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Development of a reversed-phase high-performance liquid chromatography analytical methodology for the determination of antihypertensive peptides in maize crops

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ABSTRACT

The aim of this work was to estimate the content of three highly antihypertensive peptides (LQP, LSP, and LRP) in different maize crops. For that purpose, a method consisting of the extraction of the protein containing these peptides (α-zeins), releasing of peptides by thermolysin digestion, and separation and detection of peptides was designed. The rapid and efficient ultrasound assisted extraction of α-zeins proteins from whole maize kernels was achieved using 70% of ethanol followed by precipitation with acetone. A 10 mM Tris–HCl (pH 8.0) buffer containing 8 M urea enabled to dissolve the precipitated α-zeins. This buffer was diluted to reach a 6 M urea concentration before digestion to keep active the enzyme. Other digestion parameters that were optimized were: enzyme to substrate ratio (5:100 was selected), digestion temperature (50 °C) and digestion time (6 h). The RP-HPLC separation in a fused-core column was also optimized allowing the separation of the three peptides extracted from maize kernels in 6 min. The presence of the three antihypertensive peptides in the digested extract was confirmed using HPLC–Q-TOF-MS analysis and by comparison with peptide standards. Clear differences were observed in the content of the three antihypertensive peptides and, thus, in the antihypertensive activity of the analyzed crops. The content of LRP peptide was very low regardless of the maize variety while the content of LQP and LSP significantly varied among studied maize lines.

1. Introduction

Hypertension appears as a leading cause of cardiovascular diseases (CVDs) and is known as 'silent killer' since over 50% of hypertensive population is unaware of their condition [1]. According to the World Health Organization, hypertension is a highly prevalent cardiovascular risk factor worldwide and its treatment has been shown to prevent CVDs [2]. Different therapies can be applied to prevent hypertension being the use of angiotensin converting enzyme inhibitors (ACE inhibitors (e.g. captopril)) the first choice [3]. ACE inhibitor compounds act on the renin–angiotensin system associated in the control of blood pressure in living organisms. Angiotensin I is hydrolyzed in the presence of angiotensin I converting enzyme (ACE) to angiotensin II, leading to an increase in blood pressure. The ACE also removes a dipeptide from the C-terminus of bradykinin resulting in the inactivation of this vasodilator. As a consequence, ACE inhibitors cause effective reduction of blood pressure by decreasing the angiotensin II level and rising up bradykinin level [4–6]. Most antihypertensive drugs employ mechanism of ACE inhibition. Synthetic drugs are very potent but they also provoke several adverse effects [5,7]. An alternative can rise from those foods naturally containing antihypertensive peptides which do not yield adverse effects [8,9].

Most ACE inhibitory peptides contain 2–12 amino acids residues with a noticeably amount of hydrophobic amino acids such as proline, especially at C-terminal position [10]. ACE inhibitors were found in marine foods [11], fishes [8], meat [12], vegetable foods [13], mushrooms [14], and processed products [15]. Most studied antihypertensive peptides are from foods of animal origin, specially dairy products [16–18], although the most active peptides were found in maize. Indeed, maize contains three peptides (Leucine-Glutamine-Proline (LQP), Leucine-Serine-Proline (LSP), and Leucine-Arginine-Proline (LRP)) derived from the α-zein protein fraction with extremely high antihypertensive activity (IC_{50} value (the half maximal inhibitory concentration) 2.0, 1.7, 0.29 μM, respectively) [19] which is much higher than the popular Valine-Proline-Proline (VPP) (IC_{50} = 9.13 μM) and Isoleucine-Proline-Proline (IPP) (IC_{50} = 5.15 μM) found in milk. Taking into account that the protein content of maize crops can vary [20] and that antihypertensive activity of peptides is highly dosage dependent [21,22], the development of analytical methodologies for estimating peptide contents in different crops is required.

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Worldwide corn or maize (*Zea mays* L.) is a major crop for both livestock feeding and human nutrition [23]. Maize protein content is in the range 6–12% (as dry basis) [20]. Zeins, according to Osborne nomenclature, are the prolamin fraction of maize, representing 60% of total maize proteins [24]. Zeins can be classified as: α-zeins (21–25 kDa), β-zeins (17 kDa), γ-zeins (18 and 27 kDa), and δ-zeins (10 kDa). The most abundant zein is the α fraction accounting for 75–85% of total zeins [25]. Two major groups of α-zeins can be separated using SDS-PAGE: Z19 zein migrating at 19 kDa and Z22 zein 22 kDa. Nevertheless, the studies of zein sequences obtained from cloned cDNAs and genes, have shown that those two groups of zeins had a Mw around 23–24 and 26–27 kDa, respectively [26].

Several different attempts were made in order to obtain total-zeins from maize kernels, where extraction using aqueous solutions of ethanol or isopropanol with or without a reducing agent are the most frequent [27–29]. Moreover, despite there is one methodology enabling α-zeins extraction, it was applied to maize product with high protein content (corn gluten meal, CGM) [19]. Nevertheless, to our best knowledge, none of these procedures have been applied to exclusively extract the α-zeins from maize kernels.

