Food Chemistry 124 (2011) 1316–1321

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate

Žužana Vaštag [a,*], Ljiljana Popović [a], Senka Popović [a], Vera Krimer [b], Draginja Peričin [a]

[a] Faculty of Technology, University of Novi Sad, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia
[b] Public Health Institute, Zmaj Jovina 30, Subotica, Serbia

ARTICLE INFO

Article history:
Received 21 December 2009
Received in revised form 14 July 2010
Accepted 20 July 2010

Keywords:
Pumpkin oil cake
Protein isolate
Alcalase
Flavourzyme
Protein hydrolysates
Antioxidant activity
ACE-inhibitory activity

ABSTRACT

Protein isolate from pumpkin oil cake (PuOC PI) was hydrolysed by alcalase, flavourzyme and by sequential use of these enzymes, respectively, and the antioxidant properties and angiotensin-I converting enzyme (ACE) inhibitory activities of hydrolysates were evaluated. Under the same reaction conditions, alcalase hydrolysates showed a higher degree of hydrolysis (DH) than did flavourzyme hydrolysates. The highest DH's by individual enzymes were 53.23 ± 0.7% and 37.17 ± 1.05%, respectively, both at 60 min. The increase of radical scavenging activity (RSA) in hydrolysates was positively correlated with the increase of DH, for both enzymes, though hydrolysates with flavourzyme showed two- or three-fold lower RSA than with alcalase. The highest bioactive potential was determined in the alcalase hydrolysate at 60 min, with RSA being 7.59 ± 0.081 mM TEAC/mg and ACE-inhibitory activity 71.05 ± 7.5% (IC_{50} = 0.422 mg/ml). When this hydrolysate was further hydrolysed by flavourzyme, DH increased up to 69.29 ± 0.9%, but lower RSA (4.82 ± 0.21 mM TEAC/mg) and ACE-inhibitory activity (55.81 ± 6.196%) were determined in the final hydrolysate. This study suggested that the PuOC proteins could be converted into protein hydrolysates with antioxidant and ACE-inhibitory activities by enzymatic hydrolysis. Alcalase was shown as promising enzyme in further development of bioprocesses for the production of new bioactive food ingredients.

1. Introduction

Pumpkin (*Cucurbita pepo* L.) seed is an important as oil crop in Austria, Hungary, Slovenia and Serbia (province of Vojvodina) and has been used for edible oil production for a long time. In the oil industry, the hull-less (naked) pumpkin seed varieties are more favourable compared to hulled varieties, since they have higher oil content (up to 50%) (Murković, Hillebrand, Winkler, Leitner, & Pfannhauser, 1996) and can be more easily crushed to extract the oil. The main byproduct of the oil extraction process is a defatted pumpkin oil cake (PuOC), which has a poor utility and the majority is used for animal feeding. PuOC has high quality components that may be used for human consumption, such as phenolic acids (Peričin, Krimer, Trivić, & Radulović, 2009), iodine and selenium (Kreft, Stibilj, & Trkov, 2002). Also, PuOC has high protein content (60–65%), making it as attractive and promising source of plant proteins. One of the possible ways to utilise PuOC proteins is to produce enzymatic hydrolysates, which may have useful functional properties or possess bioactivities.

Plant and animal proteins have been well known as sources of bioactive peptides. Peptides might exhibit diverse bioactivities, including: anticancer (Mora-Escobedo, Robles-Ramírez, Ramón-Gallegos, & Reza-Alemán, 2009), anti-inflammatory (Udenigwe, Lu, Han, Hou, & Aluko, 2009), antioxidant (Zhu, Zhou, & Qian, 2006) activities, cholesterol-lowering ability (Cho, Juillerat, & Lee, 2007) and blood pressure-lowering (ACE inhibitory) effect (Hong et al., 2008). Bioactive peptides could be released from parent proteins during digestion, food processing or enzymatic hydrolysis *in vitro*.

