Antihypertensive effect of insect cells: in vitro and in vivo evaluation

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ABSTRACT

In this study, we investigated the *in vitro* ACE inhibitory and *in vivo* antihypertensive effect of insect cell extracts. The IC_{50} of three insect cell lines from different type and insect species origin: S2 (embryo, *Drosophila melanogaster*), Sf21 (ovary, *Spodoptera littoralis*) and Bm5 (ovary, *Bombyx mori*), were evaluated. Most interesting results were that the IC_{50} values ranged between 0.4-0.9 mg/ml, and that an extra hydrolysis with gastrointestinal enzymes did not increase the ACE inhibitory activity conspicuously. Finally, a single oral administration with a gavage of 150 mg cell extract/kg BW to spontaneous hypertensive rats (SHR) significantly decreased ($p < 0.05$) their systolic blood pressure (SBP) with 5-6% (9-12 mm Hg) compared to the controls at 6 h post-administration. Here the undigested and digested insect S2 cell extracts were equal in activity to lower the SBP. To the best of our knowledge, this is the first report of *in vivo* antihypertensive activity of insect cell extracts and this without an extra digestion requirement.

KEYWORDS

Insect cells, bioactive peptides, hydrolysis, ACE inhibition, blood pressure, SHR

INTRODUCTION

Hypertension or high blood pressure [systolic blood pressure (SBP) >140 mm Hg or diastolic blood pressure >90 mm Hg] is an important worldwide problem and forms an important risk factor for the development of cardiovascular diseases [21]. Cardiovascular diseases are one of the major causes of death in the Western world [12]. High blood pressure nowadays is treated by a combination of antihypertensive medication and a healthier lifestyle [15]. Blood pressure is regulated by several mechanisms and one of these mechanisms is the renin-angiotensin-aldosteron system (RAAS) which involves the angiotensin converting enzyme (ACE), a zinc metallopeptidase. ACE increases blood pressure via two major pathways: it converts the inactive angiotensin I into angiotensin II, a vasoconstrictor, and breaks down bradykinin, a vasodilator [30].

To date, synthetic ACE inhibitors like captopril are widely used, but these may cause severe side effects like cough and angio-oedema [3]. In this context the ambition exists that some food proteins possess the ability to release ACE inhibitory peptides after hydrolysis [14,42]. Such peptides might form an ingredient for functional foods or nutraceuticals which might be an alternative for the use of medication or might postpone its use.

In the continuation of our research with insects, we have recently shown the unique concept that insects can serve as a source for ACE inhibitory peptides [38,39,41]. Interestingly, insects are being used for human consumption in a wide range of regions over the world [5,11,32]. Hence, Vercruysse et al. [37] reported that enzymatic hydrolysis is important and necessary for releasing bioactive peptides with ACE inhibitory activity. For example gastrointestinal digestion of a protein extract of silk moth *Bombyx mori* caterpillars, using pepsin, trypsin and chymotrypsin, decreased the IC_{50} values about 100 fold; namely from 73 mg/ml, which represents a very low to no activity, to 0.7 mg/ml, representing high

activity [38]. Besides, the potency of the ACE inhibitory peptides from insect protein hydrolysate was confirmed *in vivo* [41]. However, it needs to be mentioned that a mass culture maintenance of whole insects is posing different disadvantages such as high labor costs for insect feeding and cleaning of the cages on a regularly basis, insect health concerns, exploitation facilities and permits [10,11]. It can therefore be proposed to use insect cell cultures as a valuable alternative source of insect protein/polypeptides [43]. Indeed, insect cells can be cultured in standardized biotechnology bioreactors, in suspension or immobilized on substrates, to create a large biomass [1,13,33,47], and they can so be used as a food and protein source [43]. Another advantage of insect cell cultures over whole insects is the characteristic of a very homogenous product with a stable quality and high protein content.

This paper describes the antihypertensive capacity of insect cells based on ACE inhibition. At first, the potential of a protein extract of different insect cells to inhibit ACE activity was tested *in vitro.* Three different cells lines and types were selected: (a) the dipteran *Drosophila melanogaster* S2 cells that are from embryonic origin and can be characterized as small, round and fast growing, (b) the lepidopteran *Bombyx mori* Bm5 cells that are from ovarian origin and typically large, and (c) the ovarian cloned Sf21 cells of the fall armyworm *Spodoptera frugiperda* that are widely used in biotechnology industry for *in vitro* production. Second, we investigated the benefit of hydrolysis to increase ACE inhibitory activity by release of more bioactive peptides from the protein extract. Based on Vercruysse et al. [37] we employed a gastrointestinal digestion protocol with use of pepsin, trypsin and chymotrypsin as this was found to work best with insect protein extracts. In a final step, the antihypertensive effect of the insect cell extracts was measured *in vivo* using spontaneously hypertensive rats (SHR) to support their potential use in lowering blood pressure at organism level.

