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Bioactivity Evaluation of Cucurbitin Derived Enzymatic Hydrolysates

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***Abstract*—**After cold pressing of pumpkin oil, the defatted oil cake (PUOC) was utilised as raw material for processing of bio-functional hydrolysates. In this study, the in vitro bioactivity of an alcalase (AH) and a pepsin hydrolysate (PH) prepared from the major pumpkin 12S globulin (cucurbitin) are compared. The hydrolysates were produced at optimum reaction conditions (temperature, pH) for the enzymes, during 60min. The bioactivity testing included antioxidant and angiotensin I converting enzyme inhibitory activity assays. The hydrolysates showed high potential as natural antioxidants and possibly antihypertensive agents in functional food or nutraceuticals. Additionally, preliminary studies have shown that both hydrolysates could exhibit modest α-amylase inhibitory activity, which indicates on their hypoglycemic potential.

***Keywords*—**Cucurbitin, alcalase, pepsin, protein hydrolysates, in vitro bioactivity.

I. Introduction

IN recent years extensive scientific evidence has been derived on food bioactive peptides, having beneficial effects upon human health. Numerous peptides exhibiting antioxidant, blood pressure-lowering (mainly Angiotensin I Converting Enzyme, ACE inhibitors), antidiabetic, immune-modulatory, etc. have been reported. In this respect, protein hydrolysates, depending on bioactivity of constituting peptides, could be considered in functional food or nutraceuticals as health promoting agents or for additional therapy of cardiovascular, immune or neurological disorders [1]. Protein hydrolysates containing antioxidant peptides have demonstrated great potential as dietary antioxidants and may be natural substituents for artificial antioxidants in food or pharmaceuticals. On the other hand, peptides with ACE inhibitory activity can have antihypertensive effect in humans, and have opened a new dietary aspect in therapy of hypertension, especially in the prevention and initial treatment in mildly hypertensive individuals [2], [3].

Food derived bioactive peptides are encrypted in the parent proteins and can be released: a) by *in vitro* hydrolysis using animal, plant or microbial proteases; or b) during food fermentation or ripening (e.g. sausage ripening) [1], [4]. Many animal and plant proteins have been shown as sources of bioactive peptides, so far the most being isolated from milk and soyaproteins. However, research continues to uncover novel sources and develop specific bioprocesses to enhance their availability from food or to create novel food through the addition/fortification of enriched fractions of bioactive peptides.

Cultivation of pumpkin (*Cucurbita pepo L.*) in Austria, Hungary, Slovenia, Croatia and Serbia is widespread, primarily for pumpkin oil production. Defatted pumpkin oil cake (PUOC) is the main by-product after oil extraction, usually used only as animal feed. However, PUOC contains about 60% protein that could be utilized in human nutrition. The protein exploitation from defatted oil cakes/meals has been described as a way to valorise these agricultural by-products [5], [6]. Diverse enzymatic processes for modification of isolated PUOC proteins, including protein isolate and the major 12S seed globulin (cucurbitin) have been developed in our laboratory. The modifications have been aimed at production of functional hydrolysates, either using proteases or cross-linking enzymes [7], [8]. The improved functionality of PUOC hydrolysates can involve improved techno-functional properties (solubility, emulsifying or foaming activity), and bioactivity (antioxidant, ACE inhibitory activity) [9]-[11]. Depending on the enzyme and process conditions used diverse biofunctional hydrolysates can be obtained.

In this study, the bioactivity of two cucurbitin's hydrolysates is demonstrated. The hydrolysates were produced under the optimal reaction conditions (temperature, pH) for selected proteases, at the same enzyme/substrate ratio, during 60 min. In this way, the effect of the type of protease on the properties of hydrolysates could be considered. The bioactivity tests included free radical scavenging activity, reducing power and ACE inhibitory activity assay, in order to evaluate the antioxidant and antihypertensive potential of alcalase (AH) and pepsin hydrolysate (PH). Additionally, the α-amylase inhibitory activity was also included.

