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RESEARCH ARTICLE

Enzymatic deglycosylation of soy proteins as a method to increase the efficiency of their hydrolysis

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Abstract

Objectives. Soy protein hydrolysates are now widely used in the food industry, fish farming, poultry farming, livestock farming, as well as in medical preparations. The most effective method for their production is enzymatic hydrolysis. However, even with optimal proteolysis parameters, it is not always possible to achieve the required degree of hydrolysis. For this reason, various technological approaches are used to more intensively break down soy proteins, including the addition of enzyme preparations and pretreatment of the protein substrate. β-Conglycinin, one of the main soy proteins, is a glycoprotein whose carbohydrate portion consists primarily of mannose residues. We hypothesize that deglycosylation of β-conglycinin by an enzyme preparation with mannanase activity as a pretreatment of the soy substrate will lead to change in the structure of its protein portion due to the destruction of the carbohydrate component to increase the accessibility of peptide bonds to proteolytic enzymes. Thus, the work sets out to study the effect of enzymatic deglycosylation on the efficiency of soy protein hydrolysis.

Methods. Deglycosylation of β-conglycinin, hydrolysis of polysaccharides and lipids were performed by the Complex-concentrate enzyme preparation (*Ferment*, Republic of Belarus). Protein hydrolysis was carried out by the Protozyme C330 enzyme preparation (*Ferment*, Republic of Belarus). The formation of reducing sugars was confirmed by the Miller method. The degree of protein hydrolysis was determined by the pH-stat method. The molecular weight distribution of peptide fractions was analyzed by low-pressure liquid gel chromatography on a column with Sephadex® G-50 Medium. Computer processing of the elution profile of peptide fractions was performed in the OriginPro 8.5.1 program using the Gauss function.

Results. It is established that the treatment of soy flour by the Complex-concentrate enzyme preparation (enzyme-substrate ratio 1 : 40, hydromodule 1 : 10) promotes the breakdown of both free oligo- and polysaccharides, as well as the carbohydrate component β-conglycinin. Proteolysis by the Protozyme C330 enzyme preparation (enzyme-substrate ratio 1 : 20, pH 7.5, 50°C, 3.5 h) carried out following 20 h of deglycosylation results in a product with a degree of hydrolysis of 56.3%. The content of low-molecular-weight peptides in soy hydrolysate is 83.9%. Proteolysis without enzymatic destruction of the carbohydrate part of β-conglycinin is shown to be characterized by a degree of hydrolysis of 9.2%.

Conclusions. A pretreatment approach involving deglycosylation of enzymatic β-conglycinin can be used to significantly increase the degree of hydrolysis of soy proteins.

Keywords

soy flour, enzyme preparation with mannanase activity, deglycosylation of β-conglycinin, proteolysis, degree of hydrolysis, low-molecular-weight peptides, gel chromatography

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НАУЧНАЯ СТАТЬЯ

Ферментативное дегликозилирование соевых белков как способ повышения эффективности их гидролиза

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Аннотация

Цели. В настоящее время гидролизаты соевых белков находят широкое применение в пищевой промышленности, медицине, рыбоводстве, птицеводстве и животноводстве. Наиболее эффективным способом их получения является ферментативный гидролиз. Однако даже при оптимальных параметрах протеолиза не всегда возможно достичь требуемой степени гидролиза, поэтому для более интенсивного расщепления соевых белков используют различные технологические подходы: внесение нескольких ферментных препаратов и предварительную обработку белкового субстрата. β-Конглицинин — один из основных белков сои — представляет собой гликопротеин, углеводная часть которого состоит преимущественно из маннозных остатков. Предполагаем, что дегликозилирование β-конглицинина ферментным препаратом с маннаназной активностью в качестве предварительной обработки соевого субстрата приведет к изменению структуры его белковой части за счет разрушения углеводного компонента и позволит повысить доступность пептидных связей к действию протеолитических ферментов. Таким образом, целью работы является изучение влияния ферментативного дегликозилирования на эффективность гидролиза соевых белков.

