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ACE Inhibitory Activity in Enzymatic Hydrolysates of Insect Protein

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In this paper, ACE inhibitory activity in insect protein hydrolyzed by various enzymes (gastrointestinal proteases, alcalase, and thermolysin) is reported for the first time. Four insects of different insect orders were tested: *Spodoptera littoralis* (Lepidoptera), *Bombyx mori* (Lepidoptera), *Schistocerca gregaria* (Orthoptera), and *Bombus terrestris* (Hymenoptera). ACE inhibitory activity was measured by two different methods: a spectrophotometric method using FAPGG (2-furanacryloyl-phenylalanyl-glycyl-glycine) as substrate, and an HPLC method using dansyltriglycine (DTG) as substrate. Hydrolysis of the insect protein resulted in an increased ACE inhibitory activity. Overall, the highest ACE inhibitory activity was obtained after gastrointestinal digestion. These results suggest a role for insect protein as antihypertensive component in functional foods and nutraceuticals. Furthermore, the ACE inhibitory activity differed according to the method used. As a consequence, there is a need to standardize methodologies to evaluate ACE inhibitory activity.

KEYWORDS: Insects; ACE inhibitory activity; hydrolysis

INTRODUCTION

"Angiotensin converting enzyme" (ACE) is a zinc metallopeptidase, found as a membrane bound ectoenzyme in various mammalian tissues such as lung, kidney, and the intestine (*1*). It acts mainly as a peptidyl dipeptidase by removing a dipeptide from the C-terminus of peptides such as angiotensin I. Furthermore, it can act as an endopeptidase by cleaving of peptides with amidated C-termini like substance P (*2*). In humans, ACE plays a key role in the regulation of blood pressure as well as in the water and fluid balance. By converting the inactive decapeptide angiotensin I into the octapeptide angiotensin II, and by inactivating the vasodilator bradykinin, ACE raises blood pressure (*3*).

Hypertension is one of the most common chronic medical conditions in the developed world and is a major risk factor for coronary heart disease, congestive heart failure, stroke, and renal disease. Cardiovascular diseases are still one of the main causes of early death in Western countries (*4*). Nowadays, hypertension is mainly treated by lifestyle modification and pharmacological treatment with antihypertensive drugs (*5*). Synthetic ACE inhibitors such as captopril are often used as medicine against hypertension, but they can cause serious side effects such as cough and angioedema (*6*). ACE inhibitory peptides, present as natural ingredients in both animal and vegetable proteins, provide valuable alternatives for the synthetic drugs. For example, Calpis sour milk, a fermented milk containing two ACE inhibitory peptides (VPP and IPP), significantly reduces blood pressure of hypertensive subject following a daily ingestion for 2 months (*7*). Therefore, functional foods have great potential to play a role in the treatment and prevention of hypertension.

Antihypertensive activity has been detected in various food proteins by hydrolysis with various enzymes (*8*−*10*). The food protein sources include casein (*11*), whey, and pea protein (*12*). Yet also fish protein, like sardine (*13*) and bonito (*14*), and porcine and chicken muscle (*15*, *16*) are known sources of ACE inhibitory peptides.

Until now, insect protein as a source of ACE inhibitory peptides has not been investigated. As insects/invertebrates possess enormous biodiversity and represent a large biomass (95% of the animal kingdom), they offer a tremendous potential as "natural resources" for new bioactive peptides. Although there is a negative attitude against the use of insects as food in the Western world (*17*), insects are a part of the daily diet in tropical and subtropical countries. Over 2000 different edible insect species have been recorded (*18*). Insects are consumed in Latin America, Africa, and Asia (*17*). As reported by Ramos-Elorduy et al. (*18*), insects form an important source of high quality protein for humans in Mexico.

