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ENZYMATIC HYDROLYSIS FOR FUNCTIONALISATION OF WATERMELON SEED PROTEIN FROM ULTRASOUND-ASSISTED EXTRACTION IN COMPARISON TO SOY PROTEIN ISOLATE

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Abstract: The functional characteristics of a watermelon seed protein isolate (UA-WSPI), which resulted from an underutilised protein source by ultrasound-assisted extraction and freeze-drying, and a commercial spray-dried soy protein isolate were compared. Both were enzymatically treated with pepsin plus trypsin (PT) or additionally with PT plus Alcalase (PAT) for functionalisation by obtaining different partial hydrolysates. Composition, DPPH scavenging activity, digestibility and technological functionality (such as dispersibility, water-/oil-holding capacity, and zeta potential) were analysed for each protein isolate and the hydrolysates. UA-WSPI had advantages such as higher protein solubility. Enzymatic digestion of UA-WSPI resulted in the highest peptide yields and degrees of hydrolysis. Hydrolysis by the PT treatment even increased the antioxidant activity and emulsifying properties of the hydrolysate compared to UA-WSPI, whereas the soy protein isolates yielded hydrolysates of lowered antioxidant activity. Watermelon seeds proved to yield protein isolates as new functional food components, especially when applying appropriate enzyme combinations.

Keywords: watermelon seed protein isolate, ultrasound-assisted extraction, functional food ingredients, bioactive peptides, protein hydrolysates, enzymatic hydrolysis, pepsin-trypsin treatment, Alcalase, antioxidant activity, emulsifying properties, protein solubility, plant-based protein, watermelon seed by-products, WSPI, soy protein isolate, DPPH scavenging activity, technological functionality, water-holding capacity, oil-holding capacity, zeta potential, rational nutrition, balanced composition,

vegetable pastes, sustainable processing, food technology, functional confectionery products.

Introduction. Watermelon (Citrullus lanatus (Thunb.) Matsum. & Nakai) is a member of the cucurbit family (Cucurbitaceae). It is grown commercially in agricultural areas having warm periods. In the year 2021, the global annual production of watermelon fruit was 101.6 Mt, with 3.5 Mt thereof being produced on 729 km^2 in Turkey as one of the major cultivation countries Watermelon is widely appreciated for its nutritious properties, including vitamins and minerals, for its sweet taste and for being refreshing. It has a moisture content of almost 90 g hg^{-1} (Abu-Nasser & Abu-Naser, 2018). As it is mostly consumed as fresh fruit or processed into drinks on the small scale, e.g., in gastronomy, most of the seeds are turned into food waste, besides some seeding material for fruit cultivation. However, there is a growing demand for products containing industrially processed watermelon juice. In the process, 25%–60% of the raw materials are altered into food processing by-products. Nonetheless, industrial processing of watermelon seeds (WMS) still plays an inferior role, yielding, e.g., roasted and salted seeds as popular food snacks or watermelon seed oil for cosmetic applications. As readily available, but still underutilised by-products, WMS can be used to produce further beneficial goods cost-effectively. They are a potential protein source owing to their high protein content and the balanced amino acid composition (Arg, Asp, Leu, Glu, etc.; Wen et al., 2019). These properties make WMS a promising source to produce protein isolates (WSPI) and hydrolysates (WSPH), in particular, if the seedy fruit is grown organically. In terms of abundant contents of protein, amino acids and bioactive peptides, WMS come close to soy, being the most widely used plant protein source so far.

Broad industrial utilisation of the seeds is additionally restricted by limiting factors of existing methods for protein extraction, such as high energy consumption, effects of chemical solvents, long extraction times and low separation yields . In the last decades, various novel extraction techniques have been introduced for protein extraction from plant-based sources, including ultrasonic, microwave-assisted, pressurised liquid and subcritical extraction methods. Ultrasound, being a green, innovative and inexpensive technology, is widely used for the extraction of proteins and antioxidant compounds. Gadalkar & Rathod explored the ultrasound-assisted extraction of watermelon seed proteins with different sonication parameters. The combination of watermelon seed protein extraction with the ultrasound method enhanced the protein extraction yield by around 87% and reduced the extraction time to 9 min. Ultrasound could improve later enzymatic hydrolysis and protein properties. Furthermore, the development of

ultrasound devices can play an important role in the preparation of bioactive protein hydrolysates.

