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ARTICLE

Angiotensin I-Converting Enzyme Inhibitory Activity of Gelatin Hydrolysates and Identification of Bioactive Peptides

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In this project we report on the angiotensin I-converting enzyme (ACE)-inhibitory activity of a bovine gelatin hydrolysate (Bh2) that was submitted to further hydrolysis by different enzymes. The thermolysin hydrolysate (Bh2t) showed the highest *in vitro* ACE inhibitory activity, and interestingly a marked *in vivo* blood pressure-lowering effect was demonstrated in spontaneously hypertensive rats (SHR). In contrast, Bh2 showed no effect in SHR, confirming the need for the extra thermolysin hydrolysis. Hence, an angiotensin I-evoked contractile response in isolated rat aortic rings was inhibited by Bh2t, but not by Bh2, suggesting ACE inhibition as the underlying antihypertensive mechanism for Bh2t. Using mass spectrometry, seven small peptides, AG, AGP, VGP, PY, QY, DY and IY or LY or HO-PY were identified in Bh2t. As these peptides showed ACE inhibitory activity and were more prominent in Bh2t than in Bh2, the current data provide evidence that these contribute to the antihypertensive effect of Bh2t.

KEYWORDS: Gelatin hydrolysate; antihypertensive peptides; angiotensin I-converting enzyme (ACE); blood pressure; gastrointestinal and mucosal digestion; spontaneously hypertensive rats; aortic rings

INTRODUCTION

Hypertension, a major risk factor for cardiovascular diseases, is estimated to affect one-third of the Western population (*1*). Next to drug therapy, lifestyle modifications including diet therapy are one of the most important tools for effective lowering of blood pressure (*2*). Even small decreases in blood pressure result in significant lower risks for cardiovascular diseases (*3*). Also foods containing angiotensin I-converting enzyme (ACE) inhibitory peptides may be part of this lifestyle approach, as they have proven to be effective in both the prevention and treatment of hypertension (*4*−*6*). ACE (EC 3.4.15.1) is a dipeptidyl carboxypeptidase, involved in different blood pressure regulating mechanisms. In the renin−angiotensin−aldosterone system (RAAS), it converts the inactive decapeptide angiotensin I (Ang I) into the potent vasoconstricting octapeptide angiotensin II (Ang II). It also inactivates the vasodilatory bradykinin (*7*), resulting in an overall increase in blood pressure (*8*). The levels of both Ang II and bradykinin are mainly dictated by ACE, making this enzyme a popular target for blood pressure regulation (*3*, *8*).

To exert an antihypertensive effect *in vivo*, ACE inhibitory peptides have to reach the target sites in an active form. Human proteases and peptidases may metabolize the ingested ACE inhibitory peptides, resulting in either the release of more potent bioactive peptides or degradation into inactive fragments (*9*, *10*). Small peptides are most likely to reach the bloodstream intact. In fact, Iwai et al. (*11*) reported that, after ingestion of a gelatin hydrolysate, only free amino acids, di- and tripeptides could be recovered in the blood of humans. A variety of bioactive peptides and protein hydrolysates derived from various food proteins has been detected. On the basis of cost, economy and application, hydrolysates with ACE inhibitory and antihypertensive effect are preferable to purified peptides (*12*). As a first screening, the *in vitro* ACE inhibitory activity of the peptides or hydrolysates is usually evaluated. However, recent literature has indicated that it is difficult to establish a direct relationship between *in vitro* and *in vivo* activity (*13*). This might be explained by the bioconversion/availability of ACE inhibitory peptides after oral administration or by possible antihypertensive mechanisms other than ACE inhibition which are influenced by these peptides. Therefore, *in vivo* experiments with spontaneously hypertensive rats (SHR) are necessary to reliably determine antihypertensive activity (*3*).

Gelatin is a linear water-soluble animal protein, generally known for its unique gel-forming ability, and is therefore widely used in the food industry. It is especially important for desserts, candies, bakery products, jellied meat, ice cream and dairy products. Gelatin is a hydrolyzed form of collagen and is obtained by extraction with a heating process from animal skins, bones, tendons and loose

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connective tissues after acidic (type A gelatin) or alkaline (type B gelatin) treatment. Due to differences in collagen sources and preparation techniques, the structure has variable physical properties and chemical heterogeneity (*14*).

In the present project, different types of gelatins were screened *in vitro* for ACE inhibitory activity, and then the activity of the most active sample was optimized by hydrolysis with different enzymes. We further investigated the stability of the hydrolysate against gastrointestinal and mucosal enzymes, and the effect on bioactivity by a 3 kDa ultrafiltration. After these *in vitro* screening processes, the most active commercial sample and its optimized product were orally administered to SHR, and blood pressure was recorded by the tail-cuff method. Moreover, to evaluate the underlying blood pressure lowering mechanisms, the ACE inhibitory activity of the samples was evaluated in organ bath experiments using isolated rat aortic rings. Finally, efforts were made to identify a number of bioactive peptides in the most active gelatin hydrolysate.

MATERIALS AND METHODS

Products. Gelatins and gelatin hydrolysates were obtained from PB Gelatins (Vilvoorde, Belgium). ACE, pepsin, trypsin, α-chymotrypsin, papain, thermolysin, collagenase, peptidase from rat intestinal mucosa, hippuryl-histidyl-leucine (HHL), *o*-phthaldialdehyde (OPA), Ang I, acetylcholine (Ach), *N*-nitro-L-arginine and HCl were purchased from Sigma-Aldrich (Bornem, Belgium). $Na_2B_4O_7 \cdot 10H_2O$ was obtained from Acros Organics (Geel, Belgium). NaOH, NaCl, $NaH_2PO_4 \cdot 2H_2O$ and other nonspecified products were obtained from Chem Lab (Lichtervelde, Belgium).

