Characterization of Antidiabetic and Antihypertensive Properties of Canary Seed (*Phalaris canariensis* L.) Peptides

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Supporting Information

**ABSTRACT:** Canary grass is used as traditional food for diabetes and hypertension treatment. The aim of this work is to characterize the biological activity of encrypted peptides released after gastrointestinal digestion of canary seed proteins. Canary peptides showed 43.5% inhibition of dipeptidyl peptidase IV (DPPIV) and 73.5% inhibition of angiotensin-converting enzyme (ACE) activity. An isolated perfused rat heart system was used to evaluate the canary seed vasoactive effect. Nitric oxide (NO), a major vasodilator agent, was evaluated in the venous effluent from isolated perfused rat heart. Canary seed peptides (1 μg/mL) were able to induce the production of NO (12.24 μM) in amounts similar to those induced by captopril (CPT) and bradykinin (BK). These results show that encrypted peptides in canary seed have inhibitory activity against DPPIV and ACE, enzymes that are targets for diabetes and hypertension treatments.

**KEYWORDS:** angiotensin-converting enzyme, canary seed, dipeptidyl peptidase IV, encrypted peptides, nitric oxide

**INTRODUCTION**

The canary seed (*Phalaris canariensis* L.) belongs to the Gramineae family with production practices and life cycle similar to those of other winter cereal crops such as wheat and oats.1,2 The grain is cultivated in many areas of the temperate climates and, currently, its production is concentrated in the southwestern provinces of Canada and, on a smaller scale, in Argentina, Thailand, and Australia.3–5 Its main use is as food for birds, alone or mixed with other grains such as millet, sunflower seeds, and flaxseeds.3,4

Due to its good nutritional value, interest in canary seed has increased as an alternative nongluten cereal.1,2 Compared to common cereals, canary seed contains high levels of protein (21%), although more deficient in both lysine and threonine than wheat proteins. Canary seed is very rich in cysteine, tryptophan, and phenylalanine.6 The groats contain 61% starch, which are composed by small polygonal granules with diameters of 1.5–3.5 μm with lower amylose contents than wheat and high pasting temperatures forming rigid and highly stable gels when cooked or frozen.7 Canary seed groats are rich in crude fat (8.7%), which is mainly composed of linoleic (55%), oleic (29%), palmitic (11%), and linolenic acids (2.5%).8 The sterol and triterpene alcohol esters of caffeic acid are the major effective antioxidant components in the crude oil of a canary seed.9 The major carotenoid compounds identified in canary seed are lutein, zeaxanthin, and β-carotene.10

Traditionally, canary seeds are also used as a “folk medicine” for diabetes and hypertension treatments; however, there is no scientific information about the possible bioactives responsible for such effects. Type 2 diabetes mellitus is a metabolic disorder characterized by a hyperglycemic chronic state due to several fundamental defects such as insulin resistance in muscles and liver and an impaired insulin secretion by pancreatic β-cells.11 The incretin-based therapy is actually used as diabetic treatment. Incretins are responsible for >50% of postprandial insulin secretion, especially the hormone glucagon-like peptide 1 (GLP-1).12 However, the half-life of incretins is very short due to the cleavage and inactivation of these proteins by dipeptidyl peptidase IV (DPPIV).13 Hence, DPPIV inhibitors are the actual therapies that have shown promising results as antidiabetic agents.14 Hypertension is about twice as common in a diabetic as in nondiabetics and has become the most common public health problem worldwide.15 Hypertension treatments are based on inhibition of the angiotensin converting enzyme (ACE). ACE is a peptidylpeptidase hydrolase that plays an important physiological role in the regulation of blood pressure. ACE catalyzes the conversion of the inactive decapaptide angiotensin I into a powerful vasoconstrictor octapeptide, angiotensin II.16 ACE also catalyzes the inactivation of the vasodilator bradykinin (BK) that regulates different biological processes including vascular endothelial nitric oxide (NO) release mediated by the activation of eNOS. NO exerts its effects on the vascular smooth muscles, promoting vascular relaxation.17

Peptides with inhibitory activity of DPPIV and ACE have been found in several food proteins,18,19 but there are no reports about the presence of these peptides encrypted in canary seed proteins that could be responsible for its claimed health benefits. Thus, the aim of this work was to test the
hydrolysis conditions to release the canary seed peptides and to evaluate the inhibitory activity of these peptides against DPPIV and ACE. In addition, the antihypertensive function of canary seed peptides was tested on isolated perfused rat heart system.