MATERIALS AND METHODS

Products

Pepsin, trypsin, α-chymotrypsin, hippuryl-L-histidyl-L-leucine (HHL), ACE (from rabbit lung), o-pthaldialdehyde (OPA), antibiotics (antibiotic-antimycotic stabilized (AAS) contains 10000 units/ml penicillin G, 10 mg/ml streptomycinsulfate and 25 µg/ml amphotericine B), IPL-41 insect medium, tryptose phosphate, sucrose, $ZnSO_4.7H_2O$, $AlK(SO_4)2$, cytochrome C, insulin, substance P and Val-Tyr were purchased from Sigma-Aldrich (Bornem, Belgium; St. Louis, IL). SFX insect medium was purchased from Hyclone (Logan, UT). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA).

Insect cell cultures

The insect cell lines S2 [34] and Sf21 [37], that originated from the embryo of the fruitfly (*D. melanogaster*, Diptera) and from ovarian tissue of the fall armyworm (*S. frugiperda*, Lepidoptera), respectively, were cultured in SFX medium with 10 ml AAS/l. The Bm5 cell line that originated from *B. mori* ovary (Lepidoptera) [35] was kept in IPL-41 medium, supplemented with 10% FBS, 2.6 g/l tryptose phosphate, 9.0 g/l sucrose, 0.069 mg/l $ZnSO_4.7H_2O$, 7.59 mg/l $AlK(SO_4)_2$ and 10 ml/l AAS at pH 6.3. All cells were incubated at 27°C.

Preparation of protein/polypeptide extracts from insect cells, including hydrolysis with gastrointestinal enzymes

After insect cell harvesting by gentle centrifugation (300 g, 20 min), the pelleted cells were resuspended in distilled water, and then frozen and thawed for obtaining a cell protein extract. The resulting extract was lyophilized.

For a gastrointestinal digestion with pepsin (pH 2) followed by trypsin/α-chymotrypsin (pH 6.5), that was found as best to release ACE bioactive peptides of whole insects and increase ACE inhibitory activity [37], the powdered lyophilized protein/polypeptide insect cell extracts as obtained above were dissolved in distilled water (200 mg of sample in 5 ml distilled water), the pH was lowered to 2 (HCl, 0.1 M), pepsin was added (1 g enzyme per 250 g of sample), and the solution was incubated for 2.5 h at 37°C. Subsequently, the pH was set at 6.5 (NaOH, 0.1 M), trypsin and α-chymotrypsin were added (1 g enzyme per 250 g of sample) and after incubation for 2.5 h at 37°C, the solution was heated to 80°C for 15 min to stop the enzymatic reaction, and the resulting hydrolysate was lyophilized as previously described [37].

ACE inhibitory activity *in vitro*

ACE inhibitory activity was measured according to the colorimetric method of Chang et al. [8] with slight modifications. In brief, the substrate hippuryl-histidyl-leucine (HHL) is cleaved by ACE into hippuric acid (H) and L-histidyl-L-leucine (HL). At pH >11, the dipeptide HL reacts with o-phtaldialdehyde (OPA) forming a yellowish product, of which the absorbance can be measured at 390 nm. The ACE catalyzed reactions were performed in cuvettes containing 100 µl of sample solution (containing the powdered lyophilized protein/polypeptide insect cell extracts), 100 µl of ACE solution, and 100 µl of HHL solution for 2 h at 37°C. The enzymatic reactions (pH 5-10) were terminated by adding 2 ml of the alkaline OPA reagent. Absorbance was measured after 20 min-incubations at 25°C.

Concentration-response curves were generated for the logarithm of the concentration of the sample (mg/ml) versus ACE inhibitory activity (%) with use of the nonlinear sigmoid regression in Prism v4 software (GraphPad Prism, La Jolla, CA); the goodness of fitness was evaluated based on R^2. The IC_{50} values, referring to the concentration of sample inhibiting

50% of ACE activity, together with corresponding 95% confidence limits (95% CL), were calculated as previously described [38]. The concentration-response curves are made with 10 concentrations of sample, and each value is expressed as mean ± SD based on 3 repeated measurements.

