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CONTENT OF BOWMAN-BIRK INHIBITOR IN SEEDS OF SOYBEAN VARIETIES OF ODESA SELECTION

Abstract

The article shows that proteolysis (enzymatic hydrolysis of proteins) is a fundamental biological reaction that underlies such biological processes as digestion, reutilization of tissue proteins, immunity, neuroendocrine regulation, inflammation and dystrophy, and in some cases, as defense against pathogens. The most important regulators of proteolysis are inhibitors of proteolytic enzymes, which make them effective factors influencing the state of a significant number of biological processes. There are 2 inhibitors of proteolytic enzymes in soybeans: Kunitz inhibitor (KI), which inhibits trypsin, and Bowman-Birk inhibitor (BBI), which inhibits trypsin, chymotrypsin, elastase, cathepsins, and microbial proteases. Unlike the KI inhibitor, the BBI inhibitor is thermostable and remains in soy products (meal, cake) after defatting. Therefore, it is important to use soy products with a minimum content of BBI in feeding animals. Goal of work. To determine the content of BBI in promising soybean varieties of Odesa selection. Materials and methods. 12 soybean varieties of the Odesa selection were studied (Euridika, Orpheus, Aurora, Pivdenna zorya, Ariadna, Phoenix, Vasylkivska, Taverna, Serenada, Odesitka, Zmina, Zvytyaga). After grinding the beans and defatting the flour with acetone, the inhibitor was extracted with acetate buffer (0.05 M, pH 4.5) and the extract was mixed with three volumes of acetone. After keeping the mixture at a temperature of -16°C for 2 days, the precipitate was centrifuged and dissolved in 0.5% $NaHCO_3$. The content of BBI was determined by the effect on chymotrypsin, the activity of which was assessed by the rate of casein hydrolysis by Kunitz-Levytskyi method. Results. It was established that the content of BBI in the investigated soybean varieties varies from 93.6 μg/g (Euridika) to 368.8 μg/g (Serenada). The soybean varieties Taverna (323.8 μg/g), Vasylkivska (290.9 μg/g), as well as Zmina (282.0 μg/g) and Ariadna (263.0 μg/g) are characterized by a high content of BBI. Conclusion. New soybean varieties of the Odesa selection contain a small amount of BBI and can be used in animal feeding after conventional heat treatment without additional BBI removal.

Key words: soybean varieties, protease inhibitors, Bowman-Birk inhibitor.

Introduction

Proteolysis (enzymatic hydrolysis of proteins) is a fundamental biological reaction that underlies such biological processes as digestion, reutilization of tissue proteins, immunity, neuroendocrine regulation, inflammation and dystrophy, and in some cases, as defense against pathogens.

The most important regulators of proteolysis are inhibitors of proteolytic enzymes, which make them effective factors influencing the state of a significant number of biological processes [1].

It has been established that the largest number of protease inhibitors is found in the seeds of leguminous crops, especially in soybeans, where 2 protease inhibitors have been identified: the first is a Kunitz inhibitor (KI), the second is a Bowman-Birk inhibitor (BBI) [1, 2].

KI has a molecular weight of 21 kDa, inhibits only trypsin, easily denatures when heated [3].

BBI has a molecular weight of 8 kDa, inhibits trypsin, chymotrypsin, elastase, microbial proteases, cathepsins and, unlike KI, does not denature when heated [2]. BBI as a polyvalent (polyfunctional) inhibitor has found its application as a treatment for cancer [4], as well as for the prevention of Alzheimer's disease, AIDS and some others [5-7].

The content of IBB in soybean seeds largely depends on the variety. Thus, according to Indian researchers [8], almost 80% of Indian varieties contain BBI from 5 to 15 mg/g of defatted bean flour, and only 6-7% contain BBI more than 15% mg/g.

Aim of the study

The purpose of our work is to determine the content of BBI in the seeds of soybean varieties of Odesa selection, which are recommended for cultivation in different climatic zones of Ukraine. According to the duration of the growing season, they belong to the early-ripening, mid-early and mid-ripening groups, they differ in the amount of protein and fat content in the seeds, and they react differently to adverse environmental factors.

Materials and methods

Breeding material

The content of BBI in the seeds of such new soybean varieties as Euridika, Orpheus, Aurora, Pivdenna zorya, Ariadna, Phoenix, Vasylkivska, Taverna, Serenada, Odesitka, Zmina, and Zvytyaga was analyzed.

Chemical reagents and enzymes

Crystalline chymotrypsin in vials of 10 mg and packaging of 10 pieces was obtained from “Biopharma” LLC (Ukraine); casein according to Hammersten, trichlo-

roacetic acid, and Folin–Ciocalteu reagent, analytical grade, were from "Khimlaborreaktiv" LLC (Ukraine); sodium hydroxide, sodium carbonate anhydrous, purified grade, as well as disodium hydrogen phosphate, reagent grade, sodium dihydrogen phosphate, analytical grade, and glacial acetic acid, reagent grade, were supplied by "TOR" Chemreactives (Odesa, Ukraine).

