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ORIGINALPAPER

Biologically Active Digests from Pumpkin Oil Cake Protein: Effect of Cross-linking by Transglutaminase

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Abstract The objective of this study was to show that biologically active hydrolysates can be obtained by simulated human gastrointestinal digestion (HGD) of transglutaminase cross-linked pumpkin oil cake protein (Tg-C) which was previously reported as a potential functional food additive. A two-stage in vitro digestion model system (by pepsin and α chymotrypsin and trypsin, simultaneously) was used to simulate the process of HGD on native and Tg-C major storage pumpkin oil seed/cake protein, cucurbitin (C). The biologically active potential of the digests was evaluated, measuring the angiotensin-converting-I enzyme (ACE) inhibitory and anti-oxidant capacity. The ACE inhibitory activity was determined in both final digests, with $IC_{50} = 0.30 \pm 0.04$ mg/ml for C and $IC_{50} = 0.28 \pm 0.01$ for Tg-C. The anti-oxidant potency of the examined proteins was enhanced by the digestion process. The 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation activities and reducing power testing showed that all the hydrolysates act as a radical quencher and reducing agents. Overall, the results showed that the cross-linking by Tg did not influence the digestion process, as well as having no effect on the biological activity of the hydrolysates. These also indicate that Tg-C, if used as functional food additive, after food consumption can be digested and become a source of peptides exerting positive effects on human health.

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Introduction

In recent decades, oilseeds have had primacy over cereals as protein sources for several reasons: two to four times higher protein content [1] and higher nutritional quality [2], while oil cakes, a by-product of the oil extraction process from seeds, are an unused protein source with protein content ranging from 30 to 60% [3]. Pumpkin (*Cucurbita pepo*) seed oil is widely used in the middle-south European region (Austria, Hungary, Slovenia, Croatia and Serbia). Pumpkin oil cake, which remains from the oil extraction process is still underused in economic terms. The protein exploitation from this source has been described as a way to increase the value of this agricultural by-product [4–6].

Generally, food proteins are of great importance, not only in contributing to the improvement of the functional roles in food preparation, processing, storage and consumption, but also in increasing the quality of sensory and biological properties of food products (e.g. anti-oxidant, angiotensin-converting-I enzyme (ACE)-inhibition, opioid activity) [7]. Rarely, native proteins possess all the desirable properties for application in different food systems. An attractive method to produce novel, tailored food components, with different techno-functional and bioactive properties from different proteins, is enzymatic modification. Microbial transglutaminase (Tg, EC 2.3.2.13) is a cross-linking enzyme which is used for such purposes. Tg can catalyze acyl-transfer reactions introducing covalent cross-linking between proteins, peptides and various primary amines by forming ε-(γ-glutamyl)-lysine bonds both intra- and inter-molecularly [8]. They have been widely used to improve

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the gelation and texture properties of food proteins such as soya, wheat, pea and whey proteins, casein, myosin, gluten, etc. [9–12].

Recent research carried out in our laboratory has focused on the application of Tg for the modification of the functional properties of major storage proteins in pumpkin seed/cake (called cucurbitin). The reason is cucurbitin's low solubility, thus methods for improving the solubility and other functional properties aree very important for better use of cucurbitin as a food ingredient. The results obtained from this research showed that the solubility of polymerized proteins was increased over the whole studied pH range, and also the gelation properties of the cross-linked protein were improved thus showing that the functional properties of cucurbitin can be tailored by Tg cross-linking to specific needs [13].

However, enzymatic cross-linking does not only affect protein structure but it may also affect their safety (i.e. allergenicity) [14] and nutritive properties such as digestibility. The Tg-mediated isopeptide bond (G-L) is highly resistant to mechanical stress and proteolytic degradation [15], thus the effect of cross-linking by Tg on the digestion by human gastrointestinal proteases has been studied in a variety of protein substrates, and it has been reported that, generally, cross-linking decreases protein digestibility [9–11].

Moreover, during human gastrointestinal digestion of food proteins, bioactive peptides can be released from the inactive parent proteins. These peptides can possess multiple activities, including antihypertensive, anti-oxidant, anti-inflammatory and hypocholesterolemic. Elevated blood pressure is one of the major independent risk factors for cardiovascular diseases. 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. The inhibition of ACE activity can lead to an overall antihypertensive effect, and ACE inhibitory peptides have shown great promise in dietary strategies in the therapy of hypertension, especially in its prevention and in the initial treatment of mildly hypertensive individuals [12, 16]. Also, oxidative stress is one important factor underlying hypertension [17]. Therefore, there has been strong interest in the production of functional protein hydrolysates containing bioactive peptides with both ACE inhibitory and anti-oxidant activity for functional foods with beneficial health effects.