**MATERIALS AND METHODS**

**Biological Material.** Canary seed (Birdseed “Marubu”) and commercial canary seed flour (“more Lait”), used as folk medicine, were purchased in local markets. Seeds were ground in a mill (Krup Coiffe, Medford, MA, USA) and passed through 80 mesh. Defatted flours were obtained according to a reported protocol.20 Canary seeds were placed in water (1:5 w/v, flour/water) overnight at room temperature. The soaking water was removed, the seeds were dried and milled, and the resultant flour was named canary seed milk.

**Seed Storage Protein Fractionation.** Proteins from (a) canary seed flours, (b) commercial flour “more Lait”, and (c) canary seed milk were fractionated on the basis of their solubility.20,21 The albumin fraction was solubilized with distilled water, and the suspension of flour/water (1:10 w/v) was under agitation for 1 h and centrifuged at 20000g for 30 min. The resulting pellet was resuspended in 0.1 M NaCl, 0.01 M KH₂PO₄, and 1 mM EDTA, pH 7.5, agitated and centrifuged as above, and the soluble protein was named 75 globulins. The pellet was resuspended in 0.8 M NaCl solution, 0.01 M KH₂PO₄, and 1 mM EDTA, pH 7.5, to obtain the 11S globulin fraction. Prolamins were extracted from the resulting pellet with 70% ethanol, and the final pellet was extracted with 0.1 M NaOH to obtain the glutenin fraction. Total proteins were extracted from canary seed flours (1 g) with 10 mL of buffer containing 7.5 M urea, 63 mM CHAPS, 2.2 M thiourea, 22 mM Tris-HCl, 17.3 mM trizma base, and 0.25% (v/v) Triton X-100, pH 3.1; the suspension was mixed with vortex and centrifuged for 10 min at 20000g, and the supernatant was named total protein. All extractions were performed in triplicate.

**Tryptic Digestion of Canary Seed Proteins.** Total protein extracts were used for protein digestion using trypsin from porcine pancreas (Sigma-Aldrich, St. Louis, MO, USA) at a ratio of enzyme/substrate 1:5 (w/w). Hydrolysis was carried out in a 100 mM Tris buffer, pH 8, at 37 °C for 6 h. The reaction was stopped by freezing, and the enzyme was removed by ultrafiltration using 10 kDa MWCO filters (Millipore, Billerica, MA, USA).

**Simulation of Gastrointestinal Digestion Method.** The protein hydrolysis of canary seed samples was also performed using the simulated gastrointestinal method.19 Flour (1 g) was resuspended in 20 mL of 0.03 M NaCl, pH 2, heated in a water bath at 80 °C for 5 min, and allowed to cool to room temperature. Porcine pepsin (Sigma-Aldrich) previously dissolved in 0.03 M NaCl, pH 2.0, was added in a 1:40 ratio (w/w enzyme to substrate). The samples were digested at a constant pH 3 for 3 h at 37 °C under constant agitation. Next, the pH was adjusted to 7.5, and a mixture of trypsin/pancreatin from porcine pancreas (Sigma-Aldrich) was added at a ratio of 1:1 w/w; enzymes were previously dissolved in 0.1 N NaHCO₃ at a ratio of 1:5000 (w/w enzyme/substrate). Samples were incubated at a constant pH 3 for 3 h. Digestion was stopped by heating the samples at 75 °C for 20 min; the samples were allowed to cool and centrifuged at 20000g for 30 min. To remove the enzymes, the digests were ultrafiltered using 10 kDa MWCO membranes (Millipore).

**Protein Quantification.** Canary seed protein fractions were quantified using the kit Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). The concentration of peptides released by the trypsic and gastrointestinal digestion methods was determined using the Lowery-based DC Protein Assay (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as a standard.

**Electrophoresis.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli.22 Protein samples (5 μg) were dissolved in 0.1 M Tris-HCl, pH 6.8, and 2% SDS. Electrophoresis was conducted at a constant current of 20 mA per gel for 2–3 h. After electrophoresis, the gel was stained with Phast Gel Blue R-350 (GE Healthcare Bio-Science AB, Uppsala, Sweden) in a final concentration of 0.02%. Destaining was accomplished by washing the gel with a solution of 30% methanol, 10% acetic acid, and water. Gels were digitized in a Gel-Doc XR (Bio-Rad).

**Assay for Inhibitory Activity of Dipeptidyl Peptidase IV.** The assay for in vitro activity of DPP IV was carried out as reported previously.19 The chromogenic substrate Gly-Pro-pNA (500 μM) and 100 ng/mL dipeptidyl peptidase IV isolated from a porcine kidney (Sigma) were used. Different concentrations (0, 200, 400, 600, 800, 1000, 1200, and 1400 μg/mL) of canary seed peptides were tested. The reaction was carried out for 1 h at 37 °C, and the absorbance of p-nitroaniline (pNA) released was read at 415 nm in a microplate reader (Bio-Rad). Diprotin A (Ile-Pro-He) was used as positive control of inhibition. All assays were performed in triplicate.

