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Antihypertensive Properties of Spinach Leaf Protein Digests

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Leaf protein containing approximately 50% rubisco (ribulose bisphosphate carboxylase/oxygenase) was obtained from fresh spinach leaf with the use of a simple extraction method. Pepsin and pepsin−pancreatin digests of spinach leaf protein have potent angiotensin-I converting enzyme inhibitory properties with IC_{50} values of 56 and 120 μg/mL, respectively. Both digests of leaf protein have antihypertensive effects after oral administration to spontaneously hypertensive rats (SHR) with minimum effective doses of 0.25 and 0.5 g/kg, respectively. The maximum antihypertensive effect for the pepsin digest was observed 4 h after oral administration, while for the pepsin−pancreatin digest, the maximum effect was observed 2 h after oral administration. Undigested spinach leaf protein did not exert any significant antihypertensive effect after oral administration to SHR at doses of 0.5 and 1 g/kg. Obtained results show that the pepsin digest of leaf protein may be useful in treatment of hypertension.

KEYWORDS: Leaf protein isolation; spinach; leaf protein digest; ACE inhibitors; antihypertensive effect; spontaneously hypertensive rat

INTRODUCTION

Hypertension, which is one of the most common lifestyle-related diseases, has become a significant problem in recent years. The number of people considered to be suffering from hypertension depends on levels of "normality" for blood pressure given by different health organizations. Generally, 20−45% of a population and nearly 50−60% of elderly people have elevated blood pressure (*1*−*3*). Epidemiological studies show that both arteriosclerosis and essential hypertension rank among the most common causes of cerebrovascular, cardiac, and renal pathology. Therefore, it is very important to develop natural food-related compounds for treatment and prevention of hypertension (*4*). In a previous study, four angiotensin-I converting enzyme (ACE) inhibitory peptides, MRW, MRWRD, IAYKPAG, and LRIPVA, were isolated from a spinach rubisco pepsin−pancreatin digest (*5*). Three of the isolated peptides lowered blood pressure after oral administration to spontaneously hypertensive rats (SHR). Rubisco (ribulose bisphosphate carboxylase/oxygenase), which catalyzes the primary step in photosynthetic CO_2 fixation, is the most abundant protein on earth. Being a constituent of all green parts of plants, rubisco is important as a source of food protein. It was hypothesized that rubisco may have importance in prophylaxis of hypertension.

The initial aim of this study was to demonstrate whether the pepsin−pancreatin digest of rubisco has antihypertensive activity. Unfortunately, pure spinach rubisco is difficult to obtain in large amounts, which makes it difficult to test its physiological effect. Considering this, an attempt was made to extract spinach leaf protein and optimize extraction conditions to obtain leaf protein with high rubisco contents. Digests of such isolated leaf proteins were then prepared, and their ACE inhibitory activity was determined. These digests were checked for antihypertensive properties through oral administration to SHR. Undigested leaf protein was also examined for antihypertensive effect in SHR.

MATERIALS AND METHODS

ACE (EC 3.4.15.1) was obtained from Sigma; hippuryl−histidyl−leucine was obtained from Peptide Institute, Inc. (Osaka, Japan), and synthesized peptides were obtained from American Peptide Company (Sunnyvale, CA). Fresh spinach leaf was obtained from a local supermarket.

Preparation of Spinach Leaf Protein. Spinach leaf protein was extracted using the method described by Satoh and others (*6*) with slight modification. Fresh spinach leaves were homogenized with approximately equal amounts of aqueous 0.003 N sodium hydroxide by weight at 5 °C in a Waring blender. The homogenate obtained (pH 11) was filtered through two pieces of gauze, and the filtrate was centrifuged

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at 13 500*g* for 50 min at 5 °C with the use of a SRC20B centrifuge from Hitachi (Japan). The supernatant was filtered through Whatman No. 2 filter paper and was adjusted with 5% acetic acid to a pH of about 4.5 (final pH) to produce protein precipitate. The precipitate obtained was washed successively with acetone, ethanol, and diethyl ether on a glass filter until the filtrate was colorless. In the same way, leaf protein was extracted under different pH conditions from fresh spinach leaf to check the effect of pH. Sodium hydroxide solutions of 0.05, 0.003, and 0.001 N and water were used for homogenization with spinach leaf to obtain pH of the homogenate of 13, 11, 9, and 7, respectively.