In the present study, the ACE inhibitory activity of enzymatic hydrolysates of protein from insects of different insect orders, Lepidoptera, Orthoptera, and Hymenoptera, was investigated. Various enzymes were used to hydrolyze the insect protein, and

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Table 1. Parameters of Enzymatic Hydrolysis

enzyme	pH	T (°C)	incubation time (h)	enzyme/substrate
pepsin	2	37	2	1/250 (w/w)
trypsin:α-chymotrypsin (1:1)	6.5	37	2.5	1/250 (w/w)
alcalase	8	55	3	48 U/kg
thermolysin	8	37	5	1/1600 (w/w)

ACE inhibitory activity was measured by two different methods. The potency to screen for ACE inhibitory activities with a spectrophotometric method using FAPGG (2-furanacryloyl-phenylalanyl-glycyl-glycine) as substrate and a HPLC method using dansyltriglycine (DTG) as substrate was compared. The HPLC method was applied using two different amounts of ACE-extract.

MATERIALS AND METHODS

Products. ACE reagent, ACE control-E, pepsin, trypsin, α-chymotrypsin, alcalase, thermolysin, HCl, Trizma base, dansylglutamine, dansyl chloride, triglycine, dansylglycine, and rabbit lung acetone powder were purchased from Sigma-Aldrich (St. Louis, MO). NaOH, $NaH_2PO_4 \cdot 2H_2O$, and Na_2EDTA were obtained form Chem Lab (Lichtervelde, Belgium). Trichloroacetic acid was purchased from Acros Organics (Geel, Belgium), HPLC grade acetonitrile from Fisher Scientific (Loughborough, UK), and the Alltech Prevail C18 Column from Alltech Associates (Lokeren, Belgium).

Insects. *Bombyx mori.* Newly laid eggs of the silkworm *Bombyx mori* (Lepidoptera) were incubated at 25 °C for approximately 40−60 h after oviposition. The egg color changed during this period from pale yellow to reddish-brown. These eggs were kept for 1 week at 4 °C, followed by activation with 20% HCl (48 °C, 6 min). Approximately 12 days after activation, first-instar larvae hatched, and larvae were grown on fresh mulberry leaves. All stages of *B. mori* were maintained under standard conditions, 28 ± 1 °C, 70 ± 5% RH, and a light:dark (16:8) photoperiodic regimen (*19*). Fifth-instar larvae were collected.

Spodoptera littoralis. All stages of a continuous colony of the cotton leafworm *Spodoptera littoralis* (Lepidoptera) were maintained under standard conditions of 23 ± 1 °C, 70 ± 5% RH, and a light:dark (16:8) photoperiodic regimen as described previously (*20*). Larvae were fed on an agar-based artificial diet that had been placed in multiwell culture plates, and adults were fed a 20% honey water solution. Larvae of the last instar (sixth) of *S. littoralis* were collected.

Schistocerca gregaria. The desert locust *Schistocerca gregaria* (Orthoptera) was raised under crowded conditions ("gregarious") of 32 ± 1 °C, RH between 40% and 60%, and with a 13 h-light photoregime. All developmental stages were fed with oat flakes and fresh cabbage (*21*); adults were collected at random.

Bombus terrestris. Bumble bees *Bombus terrestris* (Hymenoptera) were kept in the dark in a controlled climate chamber and were fed with 50% sucrose solution and bee-collected pollen (*22*). Adults were collected.

Extract Preparation and Enzymatic Hydrolysis. Insects (300 g) were homogenized in a Waring-type blender with 3 L of Tris/HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged (10 000*g*, 15 min, 4 °C), and the supernatant was filtered and lyophilized.

Enzymatic hydrolysis of the insect extract (substrate) was performed using various enzymes. Subsequent hydrolysis with pepsin, trypsin, and α-chymotrypsin was conducted to simulate the human gastrointestinal digestion process. The digestion in the stomach was simulated by lowering pH to 2 with HCl (4 M), adding pepsin, and incubating for 2 h at 37 °C. Trypsin and α-chymotrypsin at pH 6.5 (with NaOH, 10 M) and incubation for 2.5 h at 37 °C simulated the small intestine phase (*12*). Furthermore, a hydrolysis using thermolysin and using alcalase was performed, followed by pH adjustment to 5 with HCl (4 M). The parameters of hydrolysis are shown in **Table 1**. All samples were centrifuged at 10 000*g* for 10 min at 4 °C, and the supernatant was filtered and lyophilized.

