Beneficial Effects of Hydrolysates of Whey Proteins in Spontaneously Hypertensive Rats

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Abstract We compared the effects of whey protein concentrate (WPC, control) with its hydrolysates either by protease M or protease S (the hydrolysates) in spontaneously hypertensive rats (SHR) in comparison to normotensive Wistar Kyoto rats (WKY). First, the hydrolysates demonstrated higher in vitro angiotensin converting enzyme (ACE) inhibition than WPC. In an 8 week of animal study, the hydrolysates decreased systolic blood pressure (SBP) and ACE activity in aorta with a greater reduction than WPC. A significant reduction in tail moment, an indicator of oxidative DNA damage was also detected in the hydrolysates compared to the WPC. Especially protease M-treated hydrolysate show low superoxide dismutase (SOD) activity. Our data indicated that the hydrolysates appeared to have greater blood pressure lowering effects than WPC possibly by greater inhibition of aorta ACE activity along with significant antioxidant roles.

Keywords: hypertension, whey protein concentrates, whey protein hydrolysates, angiotensin-converting enzyme inhibition, chromosomal DNA damage, antioxidants


1. Introduction

Hypertension is a risk factor for the incidence of cardiovascular disorders especially when accompanied by renal failure, diabetes and hyperlipidemia [1]. Although it contributes to deaths every year, hypertension is often inadequately treated [2]. In our body, renin-angiotensin system (RAS) plays a central role in the maintenance of blood pressures partly by controlling blood vessel constriction and heart rates and the secretion of antiuretic hormone. Angiotensin-1-converting enzyme (ACE) in the RAS converts angiotensin I into angiotensin II, which constricts the blood vessel [3]. To decrease blood pressure, the RAS system and/or the function of ACE need to be inhibited. Drugs such as angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers and direct renin inhibitors are aimed to inhibit the RAS system. However, the use of drugs often accompanies the side effects such as cough and headache [4]. Therefore, searching for a naturally occurring products exerting anti-hypertension has become of interest in the field of functional foods to enhance the efficacy of the anti-hypertensive drugs and to reduce the side effects.

On the other hand, close relationships between hypertension and antioxidants have been implicated. Reactive oxygen species (ROS) constrict blood vessels, which may contribute to the progress of hypertension. In studies of both human and animals, elevated levels of ROS and its induced oxidative stress were reported in hypertension [5,6]. Antioxidants-containing diets significantly reduced hypertension in rats [7,8]. Conversely, hypertension was developed when antioxidant capacity was compromised in animal models [9]. Blood pressure was decreased when ROS-generating enzymes were deficient [10].

Whey is abundantly found in milk next to casein and remains in soluble fraction after cheese manufacturing. Its high molecular weight fraction and whey protein hydrolysates were proven to contain antioxidant property in vitro system [11,12]. These antioxidant properties were suggested to be derived in part from the presence of lactoferrin chelating metals [13] and of amino acids scavenging free radicals [14]. Whey protein concentrates and its hydrolysates have anti-hypertension properties [15,16]. Low molecular weight fractions of whey have been implicated in ACE inhibition in vitro assays. Peptides derived from in vitro-digested whey protein were purified and characterized for antihypertension properties, among which lactokinins exerted the greatest effects [17].

Although ACE inhibitor peptides derived from whey were well studied in vitro models, the hypotensive effects and possible mechanisms of action of whey proteins and their hydrolysates have not been extensively investigated in vivo models. In addition, several in vitro hypotensive peptides from casein have been reported to lose their potency in vivo [18,19,20], further suggesting that testing in animals and/or human are necessary steps. In...
the current study, we investigated whether WPC and its hydrolysates might improve hypertension and hypertension-associated oxidative stress in hypertension animal models. The effects on SBP, ACE activities in kidney, aorta and blood, the ratio of nitrate to nitrite, kidney chromosomal DNA damage and the superoxide dismutase (SOD) and the activities of glutathione peroxidase (GPx) and catalase of erythrocytes were investigated in SHR rats after 8 weeks of diets with either WPC or its hydrolysates prepared by protease M (Protease M) or protease S (Protease S).