***In Vitro* ACE Inhibition Assay.** ACE inhibitory activity was measured according to the colorimetric method of Chang et al. (*15*). Briefly, the substrate HHL is cleaved by ACE into hippuric acid (H) and histidyl-leucine (HL). At pH > 11, the dipeptide HL reacts with OPA forming a yellowish product, of which the absorbance can be measured at 390 nm.

Dry samples were dissolved and diluted to different extents with distilled water for the ACE inhibitory assays. The ACE catalyzed reactions (37 °C for 2 h) were performed in cuvettes containing 100 μL of sample solution, 100 μL of ACE solution, and 100 μL of HHL solution (mixture 1). Another mixture containing 100 μL of sample solution and 200 μL of buffer A, a borate buffer with pH 8.3 (mixture 2), was used to obtain the background absorbance of the sample solutions for the colorimetric method. The third mixture containing 100 μL of buffer A, 100 μL of ACE solution, and 100 μL of HHL solution (mixture 3) was used to obtain the data for 100% reaction. The fourth mixture containing 300 μL of buffer A (mixture 4) was used to obtain the background absorbance of the OPA reagent. The enzymatic reactions were terminated by adding 3 mL of the alkaline (pH 12.0) OPA reagent. The absorbance at 390 nm of mixture 1 (A1), mixture 2 (A2), mixture 3 (A3), and mixture 4 (A4) was measured after 20 min incubation at 25 °C. The inhibitory ratios were calculated by the following equation.

$$I\ (\%) = [1 - (A1 - A2)/(A3 - A4)] \times 100$$

Dose activity curves were generated for the logarithm of the concentration of the sample (mg/mL) versus ACE inhibitory activity (%). The data were fitted by a 4 parametric logistic model using Prism v4 (GraphPad Software, Inc., La Jolla, CA) as described by Vercruysse et al. (*16*). Inhibition was expressed as the sample concentration that, according to the fitted model, inhibits 50% of ACE activity (IC_{50}). Values are based on three repeated measurements of each concentration in each dose−activity curve, and expressed as means ± SEM.

Digestion and Hydrolysis. The human gastrointestinal digestion process was simulated according to the method of Vermeirssen et al. (*17*). Samples were dissolved in distilled water at a ratio of 1 g per 25 mL. The digestion in the stomach was simulated by lowering the pH to 2 with HCl (4 M), adding pepsin in a 1/250 (w/w) enzyme/substrate ratio and incubating at 37 °C for 2 h. The small intestine phase was simulated by adding trypsin and α-chymotrypsin (each 1/250) at pH 6.5 (with NaOH, 10 M) and incubating at 37 °C for 2.5 h. Mucosal digestion was simulated by altering the pH to 7.4 (with NaOH, 10 M), adding peptidase from rat intestinal mucosa (1/500) and incubating at 37 °C for 2 h. In parallel, hydrolysis using different enzymes and different hydrolysis times was performed on Bh2. The different conditions are shown in **Table 1**. All samples were boiled for 5 min to inactivate the enzymes and centrifuged at 10000*g* for 10 min at 4 °C, and the supernatant was filtered and lyophilized.

Table 1. Different Conditions of Enzymatic Hydrolysis

enzyme	pH	*T* (°C)	incubation time (h)	enzyme/substrate ratio (w/w)
papain	6.5	65	2, 4, 6	1/100
thermolysin	8	65	0.5, 1, 2, 4, 6	1/250
pepsin	2	37	2, 4, 6	1/250
trypsin + α-chymotrypsin	6.5	37	2, 4, 6	1/250 (each)
collagenase	7.5	37	0.5, 1, 2, 4, 6	1/250

Ultrafiltration. Ultrafiltration was performed with a 3 kDa MWCO membrane, using a Centriplus YM-3 centrifugal filter unit (Millipore Corporation, Billerica, MA). The sample (0.4 g) was dissolved in 10 mL of distilled water. The filter unit with sample was centrifuged at 3000*g* for 6 h at 25 °C, and the permeate was lyophilized.

Antihypertensive Effect in SHR. Male SHR between 10 and 14 weeks old (SBP 185−230 mmHg) were purchased from Charles River Laboratories (France). The SHR were housed in a room kept at 24 °C with a 12 h dark−light cycle. They were fed a standard laboratory diet, and tap water was available *ad libitum*. The gelatin hydrolysates were dissolved in 0.5 mL of tap water, and injected orally using a plastic gavage at a dose of 300 mg/kg body weight (BW). Control rats were administered the same volume of tap water. Following oral administration of the sample, systolic blood pressure (SBP) was measured by the tail-cuff method. Before each series of measurements, SHR were conditioned on a 37 °C hot plate for 10 min. SBP was measured before and 2, 4, 6, 8, and 24 h after the gavage using a Physiograph desk model with programmed electro-sphygmomanometer and pneumatic pulse transducer (Narco biosystems, Austin, TX).

Change in SBP was considered as the difference in SBP before and after administration and was expressed as means ± standard error. The unpaired Student's *t* test was used for all statistical analysis. Statistical significance was considered $p < 0.05$.

The experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the local Ethics Committee.