ACE Inhibitory Activity. Two methods were applied to measure ACE-inhibitory activity, that is, a spectrophotometric method using FAPGG as substrate, and a HPLC method using DTG as substrate.

The FAPGG method was performed as described by Vermeirssen et al. (*23*). Briefly, the substrate FAPGG is hydrolyzed by ACE to FAP and GG. The resulting decrease in absorbance is a measurement for the ACE activity. By addition of an inhibitory compound in the reaction mixture, the assay was modified to determine ACE inhibitory activity.

The ACE inhibitory activity measured by HPLC was done according to the method of Elbl and Wagner (*24*) with modifications by Hansen et al. (*25*). The assay is based on the cleavage of the chromophore- and fluorophore-labeled tripeptide DTG by ACE into dansylglycine (DG) and diglycine. The method was slightly modified in the present study. A sample solution (25 μL) was mixed with the ACE-extract (50 or 25 μL) and then incubated for 5 min at 37 °C. The ACE-extract is prepared from rabbit lung acetone powder, as described by Murray et al. (*26*). Then, 10 μL internal standard (dansylglutamine, 0.354 mM) was added, and the reaction was initiated by adding the substrate DTG (25 μL, 0.142 mM). After an incubation of 100 min at 37 °C, the reaction was stopped by adding 40 μL of Na_2EDTA (0.05 M). The substrate DTG was synthesized by mixing dansyl chloride and triglycine, followed by purification on column chromatography as described by Hansen et al. (*25*). The amounts of DG formed and DTG not cleaved by ACE were determined by isocratic reversed phase chromatography using an Alltech Prevail C18 column (5μ, 250 mm × 4.6 mm). The instrumentation consisted of a Gilson 305 HPLC-pump, a Gilson UV/vis detector, the Gilson Unipoint System Software (version 2.0), and a 20 μL injector loop. The elution buffer was NaH_2PO_4 (50 mM, pH 6) and acetonitrile, in a ratio of 70/30. The flow rate was 1 mL/min, and the temperature of the column 30 °C. The absorbance was measured at 250 nm. The percentage of inhibition was calculated from the equation:

$$\text{inhibition (\%)} = \left(100 - \frac{[\text{DG}]_s}{[\text{DG}]_c}\right) \times 100$$

where DG = dansylglycine, s = sample, and c = control.

Inhibition, in both tests, was expressed as the concentration of protein sample that inhibits 50% of ACE activity (IC_{50}), assuming that the activity of the blank is equal to 100%. Dose−activity curves were generated for doses of sample versus ACE inhibitory activity. The data were fitted by a 4 parametric logistic model using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The IC_{50}-value was obtained by the parameters of the fitted function:

$$y = \min + \frac{\max - \min}{1 + 10^{[\log IC_{50} - x]\text{hillslope}}}$$

Parameter y is the % of ACE inhibition, and x is the logarithm of the concentration (mg/mL). Parameter max equals the baseline of 100% inhibition, min the plateau of 100% activity (= 0% inhibition). Parameter IC_{50} gives the transition center, the concentration of protein sample that causes 50% inhibition. The hill slope determines the slope of the curve at the transition center. IC_{50}-values are based on two repeated measurements of all of the dose−activity curves and expressed as means ± SD. The significance of differences was tested with a comparison of fit at $p < 0.05$ (GraphPad Prism).

Degree of Proteolysis. The degree of proteolysis (DP) was determined by the ratio of the nonprotein Kjeldahl nitrogen to the total Kjeldahl nitrogen (*12*). The nonprotein Kjeldahl nitrogen was determined by treating the samples with trichloroacetic acid solution (15%), shaking at 170 rpm for 10 min, and filtration. For the calculation of the protein, a conversion factor of 6.25 was used. Values are based on four repeated measurements and expressed as means ± SD. The significance of differences was tested with a Student's t-test at $p = 0.05$.