2. Materials and Methods

2.1. Preparation of 5% WPC and Its Hydrolysates

One hundred liters of unripened cheese whey (Seoul Milk Co. Seoul, Korea) was pH 7.0 adjusted and then heated for 5 min at 70°C and cooled to 50°C, then followed by ultrafiltration at 50°C using a spiral wound type ultrafiltrator (DDS LabUnit M20, Denmark) equipped with membrane with molecular cut off rate of 20,000 daltons. After removing 80~85 liters of permeate 15~20 liters of retentate was concentrated and spray-dried (Buechi, Mini Spray Dryer B-191, Swiss) to produce WPC with protein content of 35.00%(w/w) and lactose content of 53.32%(w/w). The 10 % of WPC solution was used for the enzyme digestion by protease M (Amano Enzyme, Japan) or protease S (Amano Enzyme, Japan) with a mass ratio of 25:1 at 37°C in an incubator (iWKYubator VS-8480S, VISION) while shaking at the speed of 180 rpm. In order to inactive the enzymes the reaction was heated at 95°C in water bath for 10 min. After cooling at room temperature, the centrifugation at 3,000 rpm was followed for 30 min. The supernatant was collected and dried in a freeze dryer (FDU-1200, EYELA) 3,000 rpm was followed for 30 min. The supernatant was collected and dried in a freeze dryer (FDU-1200, EYELA). The supernatant was then blended, centrifuged at 44,000g for 90 mins and the supernatant was diluted to 4mg/ml of concentration. The identical protease solution was used for the enzyme digestion by protease M (Amano Enzyme, Japan) or protease S (Amano Enzyme, Japan) at same condition. Pellet was re-dispersed by 0.5% Triton X-containing Tris-HCl buffer and left at rest for 1 h our.

2.2. Animals and Diets

Eight-week old male normotensive WKY (Wistar Kyoto) rats (n = 5) and SHR (n = 24) were purchased from Bridge, acclimated to pellet for 1 week and trained to adapt to blood pressure measurement. Rats were randomized by weight into 5 groups, which were WKY, SHR, SHR fed with WPC, SHR fed with Protease M and SHR fed with Protease S. All animals were housed individually in separate cages. Each group had 5 rats except SHR (n = 9). Rats were allowed to have free access to water and were pair-fed with experiment diets for 8 weeks. The diet composition was shown in Table 1. Dietary intake was measured daily, weight was measured weekly at constant time and blood pressure was taken by tail cuff method using BP-89A (Sofron, Tokyo, Japan) as indicated time points in the Fig. 2. Cage temperature (22±2°C), humidity (50±5%) and a 12-hr light and dark cycle were constantly monitored and automatically maintained. The study was approved by the Institutional Animal Care and Use Committee of Kyungnam University (KUICA-11-001). Feed efficiency ratio (FER) was calculated by dividing weight increase rate during experimental period by total dietary intake of same time period.

### Table 1. Composition of the Experimental Diet (%)

<table>
<thead>
<tr>
<th></th>
<th>WKY1)</th>
<th>SHR</th>
<th>WPC</th>
<th>Protease M</th>
<th>Protease S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
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<td>20.0</td>
<td>18.3</td>
<td>18.9</td>
<td>18.5</td>
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<tr>
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<td>53.0</td>
<td>52.5</td>
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<tr>
<td>Sucrose</td>
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<td>10.0</td>
<td>7.3</td>
<td>7.8</td>
<td>8.4</td>
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<tr>
<td>Soybean oil</td>
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<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mixture2)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Mineral mixture2)</td>
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<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
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<td>Choline bitartrate</td>
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<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>L-cysteine</td>
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<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
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</tr>
<tr>
<td>t-butylhydroquinone</td>
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<td>0.014</td>
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<td>0.014</td>
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<tr>
<td>BHT3)</td>
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<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>WPC or hydrolysates</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

1)WKY: normotensive Wistar Kyoto rats, SHR: spontaneous hypertensive rats, WPC: SHR fed WPC, Protease M : SHR fed protease M-hydrolyzed WPC, Protease S: SHR fed protease S-hydrolyzed WPC
2)AIN 93 vitamin mixture
3)AIN 93 mineral mixture
4)BHT: butylated hydroxytoluene.