Organ Baths. In order to investigate the effect of the samples on vascular reactivity, thoracic aorta rings from male Wistar rats were used. The experimental protocols were performed according to Vercruysse et al. (*18*). Rats were killed by decapitation using a small animal guillotine. The aorta was rapidly removed, immersed in physiological solution and carefully cleaned of adhering fat and connective tissue and cut into rings of approximately 2 mm length. The aortic rings were mounted in 12.5 mL organ baths filled with a physiological solution (composition in mM: NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; glucose, 11; $CaCl_2$, 1.25; $MgCl_2$, 1.2), continuously bubbled with a gas mixture of 95% O_2−5% CO_2 and maintained at 37 °C. Resting tension was adjusted to 2 g. Tension was measured with an isometric force transducer (Gould Statham, Cleveland, OH). After an equilibration period of 60 min, the vessels were contracted by changing the physiological solution in the bath to a depolarizing 100 mM KCl solution (composition in mM: NaCl, 27; KCl, 100; $NaHCO_3$, 15; glucose, 11; $CaCl_2$, 1.25; $MgCl_2$, 1.2), in order to test their contractile capacity. The presence of functional endothelium was assessed by determining the ability of acetylcholine (1 μM) to induce relaxation in KCl-precontracted rings. After washing and returning to baseline tension, aortic rings were further incubated for 30 min in physiological solution supplemented with *N*-nitro-L-arginine (100 μM) with or without the sample. The NO-synthase inhibitor *N*-nitro-L-arginine was added to avoid any bias due to interaction with NO-evoked relaxation. After the incubation period, contraction of the rings was evoked by adding cumulative concentrations of Ang I (1, 3, 10, 30, 100 nM).

All results were based on at least 4 repetitions, using aorta rings from at least 2 different rats. Agonist potency is expressed as $pD_2 \pm$ SEM, which is the negative logarithm of the EC_{50} value (concentration of agonist resulting in 50% of the maximum response) determined by a 4 parametric

Table 2. ACE-Inhibitory Activity of Hydrolysates of Bh2, Obtained with Different Enzymes and Different Hydrolysis Times[a]

	2 h hydrolysis		4 h hydrolysis		6 h hydrolysis	
enzyme	IC_{50}	log IC_{50}	IC_{50}	log IC_{50}	IC_{50}	log IC_{50}
papain	1.84	0.27 ± 0.04 a,A	2.05	0.31 ± 0.01 a,A	2.34	0.37 ± 0.07 ac,A
thermolysin	1.04	0.02 ± 0.03 b,A	0.82	−0.09 ± 0.03 b,AB	0.80	−0.10 ± 0.02 b,B
pepsin	4.20	0.62 ± 0.08 c,A	3.58	0.55 ± 0.12 a,A	3.77	0.58 ± 0.13 a,A
trypsin + α-chymotrypsin	ND[b]	ND	1.59	0.20 ± 0.03 c,A	1.63	0.21 ± 0.01 c,A
collagenase	1.03	0.01 ± 0.05 b,A	0.97	−0.02 ± 0.05 b,A	0.99	0.00 ± 0.05 b,A

[a] IC_{50} are expressed as means in mg/mL, log IC_{50} as means ± standard error. The significance of difference was tested with ANOVA followed by a *post hoc* Tukey test at $p = 0.05$. Letters (a−c) indicate a significant difference in the same column. Capital letters (A, B) indicate a significant difference in the same row. [b] Not determined.

logistic model that was fitted to the data using Prism v4. The unpaired Student's *t* test was used for all statistical analysis. Statistical significance was considered $p < 0.05$.

Identification of Small Peptides in Bh2t Using MALDI-MS. In order to detect di- and tripeptides more efficiently by MALDI mass spectrometry, we decided to increase their masses (such that they fell into the mass range that is typically detected) by fusing them to the acetyl-Arg-Arg-Gly peptide which was synthesized on an ABI 433A peptide synthesizer using Fmoc chemistry. One micromole of this peptide was activated with 3 equiv of disuccinimidyl carbonate in 16 μL of DMSO/pyridine (4/1) and added to 10 μg of dried collagen digestion mixture. After 60 min of incubation, the mixture was acidified and one twentieth of each analyte mixture was loaded onto a capillary RP-HPLC column (Vydac c18 0.075 × 150 mm) and separated in an acetonitrile/formic acid gradient (1.2% acetonitrile/min at a flow of 270 nL/min). One minute fractions were directly collected onto a Bruker AnchorChip (600 μm diameter, Bruker Daltonics, Bremen, Germany) target on which, after sample drying, 0.6 μL of α-cyano-4-hydroxycinnamic acid matrix solution (0.1 mg/mL (*R*)-cyano-4-hydroxycinnamic acid in ethanol/acetone/0.1% TFA (6/4/1)) was applied. Once the MALDI matrix solution was dried, the whole plate was briefly submerged in 10 mM ammonium citrate and excess of this wash solution was removed under a gentle nitrogen stream. The masses of the ionized peptides were automatically measured on a Bruker Ultraflex II TOF/TOF MALDI mass spectrometer (*19*).

Differential Analysis of Bh2 and Bh2t. One milligram of the thermolysin-treated collagen digest (Bh2t) and a control solution (Bh2) was reacted with either 8 mg of the light ($^{12}C_4$) or the heavy ($^{13}C_4$) butyl-*N*-hydroxysuccinimide ester, respectively, as published in Van Damme et al. (*20*). The reagents were prepared as previously described by Pöchlauer et al. (*21*). Butyrylation of primary amino groups was allowed for 120 min at 30 °C. Further, a label swapping experiment was performed in which the thermolysin-treated digest was labeled with $^{13}C_4$-butyrate. Excess reagents were quenched by adding 60 μL of 1 M glycine and samples were incubated for 15 min at 25 °C. This step was followed by heating the sample for 60 min at 95 °C to reversing the unwanted *O*-butyrylation. After acidification with 20 μL of 50% acetic acid, 10 μL (corresponding to 10 μg of the original peptide mixture) was introduced in a spray needle for ESI nanospray analysis (BG12-69-2-CE-20 PicoTip emitter; New Objective Inc., Woburn, MA). The nanospray source was connected to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) for further peptide analysis.