Using various enzymes and plant proteins, a variety plant protein hydrolysates with potent antioxidant activities have been reported; from wheat germ (Zhu et al., 2006) using alcalase; from canola seeds by flavourzyme (Cumby, Zhong, Naczk, & Shahidi, 2008), from sesame seeds using trypsin (Liu & Chiang, 2008). Nowadays, naturally antioxidants have received considerable interest from the food industry due to consumer preference because of the concern over the safety of synthetic antioxidants. Carotenoids, vitamins C, E and other phytochemicals have been well characterised for antioxidant activities and potential health benefits. Besides, the demand for the use of peptides and proteins as antioxidants in food is increasing due to the low costs, safety and their inherent nutritional and functional values.

* Corresponding author. Tel.: +381 214853679; fax: +381 21 450 413.
E-mail address: zsuzsanna_vastag@yahoo.com (Ž. Vaštag).

0308-8146/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2010.07.062

On the other hand, protein hydrolysates with angiotensin-I converting enzyme (ACE) inhibitory activity have shown great promise in the development of a novel therapeutics and for functional food for preventing hypertension. ACE (EC 3.4.15.1) plays a central role in the regulation of blood pressure through the production of the potent vasoconstrictor, angiotensin-II, and the degradation of the vasodilator, bradykinin. Captopril, lisinopril and other synthetic ACE inhibitors, which have been used in the clinical treatment of hypertension, could have some undesirable side effects in humans (Acharya, Sturrock, Riordan, & Ehlers, 2003). A large number of ACE-inhibitory protein hydrolysates have been produced from various plant proteins; such are chickpea (Pedroche et al., 2002), sunflower (Megías et al., 2009).

To the best of our knowledge, the pumpkin protein hydrolysates have not been the subject of study for bioactivity elsewhere. The aim of this study was to evaluate the bioactive properties of PuOC PI hydrolysates produced by alcalase, flavourzyme and by sequential use of these enzymes, respectively. The hydrolysis process and the *in vitro* antioxidant and angiotensin-I converting enzyme (ACE) inhibitory activities of hydrolysates were investigated. Furthermore, possible relationships between biological activities of the hydrolysates and the DH were also evaluated.

2. Materials and methods

2.1. Materials

The hull-less pumpkin (*C. pepo* L. c. v. "Olinka") oil cake (PuOC) was acquired by the "Pan-Union", Novi Sad, Serbia. It was stored at temperature of 4 °C and ground in a coffee-grinder before use. Alcalase (protease from *Bacillus licheniformis*, 2.4 AU/g), flavourzyme (protease from *Aspergillus oryzae* 518.8 LAUP/g), Angiotensin-I Converting Enzyme (ACE) from rabbit lung, N-Hippuryl-His-Leu hydrate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma (St. Louis, MO, USA). All the other chemicals used for the experiments were of analytical or better grade.

2.2. Preparation of the protein isolate (PI) and enzymatic hydrolysis

The protein isolate (PI) was prepared according to the same process described in detail in our earlier report (Peričin, Radulović-Popović, Vaštag, Mađarev-Popović, & Trivić, 2009). The hydrolysis was performed in a glass reactor under controlled conditions (temperature and stirring speed). The PI was dissolved in 0.1 mol/L Tris/HCl buffer pH 8.00 and pre-incubated at temperature of 50 °C. For the hydrolysis by individually enzymes, alcalase or flavourzyme was added to enzyme/substrate ratios (E/S) at 1/757, 1/385 and 1/250, for both enzyme and the hydrolysis was monitored during 60 min. For the hydrolysis with combined enzymes, alcalase (E/S 1/250) was used within first 60 min and then, flavourzyme was added (E/S 1/385) and the hydrolysis prolonged up to 120 min. At the end of hydrolysis the reaction mixture was immediately heated (100 °C, 5 min) to inactivate the enzyme. The mixture was centrifuged using Eppendorf Mini spin plus at 12×4 g for 10 min and the supernatant was used for further analysis. The degree of hydrolysis (DH) in PuOC PI hydrolysates was determined as the ratio of 0.22 mol/L TCA-soluble protein to total protein in the supernatant of reaction mixture, expressed as a percentage (Peričin, Radulović-Popović, et al., 2009). The protein content was assayed by the method of Lowry, Rosenbrough, Fair, and Randall (1951), using bovine serum albumin as the standard protein.