2.3. Collection of Blood and Organ Samples

Rats were fasted for 12 hours, anesthetized with ethyl ether for blood collection via abdominal aorta and blood clotting was prevented using heparin. After centrifugation at 3000 rpm for 30 min to separate plasma and red blood cell, blood was frozen at -70°C until next analysis. Organs were obtained, washed with saline solution, dried with blotter, weight was measured and quick-frozen with liquid nitrogen then stored at -70°C until next analysis.

2.4. In Vitro and in Vivo ACE inhibitory Activity Analysis

Both in vitro and in vivo ACE inhibitory activities were assayed by the modified method of Cushman and Cheung [23]. This was performed based on the principle of measuring hippuric acid, which is formed when ACE acts on the substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu). For in vivo ACE activity measurements, 1 g of kidney and 0.02g of aorta were homogenized prior to the experiments. The kidney was pulverized in cold 50mM Tris-HCl buffer, centrifuged at 44,000g for 90 mins to remove the upper layer. Tris-HCl buffer was added to the remnant pellet, dispersed, and centrifuged at same condition. Pellet was re-dispersed by 0.5% Triton X-containing Tris-HCl buffer and left at rest for 1 hour. After centrifugation at 1,000g for 10 min, upper layer was used as sample. Aorta was pulverized, centrifuged at 1,500rpm for 3 min. The upper layers of samples were diluted to 4mg/ml of concentration. The identical concentration of ACE inhibitor was used as standard.
material. The reaction mixture contained 12.5 mM Hip-His-Leu as substrate in borate buffer (0.2 M H₂BO₃ and 0.05 M Na₂B₄O₇; v:v = 5.5:4.5) containing 0.4 M NaCl. An extracted sample (50 μl) was added to the above reaction mixture and incubated at 37°C for 30 min. Then, 150 μl of ACE (100 mU/ml) was added and set for enzymatic reaction for 30 min. After reaction, 250μl of 0.5 M HCl was added to stop further reaction. The resulting hippuric acid was extracted by the addition of 1.5 ml ethyl acetate. After centrifugation (800×g, 15min), 500μl of the upper layer was transferred into a glass tube and completely evaporated the ethyl acetate in dry oven for about 40 min. 1.5ml of 1M NaCl was added and absorbance was measured at 228 nm. ACE inhibitory activity was calculated as follows: ACE inhibitory activity = (1-(SA-SB)/C)×100, SA= absorbance of sample, SB=absorbance of blank, C=absorbance of control reaction. 

2.5. Measurements of Plasma Nitrate and Nitrite 

Plasma nitrate and nitrite were determined using Schmidt et al. method and the Griess reaction [24,25,26]. For nitrite determination, plasma was diluted fourfold with distilled water and mixed with zinc sulfate. After centrifugation, 100 μl supernatant was mixed with 100 μl Griess reagent (5.8mM sulfanilamide, 25 g/l phosphoric acid, 5.3 mM N-1-naphthylethylenediamine) and was left for 10 min at room temperature. Optical density was measured with spectrophotometer at 540 nm. 25g/L phosphoric acid was used as a control. Calibration curves were generated using sodium nitrate and potassium nitrate in distilled water. Nitrate was determined as nitrite as described by Schmidt et al. Plasma was diluted fourfold with distilled water. 300μl of diluted sample was incubated at 37°C for 20 min after the addition of 200 U/L nitrate reductase, 50 μl of 500 mM NADPH and 50 μl of 50 mM FAD. After that, 50 μl of 100 units/ml lactate dehydrogenase and 50 μl of 100 mM sodium pyruvate were added to the mixture and the mixture was incubated at 37°C for 5 min. After the addition of 25 μl of 930 mM zinc sulfate, the mixture was centrifuged at room temperature at 1000 × g for 15 min. 100 μl supernatant was assayed with 100 μl Griess reagent, as described above.