Next step in the isolation of antihypertensive peptides would be the digestion of α-zeins. Different enzymes have been employed for the digestion of proteins containing antihypertensive peptides being thermolysin the most preferred due to its broad specificity to hydrophobic amino acids [30]. Reports about digestion of CGM by trypsin [31], alcalase [32], thermolysin [19], and six different commercial proteases [33] or zeins by trypsin or thermolysin [34,35] can be found in the literature. Nevertheless, in all cases CGM or zeins were purchased, and no extraction procedure was previously applied. The lack of methodologies where zein proteins were digested after extraction from whole maize kernels need to be highlighted since the selection of a suitable buffer enabling to dissolve the alcohol soluble α-zeins and to keep active the enzyme had to be overcome. Moreover, comparison of digestion protocols using thermolysin revealed that digestion conditions differed significantly from one work to the other.

Regarding peptide separation, a new trend in HPLC is focused to the development of stationary phases enabling high sample throughput analysis of peptides. Several strategies have been developed being the use of fused-core or superficially porous silica particles very interesting for the reduction of analysis times while keeping column efficiency and low back pressure. Columns with 2.7 μm fused-core particles produce approximately half of the back pressure of the 1.8 μm conventional columns allowing the use of traditional HPLC systems [36]. This fused-core particles start to play important role in chromatography and their use in bio-analytical methods have already been reviewed [37]. Nevertheless, the use of this innovative approach is still not common, and has scarcely been employed for peptide separation.

The aim of this work was to develop an analytical methodology for the rapid extraction of α-zeins from maize kernels allowing their further digestion by thermolysin and their separation by RP-HPLC in order to evaluate the content of three highly active antihypertensive peptides (LQP, LRP, and LSP) in maize crops.

2. Materials and methods

2.1. Chemicals and samples

Water, was freshly taken every day from a Milli-Q system (Millipore, Bedfore, MA, USA). All used reagents were of analytical grade purity. Acetic acid (AA), AA with purity for LC–MS, acetone, acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), isopropanol (IPA), and urea were supplied from Scharlau Chemie (Barcelona, Spain). Formic acid (FA), hydrochloric acid, sodium dodecyl sulfate (SDS), sodium hydroxide, tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl), and β-mercaptoethanol (B-ME) were purchased from Merck (Darmstadt, Germany). Ammonium hydroxide, dithiothreitol (DTT), iodoacetamide (IAM), thermolysin, and trifluoroacetic acid (TFA) were from Sigma (St. Luis, MO, USA). Heptafluorobutyric acid (HFBA), and sodium acetate were acquired from Fluka (Burchs, Switzerland) and trichloroacetic acid (TCA) was from Panreac (Barcelona, Spain). All chemicals and gels for SDS-PAGE analysis were acquired in Bio-Rad (Hercules, CA, USA): Laemmli buffer (62.5 mM Tris–HCl, 25% (v:v) glycerol, 2% (m:v) SDS, 0.001% (m:v) bromophenol blue), Mini-Protean Precast Gels, running buffer (25 mM Tris–HCl, 192 mM glycine, and 0.1% SDS, pH 8.3), Precision Plus Protein Standards, and Bio-Safe Coomassie stain. Standards and samples employed were: corn gluten meal (CGM) (Sigma, St. Luis, MO, USA), peptides LQP, LSP, and LRP (GeneScript Corp., Piscataway, NJ, USA), standard of zeins (Sigma, St. Luis, MO, USA), and maize lines that were kindly donated by a Maize Germplasm Bank (Experimental Station of Aula Dei, CSIC, Zaragoza, Spain): EZ6, B73, EZ11A, EZ9, A632.

Prepared solutions were stored in the fridge at 4 °C with the exception of urea, IAM, and DTT solutions that were always freshly prepared. Additionally, thermolysin powder or thermolysin stock solution (2.5 mg/mL in water) and peptides were stored always at −20 °C. Standards of peptides (1 mg/mL or 0.1 mg/mL) were dissolved in water (LRP, LQP) or in ACN (LSP) according to the recommendation guide supplied by Genscript. Standard of zeins (100 mg/mL) was dissolved in 70% of ethanol prior to analysis. Maize kernels (around thirty whole kernels for each line) were grounded with a domestic miller during 1 min at ambient temperature. All milled maize powders were stored at 4 °C.

2.2. Extraction and fractionation of zeins from CGM

Main maize proteins (zeins) were fractionated following a method developed by Parris and Dickey [38] and improved by Rodríguez-Nogales et al. [39] with some modifications. The method was applied to fractionate zeins from CGM. The method consisted of extracting 2 g of CGM with 20 mL of 60% IPA containing 1% B-ME at room temperature and centrifuging for 1 min at 4000 × *g*. This extraction was repeated three additional times. Next, three volumes of 100% IPA were added and the resulting solution was left overnight at 4 °C. Afterwards, the solution was centrifuged for 10 min at 4000 × *g* and 4 °C. The resulting pellet containing β- and γ-zeins was separated from the supernatant. The supernatant was mixed with two volumes of water and 0.01 volumes of sodium acetate (pH 6.0). The solution was left for 2 h followed by centrifugation for 10 min at 4000 × *g* and 4 °C. The pellet containing α-zeins was separated.

2.3. Extraction and purification of α-zeins from maize lines

α-zeins from the EZ6 maize line were extracted using a method developed by Yano et al. [19] to isolate α-zeins from CGM. The method was carried out by extracting 1 g of pulverized maize line with 10 mL of 70% EtOH. For a more efficient and fast extraction, an ultrasonic probe (VCX.130, Sonic Vibra-Cell, Hartord, CT, USA) was employed for 10 min (amplitude 90%) followed by centrifugation (4000 × *g*, 10 min, 20 °C). α-Zeins in the previous extract were purified by precipitation with 80 mL of acetone containing 0.07% B-ME followed by centrifugation (4000 × *g*, 15 min, 4 °C). The resulting