqueous phase (15 mL) was dialyzed against 1 L distilled water using an ultrafiltration membrane with a 12 kDa molecular weight cutoff. The water was exchanged every 3 h for 24 h. The high-molecular-weight melanoidins obtained were freeze-dried and stored at −20°C until analysis.

2.2. Analytical Procedures. Total phenolics and flavonoids were measured using commercial plant test kits (Jiancheng Bioengineering, Nanjing, China). Total sugars were measured using a total sugar test kit from Solarbio Science (Beijing, China). All three procedures used the protocols suggested by the manufacturers. The specific extinction coefficients for purified melanoidins were determined at 280, 325, and 405 nm as previously described using the Beer–Lambert equation [17]. In brief, lyophilized samples were dissolved in distilled water to 1.5 mg/mL by weighing to generate absorbance values between 0.1 and 1.3. Absorption was measured using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The specific extinction coefficient (K_{mix}) was calculated by the law of Lambert–Beer: $E = K_{mix} \times C \times B$, where E was the absorbance of the sample solution, C was the concentration of the sample solution (gL^{-1}), and B was length of light path (cm^{-1}).

Elemental analysis for total carbon, hydrogen, and nitrogen was determined using a Vario EL Cube instrument (Elementar, Langenselbold, Germany). Oxygen content was determined using a Flash Smart element analyzer (Thermo Fisher, Shanghai, China). The protein content was determined by calculation using a factor $5.5 \times N$ content as described [18]. The results are shown in percentages by weight. Fourier-transform infrared spectroscopy (FT-IR) analysis was performed using a Nicolet 6700 instrument (Thermo Scientific, Pittsburg, PA, USA) with the KBr pellet method. Spectra were recorded from 400 and 4000 cm^{-1} at a 2 cm^{-1} resolution with 100 scans.

Antioxidant activity in samples were measured in aqueous solution at 5 mg/mL. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity (DPPH%) was performed as previously reported [19]. In brief, 100 μL of each sample was added to 900 μL of 0.1 mM DPPH in 95% ethanol and allowed to react in the dark for 30 min. The absorbance was measured at 517 nm using water (A_c) instead of sample (A_s) as a blank. The DPPH radical-scavenging ability was calculated as $(1-A_s/A_c) \times 100$. ABTS (2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]) radical-scavenging activity (ABTS%) was performed as previously described [20]. In brief, 7 mM ABTS was mixed with 2.45 mM potassium persulfate solution for 12 h in the dark. The mixture was diluted with phosphate buffer (5 mM, pH 7) to make its absorbance 0.7 ± 0.02 at 734 nm to obtain the $ABTS^{\bullet+}$ stock solution. Sample solution (20 μL) was added into 980 μL of $ABTS^{\bullet+}$ stock solution, and absorbance was measured at 734 nm after 6 min. Distilled water was used as a blank and calculated as DPPH%.

Reducing power was measured as previously described [19]. In brief, 250 μL phosphate buffer (0.2 mM and pH 6.6) and 250 μL potassium ferricyanide (10 g/L) were added into 100 μL sample solution, and the mixture was heated at 50°C for 20 min. 250 μL trichloroacetic acid solution (0.1 kg/L) was added, and the reaction was continued for another 10 min. Then, 250 μL of the mixture was added to 250 μL distilled water and 50 μL ferric chloride (1.0 g/L). The reaction was allowed to progress for 10 min, and absorbance at 700 nm was measured.

2.3. Biological Assays. Angiotensin-I converting enzyme (ACE) inhibitory activity was measured using a commercial kit (Dojindo Chemical Technology, Shanghai, China) as described previously [21, 22]. In brief, the amount of 3-hydroxybutyric acid generated from the substrate 3-hydroxybutyryl-Gly-Gly-Gly was measured at 450 nm using a microplate reader (Thermo Scientific, Pittsburg, PA, USA). The results were expressed as the percentage of ACE inhibitory activity (%) as follows: ACE inhibitory activity $(\%) = 100\% \times (A_{blank1} - A_{sample})/(A_{blank1} - A_{blank2})$, where A_{blank1} was the absorbance of control wells, A_{sample} was the absorbance of sample-treated wells, and A_{blank2} was the absorbance of blank wells.