RESULTS AND DISCUSSION

In this paper, we elicited, for the first time, the presence of ACE inhibitory activity in enzymatic hydrolysates of insect

Table 2. ACE Inhibitory Activity, Expressed as IC_{50}-Values (in mg/mL) Measured by the HPLC-Method Using 50 and 25 μL of ACE-Extract, and Degree of Proteolysis (% DP) of the Hydrolysates of *Bombus terrestris*, *Schistocerca gregaria*, *Spodoptera littoralis*, and *Bombyx mori*[a]

species	hydrolysis	DP (%)	HPLC 50 μL of ACE-extract IC_{50}	HPLC 25 μL of ACE-extract IC_{50}
Bombus terrestris	nonhydrolyzed	52.60 ± 1.02^{a}	22.465 ± 0.615^{a}	43.220 ± 12.66^{a}
	gastrointestinal	96.23 ± 3.70^{b}	4.969 ± 0.622^{b}	1.253 ± 0.120^{b}
	alcalase	90.94 ± 2.12^{c}	2.970 ± 0.770^{b}	ND
Schistocerca gregaria	nonhydrolyzed	59.35 ± 3.99^{a}	12.402 ± 3.406^{a}	8.157 ± 1.027
	alcalase	91.41 ± 6.51^{b}	19.670 ± 1.584^{a}	ND
Spodoptera littoralis	nonhydrolyzed	62.65 ± 12.67^{a}	6.266 ± 0.267^{a}	2.576 ± 0.367^{a}
	gastrointestinal	105.55 ± 2.51^{b}	2.864 ± 0.241^{b}	0.765 ± 0.073^{b}
	alcalase	101.92 ± 3.71^{c}	3.861 ± 0.830^{ab}	0.591 ± 0.341^{b}
	thermolysin	93.73 ± 1.06^{d}	4.539 ± 1.100^{ab}	19.240 ± 1.29^{c}
Bombyx mori	nonhydrolyzed	56.52 ± 0.36^{a}	72.470 ± 23.858^{a}	17.450 ± 6.17^{a}
	gastrointestinal	96.37 ± 1.83^{b}	0.697 ± 0.013^{b}	0.588 ± 1.134^{b}
	alcalase	101.71 ± 4.51^{b}	6.491 ± 3.372^{c}	2.630 ± 1.134^{c}
	thermolysin	78.84 ± 3.49^{c}	1.519 ± 0.380^{b}	2.834 ± 1.134^{c}

[a] DP-values are expressed as means ± SD based on four replicates. The significance of differences was tested with a Student's t-test at $p = 0.05$. IC_{50}-values are based on two repeated measurements of all of the dose–activity curves and expressed as means ± SD. Per insect species, letters (a–d) in the same column indicate a significant difference determined by a comparison of fit ($p < 0.05$). "ND" indicates that this analysis was not determined.

protein. The ACE inhibitory activity was measured using two different methods: a spectrophotometric method (FAPGG) and an HPLC method. The HPLC method was performed using two different amounts of ACE-extract, 50 and 25 μL of ACE-extract.