The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS/MS acquisition for the six most abundant peaks in an MS spectrum. In the LTQ-Orbitrap XL, full scan MS spectra were acquired in the Orbitrap at a target value of 1E6 with a resolution of 30,000. The six most intense ions were then isolated for fragmentation in the linear ion trap. In the LTQ, MS/MS scans were recorded in profile mode at a target value of 5,000. Peptides were fragmented after filling the ion trap with a maximum ion time of 10 ms and a maximum of 1E4 ion counts. From the MS/MS data in each analysis, Mascot Generic Files were created using the Mascot Distiller software (version 2.2.1.0; Matrix Science, London, U.K.). No deisotoping was employed, and the relative signal-to-noise limit was set at 2. MS/MS peak lists were searched with Mascot using the Mascot Daemon interface (version 2.2.0; Matrix Science). Spectra were searched against the Swiss-Prot database (version 15.3), and taxonomy was set to mammals. Variable modifications were set to methionine oxidation. No fixed modifications were set. Mass tolerance of the precursor ions was set to ±10 ppm and of fragment ions to ±0.5 Da. The peptide charge was set to 1+, 2+ or 3+, and one missed tryptic cleavage site was allowed. Also, Mascot's C13 setting was to 1. Only peptides that were ranked one and scored above the identity threshold score set at 99% confidence were withheld and manually validated.

Mascot Distiller Quantitation Toolbox was used in the "precursor" mode for quantification of the identified peptides. Mascot Distiller detects peaks by trying to fit an ideal isotopic distribution on experimental data. This distribution is predicted using average amino acid compositions for a peptide. This is followed by extraction of the XIC signal of both peptide components (light and heavy) from the raw data. Ratios are then calculated from the area below the light and heavy isotopic envelope of the corresponding peptides (integration method "trapezium" and integration source "survey"). To calculate this ratio value, a least-squares fit to the component intensities from the scans in the XIC peak was created. MS scans used for this ratio calculation are situated in the elution peak of the precursor determined by the Distiller software (XIC threshold 0.3, XIC smooth 1, max. XIC width 250). To validate the calculated ratio, the standard error on the least-squares fit has to be below 0.16 and correlation of the isotopic envelope should be above 0.97.

Synthesis of Peptides and Their Potential To Inhibit ACE Activity *in Vitro*. Peptides were in-house synthesized using Fmoc-based chemistry on an Applied Biosystems 433A peptide synthesizer (Department of Medical Protein Research, VIB, Ghent, Belgium). ACE inhibitory activity was determined as described above. Inhibition was expressed as the sample concentration (μM) that, according to the fitted sigmoid regression curve in Prism v4, inhibits 50% of ACE activity (IC_{50}) as described above. Values are based on three repeated measurements, and expressed as means together with the corresponding 95% confidence interval.

RESULTS AND DISCUSSION

Hydrolysis. Different bovine-, pig- and fish-originated gelatins and commercial gelatin hydrolysates were screened *in vitro* for ACE inhibitory activity. For the different gelatins, IC_{50} values ranged between 15.9 and 29.05 mg/mL, while for the gelatin hydrolysates, IC_{50} values were between 3.39 and 5.54 mg/mL (data not shown). Interestingly, the highest ACE inhibitory activity was found for a gelatin hydrolysate originating from bovine hide (Bh2) (log IC_{50}: 0.59 ± 0.20). This commercial hydrolysate was produced by enzymatic hydrolysis of a type B bovine hide gelatin, and has a protein content of 90−94%. Moisture and ash content are < 8% and < 2%, respectively.

In order to lower the IC_{50} value, an extra hydrolysis step was conducted on Bh2. Five different enzymes were used at 3 different hydrolysis times (**Table 2**). After 2 h of hydrolysis with thermolysin or collagenase, the log IC_{50} value was significantly lower compared to the nonhydrolyzed sample Bh2. Pepsin failed to significantly lower the log IC_{50} value even after 4 and 6 h of hydrolysis. For papain, a longer hydrolysis time resulted in a loss of ACE inhibitory activity. Probably, the active peptides produced after 2 h of hydrolysis were further broken down. The hydrolysate prepared with thermolysin revealed the highest ACE inhibitory activity showing an IC_{50} value of 0.80 after 6 h of hydrolysis. Interestingly, these IC_{50} values are in the same order of magnitude as other IC_{50} values, ranging from 0.56 to 1.5 mg/mL, that were

Table 3. Effect of *in Vitro* Gastrointestinal and Mucosal Digestion and Ultrafiltration on Bh2 and Bh2t[a]

	gastrointestinal digestion		mucosal digestion		ultrafiltration 3 kDa	
	IC_{50}	log IC_{50}	IC_{50}	log IC_{50}	IC_{50}	log IC_{50}
Bh2	1.76	0.25 ± 0.03 a,A	1.33	0.12 ± 0.02 a,A	0.66	−0.18 ± 0.03 a,B
Bh2t	0.71	−0.15 ± 0.06 b,A	0.73	−0.14 ± 0.03 b,A	0.68	−0.17 ± 0.12 a,A

[a] IC_{50} values are expressed as means in mg/mL, log IC_{50} as means ± standard error. The significance of difference was tested with ANOVA followed by a *post hoc* Tukey test at $p = 0.05$. Letters (a−c) indicate a significant difference in the same column. Capital letters (A, B) indicate a significant difference in the same row.