**Assay for Inhibitory Activity of Angiotensin Converting Enzyme.** The ACE inhibition assay was conducted following the protocol described previously.18 The reaction was performed at 37 °C for 30 min in 100 μL of 0.1 M potassium phosphate buffer, pH 8.3, containing 0.3 M NaCl, 5 mM hippuryl-histidyl-leucine, 2 μM of ACE, and 25 μL of canary seed peptides at different concentrations (0, 83, 166, 249, 332, 415, 498, and 581 μg/mL). The reaction was stopped with the addition of 100 μL of 1 M HCl. The hippuric acid that formed was extracted with 1 mL of ethyl acetate, evaporated, and dissolved in 1 mL of distilled water; the hippuric acid absorbance was measured at 228 nm. The activity of each sample was tested in triplicate. Captopril (CPT) was used as a positive control. The IC₅₀ value was defined as the peptide concentration (μg/mL) needed to inhibit 50% ACE activity, calculated by the ACE inhibition (%) versus log peptide concentration (μg/mL) linear regression.

**Bioassay of Isolated Perfused Rat Heart (Langendorff Perfusion System).** To evaluate the effect of vasoactive canary seed peptides, the Langendorff method was used.23–25 Briefly stated, male Wistar rats (300–350 g) were euthanized with 50 mg/kg sodium pentobarbital intraperitoneally and 500 IU of heparin following the Animal Care Protocols (UASLP, Mexico). The rats’ heart were removed and cannulated via the ascending aorta in a retrograde perfusion system (IsoHearth-SR Harvard Apparatus, Holliston, MS, USA) and were maintained at a constant flow rate of 10 mL/min. The perfusion medium was Krebs–Henseleit solution, pH 7.4,21 equilibrated with 95% O₂ and 5% CO₂ at 37 °C. Increasing concentrations (0.01, 0.1, and 1 μg/mL) of canary seed peptides were administered intracoronary. BK at 10 μM and CPT at 50 μM were used as controls of vasodilation. Angiotensin I (ANG I) and/or angiotensin II (ANG II) at 1 μM were used as controls of vasoconstriction.

**Quantiﬁcation of Nitric Oxide in the Venous Eﬄuent of Isolated Perfused Rat Heart.** The concentration of NO, as nitrite and nitrate, was determined by using the Griess method.18 Venous eﬄuents from isolated hearts (100 μL) were collected and incubated at 37 °C for 30 min in the presence of 80 μL of vanadium chloride (VCl₃) followed by the addition of 20 μL of Griess reagent (1% sulfanilamide, 0.1% of naphthylethylenediamine in 2.5% phosphoric acid). Absorbance was read at 540 nm, and sodium nitrate (NaNO₃) was used as a standard.

**Statistical Analysis.** All experiments were carried out in triplicates, and results are reported as the mean values ± standard deviation. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA and Tukey’s test (P < 0.05) were performed to detect statistical differences.

**RESULTS AND DISCUSSION**

**Extraction and Quantification of Canary Seed Storage Proteins.** As shown in Figure 1, no differences were observed in protein fractions among canary seed flour (white bars), commercial flour “more Lait” (gray bars), and canary seed milk (black bars). The main fraction of canary seed storage proteins were prolamins with values ranging from 31 to 36% and globulins (35–39%). Albumins fraction represented around 16%, and globulins were found in low concentrations (6%). The protein fraction values were similar to those in previous studies,19 however, the electrophoretic profile has not been reported, and
then we proceeded to characterize the protein profile of the canary seed storage protein fractions.

**Figure 1.** Distribution of canary seed storage proteins fractions expressed in percentage: canary seed flour (white bars); flour obtained from soaked seeds in water (black bars); commercial flour “more Lait” (gray bars). In each case the bar is the average of three replicates and standard deviation is indicated. The same letters mean no significant differences at \( P < 0.05 \) in the same fraction.

**Figure 2.** Electrophoretic profile of canary seed storage proteins. Canary seed storage proteins from commercial flour “more Lait” were extracted according to their solubility in different solvents. Lanes: 1, molecular weight marker (MW); 2, albumins; 3, 7S globulins; 4, 11S globulins; 5, prolamins; 6, glutelins; 7, total seeds proteins.