Trypsin inhibitory activity was measured as previously described with some modifications [23]. In brief, 6 mg bovine trypsin was dissolved in 10 mL of Tris-HCl (pH 7.4) buffer to obtain a 1 mM trypsin stock solution. The 0.03 mM substrate solution was 13 mg/mL BApNA (*N*-*α*-benzoyl-dl-arginine-*p*-nitroaniline) in 1 mL DMSO. Equal parts (50 μL) of Tris buffer, substrate, and sample solutions were added to 96-well plates successively. The plates were incubated at 37°C

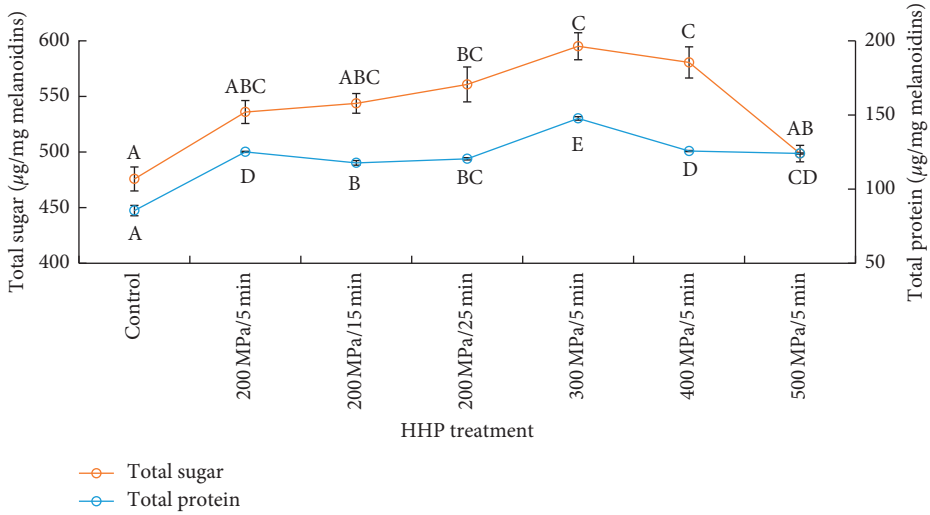

FIGURE 1: Total sugar and total protein contents in melanoidins after different HHP treatments. Different uppercase letters in the same line indicate significant differences at the $P < 0.05$ level.

for 10 min, 50 μL trypsin stock solution was added to each well, and the reaction was continued at 37°C for 10 min with stirring. Acetic acid (33% v/v) was added to stop the reactions, and the absorbance was measured at 410 nm. The inhibitory activity was calculated as follows: trypsin (%) = $100 \times (A - B)/(C - D)$, where A was absorbance of the sample in the total reaction mixture, B was the absorbance of the group where Tris-HCl buffer was instead of trypsin, C was the absorbance of the group where Tris-HCl buffer was instead of samples, and D was the absorbance of the group where Tris-HCl buffer was instead of trypsin stock solution and substrate solution.

Protein tyrosine phosphatase 1B inhibitory activity (PTP-1B) was performed using the Human PTP1B ELISA kit (MLBIO Biotechnology, Shanghai, China) and was performed according to procedures suggested by the manufacturer. The absorbance was measured at 450 nm using a microplate reader to calculated as follows: PTP-1B (%) = $100 \times (1 - A_s/A_c)$, where A_s was the absorbance of test groups and A_c was the absorbance of blank where distilled water was used instead of samples.