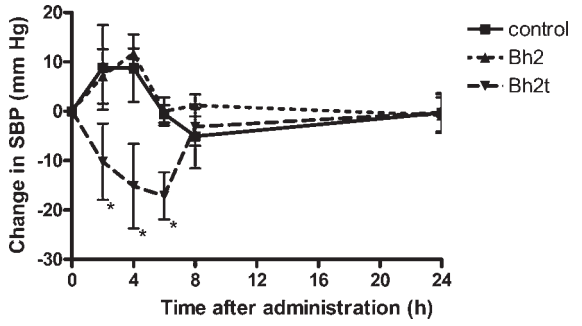

Figure 1. Change in SBP (mmHg) of SHR before and after 2, 4, 6, 8, and 24 h of administration by gavage of a single dose of tap water for control ($n = 4$), 300 mg of Bh2/kg BW ($n = 3$) and 300 mg of Bh2t/kg BW ($n = 4$). Data are expressed as means ± SEM (*: significant difference compared to control $p < 0.05$).

obtained for gelatin hydrolysates after treatment with two different enzymes (*22*, *23*).

Stability toward Gastrointestinal and Mucosal Enzymes and Ultrafiltration. Between oral intake and reaching the bloodstream, protein hydrolysates are exposed to a series of enzymes in the gastrointestinal tract. In these experiments, the stability of Bh2 and Bh2 hydrolyzed with thermolysin for 2 h (Bh2t) toward gastrointestinal digestion followed by mucosal digestion was evaluated *in vitro*. IC_{50} and log IC_{50} values are shown in **Table 3**. For Bh2, the log IC_{50} value was significantly lowered by both gastrointestinal and mucosal digestion, suggesting extra cleavage by the digestive enzymes. For Bh2t, no significant difference in ACE inhibitory activity could be seen after gastrointestinal and mucosal digestion. The log IC_{50} value of Bh2 after gastrointestinal and mucosal digestion is still significantly higher than for Bh2t. These results indicate that, first, gelatin hydrolysates with high ACE inhibitory activity and resistant toward degradation processes in the gastrointestinal tract could be obtained by hydrolysis with thermolysin and, second, this high ACE inhibitory activity could not be reached with gastrointestinal and mucosal hydrolysis.

However, in order to exert an antihypertensive effect *in vivo*, the ACE inhibitory peptides not only have to be resistant to gastrointestinal degradation but also have to be absorbed into the bloodstream in their intact form. Therefore, small ACE inhibitory peptides (2−20 amino acids) are the most interesting ones, as they are most likely to be absorbed into the bloodstream (*11*, *17*). To investigate the activity of the small peptides, Bh2 and Bh2t were, after digestion with gastrointestinal and mucosal enzymes, subjected to a 3 kDa ultrafiltration. In literature, it is reported that bioactive peptide enrichments using cutoff membranes of 3, 5, or 10 kDa give good results (*1*). With a membrane of 1 kDa, some of the active peptides may be lost (*24*). Here, it was found that both samples had 3 kDa peptide fractions that were equally active (**Table 3**). For Bh2, the log IC_{50} was significantly lowered by the 3 kDa ultrafiltration, indicating that in the nonfiltered mucosal digest, less active larger fragments are still present. For Bh2t, the ultrafiltration did not result in a significant difference in ACE inhibitory activity. Although the *in vitro* models used in this study can only be considered as an estimate of the *in vivo* situation, they provide a true indication that these gelatin hydrolysates, after gastrointestinal and mucosal digesting processes, contain small, highly active peptides that are likely to be absorbed into the bloodstream.

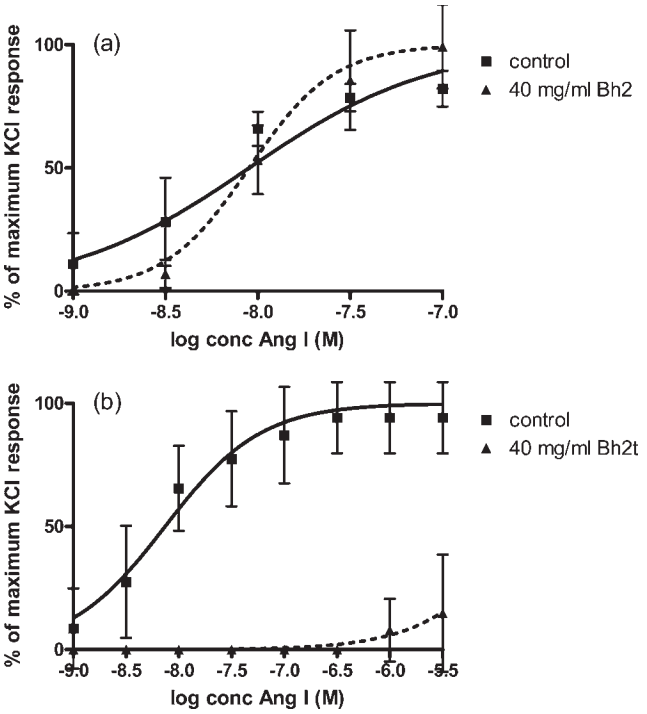

Figure 2. Effect of Bh2 (**a**) and Bh2t (**b**) on the contraction evoked by angiotensin I in rat aortic rings. Values are expressed as percentage of the maximum response to 100 mM KCl. Experimental data were fitted by nonlinear regression and are expressed as means ± SEM.