Based on all the above-mentioned facts, our investigations have been to find out whether polymerized cucurbitin, which possesses desirable functional properties for application as food ingredients, can be digested and realise bioactive hydrolysates. Hence, the objective of the present research was the application of the main gastrointestinal proteases, pepsin, α-chymotrypsin and trypsin, for the digestion of cross-linked cucurbitin, using cucurbitin (C) for control. The generated hydrolysates were assayed to determine their degree of hydrolysis, molecular weight by sodium dodecyl sulfate electrophoresis, and their anti-oxidant and ACE inhibitory properties.

Materials and Methods

Materials

The hull-less pumpkin (*Cucurbita pepo*. c. v. Olinka) oil cake (PuOC) was collected from a local Industry Pan Union, Novi Sad, Serbia. Oil cake was obtained after seed pressing in a worm press (6 kg/h capacity). It was stored at 4 °C and ground in a coffee-grinder before use. The microbial transglutaminase (ACTIVA WM 100 U/g) was a gift from Ajinomoto Foods Europe (Hamburg, Germany). ACE from rabbit lung, N-hippuryl-His-Leu (HHL) hydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and trypsin, from bovine pancreas, with declared activity of 12,885 U/mg (0.03 N-benzoyl-L-tyrosine ethyl ester, BTEE units/mg), were obtained from Sigma (St Louis, MO, USA). Pepsin from porcine stomach and α-chymotrypsin from bovine pancreas were purchased from AppliChem (Darmstadt, Germany), with declared activity of min 0.7 FIP (Fédeération Internationale de Pharmacie) U/mg and min 1500 U/mg, respectively. All the other chemicals used for the experiments were of analytical or better grade.

Preparation of Cucurbitin

Defatted ground PuOC meal (10 g) was extracted by stirring sequentially with 200 ml of water and 200 ml of 10% sodium chloride solution for 3 h at room temperature. Cucurbitin was precipitated from the clarified extract by the gradual addition of 800 ml of water. After centrifugation (20 min, 25,000*g*, 4 °C) (Sorvall RC-5B; GMI, MN, USA) the precipitate was dissolved in 100 ml standard buffer (1 mol/l KCl in 0.1 mol/l sodium phosphate buffer, pH 7.0), the solution was clarified by centrifugation and the cucurbitin was precipitated by the addition of 200 ml of water. This procedure was repeated twice. The final protein residue was dried at 30 °C for 48 h. Finally, the dried protein was ground in a coffee grinder to obtain a powder. The yield of cucurbitin was 1.2/10 g oil cake, and the protein content in obtained powder was 94.44/100 g.

Protein Cross-linking

A procedure described previously [13] was used to prepare cross-linked cucurbitin Tg-C. The protein suspension (concentration of 10 mg/ml in 0.1 mol/l phosphate buffer pH 7.0) was incubated at 50 °C with Tg and an enzyme/substrate (E/S) ratio 0.025 g/g. After 120 min, the reaction mixture was immediately heated (80 °C, 20 min) to inactivate the enzyme, after which the suspension was cooled to room temperature (25 °C), vacuum-filtered and the filtrate was dried on a Büchi 190 spray drier (Flawil, Switzerland) at an inlet temperature of 120 °C and outlet temperature of 70 °C to obtain the modified protein powder. The control sample of C was treated in the same way without Tg addition.

In Vitro Digestion

The in vitro hydrolysis with gastrointestinal proteases was performed by adaption of the method described by Vaštag et al. [18]. The Tg-C and C were initially treated with pepsin, at pH 2.00 and 37 °C, with the E/S ratio of 0.04 g/g, for 120 min. Then, the pH was adjusted to 7.50 and the solutions of α-chymotrypsin (E/S ratio 0.02 g/g) and trypsin (E/S ratio 0.08 g/g) were added; this reaction lasted 120 min. At the end of the two-stage hydrolysis, the reaction mixture was immediately heated (100 °C, 5 min) and centrifuged (23,500*g*, 10 min, room temperature) (Eppendorf Mini Spin Plus; Ontario, Canada). The collected supernatants were further analyzed.