2.4. Statistical Analysis. Data were presented with means and standard deviations (SD) calculated using SPSS software v. 19 (IBM, New York, NY, USA). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine the significance of the mean difference. Difference were considered statistically significant at $P < 0.05$.

3. Results and Discussion

3.1. Composition. We initially examined the effects of varying HHP pressures and times on recovery of sugars, phenolics, and flavonoids. HHP treatment significantly increased the total sugar content that began to increase at a pressure of 200 MPa until reaching the maximal release at 300 MPa (595.14 ± 12.14 μg/mg melanoidins). The release declined at higher pressures and showed a biphasic pattern. Interestingly, the treatment times at 200 MPa had no significant effect on total sugar release (Figure 1). The total phenolics and flavonoids were both maximal at 300 MPa for 5 min with 16.6 and 5.4 μg/mg melanoidins, respectively (Figure 2). HHP has been shown to enhance mass transfer rates, and this is most likely the reason for these results. Components including phenolics are incorporated into high-molecular-weight melanoidins, and disrupting these structures facilitates their extraction [17, 24].

The melanoidins in black garlic are present in a complex matrix, and their own composition can vary with the extraction procedure. In these cases, the specific extinction coefficient (K_{mix}) is a unique identifier for melanoidins in mixtures. Absorption at 280 nm is primarily due to the presence of protein and phenolic acids, while 325 and 405 nm peaks can be attributed to the presence of phenolic acid-like compounds such as chlorogenic acid and melanoidins [16, 17]. Therefore, the K_{mix} value at 280, 325, and 405 nm can provide information of the relative content of these compounds. The magnitude of $K_{mix\ 280nm}$ reached a maximum at 400 MPa for 5 min, while both $K_{mix\ 325nm}$ and $K_{mix\ 405nm}$ were maximal at 300 MPa for 5 min. In addition, the K_{mix} values also increased with time at 200 MPa (Table 1). Overall, HHP treatment increased protein, phenolic acids, and melanoidin extraction approximately to 1.75-fold. Interestingly, elemental oxygen significantly decreased over all the treatment levels while carbon and hydrogen remained similar to control levels. However, elemental nitrogen was significantly increased with all the treatments and was maximal at 300 mPa/5 min (Table 1). This increase was most likely the result of an alteration in protein content. Melanoidins are composed of protein and polysaccharides, and total sugars, protein, and melanoidins changed synchronously. This indicated a higher level of purification after HHP treatment. Furthermore, the total sugar content increased, while the total carbon decreased, suggesting that noncovalently bound carbon-containing impurities were

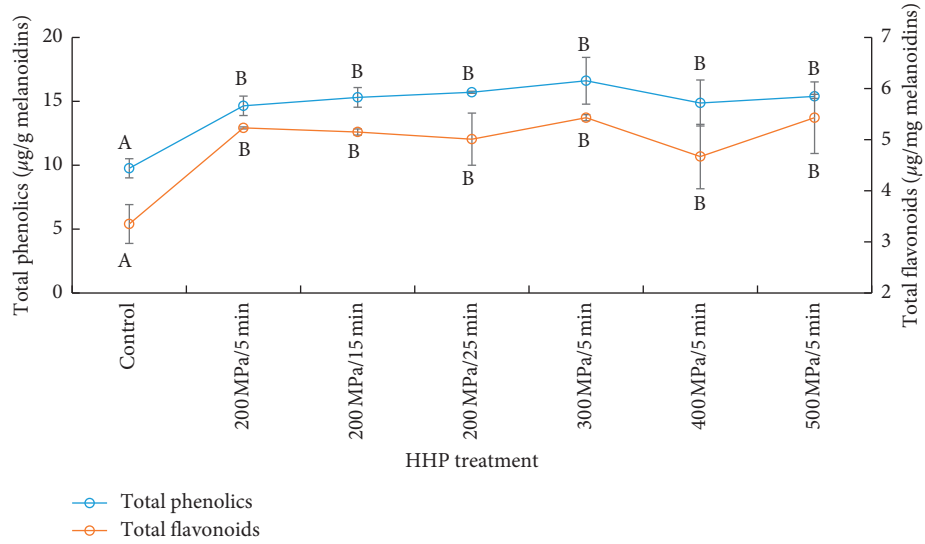

Figure 2: Total phenolic and total flavonoid contents in melanoidins after different HHP treatments. Different uppercase letters in the same line indicate significant differences at the $P < 0.05$ level.