Table 4. Effect of Bh2 and Bh2t on the pD_2 Values of Angiotensin I in Rat Aortic Rings

sample	dose	pD_2 (M)[a]
Bh2	control	8.142 ± 0.096
	40 mg/mL	8.004 ± 0.029
Bh2t	control	8.130 ± 0.068
	40 mg/mL	<5.500

[a] Data are expressed as means ± SEM for $n \geq 4$.

Antihypertensive Effect in SHR. The most active hydrolyzed gelatin sample Bh2t was investigated for *in vivo* antihypertensive effects in SHR. To evaluate the effect of the extra hydrolysis with thermolysin *in vivo*, the blood pressure lowering activity of Bh2 was also tested. Both samples were administered by gavage to SHR in a single oral dose of 300 mg sample/kg BW, and blood pressure was measured with the tail-cuff method before and 2, 4, 6, 8, and 24 h after administration. Data of the SBP measurements of both

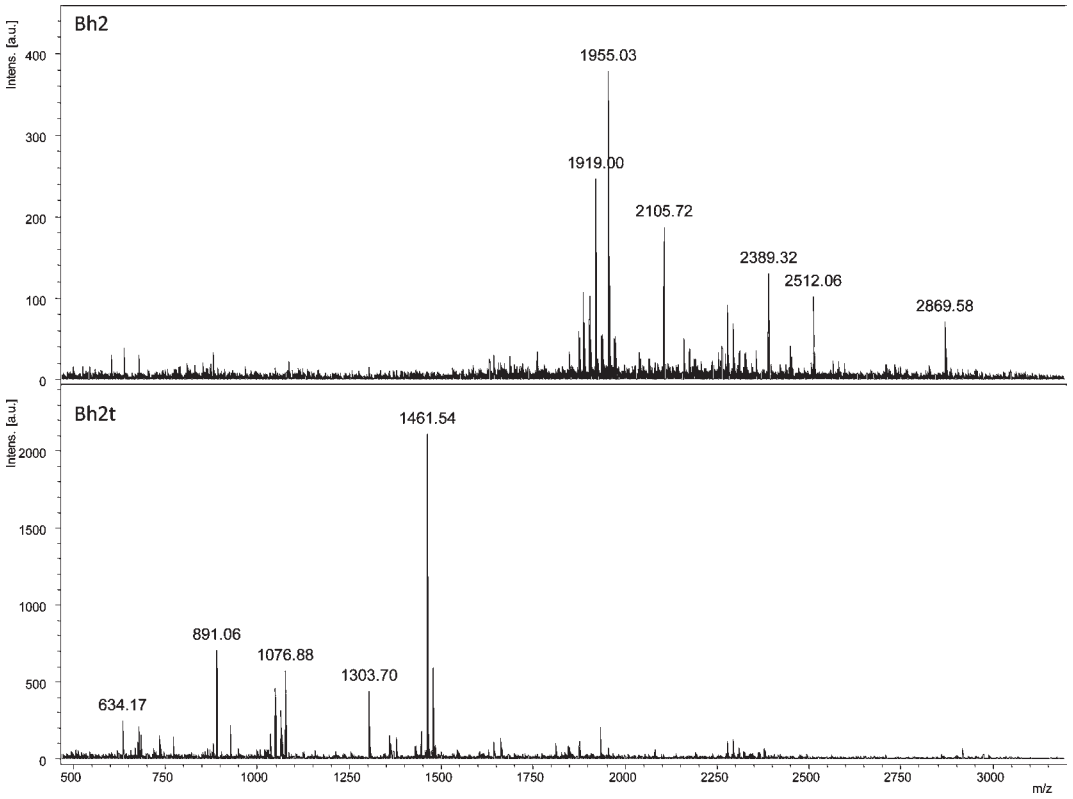

Figure 3. MALDI mass spectrum of the entire peptide mixture from Bh2 (upper panel) and Bh2t (lower panel), showing a shift toward lower masses after thermolysin treatment.

samples and a control are shown in **Figure 1**. At none of the measuring points, a difference could be observed between Bh2 and the control. But after administration of Bh2t, the blood pressure was significantly lower as compared to the control rats at 2, 4, and 6 h after administration. These results clearly demonstrate the need for the extra hydrolysis with thermolysin to exert blood pressure lowering activity, which agrees with the *in vitro* results as reported above. A maximum decrease in SBP (17 mmHg) was observed 6 h after administration of 300 mg/kg BW, while after 8 and 24 h, the blood pressure lowering effect was almost completely gone. Based on the results of the *in vivo* experiment, the bovine gelatin hydrolysate could be used as a blood pressure lowering ingredient in functional foods, but only under the condition of an appropriate hydrolysis.

