Application of assisted extraction technologies to optimize the extraction of biologically active substances from plant materials

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Abstract. There is an increased interest in obtaining amaranth grain extracts enriched with biologically active substances by deep processing of raw materials with a further prospect of their use as part of specialized multi-purpose food products. The aim of this work was to evaluate the effectiveness of membrane filtration for the extraction of biologically active substances from amaranth grains. Amaranth grain extract was obtained by enzymatic hydrolysis using a proteolytic enzyme followed by membrane treatment. the developed approach made it possible to obtain an extract from amaranth grains - a source of biologically active peptides, 20-hydroxyecdysone and polyphenols (including saponins). In total, triterpene saponins were concentrated in the extract by 8 times, and the phytoecdysteroid 20-hydroxyecdysone was more than 30 times concentrated. The high content of low molecular weight peptide fractions (more than 80%) in the composition of the extract makes it promising to evaluate its antihypertensive and antidiabetic properties in vivo. The presence of 20-hydroxyecdysone in the extract, an adaptogen with a proven effect, opens up the possibility of its use as a functional food ingredient in specialized food products, including for athletes.

1 Introduction

Amaranth grain contains a unique set of phytonutrients - compounds that protect plants from aggressive environmental influences, including fungal, bacterial and viral infections. Phytonutrients (minor biologically active components) of amaranth grain are saponins, phenolic compounds (including flavonoids), phytosterols and some other compounds.
Polyphenolic compounds are found in amaranth grain in the form of free molecules, their glycosylated forms and polymer molecules (for example, oligomeric flavonoids - proanthocyanidins, condensed tannins). In the most cultivated amaranth species: Amaranthus caudatus, A. cruentus and A. hypochondriacus, the following main groups of phenolic compounds have been identified: phenolic acids (ferulic, p-coumaric, p-hydroxybenzoic), flavonoids (rutin, quercetin), and tannins, both individually and in the composition of extracts [1].

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The triterpene saponins identified in grain are secondary metabolites consisting of a non-polar triterpene aglycone covalently bound to one or more carbohydrate residues. Saponins stimulate the immune system, physical and mental performance, improve digestion, accelerate the body's ability to absorb calcium and silicon, thereby maintaining bone strength, have an antioxidant effect [2].
Amaranth grain can also be a source of 20-hydroxyecdysone (20E). Scientific publications indicate the use of phytoecdysteroids to relieve chronic fatigue syndrome, reduce nervous and muscle fatigue, improve memory and attention processes [3]. The use of 20E in the composition of biologically active food additives and specialized food products for nutrition of athletes is promising.
Amaranth grain proteins, in addition to high biological value, also exhibit biological properties on their own and/or as a source of bioactive peptides. The authors of the study [4] managed to identify 14 different potential bioactive peptides of amaranth proteins with the following properties: ACE inhibitors, DDP-IV inhibitors, antioxidants, glucose uptake stimulators, with antithrombotic, hypotensive, antiamnesic, anxiolytic, antidiabetic and other effects.
Accordingly, there is an increased interest in obtaining amaranth grain extracts enriched with biologically active substances (BAS) by deep processing of raw materials with a further prospect of their use as part of specialized multi-purpose food products.
Currently, the extraction of biologically active substances with the use of solvents such as water, ethanol, methanol or alkaline, acidic, or enzymatic hydrolysis is widely used [1, 5–8]. The main advantages of this approach are simplicity and low cost of the equipment and reagents used. However, the yield of biologically active substances when using these methods is not always high. To increase the degree of extraction of biologically active substances from plant materials, it is possible to use the so-called assisted extraction technologies. Among this class of methodological approaches, membrane filtration [9], ultrasonic [5, 10], and microwave [11] treatments are gaining popularity.
The aim of this work was to evaluate the effectiveness of membrane filtration for the extraction of biologically active substances from amaranth grains.

2 Materials and methods

2.1 Object of the study

Amaranth grain (Russia) was preliminarily ground on a Bosch MUMXL 40G laboratory blender (Robert Bosch Hausgerate GmbH, Germany) and sifted through a sieve with a pore diameter of 0.35 mm.