Materials and chemicals

Watermelon seeds were procured as dried foodstuff from the local market (Diyarbakir, Turkey). Soy protein isolate (SPI, low-fat (1.5 g hg^{-1}), conventionally extracted, spray-dried) was purchased from the market (Myvegan, THG plc, Manchester, UK). All trials were performed with chemicals of analytical grade. Chemicals and enzymes were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany): hexane, hydrochloric acid, sodium hydroxide, ethanol, sodium chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), formaldehyde and proteases. These enzymes comprised pepsin (endoprotease from porcine gastric mucosa, 3200 units mg^{-1}), trypsin (endopeptidase from porcine pancreatic glands, 1000–2000 units mg^{-1}) and Alcalase (endoprotease from Bacillus licheniformis, 0.75 units mL^{-1}).

Watermelon protein extraction by ultrasound-assisted extraction (UAE) and extraction yield

Defatted watermelon seed powder (50 g) was suspended in 500 mL ultra-pure water (CLiR 5000, ResinTech Inc., Camden, US) in a flat-bottom glass reactor. The pH was adjusted to 9 by adding 2 M NaOH. The blend was sonicated for 9 min at 90 W and a frequency of 25 kHz (UP400St, Hielscher Ultrasonics GmbH, Teltow, Germany). Centrifugation followed at 10 000 × **g** for 15 min at 4 °C (Gadalkar & Rathod, 2020). The supernatant pH was adjusted to 4.5 with 1 N HCl to precipitate the soluble proteins. The residue was obtained by centrifugation 10 000 × **g** (FL40R, Thermo Scientific, Rockford, USA) for 15 min at 4 °C and freeze-dried (FDL1R-0E, LAB1ST, Shanghai, China) to collect the dried protein. The extraction yield was calculated as the percentage ratio of the obtained mass of ultrasound-extracted watermelon seed protein (UA-WSPI) to the mass of watermelon seed powder used. Prior to further use, the freeze-dried material was pulverised, using a granite mortar.

Compositional analysis

For both defatted and crude (non-defatted) watermelon seed powder, the contents of fibre, ash, total fat and moisture were determined, applying the standard AOAC methods 985.29, 942.05, 948.22 and 948.12 (AOAC, 2006). The protein contents were deduced from the total nitrogen contents, resulting from a Kjeldahl analyser (UDK 159, Velp Scientific, Deer Park, NY, USA) according to the Kjeldahl method (Kirk, 1950). The converting factors used to calculate the total protein contents from the nitrogen contents were 5.3 for watermelon seed products (seed powder, protein isolate, hydrolysates) as for oil seeds in general, but 5.71 for the soy products (protein

isolate, hydrolysates), as particularly specified for this plant species (Matissek et al., 2018). For the different protein samples (UA-WSPI, SPI and their hydrolysates), only the contents of total nitrogen and total protein were quantitated, but according to the Dumas method (Ebeling, 1968), using a Dumatherm analyser (C. Gerhardt, Gerhardt Analytical Systems, Königswinter, Germany) and the aforesaid converting factors.

Table 1. Proximate analysis of crude and defatted watermelon seed (WMS) powder

WMS source	Protein (g hg^{-1})	Ash (g hg^{-1})	Moisture (g hg^{-1})	Oil (g hg^{-1})	Crude fibre (g hg^{-1})
Crude WMS					
Own material	20.9 ± 0.6^{a}	13.4 ± 0.04^{a}	3.8 ± 0.02^{a}	38.2 ± 0.6^{a}	38.9 ± 0.03^{a}
Gadalkar & Rathod (2020)	18.7 ± 0.1^{a}	4.4 ± 0.2^{b}	5.6 ± 0.5^{b}	49.4 ± 1.2^{b}	6.2 ± 0.3^{b}
Wani *et al.* (2008)	16.3 ± 0.6^{b}	2.5 ± 0.4^{c}	7.7 ± 0.2^{c}	21.9 ± 0.6^{c}	22.2 ± 0.4^{c}
Defatted WMS					
Own material	49.0 ± 0.3^{c}	14.5 ± 0.05^{d}	4.9 ± 0.03^{d}	3.6 ± 0.06^{d}	40.3 ± 0.02^{a}
Gadalkar & Rathod (2020)	54.5 ± 0.3^{f}	5.9 ± 0.3^{e}	7.4 ± 0.5^{c}	4.6 ± 0.5^{e}	7.8 ± 0.1^{d}