Organ Baths. ACE inhibitory activity of Bh2 and Bh2t was evaluated using isolated rat aortic rings in order to verify if ACE inhibition is in fact the mechanism responsible for the effects observed *in vivo*. Although the *in vitro* screening test with HHL was designed to screen for ACE inhibition, the *in vivo* situation is much more complex. For example, in the *in vitro* test, ACE is freely available for its substrate and for possible inhibitors, while under *in vivo* conditions ACE is bound to aortic tissue. ACE, as present in the endothelium of the aortic rings, catalyzes the conversion of Ang I into Ang II, and in turn this octapeptide binds to the Ang II receptor on the smooth muscle cells, evoking a contraction of the aortic ring. Thus, inhibition of ACE leads to a decreased contractile response to Ang I. In the presence of Bh2 at 40 mg/mL, no significant difference in the contractile response to Ang I could be seen in the aorta rings compared to control rings (**Figure 2a**), and the Ang I pD_2 value in Bh2-treated rings was not significantly different from its value in control rings (**Table 4**). However, the presence of Bh2t at 40 mg/mL in the organ baths led to a marked decrease in contractile response to Ang I (**Figure 2b**, **Table 4**). These organ bath results demonstrate an effect on the RAAS by Bh2t, but not by Bh2. This is in accordance with the *in vivo* results, suggesting that the RAAS is responsible for, or at least a part of, the blood pressure lowering effect of Bh2t.

Table 5. Examples of Identified Peptides in Bh2 and Relative Quantification toward Bh2t

peptide[a]	FWD L/H[b]	REV L/H[b]
GP↓**VGP**↓VGKH	3.25	0.32
GHHGDQGAPG↓A↓**VGP**↓A	33.99	not found
GKSGDRGETGP↓**AGP**↓**AGP**↓IGP↓VG↓AR	only L	not found
KGH↓**AG**↓L↓A	only L	only H

[a] The symbol ↓ indicates a thermolysin cleavage site, *in silico* simulated by ExPASy PeptideCutter. [b] In the forward experiment (FWD), Bh2 was labeled with the light (L), and Bh2t with the heavy (H) isotope. In the reverse experiment (REV), the labeling was reversed.

In literature, little information can be found about the actual blood pressure lowering mechanisms of protein hydrolysates. Recently, Rousseau-Ralliard et al. (*25*) investigated the Ang I-induced aortic ring vasoconstriction in the presence of α(S1)- and α(S2)-casein hydrolysates. For those hydrolysates, no difference compared to the control was found, while the samples weakly decreased SBP in hypertensive rats.

Identification and Relative Quantification of Bioactive Peptides. In the section above, a blood pressure lowering effect was demonstrated for Bh2t. Since other authors reported that only di- and tripeptides are absorbed intact into the bloodstream (*11*, *26*), where they can act as antihypertensive agents, efforts were made to identify small peptides in Bh2t. **Figure 3** shows the MS spectrum of peptides present in Bh2 and Bh2t. General comparison of these complete peptide mixtures displays a shift to lower masses in the Bh2t sample, demonstrating that extra cleavage events happen during the hydrolysis with thermolysin, and in turn this results in the presence of smaller peptides.

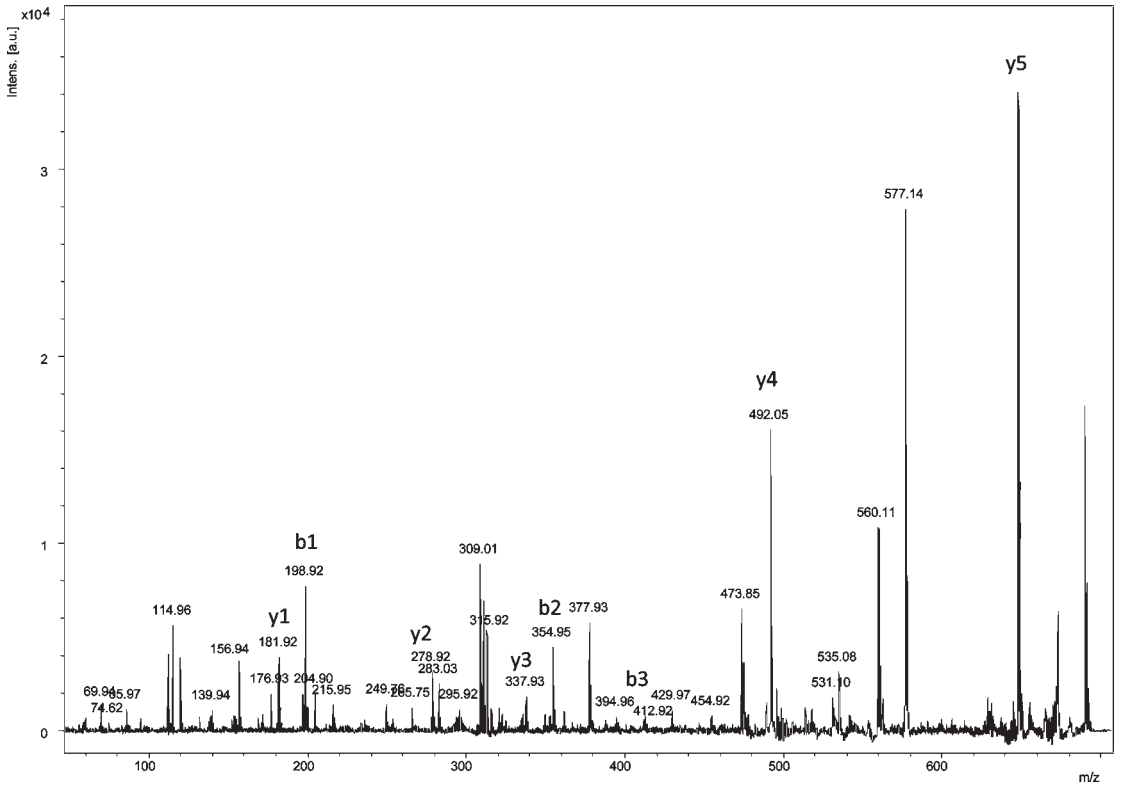

Figure 4. MALDI MS/MS spectrum from PY (Pro-Tyr) after modification of its α-amino terminus with acetyl-RRG.