II. Materials and Methods

A. Materials

Hull-less pumpkin (*Cucurbitapepo*. c. v. Olinka) oil cake (PUOC) was obtained from Pan Union, Novi Sad, Serbia. The PUOC was stored at 4°C and ground in a coffee-grinder before use. Alcalase (protease from *Bacillus licheniformis*, 2.4 AU/g), and pepsin (0.7 FIP-U/mg) were used for protein hydrolysis. Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid), ABTS (2,2'-azino-bis(3-ethyl-benzo-thiazoline-6-sulphonic acid)) radical cation, Angiotensin I

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Converting Enzyme from rabbit lung, N-Hippuryl-His-Leu hydrate were obtained from Sigma (St. Louis MO, USA). Chemicals used for HPLC analysis were HPLC grade, while all other chemicals were at least analytical grade.

B. Isolation and Enzymatic Hydrolysis of Cucurbitin

Cucurbitin was isolated by the procedure described by Peričin et al. [5]. The enzymatic hydrolysis was carried out under following controlled, reaction conditions; for alcalase at pH 8.00, temperature of 50°C, and NaCl concentration of 40 mg/ml, and for pepsin at pH 3.00 and temperature of 37°C. The enzyme to substrate (E/S) ratio was 0.02g/1g for both enzymes. After 60min, the reaction mixture was heated (100°C, 5min), centrifuged and the collected supernatants were used for further analysis. The degree of hydrolysis (DH) was determined according to the method described by Peričin et al. [7]. The protein content of each sample was determined by method of Lowry et al. [12], using bovine serum albumin as the standard protein.

C. Antioxidant Activity Assays

The radical scavenging activity of the hydrolysates was determined by the ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid)) radical cationdecolorisation assay, as described by Re et al. [13]. The bleaching rate of ABTS radicalcation solution was monitored at 734 nm. The activity of hydrolysates was compared toa water-soluble analogue of vitamin E, Trolox and results are expressed as Trolox equivalent antioxidant coefficient, TEAC (mmol/l Trolox).

The reducing power of the hydrolysates was evaluated by the method developed by Oyaizu [14], with slight modifications. The sample solution (1ml) was mixed with 2.5 ml of phosphate buffer (0.2mol/l, pH 6.6) and 2.5ml of 1g/100ml potassium ferric cyanide solution. The mixture was then kept in a 50°C water bath for 20min. The resulting solution was cooled rapidly, and then mixed with 2.5ml of distilled water and 0.5ml 0.1g/100ml ferric chloride solution. The absorbance at 700nm of the resulting mixture was measured after reaction of 10min.

D.In vitro ACE Inhibitory Assay

The ACE inhibitory test was performed according to Yoshie-Stark et al. [15]. In each assay, samples dissolved in 0.2mol/L potassium phosphate buffer (pH 8.30) was incubated at 37 ºC for 80min with hippuryl-His-Leu (HHL) in 0.2mol/L potassium phosphate buffer containing 300 mmol/L NaCl (pH 8.30) and the ACE solution. The final concentrations of the HHL and ACE were 10mmol/l and 25mU/ml, respectively. The reaction was stopped by adding 110µl of 1mol/L HCl. The amount of hippuric acid (HA) liberated from HHL by catalytic activity of ACE was quantified with reverse-phase high performance liquid chromatography (RP-HPLC). 20µl of the solution was injected directly onto Zorbax Eclipse XDB-C18 column (4.6 Id x150mm, 5µm, 80Å) to separate HA from HHL. The column was eluted with 50% methanol and 0.1% trifluoroacetic acid (in water), with flow rate of 1ml/min, at 22°C. The absorbance of the eluate was measured at 228nm. The ACE inhibition activity calculated as follows:

$$ACE\ Inhibitory\ Activity\ (\%) = \frac{(A-A_0)-(B-B_0)}{(A-A_0)} \quad (1)$$

where A is the amount of HA in reaction without an inhibitor, B is the amount of HA in reaction with potent inhibitor, while A_0 and B_0 are the respected blanks (where HCl was added in the test tube before the enzyme solution).

