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Tryptic amaranth glutelin digests induce endothelial nitric oxide production through inhibition of ACE: Antihypertensive role of amaranth peptides

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ABSTRACT

Amaranth seed proteins have a better balance of essential amino acids than cereals and legumes. In addition, the tryptic hydrolysis of amaranth proteins generates, among other peptides, angiotensin converting enzyme (ACE) inhibitory (ACEi) peptides. ACE converts angiotensin I (Ang I) into Ang II, but is also responsible for the degradation of bradykinin (BK). In contrast to Ang II, BK stimulates vasodilation modulated through endothelial nitric oxide (NO) production. The aim of the present study was to characterize the ACEi activity of amaranth trypsin-digested glutelins (TDGs) and their ability to induce endothelial NO production. An IC_{50} value of 200 μg ml^{-1} was measured for TDG inhibition of ACE. TDGs stimulated endothelial NO production in coronary endothelial cells (CEC) by 52% compared to control. The effects of TDGs were comparable to those of BK and Captopril, both used as positive controls of NO production. Consistent with these effects, TDGs induced, in a dose-dependent manner, endothelial NO-dependent vasodilation in isolated rat aortic rings. These results suggest that TDGs induce endothelial NO production and consequent vasodilation through their ACEi activity. Amaranth TDGs have a high potential as a nutraceutical food in prevention of cardiovascular diseases. Further molecular, cellular and physiological studies are currently under way and the results may contribute to a better understanding and control of cardiovascular disorders.

Introduction

Hypertension has become the most common public health problem worldwide. Around 20–45% of the active population and nearly 50–60% of elderly people have elevated blood pressure [1]. Despite its strong causal association with cardiovascular disease complications including myocardial infarction, heart failure, and stroke, most patients with hypertension do not achieve optimal control of their blood pressure [2]. Nitric oxide (NO) is a free radical generated through oxidation of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS) activation, promoting angiogenesis and vasodilation [1]. NO is activated by vasoactive substances such as acetylcholine (ACh), bradykinin (BK) or growth factors like vascular endothelial growth factor [3–5]. NO is physiologically important in maintaining vascular homeostasis, keeping vessels dilated, protecting the endothelial inner layer from platelet aggregates and leukocyte adhesion, and preventing proliferation and migration of smooth muscle cells. However, eNOS and NO bioactivity is suppressed if an endothelial dysfunction occurs during hypertensive cardiovascular diseases. This abnormality is related to blunted epicardial response and resistance to endothelium derived NO-agonists in peripheral and coronary circulation, contributing to mechanisms of altered vascular tone in hypertension [1,6].

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) is a peptidyldipeptide hydrolase that plays an important physiological role in both the regulation of blood pressure and cardiovascular function [7] through two different reactions. First, ACE catalyzes the conversion of the inactive decapeptide angiotensin I (DRVYIHPFHL) into a powerful vasoconstrictor and salt-retaining octapeptide, angiotensin II (DRVYIHPF). Thus, ACE inhibition results in lowering of blood pressure [8,9]. On the other hand, ACE catalyzes the inactivation of the vasodilator BK that regulates different biological processes including vascular endothelial NO release. NO exerts its effects on the vascular smooth muscle cells underlying the endothelium and promotes vascular relaxation [9,10]. Synthetic ACE inhibitors (ACEi), including Captopril, Enalapril, and Lisinopril, have been developed to decrease blood pressure [11,12]. However,

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some undesirable side effects, such as coughing, dizziness, headache, abnormal taste (metallic or salty taste), kidney, and liver problems have been reported for these drugs [13]. Therefore, identification and application of natural ACEi peptides can be beneficial for the treatment and/or prevention of hypertension. ACEi peptides have been isolated from animal and plant proteins after their release *in vitro* during food processing and/or *in vivo* during gastrointestinal digestion [14]. ACEi peptides derived by enzymatic hydrolysis of milk and dairy products have been extensively studied [15–19]. Several studies have focused on various ACEi peptides derived from plant [20–23], and fish muscle proteins [24,25].

Proteins stored in Amaranth seeds have an excellent balance of amino acids [26]. In addition, these proteins have been reported to be a source of peptides with useful biological properties. ACEi peptides have been reported to be released from amaranth glutelin fraction [27]. However, the biological activity of these peptides has not been tested before. The purpose of the present work was to hydrolyze amaranth glutelins with trypsin and to evaluate the effects of the peptides on ACE activity and NO production. Moreover, the action of the tryptic digests on vascular relaxation of rat aortic rings was also determined.