Table 2 shows the DP and the IC_{50}-value as determined by the HPLC-method of the hydrolysates of extracts of *B. mori*, *S. littoralis*, *S. gregaria*, and *B. terrestris*. All hydrolysates that were produced with gastrointestinal enzymes, alcalase, and thermolysin resulted in a significantly ($p < 0.05$) higher DP than the nonhydrolyzed sample. The relatively high DP obtained for all nonhydrolyzed samples is probably due to hydrolysis by proteolytic enzymes of the insect itself, which are released when making the extract (*27*). The percentage of protein of the nonhydrolyzed extracts was constant around 22% for *B. terrestris* (22.90 ± 0.77%), *S. gregaria* (22.27 ± 3.31%), and *S. littoralis* (21.69 ± 1.07%). For *B. mori*, the percentage of protein was higher, that is, 27.05 ± 0.27%. Furthermore, the percentage of nonprotein nitrogen (NPN) differed significantly ($p < 0.05$) between the species. The lepidopteran species, *S. littoralis* and *B. mori*, both had a percentage of NPN around 35% (i.e., 36.37 ± 1.10% and 35.17 ± 0.24%, respectively), while this % in *S. gregaria* and *B. terrestris* was significantly lower (32.31 ± 1.42% and 25.41 ± 0.40%, respectively).

From the results of the protein samples of *B. mori*, *S. littoralis*, and *B. terrestris* obtained with the HPLC-method, it was clear that gastrointestinal digestion with pepsin, trypsin, and chymotrypsin resulted in a significantly ($p < 0.05$) lower IC_{50}-value as compared to the nonhydrolyzed samples (**Table 2**). Interestingly, this result is observed with the HPLC-method using 50 μL as well as 25 μL of ACE-extract. Moreover, the FAPGG-method conducted on the nonhydrolyzed extract and the gastrointestinal digest of *B. terrestris* revealed the same conclusion, as the IC_{50}-values were 3.935 ± 0.014 and 0.214 ± 0.179 mg/mL, respectively. In **Figure 1**, the dose−response curves obtained by HPLC (50 μL of ACE-extract) for the nonhydrolyzed, gastrointestinal, and alcalase digest for *B. mori* are shown. After hydrolysis, the curves are shifted toward the left. Thus, in agreement with the study of Vermeirssen et al. (*23*), our results indicate an increase in ACE inhibitory activity after enzymatic hydrolysis.

The results obtained with alcalase and thermolysin also showed that hydrolysis increased the ACE inhibitory activity significantly ($p < 0.05$) as compared to the nonhydrolyzed protein samples. However, the effect was not consistent over

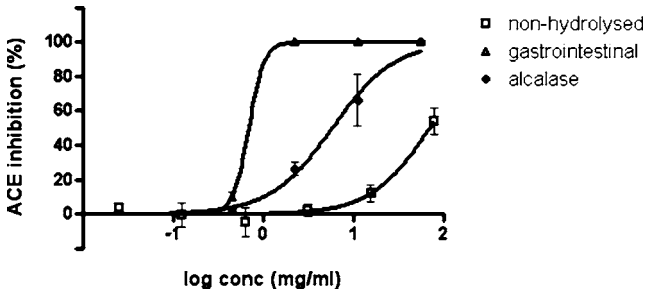

Figure 1. Dose–response curves of the nonhydrolyzed sample and of the gastrointestinal and alcalase hydrolysate of *Bombyx mori* obtained by HPLC with 50 μL of ACE-extract.

all insect species. For alcalase, lower IC_{50}-values were scored for *B. terrestris* (HPLC 50 μL), *B. mori* (HPLC 25 and 50 μL), and *S. littoralis* (HPLC 25 μL). The FAPGG-method was conducted on alcalase hydrolysates of *B. terrestris* and *S. gregaria* and resulted in respective IC_{50}-values of 0.899 ± 0.344 and 0.453 ± 0.001 mg/mL. The IC_{50}-values of the nonhydrolyzed samples were 3.935 ± 0.014 and 3.109 ± 0.546 mg/mL for *B. terrestris* and *S. gregaria*, respectively. As was the case for gastrointestinal hydrolysis, alcalase hydrolysis increased the ACE inhibitory activity.

Hydrolysis with thermolysin was tested on two species: *B. mori* and *S. littoralis* (**Table 2**). For *B. mori*, a significant increase in ACE inhibitory activity was obtained after thermolysin hydrolysis. In contrast, for *S. littoralis*, the thermolysin hydrolysate showed a significantly lower ACE inhibitory activity (HPLC 25 μL) or a nonsignificant difference as compared to the nonhydrolyzed sample (HPLC 50 μL).