E. In vitro α-Amylase Inhibitory Assay

The α-amylase inhibitory test was performed according to Yu et al. [16]. 20µL of a-amylase solution was pre-mixed with 10µL of sample solution at different concentrations. After incubation for 15min, 500µL of 1% starch solution in sodium phosphate buffer (pH=6.9) was added to start the reaction. The reaction was carried out at 37.5°C for 5min and terminated by addition of 500µL of the DNS reagent (1% 3,5-dinitrosalicylic acid, 12% Na–K tartrate in 0.4M NaOH). The reaction mixture was placed ina water bath at boiling point for 15min and then cooled down to room temperature. The absorbance was measured at 540nm and the inhibitory activity was calculated by the following equation:

$$\alpha - Amylase\ Inhibitory\ Activity(\%) = \frac{(A-B)}{A} * 100 \quad (2)$$

where A was the absorbance of reaction blank, B was the absorbance of the reaction in the presence of the hydrolysate sample.

F. Statistical Analysis

Data were expressed as mean ± standard deviation for triplicate determinations and a least significant difference test with a confidence interval of 95% was used to compare the means.

III. Results and Discussion

Under the reaction conditions used in this study, the final degree of hydrolysis (DH) in alcalase (AH) and pepsin hydrolysate (PH) were 26.944±1.1% and 18.7037±1.2%, respectively. Different DH values achieved using the same E/S ratio and reaction time indicates that these two endoproteases hydrolysed the same protein at different peptide bonds dictated by their specificity, while resulting in different peptide composition of hydrolysates.

A. Antioxidant Activity of Cucurbitin Hydrolysates

The activity against ABTS radical cation indicates the ability of substances to act as electron donors or H atom donors in free radical reactions [17].The radical scavenging activity (RSA) of cucurbitin's hydrolysates determined by the ABTS radical cation decolorisation assay is shown in Fig. 1 (a). AH and PH exhibited similar activities (in range 3.3 - 3.4 mmol/l TEAC). The ability of protein hydrolysates/peptides to donate electrons could also be evaluated using the Fe^{3+} reducing power assay. Besides, Fe^{3+} is a pro-oxidant metal ion. The ability of cucurbitin hydrolysates to reduce Fe^{3+} to Fe^{2+} is presented in Fig. 1 (b). High absorbance indicates

strong reducing power. The reducing power of PH was about two fold lower than for AH. This suggested that the AH presumably had more active amino acids or peptides able to donate electrons.

The antioxidant testing revealed that both hydrolysates exhibit significant antioxidant capacity, and showed that the overall RSA of hydrolysates were altered by the enzyme used.

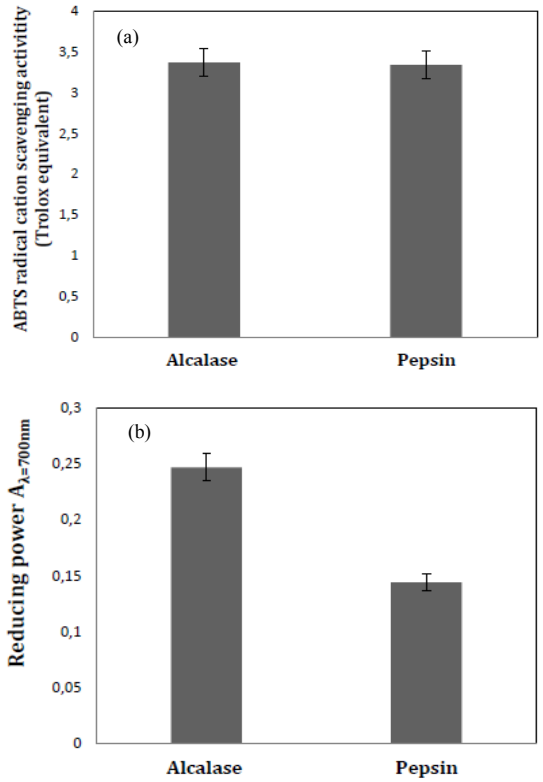

Fig. 1 Antioxidant activity of alcalase and pepsin hydrolysates determined by (a) ABTS radical scavenging activity and (b) Fe^{3+} reducing power