2.2 Obtaining BAS extracts from amaranth grain

Amaranth grain extract was obtained by enzymatic hydrolysis using a proteolytic enzyme followed by membrane treatment.
A 500 g sample of crushed grain was introduced into an FA-10 fermenter (PROINTECH, Russia) and distilled water was added in a ratio of 1/20. The mixture was stirred for 2 hours at 60°C. Next, the enzyme "Protozyme" (bacterial alkaline protease with an activity of 50,000 U/g) was added in an amount of 5% by weight of a sample of grain (25 g). Hydrolysis was carried out for 3 hours at a temperature of 60°C, the pH of the mixture was maintained in the range of 8.4-8.6 with 1M NaOH. At the end of the hydrolysis process, the pH of the mixture was adjusted to 7.5 with 1 M HCl, then the mixture was centrifuged at 3500 rpm for 30 min. The supernatant was collected and subjected to microfiltration

through a membrane with a pore diameter of 0.2 μm on a micro- and ultrafiltration unit based on an ASF-018 filter holder (Vladisart, Russia), then it was additionally concentrated on a reverse osmosis unit with a URF-1812 membrane roll filter (Vladisart, Russia). The resulting product was frozen and lyophilized.
The protein content in the experimental samples was determined by the Kjeldahl method according to GOST 26889-86. The lipid content in the samples was determined by magnetic resonance relaxometry using an EchoMRI-1100 analyzer (EchoMRI LLC, USA). Humidity was determined using an MJ33 moisture analyzer (Mettler Toledo, USA).

2.3 Quantification of the molecular weight distribution of peptide fractions in the extract

A sample in the amount of 1.5 ml is transferred into a polyethylene tube, centrifuged for 15 minutes at 15,000 rpm, the supernatant is taken, 100 μl of which is injected into a Superose-12 column (1.0 * 30 cm), using 0, 2M NaCl solution with the addition of sodium azide. The elution rate is 0.4 ml/min. The optical density of the eluted extract is recorded using a UV flow detector (UF-132, Khimavtomatika, Russia) at a wavelength of 280 nm. The results of measuring the optical density of the eluted extract solution were processed by the "weight method".

2.4 Determination of the of 20-hydroxyecdysone content

The content of 20E in experimental samples was determined by HPLC-MS/MS on an Agilent 1200 chromatograph (Agilent Technologies, USA) equipped with a Thermo TSQ Quantum Access MAX mass detector (Thermo Fisher Scientific, USA). 20-hydroxyecdysone with the main component content of 98.93% (J&K Scientific, USA) was used as a standard. Data processing was carried out using the built-in Xcalibur software (Thermo Fisher Scientific, USA).

2.5 Determination of total polyphenol content

The content of total polyphenols was determined by the Folin-Ciocalteu method [12]. Gallic acid was used as a standard. The optical density of the samples was measured at 765 nm. In solutions, the concentration of total polyphenols, expressed in mEq of gallic acid, was determined using a calibration curve prepared using a gallic acid standard (97.5%, Sigma, USA).

2.6 Determination of triterpene saponins content

The profile of triterpene saponins was determined by RP HPLC-MS on an Ultimate 3000 liquid chromatography system (Dionex, USA) with a diode array spectrophotometric detector (DAD) and a TSQ Endura triple quadrupole mass spectrometric detector (Thermo Fisher Scientific, USA). Saponins were identified by retention times and mass spectra in accordance with [12].

3 Results

The resulting extract was a fine powder of light brown color. From 1 kg of crushed and sieved grain, an experimental batch of the product with a total weight of 217.0 g was obtained (the yield is 21.7%). The protein content in the original grain was 15.1±0.2%, in

the resulting extract - 44.5±2.2%. The fat content in the original grain is 7.3±0.2%, the fat content in the extract is 0.043±0.004%. The developed technology for obtaining the extract made it possible to almost completely degrease the final product. The moisture content of the obtained extract was 5.7±0.1%.

The chromatogram of the molecular weight distribution of peptide fractions in the composition of the obtained hydrolyzate is shown in Figure 1.