Методы. Дегликозилирование β-конглицинина, гидролиз полисахаридов и липидов проводили ферментным препаратом «Комплекс-концентрат» (*ООО «Фермент»*, Республика Беларусь). Гидролиз белков осуществляли ферментным препаратом «Протозим С330» (*ООО «Фермент»*, Республика Беларусь). Образование редуцирующих сахаров подтверждали методом Миллера. Степень гидролиза белков определяли pH-статическим методом. Молекулярно-массовое распределение пептидных фракций анализировали методом жидкостной гель-хроматографии низкого давления на колонке с гелем Sephadex® G-50 Medium. Компьютерную обработку профиля элюирования пептидных фракций выполняли в программе OriginPro 8.5.1 с помощью функции Гаусса.

Результаты. Установлено, что обработка соевой муки ферментным препаратом «Комплекс-концентрат» (фермент-субстратное соотношение 1 : 40, гидромодуль 1 : 10) способствует расщеплению как свободных олиго- и полисахаридов, так и углеводного компонента β-конглицинина. Протеолиз ферментным препаратом «Протозим С330» (фермент-субстратное соотношение 1 : 20, pH 7.5, 50°C, 3.5 ч) после 20-часового дегликозилирования приводит к получению продукта со степенью гидролиза 56.3%. При этом содержание низкомолекулярных пептидов в соевом гидролизате составляет 83.9%. Показано, что протеолиз без ферментативного разрушения углеводной части β-конглицинина характеризуется степенью гидролиза 9.2%.

Выводы. Применение ферментативного дегликозилирования β-конглицинина в качестве предварительной обработки позволяет существенно повысить степень гидролиза соевых белков.

Ключевые слова

соевая мука, ферментный препарат с маннаназной активностью, дегликозилирование β-конглицинина, протеолиз, степень гидролиза, низкомолекулярные пептиды, гель-хроматография

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INTRODUCTION

At present, soy protein hydrolysates possessing antioxidant, hypotensive, immunomodulatory, antimicrobial, anti-inflammatory, and other properties are widely used in the food industry [1], medicine [2], fish farming [3], poultry farming [4], and livestock farming [5]. Soy proteins, which are comparable in biological value to meat and milk proteins, are rich in essential amino acids, especially lysine, which is often limited in other plant proteins. About 70% of soy proteins are represented by the storage proteins glycinin and β-conglycinin. Glycinin consists of a basic polypeptide B having a molecular mass of about 20 kDa along with an acidic polypeptide A whose molecular mass is about 38 kDa, which are linked by a disulfide bond to form an individual AB subunit. The quaternary structure of glycinin depends on pH and ionic strength of the solutions. At moderate temperatures and neutral pH values, glycinin forms hexameric complexes having a molecular mass ranging from 320 to 375 kDa, composed of heterogeneous subunits. Each hexamer contains about 2 free SH groups and 18–20 disulfide bonds. β-Conglycinin, having a molecular mass of 150–180 kDa, is a trimeric glycoprotein consisting of 3 subunits: α (72–76 kDa), α′ (68–72 kDa), and β (52–53 kDa), which form seven isomers. The subunits, which are linked mainly by hydrophobic interactions or hydrogen bonds, contain no free SH groups [6]. The carbohydrate moiety of one β-conglycinin molecule consists of 38 mannose and 12 glucosamine residues [7].

The most effective method for obtaining protein hydrolysates is enzymatic hydrolysis, which allows for selective cleavage of peptide bonds due to the specificity of proteolytic enzymes. Table 1 presents the parameters of soy protein hydrolysis using some enzyme preparations [8–13]. To achieve more intensive protein breakdown, a two-stage hydrolysis process is employed. For instance, the study [9] demonstrated that sequential application of alcalase and flavourzyme to effect a twofold increase in the degree of hydrolysis of soy protein isolate as compared to a single-stage proteolysis process using alcalase alone. Furthermore, preliminary treatment of the protein substrate significantly enhances the efficiency of enzymatic hydrolysis [14–16] (Table 2).