Determination of the Degree of Hydrolysis (DH)

The DH was determined according to the method by Peričin et al. [5].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE of the protein samples was carried out by the method of Laemmli [19]. The discontinuous system used consisted of a 40-mg/ml acrylamide stacking gel and a 100-mg/ml acrylamide separating gel. Samples (1 mg/ml protein) were dissolved in tris/glycine buffer (pH 6.8) containing 20 g/l SDS and 50 g/l β-mercaptoethanol. Electrophoresis (Multi Drive XL; Pharmacia, Uppsala, Sweden) was carried out at 60 mA until the tracker dye reached the bottom of each gel. After electrophoresis, the gels were stained with 2 mg/ml Coomassie brilliant blue R-250 in a mixture of acetic acid:methanol:water (1:5:4) and destained with a mixture containing 70 ml/l of acetic acid and 60 ml/l of methanol in water for 16 h.

ACE Inhibitory Assay

The test of ACE inhibitory activity of the hydrolysates was performed according to Yoshie-Stark et al. [20]. In each assay, a sample (at different concentrations) was incubated with HHL in 0.2 mol/l potassium phosphate buffer containing 300 mmol/l NaCl (pH 8.30) and ACE solution at 37 °C for 80 min. The final concentrations of the HHL 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 (HA) liberated from the HHL was quantified with reverse-phase high-performance liquid chromatography, and then 20 μl of the solution was injected directly onto a Zorbax Eclipse XDB-C18 column (4.6 Id × 150 mm, 5 μm, 80 Å; Agilent Technology, Santa Clara, CA, USA) to separate the HA from the HHL. The column was eluted with 50% methanol and 0.1% trifluoroacetic acid, with a flow rate of 1 ml/min, at 22 °C. The absorbance of the eluate was measured at 228 nm. From the chromatograms, the peak area (corresponding to HA) was integrated, and the amount of HA in the samples was quantified. The ACE inhibition activity was calculated as

$$\text{ACE inhibitory activity (\%)} = \left(A - A_0\right) - \left(B - B_0\right) / \times 100 / \left(A - A_0\right),$$

where A is the amount of HA in the reaction without an inhibitor, B is the amount of HA in the reaction with the potent inhibitor, while A_0 and B_0 are the respective blanks (where HCl was added to the test tube before the enzyme). The sample concentration required to produce 50% inhibition of ACE (IC_{50}) was used as an activity indicator.

Anti-oxidant Activities

DPPH Scavenging Activity Assay

The free radical scavenging activity was evaluated using the DPPH scavenging activity assay as described by Morales and Jimenez-Perez [21]. In brief, an aliquot of 200 μl of the sample was added to 1 ml of a daily prepared solution of DPPH (74 mg/l) in ethanol. The absorbance was measured at 520 nm (T80 UV–Vis Spectrophotometer; PG Instruments, Lutterworth, UK) after 30 min. The DPPH scavenging activity was calculated as anti-oxidant activity (AA):

$$AA\,(\%) = \left(A_{\text{control}} - A_{\text{sample}}\right) \times 100 / A_{\text{control}},$$

where A_{control} is the concentration of DPPH in the blank (in the presence of a buffer instead of the protein extract) and A_{sample} is the concentration of DPPH in the sample (in the presence of the protein extract), after 30 min of reaction.

ABTS Radical Scavenging Activity Assay

The radical scavenging activity was determined by the ABTS radical cation decolorization assay as described by Re et al. [22]. Briefly, 30 μl of the protein sample was added to 3 ml diluted ABTS radical cation solution ($A_{734\text{nm}} = 0.7 \pm 0.02$)

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and the absorbance was measured after 10 min at 734 nm (T80 UV–Vis Spectrophotometer; PG Instruments). Appropriate solvent blanks were run in each assay. The AA was calculated as:

$$\mathrm{AA}\ (\%) = \left(A_{\mathrm{control}} - A_{\mathrm{sample}}\right) \times 100/A_{\mathrm{control}},$$

where A_{control} is the concentration of ABTS in the blank (in the presence of a buffer instead of the protein extract) and A_{sample} is the concentration of ABTS in the sample (in the presence of the protein extract), after 10 min of reaction.

Reducing Power

The reducing power was evaluated by the method described by Oyaizu [23] with slight modifications. The sample solution (1 ml) was mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of 10 mg/ml potassium ferric cyanide solution. The mixture was then kept in a 50 °C water bath for 20 min. The resulting solution was cooled rapidly, and then mixed with 2.5 ml distilled water and 0.5 ml 10 mg/ml ferric chloride solution. The absorbance at 700 nm of the resulting mixture was measured after 10 min reaction (T80 UV–Vis Spectrophotometer; PG Instruments). Increase of absorbance indicates increase of reducing power.