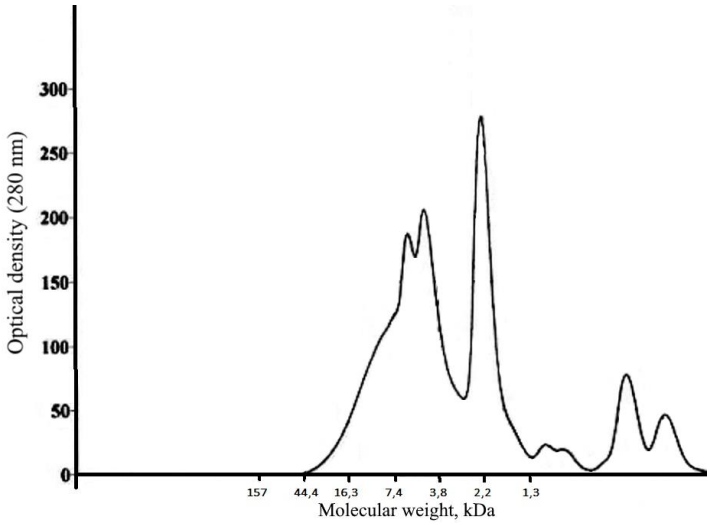

Fig. 1. Chromatogram of the distribution of peptide fractions in the composition of the amaranth grain hydrolyzate

Table 1 shows the molecular weight distribution of peptide fractions in the composition of the BAS extract of amaranth grain.

Table 1. Molecular weight distribution of peptide fractions in the extract

№	Molecular weight range (Δ MW)	Mass fraction, % (280 nm)
1	>157.0	0
2	157.0-44.4	0
3	44.4-16.3	3.0
4	16.3-7.4	16.0
5	7.4-3.8	30.8
6	3.8-2.2	21.4
7	2.2-1.3	13.5
8	<1.3	15.3

The total content of low molecular weight peptide fractions (less than 10 kDa) in the composition of the obtained extract of biologically active substances of amaranth grain was more than 80%. Accordingly, the resulting extract has a potentially low allergenicity. In addition, amaranth grain proteins are sources of biologically active peptides with antioxidant, antihypertensive and hypolipidemic properties. Most of these peptides, according to the literature, have a low molecular weight of up to 170 Da.

The approach used to obtain an extract from amaranth grain made it possible to concentrate triterpene saponins in its composition by 8 times compared to the original

grain. It can be assumed that hydrolysis contributed to the release of protein-bound saponins, and subsequent purification by microfiltration led to additional concentration of saponins in the extract.

The content of total polyphenols in the obtained extract of biologically active substances of amaranth grain was 23.6±1.1 m g-eq. of gallic acid / g of extract.

The content of 20E in the original grain was 18.0 μg/g, while in the composition of the obtained extract it was 538.7 μg/g. The developed method for the preparative preparation of the BAS concentrate of amaranth grain using proteolytic enzymes made it possible to concentrate 20E by almost 30 times compared to the initial raw material.

Figure 2 shows the chromatograms of the 20E content in the original grain and in the extract.

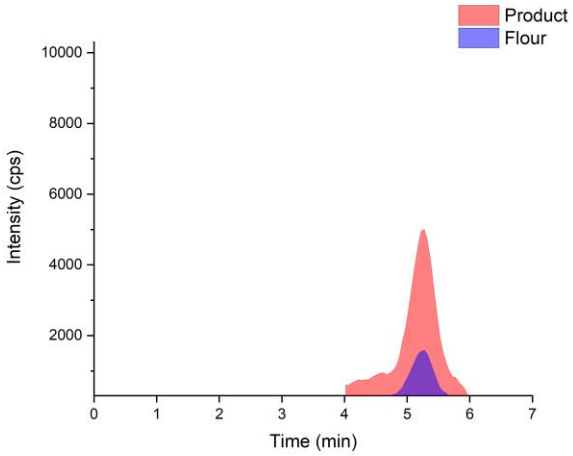

Fig. 2. Chromatograms of the original grain and BAS extract.

Thus, the developed approach made it possible to obtain an extract from amaranth grains - a source of biologically active peptides, 20-hydroxyecdysone and polyphenols (including saponins). In total, triterpene saponins were concentrated in the extract by 8 times, and the phytoecdysteroid 20-hydroxyecdysone was more than 30 times concentrated.

The high content of low molecular weight peptide fractions (more than 80%) in the composition of the extract makes it promising to evaluate its antihypertensive and antidiabetic properties in vivo. The presence of 20-hydroxyecdysone in the extract, an adaptogen with a proven effect, opens up the possibility of its use as a functional food ingredient in specialized food products, including for athletes.

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