Enzymatic Hydrolysis of Spinach Leaf Protein. Spinach leaf protein (10 mg/mL) was digested by pepsin and pancreatin (E/S = 1/100) for 5 h at 37 °C, with pH 7.5 for the pancreatin digest and pH 2.0 for the pepsin digest. The reaction was stopped by boiling for 10 min. To prepare the pepsin−pancreatin digest, the pepsin digest was adjusted to a pH of 7.5 with 1 N NaOH and was subsequently digested with pancreatin for 5 h at 37 °C. The reaction mixture was boiled to halt the reaction and then centrifuged.

Determination of ACE Inhibitory Activity of Digests. ACE inhibitory activity was determined using the method reported by Cushman and Cheung (*7*) with minor modification by Yamamoto and others (*8*) and expressed by IC_{50}.

Analysis of Protein by Sodium Dodecyl Sulfate−Polyacrylamide Gel Eelectrophoresis (SDS−PAGE). Leaf protein extracted under different pH conditions from fresh spinach leaf was analyzed by SDS−PAGE using 0.75 mm separating gel in a final concentration of 11% (*9*) and compared with pure commercially available spinach rubisco (Sigma). The electrophoresis was carried out using the Mini-PROTEAN II apparatus (Bio-Rad). Protein bands were stained with Comassie Brilliant Blue R-250, and the apparent molecular masses of proteins were estimated using prestained protein marker broad range (New England Biolabs Inc.). The gel was scanned with the use of an ATTO Densitograph, and the concentration of rubisco as a percent of total protein was calculated.

Examination of Antihypertensive Effect of Digest after Oral Administration to SHR. Male SHR (16−24 weeks old) were employed. The peptides dissolved in saline were administered orally to SHR via a gastric metal zonde in a volume of 1.0 mL. Saline was used as a control. All assays were performed with at libidum access to feed and water. Experiments were always started at the same time (10:00 a.m.) to ensure comparable conditions. Blood pressure was measured before administration of peptide or saline (time zero) and every 2 h following oral administration for 6 h by the tail cuff method using a blood pressure meter MK-2000 (Muromachi Kikai) according to the instruction of apparatus.

Data Analysis. All results are expressed as means ± SEM. Statistical comparisons of the results between the two groups were made with Student's *t*-test.

RESULTS AND DISCUSSION

Preparation of Spinach Leaf Protein. The effect of pH value during the extraction process of spinach leaf protein was examined. Analysis of the amount of obtained proteins and relative content of rubisco in isolated protein revealed that pH of extraction is a very important factor determining composition and yield of extracted protein (**Table 1** and **Figure 1**). An increase of pH of the extraction mixture from 7 (water) to 13 [0.05 N NaOH (*6*)] caused an increase in the amount of isolated protein from 230 to 281 mg per 50 g of fresh leaf. The rubisco content in isolated protein was 66.3% at pH 7 and 53.3% at pH 13. The best effect, taking into account the amount of isolated protein, was obtained by homogenization of leaves at pH 7 followed by adjusting the pH of the homogenate to 13 with 1 N NaOH before filtration (285 mg/50 g of fresh leaf). However, under these conditions, the content of rubisco in isolated protein was low (52.7%). The highest content of rubisco in isolated protein (71.6%) was obtained when extraction of fresh spinach leaf was performed at pH 11 (0.003 N NaOH). The amount of extracted protein from 50 g of leaves in this condition was 261 mg providing the highest amount of isolated rubisco.