2.6. Glutathione Peroxidase (GPx) Analysis 

Glutathione, glutathione reductase and NADPH were added to distilled water-hemolysed red blood cell and were incubated at 37°C for 10 min. The reaction was further induced by the addition of t-butyl hydroperoxide. NADPH concentration was then measured by spectrophotometer at 340 nm for 90 sec.

2.7. Catalase Analysis 

Catalase activity of hemolysed RBC was measured by the addition of 50 mM phosphate buffer (pH 7) and hydrogen peroxide. The reduction of hydrogen peroxide was measured by spectrophotometer at 240 nm for 30 sec. 

2.8. Comet Assay of Kidney DNA Damage 

The comet assay was performed with a slight modification of the methods by Singh et al. [27]. 0.5g of kidney tissue was incubated for 10 min in 150 unit collagenase/10 ml HBSS, centrifuged at 40g for 5min, and upper layer was separated. After centrifugation at 700g for 10 min, lower layer was evenly mixed in 1% low melting agarose (LMA) and added to fully frosted slides precoated with 0.5% LMA. For lysis, cold alkali lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris) prepared just before usage, was mixed with 1% Triton X-100. The slide was dipped at low temperature in dark room for 1 hr to unzip DNA double strand. After lysis, the slide was placed in electrophoresis tank and filled with 4°C buffer (300mM NaOH, 10mM Na₂EDTA). The slides went through electrophoresis at 25V/300±3mA for 20 min. Then, the slides were washed thoroughly with a neutralizing buffer (0.4M Tris buffer, pH 7.4) and nuclei were stained with 20 μl/ml ethidium bromide. A fluorescence microscope (DMLB; LEICA, Wetzlar, Germany) was used for the measurement of head and tail DNA and tail length using at least 50 cells from each of two replicate slides. Each cell nucleus image was sent through CCD camera and analyzed using computer-based comet image analyzing system (Komet 4.0; Kinetic Imaging, Liverpool, UK).

3. Results 

3.1. In vitro ACE Inhibition by WPC and the Hydrolysates

In vitro ACE assays were performed for WPC and its hydrolysates (Protease M and Protease S) (Figure 1A). The hydrolysates had higher ACE inhibitory activities than WPC. Especially, Protease M appeared to exert stronger effects than Protease S. These findings suggested that the hydrolysates had greater in vitro ACE inhibition than WPC.

3.2. Effects of WPC and the Hydrolysates on Food Intake, Body Weight, Tissue Weights and Liver Toxicity

Although there were no significant differences in body weight gains (data not shown), average daily food intake was higher in SHR group compared to the control WKY, which was lowered in WPC and Protease M groups with a decreased tendency in the Protease S (Figure 1B). FER was similar among the groups (data not shown). The weights of organs including liver, kidney, heart and aorta per 100 g of body weight were not different among the animal groups. However spleen weighed more in SHR than in WKY, which showed a decreased tendency in the WPC, Protease M and Protease S groups (Figure 1C). The elevation of GOT seen in SHR compared to WKY tended to be lower in WPC and Protease M and was significantly decreased in Protease S (Figure1D). These results indicated that WPC and its hydrolysates reverted SHR-increased food intake and blood GOT levels.
ACE inhibition rate (%) by WPC, Protease M or Protease S was compared to standard (A), average daily food intake (B), the weight of spleen (C) and plasma GOT levels (D) in WKY (n=5), SHR (n=9) and SHR fed WPC (n=5), Protease M (n=5) or Protease S (n=5). Data are presented as mean ± standard error (SE). Values sharing a common superscript letter are not significantly different (p < 0.05).

Figure 1. Effects of diet groups on in vitro ACE inhibition and average daily food intake, spleen weight and GOT in animal groups after 8 weeks of study.

Changes in SBP was monitored over the course of 8 weeks of study in WKY (○, n=5), SHR (●, n=9), WPC (△, n=5), Protease M (▲, n=5) and Protease S (□, n=5). Data are presented as mean ± SE. Values sharing a common superscript letter are not significantly different (p < 0.05).

Figure 2. Effect of WPC or its hydrolysates on SBP during the study period.