The authors of the study [17] demonstrated that the carbohydrate component of β-conglycinin, which exhibits pronounced IgE reactivity, is responsible for the allergenic properties of soy proteins. At the same time, it has been established [18] that deglycosylation of β-conglycinin by peptide-*N*-glycosidase F not only reduces its antigenicity compared to the native glycoprotein but also leads to significant changes in the secondary and tertiary structure of the protein moiety. Based on this, we hypothesize that the use of enzymatic deglycosylation as a pretreatment of the soy substrate will destroy the carbohydrate part of β-conglycinin to increase the susceptibility of peptide bonds to the action of proteolytic enzymes. It should be noted that the proposed approach of preliminary deglycosylation of soy proteins to increase the yield of low-molecular-weight peptides has not been described in the literature.

Thus, the aim of the present work is to investigate the influence of enzymatic deglycosylation on the efficiency of soy protein hydrolysis.

Table 1. Parameters of enzymatic hydrolysis of soy proteins

Substrate	Enzyme preparation	Hydrolysis conditions				Degree of hydrolysis		Reference
		E : S ratio**	pH	*t*, °C	τ, h	%	Method for determining	
One-step process								
Soy protein isolate*	Pepsin	1 : 20	1.6	39	5	60.5	Formol titration method	[8]
	Trypsin	1 : 30	7.8	39	5	55.7		
Soy protein concentrate*	Alcalase	4%	8.0	55	3	14.5	pH-stat method and 2,4,6-trinitrobenzenesulfonic acid method	[9]
	Papain	4%	8.0	50	1.5	14.5		
	Neutrase	4%	7.0	45	1.25	7.2		

Table 1. Continued

Substrate	Enzyme preparation	Hydrolysis conditions				Degree of hydrolysis		Reference
		E : S ratio**	pH	*t*, °C	τ, h	%	Method for determining	
Soy protein isolate	Alcalase	1%	8.0	50	3	8.0	*o*-Phthalaldehyde method	[10]
	Flavourzyme	1%	7.0	50	3	10.0		
Soy protein isolate	Flavourzyme	8%	7.0	55	4	57.0	Biuret method	[11]
Soy flour*	Alcalase	–	8.0	40	8	35.1	Ninhydrin method	[12]
	Flavourzyme	–	8.0	40	8	39.5		
	Novozym	–	8.0	40	8	33.3		
Two-step process								
Soy protein isolate	Pepsin	1 : 20	1.6	39	8	88.2	Formol titration method	[8]
	Trypsin	1 : 20	7.8					
Soy protein concentrate	Alcalase	4%	7.0	50	2.5	30.0	pH-stat method and 2,4,6-trinitrobenzenesulfonic acid method	[9]
	Flavourzyme	4%						
	Neutrase	4%	7.0	50	2.75	14.0		
	Flavourzyme	4%						

* Soy flour contains 50% protein on a dry matter basis, soy protein concentrate contains more than 65% protein, soy protein isolate contains more than 90% protein [13].

** E : S ratio is the enzyme-to-substrate ratio.

Table 2. Effect of pretreatment of soy protein isolate on the degree of hydrolysis

Pretreatment	Enzyme preparation	Hydrolysis conditions				Degree of hydrolysis		Reference
		E : S ratio	pH	*t*, °C	τ, h	%	Method for determining	
–	Papain	0.5%	7.0	55	3	0.9	pH-stat method	[14]
Ultrasound pretreatment 600 W						1.86		
–	Pancreatin	2%	7.0	55	3	9.6	pH-stat method	[15]
Extrusion pretreatment						16.4		
–	Bromelain	1 : 20	7.0	55	3	13.7	Formol titration method	[16]
Thermal pretreatment 90°C, 10 min						16.9		
High-pressure homogenization 30 MPa						23.5		