**Figure 3.** Digestion profile of total soluble proteins extracted from canary seed commercial flour “more Lait”: (A) tryptic digestion; (B) gastrointestinal digestion. Lanes: 1, molecular weight marker; 2, nondigested canary seed proteins; 3–7, digestions at 0.1, 0.5, 1, 5, and 6 h, respectively. (C) Lanes: 1, molecular weight marker; 2–4, 10 kDa ultrafiltrates obtained from commercial flour “more Lait”, canary seed flour, canary seed milk, respectively.

**Figure 4.** Inhibitory function of canary seed peptides against DPPIV activity. Canary seed proteins were digested by gastrointestinal digestion method, and fragments smaller than 10 kDa, obtained by ultrafiltration, were tested. The percentage of DPPIV inhibition was measured in the presence of increasing concentrations of canary seed peptides from canary seed flour (solid triangles), canary seed milk (open squares), and commercial canary seed flour “more Lait” (solid squares). Negative control used was nondigested canary seed proteins (solid circles). Error bars ± SD (N = 3).

**Electrophoretic Profile of Canary Seed Storage Proteins.** The electrophoretic profile under denaturing conditions of canary seed fractions obtained from commercial flour “more Lait” is shown in Figure 2. This pattern was similar to canary seed flour and canary seed milk (Supporting Information Figure 1S). Canary seed albumins pattern showed bands of molecular masses of 9.8, 12.8, 15.8, 19.3, 23.4, 25.9, 39.2, and 43.4 kDa similar to the components of oat albumin (14–17, 20–27, and 36–47 kDa). The 7S globulins profile was similar to that of albumin with the addition of another band of 37.8 kDa; 11S globulins showed bands at 62.8, 57.1, 46.5, 39.2, 25.8, 15.3, and 12.8 kDa. Prolamins patterns in canary seed showed a broad band containing at least two bands of 26.6 and 24.9 kDa. Prolamins patterns were similar to those of the oat (17–34 kDa), barley (15–20 kDa), and the zeins or prolamins from corn (23–24 and 26.5–27 kDa). Glutelins main bands were located at 53.3, 41.5, 34.7, and 31.9 kDa, which are within a range similar to that of barley glutelins (35 and 42–46 kDa). These results suggest that the electrophoretic profile of the canary seed storage proteins is...
characteristic of the subfamily Pooidae cereals such as barley and oats and cereal family Poaceae (grass) such as corn.

**Digestion of Canary Seed Proteins.** The trypic and gastrointestinal digestion (Figure 3) hydrolysis patterns of canary seed flour “more Lait” were similar to those of canary seed flour and canary seed milk patterns (Supporting Information Figure 2S). It was observed that at 6 h of gastrointestinal digestion, the bands of high molecular mass disappeared with the concomitant increasing of a smear below 10 kDa (Figure 3A). At this time, with trypic digestion, bands of high molecular mass are still undigested (Figure 3B). Large protein fragments were removed by ultrafiltration using membranes of 10 kDa MWCO (Figure 3C), and eluted peptides were used for enzyme inhibition assays.

**Characterization of Canary Seed Peptides Inhibitory Function against DPPIV Activity.** Canary seed peptides obtained by gastrointestinal digestion showed an inhibition of DPPIV activity in a dose-dependent manner, where the highest inhibition of 43.4% was obtained at the higher peptide concentration (1.4 mg/mL). This behavior was similar among the three samples of canary seed under study (Figure 4). Nondigested canary seed proteins showed very low inhibition (9.3%). The tryptic digests (Supporting Information Figure 3S) had a lower percentage of inhibition (23%), and from this observation followed experiments that were evaluated using peptides obtained from gastrointestinal digestion. Diprotin A (Ile-Pro-Ile), the inhibition control for DPPIV, had an IC$_{50}$ of 2 μg/mL.

**Figure 5.** Inhibitory function of canary seed peptides against ACE activity. Canary seed proteins were digested by gastrointestinal digestion method, and fragments smaller than 10 kDa, obtained by ultrafiltration, were tested. The percentage of ACE inhibition was measured in the presence of increasing concentrations of canary seed peptides from canary seed flour (light-dotted dark squares), canary seed milk (solid triangles), and commercial canary seed flour “more Lait” (gray circles). Nondigested canary seed proteins were used as negative control (gray-crossed black squares).