B. ACE Inhibitory Activity of Cucurbitin Hydrolysates

The *in vitro* activity of hydrolysates reflects potential of*in vivo* antihypertensive effect, as the inhibition of ACE results in decreased blood pressure [3]. In the ACE inhibitory assay, HHL acts as the substrate for ACE which catalyses its conversion to hippuric acid (HA) and the dipeptide, histidyl-leucine (HL). ACE activity is assumed to be directly related to the extent of HA release and hence its lower amount indicates higher inhibitory activity of extracts towards ACE. Both tested hydrolysates exhibited ACE inhibiting activity, which was dependent on their concentration. The activities were examined for sample amount range from 0.0024 to 0.37mg, i.e. from 0.016 up to 2.5mg/ml (in the test tube, based on protein content). Fig. 2 shows that logarithmic curves could be fit adequately to represent the ACE inhibitory activity in function of sample amount. The apparent IC_{50} values were determined through established equations (Fig. 2), being 0.0244mg (0.163mg/ml), and 0.0445mg (0.3mg/ml) (final concentration in the test tube), for AH and PH respectively. Based on these results, cucurbitin hydrolysates can be regarded as valuable sources of natural ACE inhibitory peptides, similarly as it has been shown for other plant protein hydrolysates.

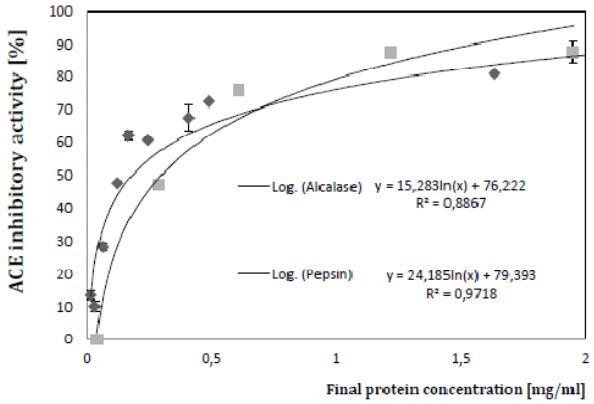

Fig. 2 The ACE inhibitory activity of cucrbitin hydrolysates vs. protein concentration in the test tube, for ♦Alcalase hydrolysate (AH); and ■Pepsin hydrolysate (PH)

C. α-Amylase Inhibitory Activity of Cucurbitin Hydrolysates

α-amylase (E.C.3.2.1.1) catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such glucose, maltose and maltotriose units. Many commercially available drugs used in the management of type-2 diabetes are inhibitors of α-amylase and α-glucosidase, as their inhibition can delay the absorption of glucose following starch and sucrose conversion, thereby moderating the postprandial blood elevation.

In general, both hydrolysates showed modest α-amylase inhibitory activity (Fig. 3).

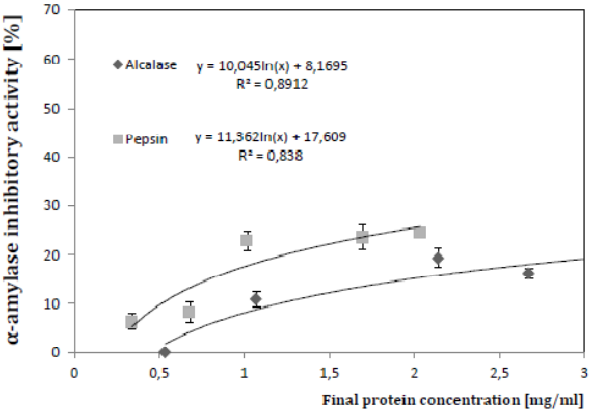

Fig. 3 The α-amylase inhibitory activity of cucrbitin hydrolysates vs. protein concentration in the test tube, for ♦Alcalase hydrolysate (AH); and ■Pepsin hydrolysate (PH)

The PH showed higher α-amylase inhibition than AH. In Fig. 3 it could also be seen that both hydrolysates caused a dose dependent inhibition of the enzyme.

IV. Conclusion

In this work the antioxidant, *in vitro* ACE and α-amylase inhibitory activity of cucurbitin hydrolysates were investigated. The results show that cucurbitin, the major storage protein of pumpkin oil cake is a potential starting material for enzyme mediated production of protein hydrolysates containing bioactive peptides with potential therapeutic properties, which can be used in functional foods or nutraceuticals. However, further studies are necessary to purify and characterise the individual bioactive peptides within these hydrolysates.

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