MATERIALS AND METHODS

The following materials were used in this work: defatted soy flour (protein content, 48%; fat, 1%; carbohydrates, 35%; *Irkutsk Maslozhirkombinat*, Russia); the Protozyme C330 enzyme preparation based on a neutral protease (≥250 units/g, 30–50°C, pH 6.0–7.5; *Ferment*, Republic of Belarus); an enzyme preparation based on an alkaline protease (204324 units/g, 40–50°C, pH 9.0–11.0; *Beijing Donghua Qiangsheng Biotechnology Co.*, China); the Complex-concentrate enzyme preparation, possessing cellulase (1452 units/g), xylanase (21256 units/g), β-glucanase (16415 units/g), pectinase (2152 units/g), phytase (1550 units/g), mannanase (10586 units/g), and lipase (1080 units/g) activity (*Ferment*, Republic of Belarus).

Deglycosylation of β-conglycinin, hydrolysis of polysaccharides and lipids were carried out using the Complex-concentrate enzyme preparation at an enzyme-to-substrate (E : S) ratio of 1 : 40 and a hydromodule of soy flour : purified water (1 : 10) for 20 h. Protein hydrolysis was performed using a proteolytic enzyme preparation at an E : S of 1 : 20 and pH 7.5. Upon completion of proteolysis, the enzyme was inactivated at a temperature of 100°C for 10 min.

The degree of protein hydrolysis was determined by the pH-stat method [19]. The pH value of the reaction medium was controlled using an HI 83141 pH meter (*Hanna*, Germany). The constancy of pH was maintained by adding a 1 M sodium hydroxide solution.

The degree of hydrolysis (*DH*, %) of proteins was calculated using the formula:

$$DH = V \cdot N \cdot \frac{1}{\alpha} \cdot \frac{1}{m} \cdot \frac{1}{h} \cdot 100, \quad (1)$$

where V is the volume of sodium hydroxide solution added during hydrolysis, mL; N is the molar concentration of sodium hydroxide, mol/L; m is the mass of protein, g; h is the number of peptide bonds in 1 g of soy proteins, $h = 7.8$ mmol/g; α is the degree of dissociation of α-amino groups at the hydrolysis pH:

$$\alpha = \frac{10^{(\mathrm{pH-pK})}}{1+10^{(\mathrm{pH-pK})}}. \quad (2)$$

Here, the pK of the formed α-amino groups depends on the hydrolysis temperature T, in Kelvin, as follows:

$$\mathrm{pK} = 7.8 + \frac{(298 - T)}{(298 \cdot T)} \cdot 2400. \quad (3)$$

Reducing sugars were determined according to the Miller method [20] with some modifications. The essence of the method is that upon interaction of reducing sugars with 3,5-dinitrosalicylic acid, the latter is reduced to 3-amino-5-nitrosalicylic acid, which has a yellow-orange color.

To 0.5 mL of the carbohydrate hydrolysate in a test tube, 1.5 mL of dinitrosalicylic acid reagent and 1.0 mL of distilled water were added. After closing the test tube with a cotton plug and placing it in a boiling water bath for 15 min, the tube was cooled to 20°C and 0.5 mL of a 40% solution of potassium sodium tartrate (Rochelle salt) was added to stabilize the color. The optical density of the solution was measured at 582 nm using a Specord 200 Plus spectrophotometer (*Analytik Jena*, Germany) against a control sample. According to the Beer–Lambert–Bouguer law, the optical density is proportional to the concentration of reducing sugars. To prepare the dinitrosalicylic reagent, 1 g of 3,5-dinitrosalicylic acid, 1 g of sodium hydroxide, 0.05 g of sodium sulfate, and 0.2 g of phenol were weighed and dissolved in distilled water with continuous stirring, bringing the solution volume to 100 mL.