Materials and methods

Amaranth seed storage protein extraction

Amaranth glutelins were obtained through sequential extraction-solubilization of albumins, globulins (7S and 11S) and prolamins according to the method of Barba de la Rosa et al. [26]. Extraction of albumin plus non-protein nitrogen (NNP) fraction was carried out on defatted flour using distilled water as an extracting agent. Suspensions of flour/extracting agent (1:10 w/v) were stirred for 30 min at 4 °C and centrifuged at 13,000*g* for 20 min. The resulting pellet was resuspended in 0.1 M NaCl, 10 mM K_2HPO_4 at pH 7.5, and 1 mM EDTA, stirred, and centrifuged as mentioned above. The supernatant corresponded to the 7S globulin fraction. The pellet was resuspended in 0.8 M NaCl, 10 mM K_2HPO_4 at pH 7.5, and 1 mM EDTA, stirred, and centrifuged as mentioned above, resulting in the 11S fraction. The prolamin fraction was extracted from the 11S fraction pellet with 60% aqueous ethanol. The pellet resulting from prolamin extraction was resuspended in 0.1 M Tris pH 8.0. The soluble proteins recovered were termed the native glutelin fraction. The concentration of protein was quantified using the BioAssay Kit (Bio-Rad, Hercules, CA, USA), using BSA as a standard.

Digestions of amaranth glutelins with trypsin

Native glutelins were digested with trypsin (Sigma-Aldrich, St. Louis, MO, USA) at an enzyme/substrate ratio of 1:10 (w/w) for 10 h at 37 °C. The digestion was verified by SDS–PAGE, using the method described below. Glutelins and trypsin-digested glutelins (TDGs) were denatured by heating in boiling water at 100 °C for 5 min.

Glutelins and TDGs electrophoretic pattern

The electrophoretic patterns of native glutelins and TDGs were analyzed by SDS–PAGE with 15% w/v polyacrylamide gel in a Mini-Protean III system (Bio-Rad). Samples of 1 mg ml^{-1} were dissolved in Laemmli sample buffer and boiled for 5 min [28]. SDS–PAGE was conducted at a constant current of 20 mA for 2–3 h. Gels were stained with Coomassie Brilliant Blue G250 at a final concentration of 0.25%. Destaining was achieved by washing the gel for 2–4 h with acetic acid/methanol/water (4.5:4.5:1 v/v/v). Gels were photo documented in a Gel-Doc System (Bio-Rad).

Characterization of TDGs fragments by mass spectrophotometry (MS)

TDGs were analyzed by LC-ESI/MS/MS as described by Silva-Sánchez et al. [27]. Briefly, 200 μg of sample was precipitated with acetone. The pellet was dissolved in 250 μl of urea buffer (6 M urea and 100 mM Tris pH 7.4). The sample was reduced with DTT and alchilated with iodoacetamide. Chromatography separation of TDGs was done on an Agilent Zorbax 300SB C18 column (150 × 0.075, 3.5 μm; Agilent Technologies, Foster City, CA, USA), loading 10 μl of sample into the column. HPLC solvents were: A, 98% (v/v) water, 2% (v/v) acetonitrile, and 0.1% (v/v) of formic acid; B, 98% (v/v) acetonitrile, 2% (v/v) water, and 0.1% (v/v) of formic acid. After 30-min equilibration with 90% buffer A, the peptides were eluted directly into the electrospray source of LC-ESI MS/MS (Applied Biosystems, Foster City, CA, USA) with a gradient from 60% to 80% of buffer B at a flow rate of 400 nl min^{-1}. Peptide identification was done using MASCOT (Matrix Sciences, http://www.matrixcience.com/). The identification of bioactive peptides was conducted by searching in the active peptide database (http://www.uwm.edu.pl/biochemia).

Measurement of ACE-inhibitory activity

ACEi activity was measured by the spectrophotometric assay as reported by Hernández-Ledesma et al. [29], with some modifications. Briefly 20 μl of sample was added to 0.1 ml of 0.1 M potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl and 5 mM hippuryl–histidyl–leucine (HHL, Sigma). ACE (5 mU) (EC 3.4.15.1, 5.1 U mg^{-1}, Sigma) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.1 ml of 1 M HCl. The hippuric acid formed was extracted with ethyl acetate, heat-evaporated at 95 °C for 10 min, redissolved in distilled water and measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate. Captopril was used as a positive control. The IC_{50} value was defined as the peptide concentration (mg ml^{-1}) needed to inhibit 50% ACE activity; it was calculated by an ACE inhibition (%) vs. log peptide concentration (mg ml^{-1}) linear regression.

Cell culture

Rat coronary endothelial cells (CEC) were obtained as previously described [30]. Cells were cultured in DMEM with 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) 100 μg ml^{-1} porcine heparin, and 50 U ml^{-1} penicillin/streptomycin, as previously described [5]. Cells were grown to confluence, placed in serum-free medium for 24 h and treated during 24 h with varying concentrations of TDGs dissolved in DMEM.

Nitric oxide assay

The NO production by CEC was indirectly determined by measuring the accumulation of nitrites (NO_2) the oxidation products of NO, in the culture medium by the Greiss reaction [31]. The absorbance was measured at 540 nm in a microplate reader spectrophotometer (Bio-Rad). Serial dilutions of sodium nitrite were used as standards [32]. Medium alone, without treatments was used as control. BK (50 μM) and Captopril (50 μM) were used as positive controls. HOE-140 (1 μM) was used as negative control. All products were purchased from Sigma.

Western blot analysis

CECs were treated in the absence and presence of TDGs, BK (50 μM) or HOE-140 (1 μM) for 1 min. Cell samples were then processed as previously described [33]. Polyclonal anti-phospho