Table 1: Elemental composition and K_{mix} values of black garlic-extracted melanoidins under different high hydrostatic pressure-processing conditions.

	*Elemental composition (wt%)				*K_{mix} values (L/g/cm)		
	C	H	O	N	$K_{mix\ 280nm}$	$K_{mix\ 325nm}$	$K_{mix\ 405nm}$
#Control	38.49 ± 0.15^{A}	6.19 ± 0.03^{A}	46.58 ± 0.49^{A}	1.56 ± 0.06^{A}	1.477 ± 0.083^{A}	1.046 ± 0.057^{A}	0.469 ± 0.018^{A}
200 MPa/5 min	37.93 ± 0.13^{B}	6.01 ± 0.08^{AB}	44.87 ± 0.28^{B}	2.28 ± 0.01^{D}	2.360 ± 0.031^{B}	1.711 ± 0.033^{B}	0.728 ± 0.022^{B}
200 MPa/15 min	38.05 ± 0.02^{B}	6.19 ± 0.04^{A}	42.62 ± 0.69^{C}	2.14 ± 0.03^{B}	2.420 ± 0.011^{BC}	1.737 ± 0.007^{B}	0.743 ± 0.010^{D}
200 MPa/25 min	37.99 ± 0.07^{B}	6.13 ± 0.10^{A}	42.74 ± 0.03^{C}	2.19 ± 0.01^{BC}	2.493 ± 0.007^{CD}	1.800 ± 0.006^{BC}	0.760 ± 0.003^{CD}
300 MPa/5 min	37.52 ± 0.33^{C}	5.90 ± 0.15^{AB}	42.31 ± 1.02^{C}	2.69 ± 0.02^{E}	2.576 ± 0.034^{D}	2.216 ± 0.035^{C}	0.840 ± 0.039^{BC}
400 MPa/5 min	37.82 ± 0.16^{BC}	6.08 ± 0.11^{AB}	42.46 ± 1.43^{C}	2.29 ± 0.01^{D}	2.584 ± 0.059^{D}	1.897 ± 0.072^{C}	0.804 ± 0.026^{B}
500 MPa/5 min	37.87 ± 0.06^{BC}	6.07 ± 0.03^{AB}	42.30 ± 0.59^{C}	2.26 ± 0.01^{CD}	2.462 ± 0.059^{BCD}	1.787 ± 0.038^{B}	0.753 ± 0.018^{BC}

*Values are expressed as mean ± standard deviation (SD). Means with different letters in a column are significantly different ($P < 0.05$). #Atmospheric pressure.