The molecular weight distribution of peptide fractions was analyzed by low-pressure liquid gel chromatography in a 1.8 × 35 cm glass column packed with Sephadex® G-50 Medium gel (separation range 1.5–30 kDa; *Pharmacia Fine Chemicals*, Sweden) and pre-calibrated using standard substances (Fig. 1): trypsin (24 kDa; *Sigma-Aldrich*, USA), cytochrome C (12.3 kDa; *Serva Fein Biochemica*, Germany), vitamin B_{12} (1.36 kDa; *Sigma-Aldrich*, USA). The void volume of the column was determined at 32.5 ± 0.1 mL by the elution volume of blue dextran 2000 (2000 kDa; *Sigma-Aldrich*, USA). Prior to loading onto the column, the hydrolysate solution was filtered through a membrane filter (pore size 0.2 μm; *Agilent Technologies*, USA). A 0.025 M Tris-HCl buffer solution (pH 8.0) was used as the eluent. Detection was performed at 280 nm.

Graphs were plotted using Microsoft Office Excel 2010 software. Computer processing of the elution profile of peptide fractions was performed in OriginPro 8.5.1 software using the Gaussian function.

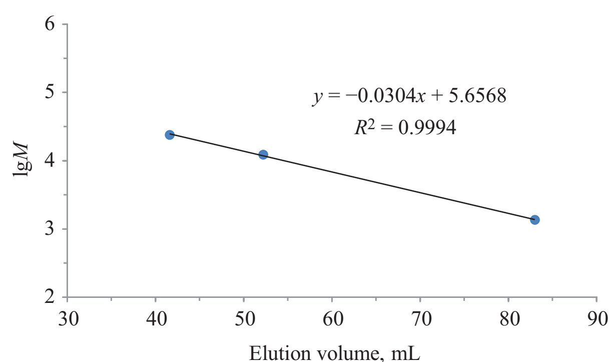

Fig. 1. Calibration curve for a column with Sephadex® G-50 Medium. *M* is molecular weight

RESULTS AND DISCUSSION

Selection of a proteolytic enzyme preparation. Protein hydrolysis was carried out with a proteolytic enzyme preparation at 40°C following preliminary deglycosylation of β-conglycinin at the same temperature. As seen from Fig. 2, when using the Protozyme C330 enzyme preparation, significant formation of proteolysis products (*DH* = 55.5%) occurs within 5.5 h, while the maximum degree of hydrolysis (*DH* = 43.3%) is achieved in 5 h with the addition of the alkaline protease-based enzyme preparation. For further research, the Protozyme C330 enzyme preparation was selected.

Influence of temperature. Protein hydrolysis was carried out using the Protozyme C330 enzyme preparation at temperatures of 40 and 50°C (the temperature range of highest catalytic activity for the neutral protease) following preliminary cleavage of the carbohydrate component of the glycoprotein at the same temperatures. As can be seen from Fig. 3, proteolysis at 40°C yields a product having a degree of hydrolysis of 55.5% over 5.5 h, while proteolysis at 50°C yields a product with the same degree of hydrolysis (*DH* = 56.3%) in 3.5 h. The results obtained indicate that the preferred temperature for protein hydrolysis is 50°C.

Influence of preliminary cleavage of the carbohydrate component of the glycoprotein. Enzymatic deglycosylation of β-conglycinin and hydrolysis of polysaccharides were carried out at 50°C. The kinetic curve of reducing sugar formation is presented in Fig. 4. Since soy flour initially contains reducing sugars (glucose, fructose) [21], their concentration on the kinetic curve is non-zero at the initial time point. The 2.5-fold increase in the concentration of reducing sugars observed 15 min after the addition of the Complex-concentrate enzyme preparation to the soy flour suspension is due to the hydrolysis of free oligo- and polysaccharides. The subsequent increase in the concentration of reducing sugars can be attributed to the slow cleavage of the carbohydrate part of the glycoprotein.

Protein hydrolysis was carried out using the Protozyme C330 enzyme preparation at 50°C both with and without preliminary deglycosylation of β-conglycinin at the same temperature. The destruction of the carbohydrate component of the glycoprotein was found to contribute to a 6-fold increase in the degree of protein hydrolysis (*DH* = 56.3% in 3.5 h) as compared to proteolysis without pretreatment (*DH* = 9.2% in 3.5 h) (Fig. 5).