Table 1. Effect of pH during Extraction on the Amount of Isolated Protein from Fresh Spinach Leaf and Concentration of Rubisco in Isolated Protein

pH	leaf protein (mg)[a]	content of rubisco in extracted protein (%)
7	230	66.3
9	260	70.2
11	262	71.6
13	281	53.3
7→13[b]	285	52.7

[a] Extracted from 50 g of fresh spinach leaf. [b] pH value was adjusted from 7 to 13 with 0.1 N NaOH immediately after homogenization.

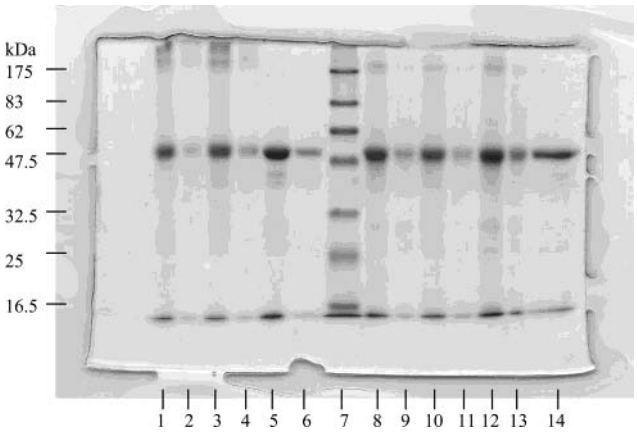

Figure 1. SDS−PAGE analysis of spinach leaf protein extracted at different pH values: lane 1, pH 13 (10 μg); lane 2, pH 13 (3 μg); lane 3, pH 7→13 (pH value was adjusted from 7 to 13 immediately after homogenizing) (10 μg); lane 4, pH 7→13 (3 μg); lane 8, pH 11 (10 μg); lane 9, pH 11 (3 μg); lane 10, pH 9 (10 μg); lane 11, pH 9 (3 μg); lane 12, pH 7 (10 μg); lane 13, pH 7 (3 μg); lane 5, spinach rubisco (10 μg); lanes 6 and 14, spinach rubisco (3 μg); and lane 7, standard marker (10 μg).

Another important factor is pH during protein precipitation (final pH). The highest concentration of rubisco in extracted protein was obtained when protein was precipitated at pH 4.5. When the pH of precipitation was lower than 4.0, it became very difficult to decolorize the leaf protein using acetone, ethanol, and diethyl ether. Moreover, in this case, the content of rubisco was also lower than when precipitated at pH 4.5 (data not shown). SDS−PAGE analysis of leaf protein extracted at different pH showed that the pattern of spinach leaf protein from fresh spinach leaf was very similar to the pattern of commercially available spinach rubisco. The highest content of rubisco in isolated leaf protein was obtained when extraction was performed at pH 11 (**Figure 1**). On the other hand, almost no spinach rubisco was extracted from commercially available spinach juice at the same conditions (data not shown). This result may be explained by denaturation or degradation of the commercial spinach juice during the heating process used for its preservation.

ACE Inhibitory Activity of Leaf Protein Digests. The pepsin and pepsin−pancreatin digests of spinach rubisco showed potent ACE inhibitory activities, inspiring an investigation to determine whether enzymatic digest of spinach leaf protein also has similar properties. Spinach leaf protein was hydrolyzed using pepsin, pancreatin, and pepsin−pancreatin, respectively. Among the obtained digests, the pepsin digest showed the most potent inhibitory activity for ACE (IC_{50} = 56 μg/mL) (**Table 2**). It

Table 2. ACE Inhibitory Activity of Digests of Spinach Leaf Protein and Rubisco

enzyme	IC_{50} (μg/mL) leaf protein	rubisco[a]
pepsin	56	64
pepsin–pancreatin	120	72
pancreatin	350	170

[a] Ref 5.