Peptide profile with gel filtration chromatography

Peptide samples were analyzed with use of a Superdex Peptide HR 10/30 column (Alltech Associates, Lokeren, Belgium) coupled to a UV detector (Thermo Surveyor Finnigan, Spectralab Scientific Inc., Toronto, Canada), measuring at 214 nm. The elution buffer (0.02 M $NaH_2PO_4.2H_2O$, 0.25 M NaCl, pH 7.2) was pumped through the column at a flow rate of 0.5 ml/min. The samples were dissolved in elution buffer (10 mg lyophilized powder/ml) and injected with a loop of 50 µl. The column was calibrated with cytochrome C (12500 kDa), insulin (5777 Da), substance P (1348 Da) and Val-Tyr (280 Da).

Antihypertensive effect with SHR *in vivo*

Male SHR of 10-14 weeks old and a fresh weight of 230-310 g were purchased from Harlan (Horst, the Netherlands), and housed in steel cages in a climatized room kept at 24°C with a 12 h dark-light cycle. They were fed a standard laboratory diet and tap water was freely available. The powdered lyophilized protein/polypeptide insect cell extracts as obtained above were dissolved in 0.5 ml of tap water at a dose of 150 mg per kg body weight (BW), and treated orally using a plastic gavage. Control rats were administered the same volume of tap water. Following oral administration with use of a gavage (0 h), the SBP was measured in conscious restrained rats after 2, 4, 6, 8 and 24 h by the tail-cuff method with a piezoelectric pneumatic pulse transducer [24]. The change of the SBP since time 0 h was expressed as mean ± SEM based on 3 measurements with 6 SHR for each sample (undigested and digested

insect cell extract) and 9 SHR for the control. The significance of differences between treatments and the controls was calculated with ANOVA and means were separated by a *post hoc* Tukey test (S-Plus, TIBCO software Inc., Palo Alto, CA). These experiments were approved by the ethical committee for animal experiments of the Faculty of Medicine and Health Sciences, Ghent University.

RESULTS

Potency of different insect cells to inhibit ACE

In this project three different insect cell lines S2 *(D. melanogaster),* Sf21 *(S. littoralis)* and Bm5 *(B. mori)* were evaluated for their ACE inhibitory activity *in vitro*. The insect cells were homogenized by freezing and thawing. As depicted in Fig. 1, the median effect concentrations (IC_{50}) of the three different undigested insect cell extracts to inhibit ACE ranged between 0.7-0.9 mg lyophilized cell extract per ml, and were not significantly different based on overlapping 95% CL.

Need for extra hydrolysis with use of insect cells as protein source to inhibit ACE

A gastrointestinal digestion was applied to the insect cell extracts of the three different cell lines. The calculated IC_{50} values of the digested insect cell extracts to inhibit ACE ranged between 0.4-0.7 mg lyophilized cell extract per ml. They have overlapping/touching 95% CL with the IC_{50} values of the undigested insect cell extracts (Fig. 1).

On the water extraction by freezing and thawing, we can confirm that this resulted in a chromatographic peptide profile with most proteins/peptides being bigger than 5 kDa; however some small peptide peaks (ranging between 150-500 Da) were present, as seen for S2 cells in Fig. 2. A similar peptide profile was observed for Bm5 and Sf21 cells (data not

shown). In addition, the extra gastrointestinal digestion with pepsin and trypsin/α-chymotrypsin caused a clear shift to more and smaller peptides, confirming effective (further) cleavage of the protein/polypeptide fraction. As depicted in Fig. 2, most peptides after digestion had a molecular weight below 5 kDa with S2 cells and this concurred with a higher fraction of polypeptides/peptides ranging between 150-500 g/mol. This trend was also found for Bm5 and Sf21 cells (data not shown).