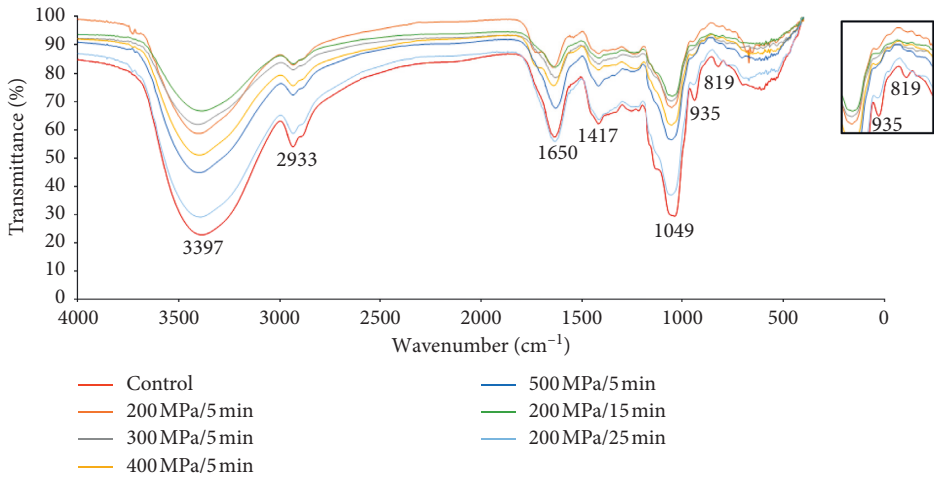

Figure 3: FT-IR spectra of black garlic-extracted melanoidins.

removed by dialysis. These findings support the idea of a dynamic equilibrium of melanoidins in foods; low-molecular-weight compounds are noncovalently linked to high-molecular-weight melanoidins as previously described [25].

3.2. Melanoidin Structural Alterations during HHP. The structure or composition of a polymer can be identified based on the differences in absorption frequencies of different functional groups or chemical bonds using FT-IR.

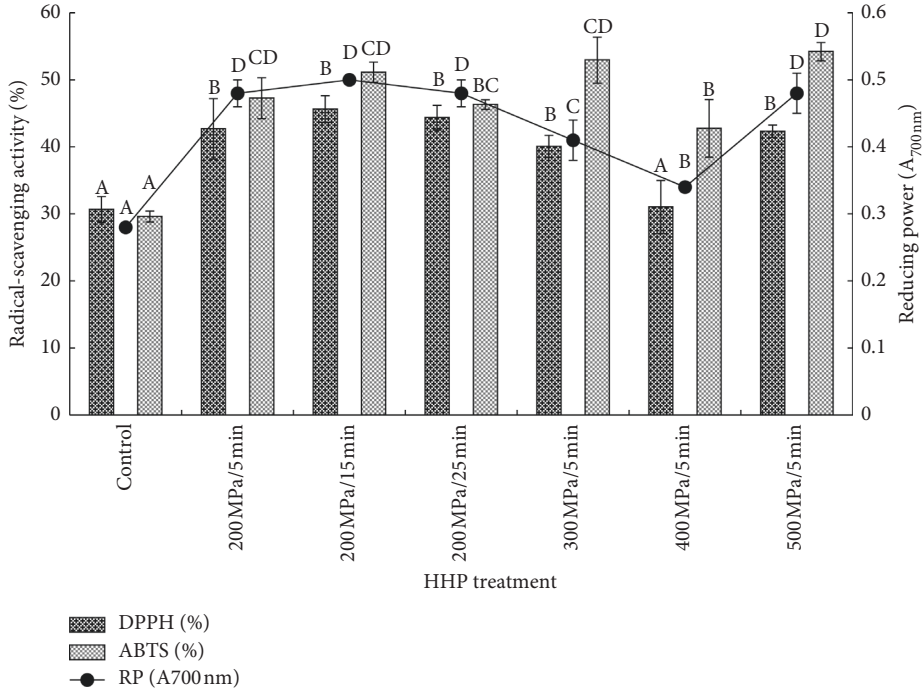

Figure 4: Antioxidant activities of melanoidins after different HHP treatments. Different uppercase letters in the same color bar and line indicate significant differences at the $P < 0.05$ level.

Our overall FT-IR results demonstrated significant changes in transmittance intensity under different HHP conditions, and the control intensity at 84.85% was increased to the maximum at 99.17% at 200 MPa/5 min. There were no significant differences in transmittance intensities at 300, 400, and 500 MPa although they decreased with duration of treatment from 5 to 25 min (Figure 3). The primary differences were in the absorption intensity, suggesting an increase in interchain interactions [26].