Composition of protein isolates and their partial hydrolysates

In terms of the total nitrogen content, being 13.61 ± 0.07 g hg^{-1} for UA-WSPI and 14.01 ± 0.02 g hg^{-1} for SPI, the two protein isolates from WMS and soy were very similar (Table 2). The different nitrogen–protein conversion factors for oil seeds and soy slightly increased the difference in the protein contents, which were 72.13 ± 0.35 g hg^{-1} for UA-WSPI and 80.01 ± 0.14 g hg^{-1} for SPI. Nonetheless, the protein contents of both isolates were comparable to those reported by Wang et al. (2004) and Wani et al. (2012). Application of UAE to defatted, but non-dehulled WMS thus enabled the recovery of a protein isolate (UA-WSPI) that showed almost the same protein content as those, which had been extracted conventionally from dehulled plus defatted WMS (79.1–83.8 g hg^{-1}, Wani et al., 2012).

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Protein isolate	Hydrolysate	Total nitrogen (g hg^{-1})	Protein (g hg^{-1})
UA-WSPI		13.61 ± 0.07^{a}	72.13 ± 0.35^{a}
	WSPH-PAT	5.03 ± 0.02^{b}	26.63 ± 0.08^{b}
	WSPH-PT	5.57 ± 0.04^{c}	29.53 ± 0.20^{b}
SPI		14.01 ± 0.02^{a}	80.01 ± 0.14^{c}
	SPH-PAT	5.12 ± 0.01^{b}	29.24 ± 0.06^{b}
	SPH-PT	5.62 ± 0.01^{c}	32.09 ± 0.08^{d}

Table 2. Total nitrogen and protein content of watermelon protein isolate, soy protein isolate, and their hydrolysates

For the protein hydrolysates from UA-WSPI and SPI, the degree of hydrolysis increased as the number of applied enzymes rose, whereas the peptide yields fell (Table 3). Accordingly, the combined use of pepsin, Alcalase and trypsin (PAT-treatment) caused higher DH percentages and lower peptide yields than the incubation with pepsin plus trypsin (PT-treatment) did. The peptide yields always ranged at ~37% after PAT-treatments and at ~40% after PT-treatments ($P < 0.05$). However, DH percentages of hydrolysates from UA-WSPI were always clearly higher (47%–56%) than those of hydrolysates from the soy protein isolate (34%–40%). Consequently, WSPH-PAT showed the highest DH (55.8% ± 0.7%).

Similar differences in DH among these enzyme combinations were reported previously for soy protein hydrolysates (Chen et al., 2020). As described by Meinlschmidt et al. (2016), incubation of soy protein for 2 h with either Alcalase, trypsin or pepsin under the individual optimum conditions of each enzyme led to soy protein hydrolysates of different DH. For mere Alcalase, peptic and tryptic hydrolysis of WMS protein, DH was reported to increase from 13% to 19% to 26% (Arise et al., 2016). Concurrently, the peptide yields found by Arise et al. (2016) after mere peptic hydrolysis were much higher than those shown in Table 3 for enzyme combinations.

Conclusions. The protein isolate produced by UAE from defatted, but non-dehulled WMS proved comparable to commercial soy protein isolate in terms of protein content, the negative ζ-potential, dispersibility, and digestibility. Most importantly, UA-WSPI turned out to be even superior to SPI as regards solubility at pH 11 and water-holding capacity, besides oil-holding

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capacity and emulsion stability. However, it could not compete with SPI in terms of foaming properties and antioxidant capacity. Partial enzymatic hydrolysis (PT- and PAT-treatments) entailed greater degradation of UA-WSPI than of SPI. For both hydrolytic treatments, protein hydrolysis turned out to be apparently too intense to maintain important technological properties of the proteins, such as water−/oil-holding capacity as well as foaming and gelling properties.

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