Statistics

Measurements were carried out in triplicate and the results are given as the mean of three measurements ± standard deviations. Analysis of variance was performed on the data, and a least significant difference test with a confidence interval of 95% was used to compare the means.

Results and Discussion

In Vitro Digestion

DH was the parameter for monitoring the protein hydrolysis. During simulated digestion, the hydrolysis rates of C and Tg-C followed a similar trend (Fig. 1). After pepsin digestion, the DH of C and Tg-C reached 44.1 ± 2.21 and 38.2 ± 1.82%, respectively. Furthermore, subsequent digestion by α-chymotrypsin and trypsin led to an increase of DH in both C and Tg-C digests, reaching similar DH values (57.3 ± 2.81 and 59.2 ± 2.48%, respectively). Unlike β-lactoglobulin, β-casein, and phaseolin, for which it was reported that cross-linking by Tg resulted in products resistant to both pepsin and pancreatic enzymes [11, 14, 24] polymerization of cucurbitin by Tg had no influence on its digestion process.

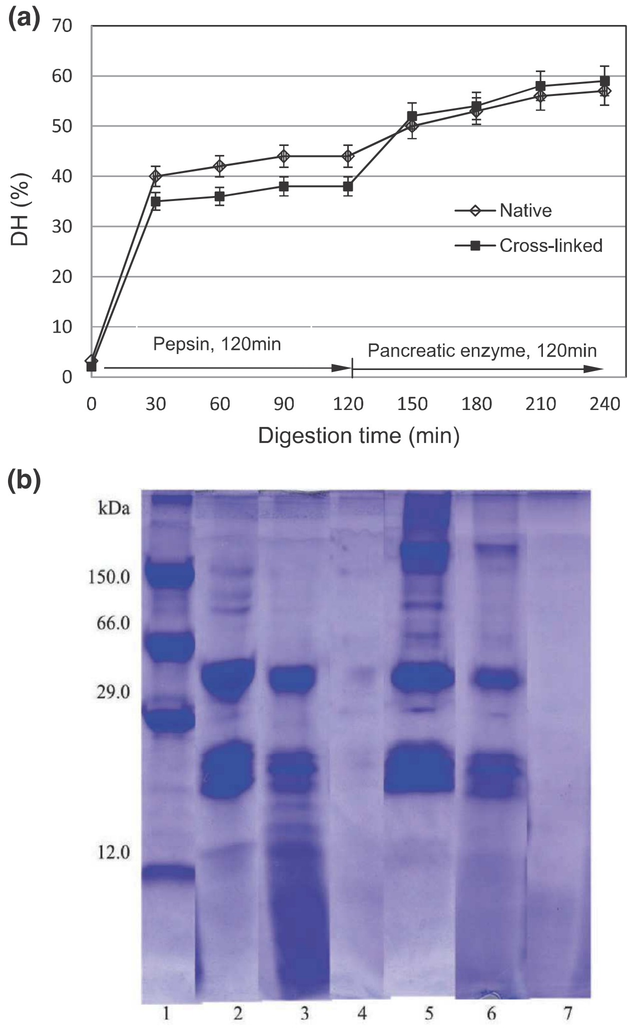

Fig. 1 Protein hydrolysis monitoring by the (**a**) degree of hydrolysis (*DH*) and (**b**) SDS-PAGE analysis of native (C) and enzymatically cross-linked cucurbitin (Tg-C) during in vitro digestion first by pepsin and then with α-chymotrypsin and trypsin, simultaneously. In (**b**), molecular weight standard (*lane 1*), C (*2*), Tg-C (*5*); cucurbitin digested by pepsin C1 (*3*), pepsin, α-chymotrypsin and trypsin C2 (*4*); enzymatically cross-linked cucurbitin digested by pepsin Tg-C1 (*6*), pepsin, α-chymotrypsin and trypsin Tg-C2 (*7*)

SDS-Page

Figure 2 shows the eletrophoretic profiles of C, Tg-C and their products of in vitro digestion by pepsin (C1 and Tg-C1) and subsequently by α-chymotrypsin and trypsin (C2 and Tg-C2). Electrophoresis of C exhibited two major bands (MW ~ 33.0 and 22.0 kDa) corresponding to acid polypeptides and basic polypeptides of cucurbitin, respectively (lane 2). The sample Tg-C showed a high MW band (>150.0 kDa) (lane 5). During in vitro digestion, both native and polymerized C were further degraded to apparent molecular weights lower than 12 kDa (lanes 3, 4, 6 and 7). After hydrolysis