Determination of Bowman-Birk inhibitor content in soybean extracts

The modified Kunitz-Levytskyi method of inhibition of the caseinolytic activity of chymotrypsin by an inhibitor was used for the analysis [9].

After preincubation of the inhibitor with chymotrypsin, the residual activity of the enzyme was determined by the hydrolysis of casein in the solution after precipitation of the uncleaved substrate with trichloroacetic acid followed by a colorimetric reaction for phenol-containing amino acids of peptides with the Folin–Ciocalteu reagent, which was calculated in μg of inhibitor per gram of soybean flour.

Reagents

1. 2% casein solution: add 4 g of Hammersten casein powder to 150 ml of 0.2 M phosphate buffer (pH 7.6) with stirring and leave in the refrigerator for 8-10 hours. After that, another 30 ml of distilled water is added and heated in a boiling water bath with stirring for 15 minutes. The solution is cooled, the pH is adjusted to 7.6 and the volume is brought to 200 ml with distilled water. Add 2-3 drops of toluene for preservation and freeze most of it fractionally. Store the working solution at +4°C for a week. In the event of an increase in "zero time" indicators during the analysis of the inhibitor, indicating microbial contamination, the next batch of previously prepared casein substrate is thawed, which, if necessary, is filtered from the sediment that may form.

2. 10% solution of trichloroacetic acid (TCA): 44 ml of a saturated solution of TCA is brought up to 500 ml with distilled water.

3. 5% TCA solution: dilute a 10% TCA solution in half with distilled water.

4. Reagent A: 8 g of NaOH and 40 g of Na_2CO_3 are dissolved in 2 l of distilled water. Store in a plastic container at room temperature.

5. Folin–Ciocalteu reagent: use a proprietary solution and dilute it twice with distilled water before analysis.

6. 0.05 M acetate buffer pH 4.5: dissolve 6 g of ice-cold CH_3COOH in 1.5 l of distilled water, raise the pH to 4.5 by adding $NaHCO_3$ (~ 3.6 g) and bring the final volume to 2 l with distilled water.

Equipment

1. UVmini-1240 spectrophotometer of the company "Shimadzu" (Japan).

2. Water bath BN-06 "Uoslab" of "RIVA-Stal" LLC (Ukraine) with a stand for test tubes, capable of maintaining a temperature of no more than 30 ± 1°C.

3. Automatic pipettes "Pipette" for 20-100 μl, 200-1000 μl and 1000-5000 μl of the company "MEDICA+" (China) with changeable plastic tips.

4. Centrifuge T-24 of the company "VEB MLW Medizintechnik" (Germany) with a bucket rotor and sockets for test tubes with a diameter of 16 mm.

5. pH-Monitor of the company "Pharmacia LKB" (Sweden).

6. Vibrating mixer of the Vortex type "VEVOR KW-3000-1L" of the company "Vevor" (China).

7. Thick-walled glass tubes 16 mm x 100 mm, type TYD1, capable of withstanding centrifugation at 3000 g, purchased from "Starlab" (Ukraine).

Progress of analysis

Chymotrypsin activity is determined at a temperature of +30 ± 1°C.

The test tubes in the tripod are divided into two groups: reference - for the analysis of free hydrolysis of casein by chymotrypsin with control for the initial presence of peptides in the substrate (the so-called "zero time") and experimental - for the analysis of the degree of inhibition of the enzyme by the inhibitor, also with the control of "zero time".

Aliquote of 0.1 ml of the solvent is added to the reference test tubes, and 0.1 ml of the appropriate dilution of the Bowman-Birk inhibitor to the experimental tubes. Then, 0.1 ml of chymotrypsin solution with a concentration of 0.5 mg/ml is carefully added to all test tubes, placed in a water bath, gently mixed in a circular motion, and the preincubation time (10 minutes) is recorded, after which 0.5 ml of 10% TCA is added to all reference "zero time" and experimental "zero time" tubes. Then, after equal time intervals, 0.4 ml of casein solution is added to all test tubes, starting with the first one, and incubated for exactly 15 minutes. After the end of this time of incubation of the enzyme with the substrate and the enzyme + inhibitor mixture with the substrate, with the exception of the "zero time" tubes where TCA had already been added, the reaction is stopped by adding 0.5 ml of 10% TCA at the same time intervals between samples.