3.3. WPC and/or the Hydrolysates Decreased SBP and in vivo ACE Activities and Increased NO Production

Changes in SBP were monitored for 8 weeks (Figure 2A). At 7 and 8 weeks SHR showed a huge increase in SBP compared to WKY, WPC, Protease M and Protease S groups (Figure 2). At 8 weeks, the hydrolysate groups had lower SBP than WPC (Figure 2). To investigate if SBP-lowering effects are mediated by inhibiting ACE activity, we measured ACE activities in kidney, blood and aorta. SHR increased ACE activity in all the tested tissues in comparison to WKY (Figure 3A-C). In the kidney WPC, Protease M and Protease S lowered SHR-elevated ACE activity (Figure 3A). In the blood, WPC decreased ACE activity but the hydrolysates groups failed to lower ACE activity (Figure 3B). On the other hand, the hydrolysates groups reduced SHR-driven ACE activity in aorta, whereas WPC did not (Figure 3C). In terms of the nitrate to nitrite ratio, which is a measurement of nitric oxide (NO) production, SHR decreased the nitrate to nitrite ratio when compared to WKY. But WPC and its hydrolysates groups reverted the SHR-driven reduction in the nitrate to nitrite ratio (Figure 3D). These data indicated that WPC and the hydrolysates decreased SHR-elevated SBP but with greater effects by the hydrolysates (protease M and S) and these SBP-lowering effects were likely to be mediated by the inhibition of ACE activity in the kidney and aorta and probably in the blood, as well as improving SHR-impaired NO production.

3.4. Effects of WPC and the Hydrolysates on DNA Damage in Rat Kidney and Antioxidant Enzyme Activity in Erythrocytes

Although there was a slightly increased tendency in SHR compared to WKY, mean tail moment in the kidney and the enzyme activities of GPx and SOD in the erythrocytes were not statistically different between SHR and WKY (Figure 4A-C). Mean tail moment was decreased in Protease M and S groups but not in WPC when compared to SHR (Figure 4A). GPx and SOD activities reduced only in Protease M group without significant difference in WPC and Protease S groups in comparison to SHR (Figure 4B and C). These data indicated that chromosomal DNA damage as indicated by tail moment if any in the SHR group was significantly decreased in the hydrolysates and in the case of Protease M reduced antioxidant enzyme activity was accompanied.
ACE activity in the kidney (A), blood (B) and aorta (C) and the nitrate to nitrite ratio (D) after 8 weeks of study in WKY (n=5), SHR (n=9), WPC (n=5), Protease M (n=5) and Protease S (n=5). Data are presented as mean ± standard error (SE). Values sharing a common superscript letter are not significantly different (p < 0.05).

Figure 3. Effects of WPC or its hydrolysates on ACE activity in the kidney, blood and aorta and nitrate to nitrite ratio

Mean tail moment in the kidney measured using comet assays (A) and the activities of SOD (B), GPx (C) and catalase (C) in the RBC of WKY (n=5), SHR (n=9), WPC (n=5), Protease M (n=5) and Protease S (n=5). Data are presented as mean ± standard error (SE). Values sharing a common superscript letter are not significantly different (p < 0.05).

Figure 4. Effects of WPC or its hydrolysates on mean tail moment in the kidney and SOD, GPx and catalase activities in erythrocytes

4. Discussion

Herein, we demonstrated that WPC and/or its hydrolysates generated by protease M or S were effective in lowering SBP and ACE activity and increasing NO production as well as decreasing oxidative stress in chromosomal DNA followed by reduction in antioxidant enzyme activity in SHR. Although there are many studies indicating the WPC’s hypotensive roles in vitro, its in vivo efficacy and its relation to antioxidant properties have not been studied extensively. Our data provide evidence to support the blood pressure-lowering and antioxidant effects of WPC and/or its hydrolysates in animal model for hypertension.

In order to generate the hydrolysates we used protease M or protease S because their degree of the hydrolysis of WPC were known to be greater than or similar to trypsin,
Hydrolysates were presumed to be better than unhydrolyzed one possibly due to the removal of any inhibitory portion of the protein when protein is digested in the gastrointestinal system or by food processing [29]. Thus, Protease M and Protease S might have exerted greater effects than unhydrolyzed WPC.