Peptides responsible or at least partly responsible for the blood pressure lowering activity of Bh2t should be more prominent in Bh2t than in Bh2, since the latter hydrolysate failed to show any antihypertensive effect in SHR. Thus, peptides in Bh2t were quantified relatively toward Bh2. For this purpose, both Bh2t and Bh2 were labeled differentially using isotopic labeling of amino groups of peptides (α- and γ-amino groups) with either light (^{12}C) or heavy (^{13}C) butyryl. In a first experiment, referred to as the forward experiment, peptides from Bh2 were labeled with the light (L) and Bh2t with the heavy (H) reagent. In the reverse experiment, labeling was swapped. Following MS analysis and peptide identification, peptide ratios were quantified using the Mascot Distiller software. A number of larger peptides were identified that were equally present in both samples (L/H ratio ~1), indicating that they were not cleaved by thermolysin. We also found peptides that were more prominent in Bh2 than in Bh2t (FWD, L/H ratio >1; REV, L/H ratio <1), demonstrating cleavage of these peptides by thermolysin. Otherwise, a number of peptides were more prominent in Bh2t than in Bh2, indicating that they are cleavage products from the thermolysin hydrolysis. Examples of cleaved peptides and their quantitative data are given in **Table 5**.

However, identification of smaller peptides like di- and tripeptides has proven to be extremely difficult by mass spectrometry. Therefore, all peptides present in Bh2t were modified at their α-amino terminus with a tripeptide (acetyl-RRG) in order to increase their overall mass and their ionization efficiency during mass spectrometry. This approach resulted in the identification of the dipeptides PY (**Figure 4**), QY, DY and IY or LY or hydroxy-PY (HypY). Of these 6 peptides, ACE inhibitory activity was determined. **Table 6** shows the measured ratios of these peptides which demonstrate that these peptides are more prominent in Bh2t than in Bh2, and their IC_{50} values and 95% confidence intervals. This latter parameter predicts the interval that, for 95% certainty, contains the true IC_{50} value, and thus gives an idea about the precision of the calculated IC_{50} value. The most active peptides were IY, LY and DY with a respective IC_{50} value of 6.4, 70, and 90 μM. The dipeptides PY and HypY were intermediate active (IC_{50} value of 2380 and 4230 μM, respectively). When expressed in mg/mL, all peptides except HypY (IC_{50}: 1.24 mg/mL) have IC_{50} values lower than the IC_{50} value of Bh2t (IC_{50}: 1.04 mg/mL), ranging from 0.002 to 0.66 mg/mL. Previously, Wu et al. (*27*) also reported on ACE inhibitory IC_{50} values for diverse peptides, although these authors used another methodology, and interestingly, of the peptides identified here, IY, DY and LY showed the lowest respective IC_{50} value of 2.1, 100, and 180 μM. In general, all these data provide strong evidence that IY is very active to inhibit ACE, followed by DY and LY. Second, QY and HypY are novel ACE inhibitory peptides, with intermediate activity.

Table 6. Identified Peptides in Bh2t, Their IC_{50} Values with 95% Confidence Intervals, and Relative Quantification toward Bh2, Expressed as Light/Heavy Isotope Ratio (L/H)

peptide	IC_{50} (μM)[a]	95% CI (μM)	FWD L/H[b]	REV L/H[b]
AG	(*2500*)		1/70	not found
AGP	(*90*)		not found	23/1
VGP	(*30*)		1/5	7/1
PY	2380	1250−4540	1/10	12/1
QY	550	300−1000	1/105	250/1
DY	90	60−130	not found	not found
IY	6.4	5.5−7.3	1/100	not found
or LY	70	40−100		
or HypY	4230	3690−4860		

[a] (*Italic*): IC_{50} values according to Wu et al. (*27*). [b] In the forward experiment (FWD), Bh2 was labeled with the light (L), and Bh2t with the heavy (H) isotope. In the reverse experiment (REV), the labeling was reversed.

In addition to the amino terminus modification, the MS identified larger peptides were cleaved *in silico* with thermolysin using the ExPASy PeptideCutter tool (www.expasy.ch/tools/peptidecutter), as demonstrated for the peptides in **Table 5**, and the resulting di- and tripeptides that were most prominent in the

collagen sequence were checked for matching MS data. This led to the identification of AG, AGP and VGP. Interestingly, the latter two are strong ACE inhibitory peptides based on the data of Wu et al. (*27*) (**Table 6**), showing IC_{50} values of 0.02 and 0.008 mg/mL, respectively. The L/H ratios for these three peptides confirm that they are more prominent in Bh2t than in Bh2 (**Table 6**). However, the absolute concentration of all the identified peptides in Bh2t or Bh2 could not be determined by the methodology used here.

In conclusion, the bovine gelatin hydrolysate Bh2, under the condition of the extra thermolysin hydrolysis (Bh2t), shows high ACE inhibitory activity and resistance toward gastrointestinal and mucosal enzymes *in vitro*, and a marked blood pressure lowering effect *in vivo*, of which the involvement of the RAAS has been shown in organ baths. Seven small peptides, AG, AGP, VGP, PY, QY, DY and IY or LY or HypY were identified in Bh2t, with IY as the most active, followed by DY and LY, and with QY and HypY as two novel ACE inhibitory peptides. As these peptides showed ACE inhibitory activity and were more prominent in Bh2t than in Bh2, the current data provide strong evidence that these contribute to the antihypertensive effect of Bh2t.

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