The control and 200 MPa/25 min groups displayed 2 strong peaks at 3397 cm^{-1} (-OH) and 1049 cm^{-1} (C-C) that were diminished at the other pressures and times. The medium intensity peak at 2933 cm^{-1} (CH_2) followed the same pattern [27]. The double bond stretching (C=O, C=C, and C=N) at 1650 cm^{-1} was most likely indicative of α-helical amide I stretching [28, 29]. This peak was similar between the control and 200 MPa/25 min and interestingly, was minimal at 200 MPa for 5 and 15 min. A similar result has been previously reported and may reflect that an increased exposure time at a lower pressure was sufficient for maximal extraction [29]. For example, the reduction in the intensities of the 2 strong peaks generated by exposure to 200 MPa for 25 min could only be partially compensated for by an increase in pressure up to 500 MPa. This same pattern was reflected across the total FT-IR spectrum and included the medium peak at 1417 (-CH) [16]. This pattern is indicative of a decrease in interchain interactions [26]. Furthermore, the control sample peaks at 933 and 819 cm^{-1} disappeared with HHP treatment and were most likely a consequence of the significant reductions in α-helix and an increase in signals corresponding to β-sheets [30]. Overall, the changes of the infrared spectra of the extracts after HHP indicated there were alterations in the structures of the associated polymers and HHP has been previously shown to alter noncovalent interactions including hydrogen and hydrophobic bonds. These would lead to an alteration in the protein structure due to unfolding [31].

3.3. Biological Activity Properties. We performed biological activity assays that are related to human physiological processes as surrogates to test the biological activities of our extracts. Overall, the antioxidant activities as well as ACE, PTP-1B, and trypsin inhibition were all markedly enhanced with HHP treatment similar to previous reports [19]. The antioxidant activity can be attributed to different mechanisms, including DPPH radical-scavenging activity, ABTS radical-scavenging activity, reducing power (RP), or a combination of these properties [21]. The DPPH%, ABTS%, and RP were enhanced at 200 MPa with the maximal levels of 45.63%, 51.14%, and 0.50 at 15 min, respectively. However, the three indexes had different patterns of change with altered pressure similar to a previous report [19] (Figure 4). The antioxidant activities of melanoidins against different types of free radicals were different because the reactions differed [21, 32]. DPPH% was improved after different pressure treatments (except for 400 MPa) compared to control. The latter value was the smallest although there were no significant differences between the other sample groups. The ABTS% and RP values were all increased after HHP, and 400 MPa for 5 min displayed the minimum, while 500 MPa for 5 min produced the maximum highest ABTS% ability above control. The RP values of the 500 MPa and 200 MPa for 5 min extracts were similar (Figure 4). Differences in antioxidant activities