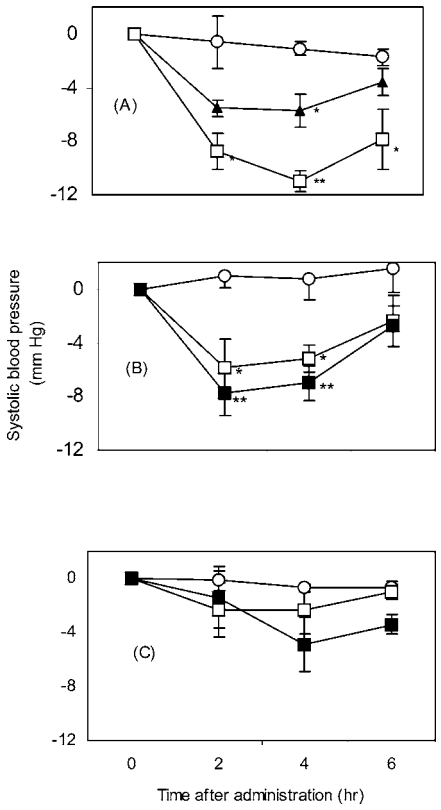

Figure 2. Antihypertensive activity of pepsin digest (**A**), pepsin–pancreatin digest (**B**), and undigested spinach leaf protein (**C**) after oral administration to SHR. Protein or digests were administered as a solution in saline at dosages: ○, saline (control); ▲, 0.25 g/kg; □, 0.5 g/kg; and ■, 1 g/kg. Changes of systolic blood pressure from time zero were expressed with means ± SEM. Asterisks indicate significant differences against control (*$p < 0.05$; **$p < 0.01$; $n = 4$).

should be noted that its activity was even higher than that of the pepsin digest of spinach rubisco. Such results indicate that other proteins present in extracted leaf protein other than rubisco might also be a source of ACE inhibitory peptides. The pepsin−pancreatin digest of leaf protein also showed fairly high inhibitory activity for ACE ($IC_{50} = 120$ μg/mL), which was about 50% that of the pepsin−pancreatin digest of spinach rubisco. The pancreatin digest of spinach leaf protein had the lowest ACE inhibitory activity ($IC_{50} = 350$ μg/mL) suggesting that pancreatin is not so effective in releasing ACE inhibitory peptides.

Antihypertensive Activity of Pepsin and Pepsin−Pancreatin Digests of Leaf Protein. Both pepsin and pepsin−pancreatin digests of leaf protein had antihypertensive effects following oral administration in SHR with minimum effective doses of 0.25 and 0.5 g/kg, respectively (**Figure 2A**,**B**). A maximum antihypertensive effect for the pepsin digest occurred 4 h after oral administration, while for the pepsin−pancreatin digest, it occured 2 h after oral administration. The pepsin digest was more effective than the pepsin−pancreatin digest at the same dosage of 0.5 g/kg. It exerted not only a higher antihypertensive effect 4 h after administration (−11.1 ± 0.75 mm Hg) than the pepsin−pancreatin digest (−7.2 ± 1.25 mm Hg) but also was effective for a longer period of time (even 6 h after administration).

Antihypertensive Activity of Undigested Leaf Protein. We also investigated whether undigested leaf protein shows antihypertensive activity after oral administration at different doses to SHR. In the case of undigested leaf protein, there was no significant antihypertensive effect at the tested dosages (0.5 and 1 g/kg). It should, however, be noticed that 4 h after oral administration of undigested protein at the dosage of 1 g/kg there was an observed decrease in blood pressure, −5 ± 2 mm Hg (**Figure 2C**). This result means that a much higher dosage of undigested leaf protein may exert a significant antihypertensive effect. The obtained results show that pepsin digestion in vivo of leaf protein is insufficient to bring about a suitable antihypertensive effect. The use of a pepsin digest of leaf protein is found to be more effective.

CONCLUSIONS

Obtained results support our thesis that leaf protein with a high rubisco content has the potential to be useful in prevention and/or treatment of hypertension. To take full advantage of the antihypertensive potential of leaf protein, it is necessary to hydrolyze it with pepsin prior to ingestion.

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