Antihypertensive activity of insect cell extracts in SHR *in vivo*

SHR is an accepted animal model to evaluate the antihypertensive effect of components with ACE inhibitory activity. To test the antihypertensive effect of the insect cells, the change in SBP of SHR after oral administration of extracts of S2 *D. melanogaster* cells was measured. We selected to work with S2 cells as the ACE inhibitory activity seemed independent of cell type and insect species (see above) and S2 cells are fast growing, which allows a rapid collection of a high biomass amount of insect cell extract for testing on SHR. The basal SBP measured at time point zero in SHR was 178 ± 3 mm Hg. As depicted in Fig. 3, the change in SBP after a single oral administration of undigested S2 cell extract at 150 mg/kg BW tended ($p = 0.051$) already to be lower as compared to the control (ΔSBP of about 6 mm Hg) after 4 h, and was significant at 6 h ($p = 0.003$: 9 ± 3 mm Hg on 178 mm Hg, representing 5% decrease) and at 8 h ($p = 0.03$; 6 ± 4 mm Hg on 178 mm Hg, representing 3% decrease) post-administration. Also the S2 cell extract after an extra gastrointestinal digestion was tested with one single dose at 150 mg/kg BW in SHR, and the ΔSBP curve for the digested sample demonstrated a high activity (Fig. 3). At 4 h post-administration, the SBP was significantly ($p = 0.02$) lowered compared to the water-treated controls and the effect increased during the course of the experiment. After 6 h, the significant ($p = 0.0007$) decrease in SBP yielded 12 ± 3 mm Hg, which represents a decrease of 6%, and the effect remained also at 8 h ($p = 0.009$).

The decrease in blood pressure caused by the digested S2 cell extract was not significantly different from that of the undigested S2 cell extract ($p > 0.05$).

DISCUSSION

In this project we investigated the ACE inhibitory potential of water extracts of three different insect cell lines (*i.e.* S2, Sf21 and Bm5), resulting in IC_{50} values ranging between 0.7-0.9 mg/ml. These values are low enough to be considered as biologically active against ACE [6,9,18-20,36,45,46]. Interestingly, the three IC_{50} values for the undigested water extracts of the insect cell lines of the three insect species were not significantly different, suggesting that insect cell extracts possess the potential to inhibit ACE independent of cell type and insect species origin. As compared to previous experiments with use of whole insect body extracts where for instance IC_{50} values of 22.5 mg/ml for bumblebees *Bombus terrestris*, 12.4 mg/ml for locusts *Schistocerca gregaria*, and 6.3 mg/ml for armyworms *Spodoptera littoralis* were obtained [38], the IC_{50} values of insect cell extracts are much lower. To explain the higher activity with use of insect cells, it should be mentioned that cell cultures contain relatively higher amounts of protein per biomass as compared to whole insect bodies which contain highly sclerotized cuticle parts like mouthparts, legs, wings and other low-protein parts [23]. In addition, we speculate that the protein/polypeptide fraction of insect cell lines is more ready available. In insect cells for instance the extraction matrix is free of the whole body exoskeleton that contains high amounts of proteins but which are complexed in the chitin polymerized matrix [22,27]. Besides, it is reasonable that intracellular enzymes, which can be activated during the homogenization by freezing and thawing, cleave proteins/polypeptides resulting in ACE inhibitory peptides. However, to date little is known about such enzymes in insect cells. As a consequence, we envisage that future research can be of interest to indicate

255 which insect cell-related enzymes are responsible for generating a high ACE inhibitory
256 activity. In conclusion, the present results provide strong evidence that a relatively simple
257 water extraction of insect cells possesses high ACE inhibitory activity and this is suggested to
258 be independent of the cell type and insect species origin. These findings are very promising
259 for using insect cells as a dietary protein source to help control hypertension.

260 In a recent study of Vercruysse et al. [37] enzymatic hydrolysis of whole insects was
261 necessary to obtain a significant increase, ranging between 5 and 100 fold, of ACE inhibitory
262 activity. In the latter study, the simulated human digestion by gastrointenstinal enzymes with
263 pepsin at pH 2 followed by trypsin/ α-chymotrypsin at pH 6.5 was found the best with an
264 increase in activity of nearby 100 fold. Similarly, other authors confirmed the necessity of
265 enzymatic hydrolysis with for instance thermolysin, proteinase A, alcalase, collagenase
266 [4,7,16,17,29,31] and the great potential of gastrointestinal digestion [2,25,26] to obtain a
267 bioactive hydrolysate/peptide fraction for ACE inhibition. Interestingly, an extra hydrolysis of
268 the insect cell extracts only caused a minor improvement of the ACE inhibitory activity, as
269 the IC_{50} values ranged between 0.4-0.7 mg/ml. This is in great contrast to many previous
270 reports in the recent decade [4,17,28,29,31,36,38,44,45] that confirmed that an enzymatic
271 hydrolysis of food protein is an important step for obtaining ACE inhibitory activity.
272 Although the values of the digested insect cell extracts are very close to the IC_{50} values of the
273 undigested insect cell extracts as they have touching/overlapping 95% CL, the current results
274 suggest that the maximum ACE inhibitory potential of the insect cell extracts is not fully
275 exploited yet with a simple water extraction by freezing and thawing. Future research can
276 focus on the optimization of the extraction procedure with an extra hydrolysis step of the
277 insect cells to increase the activity. However, this increase will probably only be to a limited
278 extent, since as stated above, cell cultures contain high amounts of protein that are readily
279 available for digestion by cell-related enzymes during the water extraction. Taken together,