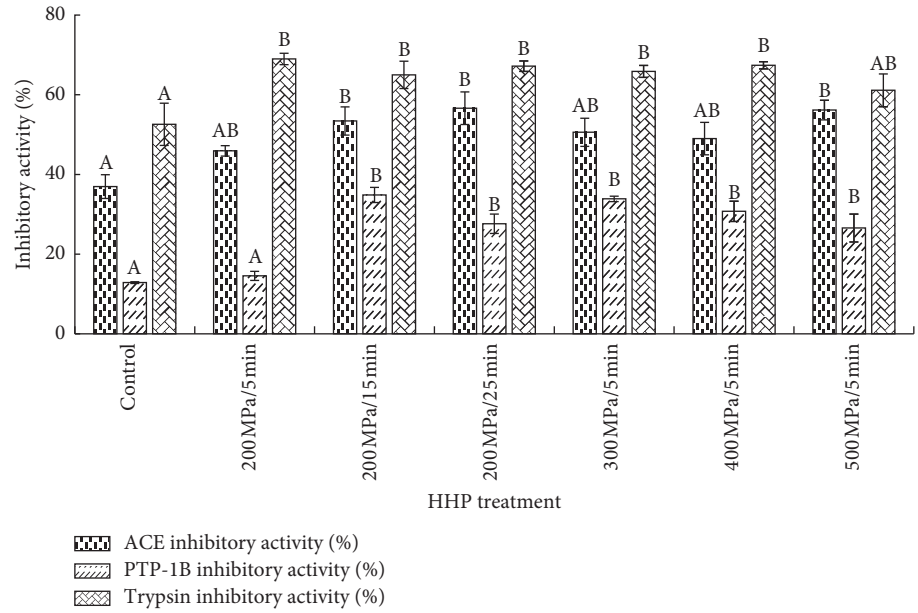

Figure 5: Enzyme inhibitory activities of melanoidins after different HHP treatments. Different uppercase letters in the same color bar indicate significant differences at the $P < 0.05$ level.

have been shown to depend on pressure, treatment duration, as well as the food substrate [15, 19, 33].

ACE levels in blood are directly related to blood pressure because of its effects on water and salt metabolism and its inactivation of the hypotensive peptide bradykinin [34]. We found that melanoidins extracted from black garlic had a strong inhibitory effect on ACE, suggesting a potential for the prevention and treatment of cardiovascular diseases [35]. Compared to the control, ACE inhibitory activity of melanoidins was improved by HHP and the maximum activity (56.62%) was observed at 200 MPa for 25 min although all the HHP treatments were significantly better than control (Figure 5). The primary inhibitory activity was located at the melanoidin core which is partially blocked by other noncovalently-linked compounds [3]. HHP altered the structure that increased core melanoidin exposure.

PTP-1B regulates insulin sensitivity and carbohydrate metabolism, and its inhibition can improve glycemic control [36]. In our study, extracted melanoidins displayed PTP-1B inhibitory activity, suggesting a potential for the prevention and treatment of type 2 diabetes. We found PTP-1B inhibitory activity was increased after HHP treatment with a maximum value at 200 MPa for 15 min (34.85%) similar to the result at 300 MPa for 5 min (33.86%). Longer treatments at 200 MPa and higher pressure reduced activity due the damage to of the melanoidin active site structure. Interestingly, 200 MPa for 5 min was similar to control (Figure 5) and could be attributed to the reversible structural alteration of melanoidins [37].

Melanoidins are also potent trypsin inhibitors and therefore act as antimetabolic factors such as those found with the trypsin inhibitor from soybeans [38]. However, melanoidins also have strong inhibitory effects on trypsin that in turn causes pancreatic hyperfunction and promotes insulin secretion, thereby facilitating the treatment of type 2 diabetes [39]. In our study, trypsin inhibitory of melanoidins was enhanced by HHP treatment. However, the change in trypsin inhibition ability was not obvious with changes in pressure and treatment times (Figure 5). This might be a consequence of the three disulfide bonds in trypsin which significantly contribute to its thermal stability [38].

4. Conclusions

HHP treatment increased the extraction efficiency of melanoidins, and the appropriate treatment pressure and time could significantly increase the content of total phenolics and flavonoids that were noncovalently linked to high-molecular-weight melanoidins. Compounds as well as sugars, protein, and melanoidins all were maximally released at 300 MPa for 5 min. HHP also resulted in significant increases in the antioxidant activities as well as overall reducing power of the extracts. However, the DPPH and ABTS radical-scavenging activities were not significantly different between the maximum value and the value at 300 MPa for 5 min. Importantly, ACE, PTP-1B, and trypsin inhibitory activities were all enhanced using HHP although this effect was relatively insensitive to treatment pressure. Furthermore, our FT-IR analysis indicated that HHP treatment significantly altered melanoidin structures. After a comprehensive consideration of all these factors, the use of HHP at 300 MPa for 5 min was optimal for melanoidin extraction from black garlic. The yields from HHP were greater than when using the traditional extraction procedures. This method can be applied to the development of novel natural food additives.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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