All tubes are removed from the thermostated water bath, dried externally and centrifuged at 3000 g for 15 min. The supernatant liquid in the amount of 0.4 ml is carefully taken from each test tube twice and transferred to clean test tubes, where 4 ml of reagent A is then added, mixed on a Vortex, and after 10 minutes, 0.4 ml of diluted Folin–Ciocalteu reagent is added. After 30 minutes, extinction readings are taken on a spectrophotometer at 750 nm in relation to the control for reagents: 0.4 ml of 5% TCA, 4 ml of reagent A and 0.4 ml of Folin–Ciocalteu reagent.

Calculation

$$I = \frac{[(Ect15t - Ect0t) - (Ect+i15t - Ect+i0t)] \cdot 10 \cdot p \cdot V}{K \cdot m \cdot 3}$$

where:

I – quantitative content of the Bowman-Birk inhibitor in μg/g of soybean flour;

Ect15*t* – extinction of the reference sample of chymotrypsin after 15 min of incubation with the casein substrate;

Ect0*t* – extinction of the reference sample of chymotrypsin at 0 min of incubation with the casein substrate;

Ect+i15*t* – extinction of the experimental sample of chymotrypsin and inhibitor after preincubation for 10 min and after 15 min of incubation with the casein substrate;

Ect+i0*t* – extinction of the experimental sample with chymotrypsin and inhibitor after preincubation for 10 min and at 0 min of incubation with casein substrate;
10 – conversion factor for 1 ml of samples introduced into the reaction in the volume of 0.1 ml;
p – sample dilution factor;
V – the volume of 0.05 M acetate buffer pH 4.5 used as an extractant;
K – coefficient of conversion of extinction into micrograms of inhibited chymotrypsin in 0.1 ml of the sample, calculated on the basis of the calibration curve;
m – weight of soybean flour in grams;
3 – a divisor for calculating the amount of Bowman-Birk inhibitor in the sample, the mass of which is 3 times less than the mass of chymotrypsin in the stoichiometric ratio in the enzyme-inhibitor reaction mol per mol.

Plotting a Calibration Curve

Solutions of chymotrypsin for constructing a calibration curve are prepared as follows: 10 mg of chymotrypsin is dissolved in a vial in 2 ml of 0.001 M HCl (pH ~ 2.0) to obtain a stock solution of the enzyme with a concentration of 5 mg/ml, from which, by diluting with distilled water, solutions with a concentration of: 0.1 mg/ml (1:50); 0.2 mg/ml (1:25); 0.5 mg/ml (1:10); and 1 mg/ml (1:5) is prepared. Next, for each dilution of the enzyme, a hydrolysis reaction of a 2% casein solution in a volume of 0.4 ml is carried out in parallel, adding 0.1 ml of the corresponding dilution of chymotrypsin to the sample at +30°C for 15 minutes. The reaction is stopped by adding 0.5 ml of 10% TCA, centrifuged at 3000 g for 15 minutes, and the amount of resulting peptides with phenolic amino acids is determined using the Folin–Ciocalteu reagent. After 30 minutes, spectrophotometer readings are taken at 750 nm. A calibration curve showing the dependence of extinction on enzyme concentration is drawn. The extinction coefficient of chymotrypsin in milligrams for each concentration is calculated using the formula: concentration/extinction of chymotrypsin, the average coefficients for all concentrations are derived and substituted into the above calculation formula.

Extraction of Bowman-Birk inhibitor from soy flour

Soybean seeds were ground on the LZMK-1 laboratory grain mill of the FOP "A.I. Klyuchkin" (Ukraine), the flour was sifted through a 100-mesh sieve with a particle size of about 0.15 mm.

Weights of flour of different varieties of soybeans in the amount of 1 gram were placed in thick-walled glass tubes 16 mm x 100 mm, defatted with 10 ml of acetone with periodic shaking during the day at room temperature, centrifuged at 2500 g for 10 min and dried in a thermostat at 70°C within 2-3 hours. Then 4 ml of 0.05 M acetate buffer pH 4.5 was poured into each test tube, closed with rubber stoppers with layers of plastic food film, mixed on a Vortex mixer periodically for 30 seconds during 30 minutes and then kept for 10 minutes in a boiling water bath. After cooling to room temperature, centrifugation was carried out at 2500 g for 10 min, and supernatants in a volume of 1 ml were transferred to other 10 ml centrifuge tubes with a narrow bottom. 3 ml of acetone was added to each test tube, mixed and placed in the freezer of the refrigerator for 12 hours. The next day, the precipitates formed were separated by centrifugation at 1000 g for 10 min, dried in a thermostat at 70°C for 1 hour and suspended-dissolved in 1 ml of 0.5% $NaHCO_3$. These solutions were used to determine the content of the Bowman-Birk inhibitor in soybean flour according to the method described above.