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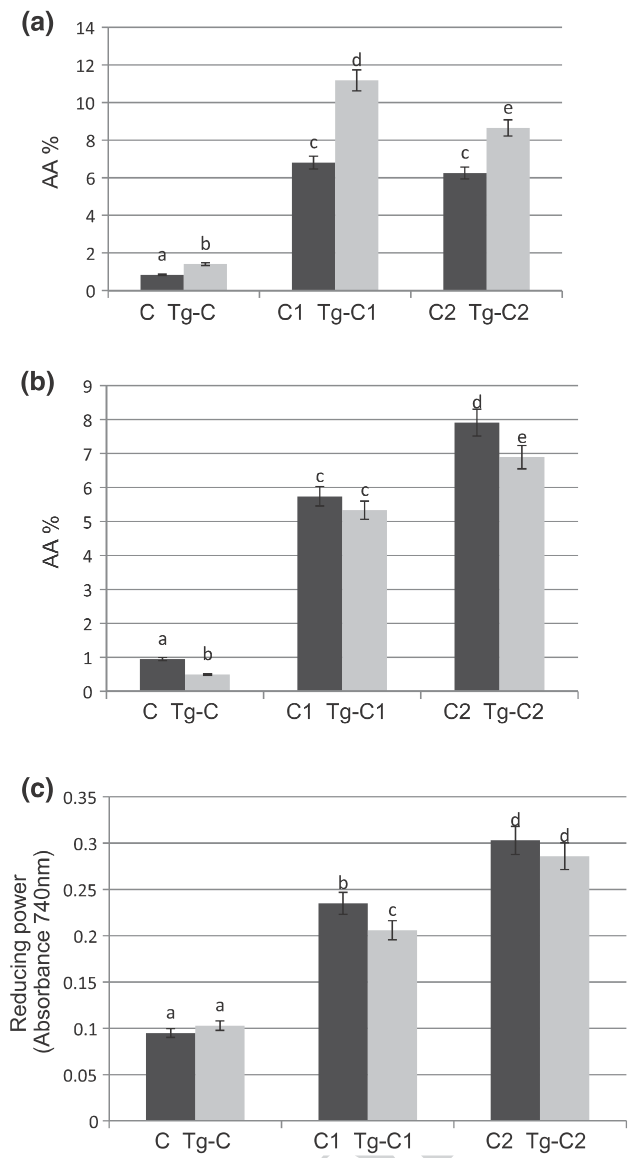

Fig. 2 Anti-oxidant activity (*AA*) of native (*C*) and enzymatically cross-linked cucurbitin (*Tg-C*) and their products of in vitro digestion (*C1* and *Tg-C1* after pepsin digestion; *C2* and *Tg-C2* after pepsin and pepsin/trypsin/chymotrypsin digestion). Radical scavenging activity measured by **a** DPPH (2, 2-diphenyl-1-picrylhydrazyl), **b** ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) methods (results expressed per 1 mg/ml proteins), and **c** reducing power (*different letters* indicating significant statistical differences at $p < 0.05$)

by pepsin, two bands were still visible, but with reduced intensity (lanes 3 and 6). In the final digests, after treatment by trypsin and α-chymotrypsin, no visible bands were obtained on the gel (lanes 4 and 7).This is in accordance with observations of other researchers, where the products of subsequent hydrolysis of peptide/proteins mixtures by pepsin and pancreatic enzymes were not detected by SDS-PAGE [25, 26].

Table 1 ACE inhibitory activity of hydrolysates obtained from native and Tg cross-linked cucurbitin by pepsin (120 min) and pepsin/trypsin/chymotrypsin (240 min)

Sample	ACE inhibitory activity IC_{50} (mg/ml)
C	n.d.
Tg-C	n.d.
Pepsin digest	
C1	0.65 ± 0.02a
Tg-C1	0.65 ± 0.01b
α-Chymotrypsin and trypsin digest	
C2	0.30 ± 0.04c
Tg-C2	0.28 ± 0.01d

IC50 the concentration required to produce 50% inhibition of ACE; *n.d.* not detected

Different letters in the rows indicate significant statistical differences at $p < 0.05$