It should be noted that the IC_{50}-values differ when using 50 or 25 μL of ACE-extract in the HPLC-method. The fact that the ACE inhibitory activity differs when using the same method but a different amount of enzyme was already reported before by Murray et al. (*26*). The latter authors showed that increasing the level of ACE activity in their assay resulted in a corresponding increase in the IC_{50}-value for captopril. The results in this paper confirm that the ACE inhibitory activity (expressed as IC_{50}-value) differs according to the method used. When using the HPLC-method to screen for ACE inhibitory activity, it is preferred to work with 50 μL of ACE-extract instead of 25 μL of ACE-extract as the area of the product peak is larger, making discrimination of an ACE inhibitory activity more accurate.

In literature, numerous studies on ACE inhibitory activity derived from vertebrate proteins are reported. Katayama et al. (*16*, *28*, *29*) showed that porcine crude myosin hydrolyzed with pepsin, chymotrypsin, and trypsin showed IC_{50}-values of 0.112, 0.121, and 0.166 mg/mL, respectively. A peptic hydrolysate of troponin showed an IC_{50}-value of 0.225 mg/mL. Matsui et al. (*13*) measured the ACE inhibitory activity of the alcalase hydrolysate and the peptic hydrolysate of sardine muscle. The IC_{50}-values were 0.250 and 0.620 mg/mL, respectively. A preheat treatment of the sardine muscle before hydrolysis with alcalase resulted in a higher ACE inhibitory activity (IC_{50} = 0.083 mg/mL). A thermolysin digest of chicken muscle showed almost the same potent ACE inhibitory activity (IC_{50} = 0.045 mg/mL) as that of dried bonito (IC_{50} = 0.029 mg/mL) (*15*). By hydrolyzing defatted muscle protein of chum salmon with 5% thermolysin, an IC_{50}-value of 0.0279 mg/mL was obtained (*30*). A gelatine extract from Alaska Pollack skin was hydrolyzed by serial protease-treatments. The hydrolysates prepared with alcalase and Pronase E revealed ACE inhibitory activity showing respective IC_{50}-values of 0.629 and 0.649 mg/mL (*31*).

It should be noted that in all of the examples from literature, ACE inhibitory activity was measured using the method of Cushman and Cheung (*32*), which is similar to the FAPGG-method, a spectrophotometric assay, but in which the rate of production of hippuric acid was followed by degradation of hippuryl-L-histidyl-L-leucine by ACE. Overall, the samples used in this paper tend to have a higher IC_{50}-value as compared to the ones in the examples given above. Seven IC_{50}-values in this paper are in the same range as the ones in the given examples; for example, the gastrointestinal digest of *B. mori* protein measured with HPLC (IC_{50} = 0.697 mg/mL) has an IC_{50}-value similar to that of the hydrolysates prepared from Alaska Pollack skin (IC_{50} = 0.649 mg/mL). However, as different tests are used, comparison of the results from this paper with the ones reported in the literature cited above should be handled with care.

In conclusion, this is the first report of ACE inhibitory activity derived from insect protein. Hydrolysis of the insect protein with different enzymes resulted in an increased ACE inhibitory activity. Overall, the highest ACE inhibitory activity was obtained with the gastrointestinal digestion. These results suggest a role for insect protein as antihypertensive component in functional foods and nutraceuticals. Furthermore, we confirmed that ACE inhibitory activity differs according to the method used, creating a need to standardize methodologies to evaluate ACE inhibitory activity.

ABBREVIATIONS USED

ACE, angiotensin converting enzyme; DG, dansylglycine; DTG, dansyltriglycine; DP, degree of proteolysis; FAP, 2-furanacryloyl-phenylalanine; FAPGG, 2-furanacryloyl-phenylalanyl-glycyl-glycine; NPN, nonprotein nitrogen; RH, relative humidity.

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