Research results

The table 1 shows the results of determining the content of BBI in the defatted flour of the investigated soybean varieties. Despite the significant variation in indicators for the determination of the Bowman-Birk inhibitor in the samples, the highest amount was found in Taverna and Serenada varieties.

Table 1 – Bowman-Birk inhibitor content in soybean seeds of Odesa selection

№	Name of soybean variety	Mass of BBI inhibitor (M±m), μg/g flour
1.	Euridika	93,6 ± 32,6
2.	Orpheus	133,2 ± 43,4
3.	Aurora	116,5 ± 19,6
4.	Pivdenna zorya	121,1 ± 61,8
5.	Ariadna	263,0 ± 100,3
6.	Phoenix	164,4 ± 70,2
7.	Vasylkivska	290,9 ± 88,6
8.	Taverna	323,8 ± 180,6
9.	Serenada	368,8 ± 112,6
10.	Odesitka	169,9 ± 107,1
11.	Zmina	282,0 ± 113,2
12.	Zvytyaga	207,7 ± 83,4

The lowest content of the Bauman-Birk inhibitor was found in Eurydice soybeans (93.6 ± 32.6 μg/g).

In soybean varieties: Aurora, Southern Star, Orpheus, Phoenix, Odesytka, this indicator is 1.2-1.8 times higher and ranges from 116.5 ± 19.6 to 169.9 ± 107.1 μg/g.

In soybean varieties Zvytyaga and Ariadna, the studied indicator is 2, 2, and 2.8 times higher, respectively, than in the Eurydice variety.

The varieties Zvytyaga, Zmena, Taverna, Serenada are distinguished by an even higher content of the Bauman-Birk inhibitor, the indicator ranges from 207.7 ± 83.4 to 368.8 ± 112.6 μg/g, which in 3.0 – 3, 9 times higher than the content of Eurydice soybeans.

Conclusion

Soybean varieties of the Odesa selection belong to the group of low-inhibitors in terms of BBI content, which can contribute to their more effective assimilation in the animal body.

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ВМІСТ ІНГІБІТОРА БАУМАНА-БІРК В НАСІННІ СОРТІВ СОЇ ОДЕСЬКОЇ СЕЛЕКЦІЇ

Анотація

В матеріалах статті показано, що протеоліз (ферментативний гідроліз протеїнів) є фундаментальна біологічна реакція, яка лежить в основі таких біологічних процесів як перетравлення, реутилізація тканинних білків, імунітет, нейроендокринна регуляція, запалення та дистрофія, а в деяких випадках, і як захист від патогенів. Найважливішими регуляторами протеолізу є інгібітори протеолітичних ферментів, що робить їх ефективними чинниками впливу на стан значної кількості біологічних процесів. У соєвих бобах існує 2 інгібітори протеолітичних ферментів: інгібітор Кунітца (ІК), який інгібує трипсин, і інгібітор Баумана-Бірк (ІББ), інгібуючий трипсин, хімотрипсин, еластазу, катепсини і мікробні протеази. На відміну від інгібітора ІК, інгібітор ІББ термостабільний і залишається в соєвих продуктах (шрот, жмих) після їх знежирення. Тому важливо використовувати у годуванні тварин соєві продукти з мінімальним вмістом ІББ. Мета роботи - Визначити вміст ІББ у перспективних сортах сої Одеської селекції. У якості дослідних матеріалів було досліджено 12 сортів сої Одеської селекції (Евридіка, Орфей, Аврора, Південна зоря, Аріадна, Фенікс, Васильківська, Таверна, Серенада, Одеситка, Зміна, Звитяга). Після механічного подрібнення і знежирення за допомогою ацетону інгібітор екстрагували ацетатним буфером (0,05 М, pH 4,5) і змішували екстракт з трьома об'ємами ацетону. Після витримки суміші при температурі -16°С протягом 2 днів осад відцентрифугували і розчиняли в 0,5% $NaHCO_3$. Вміст ІББ визначали за дією на хімотрипсин, активність якого оцінювали за швидкістю гідролізу казеїну методом Кунітца-Левицького. Результати. Встановлено, що вміст ІББ у досліджених сортах сої варіює від 93,6 мкг/г (сорт Евридіка) до 368,8 мкг/г (сорт Серенада). Високим вмістом ІББ характеризуються сорти сої Таверна (323,8 мкг/г), Васильківська (290,9 мкг/г), а також Зміна (282,0 мкг/г) та Аріадна (263,0 мкг/г). На основі отриманих даних встановлено, що нові сорти сої Одеської селекції місять незначну кількість інгібіторів Баумана-Бірка і можуть використовуватися у годівлі тварин після звичайної теплової обробки без додаткового їх вилучення.

Ключові слова: сорти сої, інгібітори протеаз, інгібітор Баумана-Бірк.

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