ACE Inhibitory Activity

The in vitro ACE inhibitory activity of protein hydrolysates/peptides reflects their potential antihypertensive effect in vivo [12]. Neither native nor Tg-modified cucurbitin prior to in vitro digestion showed ACE inhibitory activity (Table 1). As their hydrolysates exerted significant inhibitory activity, this indicates that, during the proteolysis, ACE inhibitory peptides were released from both protein substrates. The peptic hydrolysate of C and Tg-C had IC_{50} values of 0.647 ± 0.02 and 0.653 ± 0.01 mg/ml, respectively. Further hydrolysis by α-chymotrypsin and trypsin significantly ($p < 0.05$) decreased IC_{50} values to 0.30 ± 0.04 mg/ml for the C digest and to 0.28 ± 0.01 mg/ml for the Tg-C digest. According to the available literature, the IC_{50} values of a variety of food protein enzymatic hydrolysates are in the range of 0.160–3.770 mg/ml [6, 26]. Thus, the final hydrolysates of native and modified C obtained in this research can be considered as competetive ACE inhibitors compared with other food protein hydrolysates [27, 28].

Anti-Oxidant Activity

Protein hydrolysates/peptides in food are of great interest as natural anti-oxidants in the human diet. In investigations of the anti-oxidant activity of protein hydrolysates/peptides, there are various in vitro assays that should be performed, due to possible diverse anti-oxidant mechanisms. Among the common methods, DPPH and ABTS radical scavenging assays and the reducing power assay are usually carried out [29].

After pepsin digestion, the DPPH radical scavenging activity of C and Tg-C at a protein concentration of 1 mg/

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ml was 6.8 ± 0.4 and 11.2 ± 0.2%, respectively (Fig. 2a). After the next step of digestion, the activity of C did not significantly change (6.2 ± 0.1%), while that of Tg-C decreased to 8.6 ± 0.3%.

The ABTS radical scavenging activity of both C and Tg-C was significantly increased after incubation with pepsin, and at 1 mg/ml reached the values of 5.7 ± 0.2 and 5.3 ± 0.3%, respectively. After treatment with α-chymotrypsin and trypsin, the radical scavenging activities continued to increase, and in the final digest reached 7.9 ± 0.7 and 6.9 ± 0.6%, respectively (Fig. 2b).

Both assays showed that the radical scavenging activity of C and Tg-C were significantly enhanced after hydrolysis by digestive proteases compared to the non-digested samples. Generally, during in vitro digestion, C and Tg-C, were cleaved into peptides, which exert greater anti-oxidant activity than their parent proteins [18, 30]. However, in the case of the DPPH assay, the activity of Tg-C was notably reduced after the treatment with the pancreatic proteases. When the ABTS radical scavenging assay was used, the activity of both final hydrolysates significantly increased in comparison to the activity of the peptic digests. The different patterns between the DPPH and ABTS radical scavenging activities could be due to several reasons. The antiradical response of proteins/peptides in a particular assay depends on several factors, such as the solvent used in the test and the affinity between the reactive species and the anti-oxidant. DPPH is an oil-soluble, while ABTS is a water-soluble, free radical and different reaction media could have affected the anti-oxidant efficiency of the tested samples. There are reports on the disadvantages of DPPH when used for hydrophilic anti-oxidants, such as peptides [31].

In addition to this, the anti-oxidant activity of a protein hydrolysate is the result of the activity of the constituent peptides, which further depends on the polarity and amino acid composition at the individual level [32, 33]. As the structural composition of C as well as Tg-C and their corresponding hydrolysates were continuously changing during the digestion process, it could be assumed that the generated anti-oxidant peptides were of different structures at certain stages of digestion, acting differently towards the examined radicals.

The reducing power of peptides indicates their capacity to donate electrons. This is an important anti-oxidant mechanism for the stabilisation of free radicals and oxidised anti-oxidants that can accept electrons [34]. There was significant increase in the reducing power of both digests after pepsin digestion (Fig. 2c). During the second stage of digestion, the reducing power also continued to increase. This is in accordance with other authors [30, 32], who also found an increase in the reducing power of proteins caused by in vitro digestion. It should be noted that there is no difference between the overall reducing power of the C and Tg-C digests ($p > 0.05$).

Conclusion

The above results showed that the native and Tg-modified cucurbitin can be hydrolysed by the main digestive enzymes, and that the cross-linking by Tg had no significant impact on the bioactive potential of the obtained hydrolysates. Furthermore, these results showed multifunctionality of the cross-linked cucurbitin with Tg, as it has previously been reported as a functional food additive. This research indicates that this protein ingredient could also be the source of natural anti-oxidant and potent antihypertensive peptides released in the digestive system after normal consumption.

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