**Figure 6.** Representative trace recordings of the vasodilator effect induced by canary seed peptides in Langendorff system. Perfusion pressure (PP) was taken as an index of vascular tone (vasodilation/vasoconstriction) recorded from isolated perfused rat heart. PP representative trace recordings were in response to (A) increasing and independent administrations of canary seed peptides obtained by gastrointestinal digestion (CAN, 0.01, 0.1, and 1 μg/mL). Bradykinin (BK, 10 μM) was infused as control of vasodilation. (B) Vascular tone controls: Angiotensin I (ANG I; 1 μM), the precursor of the formation of ANG II. ANG II (1 μM) was administered as control of vasoconstriction, and captopril (CPT, 50 μM), an ACE inhibitor, was administered as control of vasodilation. The administration of ANG I in the presence of CPT blocked the vasoconstriction induced by ANG I. (C) ANG (1 μM) was administered as control of vasoconstriction, and the vasodilation action of CAN (1 mg/mL) was measured. ANG I in the presence of CAN blocked the vasoconstriction induced by ANG I. ANG II was infused as control of vasoconstriction. Canary seed peptides and controls were administered in all cases after washout periods. All experiments were carried out in triplicates.
Characterization of Canary Seed Peptide Inhibitory Function against ACE Activity. The antihypertensive action of canary seed peptides was evaluated as the capacity to inhibit ACE activity. Increasing concentrations of the gastrointestinal digestion derived peptides (0–581 μg/mL) were evaluated. The highest inhibition value was 73.5% (Figure 5), a value similar to that reported for the bovine whey peptides.31 The IC50 of canary seed peptides was 332 μg/mL, similar to the IC50 value of the peptides of chickpea,32 pea,32 soybean,33 wheat gliadin,34 and sardine muscle.35 All canary seed presentations had the same inhibition pattern. Nondigested samples reached only 10.7% inhibition at its highest concentration. Captopril, used as a positive control of ACE inhibition, showed an IC50 value of 4.074 μg/mL (Supporting Information Figure 4SB), similar to that reported in the literature.37

Assessment of the Vascular Tone Induced by Canary Seed Peptides in Isolated Perfused Rat Heart. In the Langendorff system, the perfusion pressure (PP) was taken as an index of vascular tone (vasodilation/vasoconstriction).17 Three different concentrations (0.01, 0.1, and 1 μg/mL) of canary seed peptides were tested. The vasodilator effect was observed in a dose-dependent manner, where the lowest pressure was observed at 1 μg/mL, similar to the vasodilation induced by BK (10 μM) (Figure 6A). It has been reported that ANG I causes vasoconstriction in biological systems in perfusion36−39 and is blocked by ACE inhibitors;37,40−42 then ANG I and ANG II (1 μM) were tested as vasoconstriction controls, and CPT (50 μM) was used as vasodilation control and blocker of ACE (Figure 6B). In Figure 6C canary seed peptides (1 μg/mL) were used instead of CPT. It was observed that both CPT (Figure 6B) and canary seed peptides (Figure 6C) counteract the vasoconstrictor effect induced by ANG I. Vasodilation by canary seed peptides (1 μg/mL) was 82.7% compared to the 100% vasodilation induced with CPT and BK (Figure 7A). Figure 7B shows the percentage of vasoconstriction of ANG I (72%) and ANG II (100%), and it was observed that both CPT and canary seed peptides completely block the ANG I-induced vasoconstriction, suggesting the endogenous activity of ACE in the coronary vessels of the isolated and perfused rat hearts. Thereby, it was proposed that canary seed peptides are potential candidates as inhibitors of ACE activity (Figure 7B).

Nitric Oxide in the Venous Effluent Isolated Perfused Rat Heart. To determine whether the vasodilator effect of the canary seed peptides was also associated with NO production, NO was determined in venous effluent from the isolated perfused rat heart. The highest amount of NO produced by canary seed peptides at 1 μg/mL was 12.24 μM, whereas CPT induced the production of 20.27 μM and BK 19.24 μM (Figure 8). These data suggest an important function of canary seed peptides in the regulation of vascular tone through NO production. The NO produced in the inner layer of the blood vessel diffuses at the cardiac cell, blocking the release of calcium needed for muscle contraction, and therefore promotes a state of relaxation.18,43 ACE inhibitors induce vasodilation due the formation of a BK−NO-dependent pathway.18 Then, canary seed peptides are ACE type inhibitors stimulating NO production by this route.

In conclusion, the protein profile of canary seed protein fractions is more similar to those of cereals such as oats and maize and is farther from wheat electrophoretic patterns. Canary seed proteins contain encrypted peptides with inhibitory activity against DPPIV and ACE. The inhibition of DPPIV is the basis of modern antidiabetic therapy, whereas inhibition of ACE is the drug therapy against hypertension. In addition we have demonstrated that canary seed peptides are able to induce the production of NO, a potent vasodilator, at levels similar to those produced by CPT and BK.
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