these results demonstrated that an extra hydrolysis is not essential to obtain an ACE inhibitory activity.

Next to *in vitro* activity for ACE inhibition, the antihypertensive potential of the undigested and digested insect S2 cell extracts was also investigated *in vivo* with SHR. The maximum decrease in blood pressure after a single oral administration amounted to 5-6% against the baseline. Indeed it was of great interest in this project that the effects of nondigested and digested S2 cell extracts were significantly equal over the course of the experiment. These results are in accordance with the expectations based on the IC_{50} values from our *in vitro* ACE inhibition tests. So in conclusion the results of this project demonstrated that a water-based protein extract of insect S2 cells exerts *in vivo* antihypertensive activity after a single oral administration and that hydrolysis is not a necessity to release the antihypertensive peptides. Moreover, the equal antihypertensive effects of the undigested and digested S2 cell extracts confirm that the ACE inhibitory peptides are (at least in part) resistant to the rat gastrointestinal digestion. However, it should also be mentioned here that we believe that it cannot absolutely be excluded that also other components than peptides can be present in the insect cell extracts and that they are involved in ACE activity and/or other blood pressure regulatory mechanisms. Here, we envisage that long term administration of the insect cell extracts to SHR is important. Studies with long term administration could provide more information whether the effect on blood pressure is directly linked with/influenced by ACE inhibition and/or other blood pressure regulating mechanisms as other factors could be evaluated such as the sodium in the urine, the organ weight (liver, kidney, heart and lungs) and serum ACE activity [40,41]. In addition, identification of the bioactive peptides in the insect cell extracts would help to confirm or generate new lead structures for ACE inhibition and antihypertension and potentially other biologically innovative physiological events.

In conclusion, to the best of our knowledge, this is the first report of *in vivo* antihypertensive activity of insect cell extracts and this without requirement of an extra digestion. As a consequence, our results confirm the potential of insect cells, which can easily be cultured in industrial bioreactors to obtain high biomass amounts, as a source of bioactive peptides for functional foods or nutraceuticals with antihypertensive activity.

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Figure legends

Fig. 1 – (A) Sigmoid concentration-response curves for ACE inhibition with protein/polypeptide water extracts of the three insect cell lines, S2, Sf21 and Bm5, when extracted by freezing and thawing (undigested) and after an extra gastrointestinal digestion (digested). The concentration-response curves are made with 10 concentrations of sample, and each value is expressed as mean ± SD based on 3 repeated measurements. (B) Tabulated IC_{50} values (in mg/ml) for undigested and digested extracts of the three insect cell lines (S2, Sf21 and Bm5) together with corresponding 95% confidence limits (95% CL), and the R^2 as goodness of fitness for each concentration-response curve.

Fig. 2 - Chromatographic profiles of undigested and digested S2 cells with Superdex Peptide column. Absorbance was measured at 214 nm.

Fig. 3 - Change in SBP with use of spontaneous hypertensive rats (SHR) after a single oral administration of 0.5 ml of tap water (control, n = 9), undigested and digested S2 cell extract (150 mg/kg BW dissolved in 0.5 ml tap water, n = 6). Values with different letters (a, b) indicate a significant difference ($p < 0.1$) between values at the same time point calculated with ANOVA followed by a *post hoc* Tukey test (df = 2; 2 h: F = 0.28, p = 0.75; 4 h: F = 2.36, p = 0.12; 6 h: F = 9.72, p = 1.54e-3; 8 h: F = 3.72, p = 0.05; 24 h: F = 0.62, p = 0.55).