Indeed, SBP-lowering effects in SHR were greater with the hydrolysates than with WPC although WPC itself showed lower SBP than WKY. Thus, the hydrolysis of WPC by protease M or protease S might have produced more blood pressure-lowering functionally active peptide fragments. In addition, the antioxidative roles of WPC and/or its hydrolysates might have further contributed to blood pressure lowering effects. The strong relation of oxidative stress to the incidence of hypertension was reported [5-10]. Previously, anti-oxidants such as ascorbic acid and glutathione were shown to increase vascular relaxation in the aorta of spontaneous hypertensive rats (SHR) endothelium-dependently [30]. We demonstrated the antioxidative role of WPC hydrolysates in terms of chromosomal DNA damage in the kidney and the activities of erythrocytes’ antioxidant enzymes. Although hypertension in SHR did not statistically increase kidney DNA damages, all the hydrolysates significantly decreased the levels of DNA damage seen SHR as indicated by the mean tail moment, and WPC showed a decreased tail moment without reaching statistical significance. These results suggest that the hydrolysates might be stronger antioxidants than WPC. Previously, others indicated that WPC contains antioxidant properties [11,12,13,14]. When erythrocyte antioxidant activities were examined, SOD, GPx and catalase were not significantly reduced by WPC and Protease S; whereas Protease M greatly lowered SOD, which might have suggested less necessity of antioxidant enzyme activities because of decreased oxidative stress by antioxidant Protease M. RBCs are used as a model to assess oxidative stress because of lack of DNA and mitochondria to produce detoxifying enzymes but of high exposure of oxygen radicals, making them susceptible to oxidative stress [31,32,33]. Overall, the hydrolysates either of protease M or S or both appeared to be more effective at defense against oxidant radicals than unhydrolyzed WPC, which then might have contributed to a further reduction in SBP compared to WPC.

The greater SBP-lowering effects induced by the hydrolysate groups might have not been due to their effects on ACE inhibition activity and NO production because of lack of differential ACE inhibition and NO production between WPC and the hydrolysate groups. SHR-impaired NO production was recovered by both WPC and its hydrolysates. As for ACE inhibition, even if WPC hydrolysates were more effective than WPC in vitro, WPC and its hydrolysates were not statistically different in the results from in vivo assays using the kidney and aorta tissues. Even in the blood, WPC showed a stronger ACE inhibition than either of the hydrolysates. In the case of casein, hydrolysates were more sensitive to protease or peptidase digestion during the gastrointestinal absorption than unhydrolysates [18,19,20]. Thereby, the efficacy in vitro might have been different from in vivo results. Similarly, WPC might have become more active in vivo after being digested in digestive tracts and/or the hydrolysates by protease M or S might have been digested too much to become less active forms. Thus, WPC appeared to exert hypotensive effects mainly through ACE inhibiting activities; but overall, the hydrolysates appeared to be stronger in blood pressure lowering effects by combinatorial effects on both ACE inhibition and antioxidants by preventing kidney DNA damage and erythrocytes’ oxidative stress.

We also noticed a reduction in SHR-increased GOT levels only in the protease S hydrolysate, suggesting that the hydrolysates have greater protective effects on the liver function. A reduction in food intake was also noticed in WPC and Protease M without affecting body weight gain. Our findings are in line with a recent report, demonstrating that whey protein lowered food intake when compared to soy protein [34].

5. Conclusions

In conclusion, SHR fed the hydrolysates for 8 weeks decreased SBP in greater extent than WPC along with a greater ACE inhibition in aorta. In addition, the hydrolysates decreased chromosomal DNA damage and the depletion of antioxidant enzyme activity. These results might suggest that the hydrolysates were better than WPC in terms of prevention and/or amelioration of hypertension.

Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

WPC: why protein concentrate; SHR: spontaneously hypertensive rats; WKY: Wistar Kyoto rats; SBP: systolic blood pressure

References