2.3. SDS–PAGE electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) of protein samples was carried out by the method of Laemmli (1970). The discontinuous system used consisted of a 4% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide separating gel. Samples (1 mg/ml protein) were dissolved in Tris/Gly buffer (pH 6.8) containing 20 g/l SDS and 50 g/l 2-mercaptoethanol. Electrophoresis (Pharmacia LKB-Multi Drive XL) was carried out at 40 mA until the tracker dye reached the bottom of each gel. After electrophoresis, the gels were stained with 0.2% (w/v) Coomassie brilliant blue R-250, in 10% (v/v) acetic acid: 50% (v/v) methanol and then, distained with 10% (v/v) acetic acid containing 40% (v/v) methanol for 16 h.

2.4. $ABTS^{+\cdot}$ radical cation decolorisation assay (TEAC)

The radical scavenging activity of the hydrolysates was determined by the $ABTS^{+\cdot}$ radical cation decolorisation assay as described by Re et al. (1999). The bleaching rate of $ABTS^{+\cdot}$ radical cation solution was monitored in the presence of hydrolysate sample at 734 nm, using T80/T80+ UV–Vis spectrophotometer (PG instruments Ltd.). Briefly, 30 μl of sample (containing 0.5, 0.75, 1.5 and 1 mg/ml proteins) were added to 3 ml of diluted $ABTS^{+\cdot}$ radical cation solution ($A_{734\ nm} = 0.7 \pm 0.02$) and the absorbance reading was taken up to 10 min. Appropriate solvent blanks were run in each assay. In the same way, a standard Trolox curve was prepared with known Trolox concentrations. The percentage decrease in absorbance at 734 nm at 10 min was calculated and plotted as function of antioxidant or Trolox concentrations. The Trolox equivalent antioxidant coefficient (TEAC) was calculated by dividing the absorbance percentage inhibition versus antioxidant concentration slope by the Trolox plot slope and was expressed as mM TEAC per mg of proteins in hydrolysates (mM TEAC/mg).

2.5. Reducing power

The reducing power of the hydrolysates was measured according to the method of Moure, Domíngeuez, and Parajó (2006). One milliliter of a protein hydrolysate (1.5 mg/ml) was mixed with 2.5 ml of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide, and the mixtures was incubated at 50 °C. After 20 min of incubation, 2.5 ml of 10% TCA was added in the mixture and then, centrifuged. The obtained supernatant (2.5 ml) was diluted with water (2.5 ml) and after addition of 0.1% ferric chloride (0.5 ml), the absorbance was measured at 700 nm (T80/T80+ UV–Vis Spectrophotometer, PG instruments Ltd.). The increase of the absorbance of the reaction mixture indicates increase of the reducing power against ferri ions.

2.6. Assay of ACE-inhibitory activity

The ACE-inhibitory activity of the hydrolysates was measured according to method described by Yoshie-Stark, Bez, Wada, and Wäsche (2004). In each assay, 60 μl of the hydrolysate sample was incubated at 37 °C for 80 min with 80 μl hippuryl-His-Leu in 0.2 mol/L potassium phosphate buffer containing 300 mmol/L NaCl at pH 8.3 and 10 μl of ACE solution. The final concentrations of the hippuryl-His-Leu and ACE were 10 mmol/L and 25 mU/ml, respectively. The reaction was stopped by adding 110 μl of 1 mol/L HCl. The hippuric acid liberated from hippuryl-His-Leu by ACE was extracted with 1.5 ml ethyl acetate. The ethyl acetate layer (1 ml) was collected, evaporated and the hippuric acid was dissolved in 3 ml distillated water. The absorbance was determined at 228 nm (T80/T80+ UV–Vis Spectrophotometer, PG instruments Ltd.).