Analysis of the molecular weight distribution of peptide fractions after proteolysis with preliminary deglycosylation of β-conglycinin. Analysis of the elution profile of peptide fractions (Fig. 6) showed that the soy hydrolysate contains 83.9% of peptides with a molecular weight ≤6 kDa, which potentially possess biological activity [22, 23]. Furthermore, the

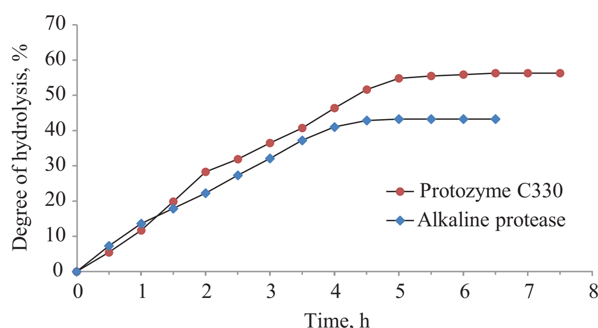

Fig. 2. Effect of proteolytic enzymes on the degree of protein hydrolysis

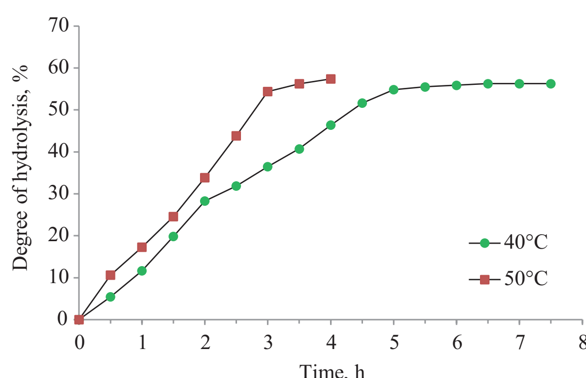

Fig. 3. Effect of temperature on the degree of protein hydrolysis by the Protozyme C330 enzyme preparation

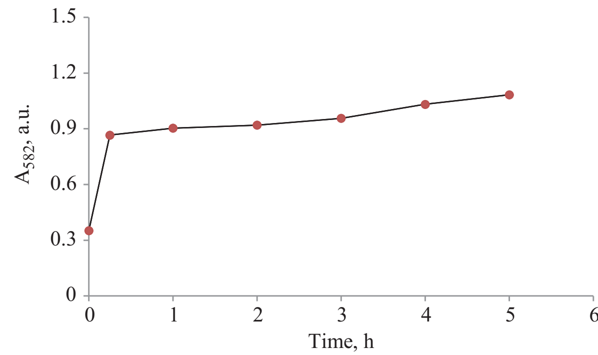

Fig. 4. Kinetic curve of reducing sugars formation

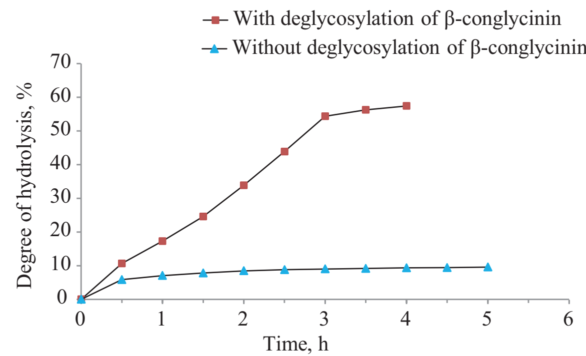

Fig. 5. Effect of preliminary cleavage of the carbohydrate part of β-conglycinin on the degree of protein hydrolysis by the Protozyme C330 enzyme preparation

peptide fraction with a molecular weight ≤1.0 kDa is predominant (58.6%), which is consistent with the degree of hydrolysis (DH = 56.3%). Polypeptides with a molecular weight >32 kDa and unhydrolyzed proteins elute in the void volume of the column (Table 3).

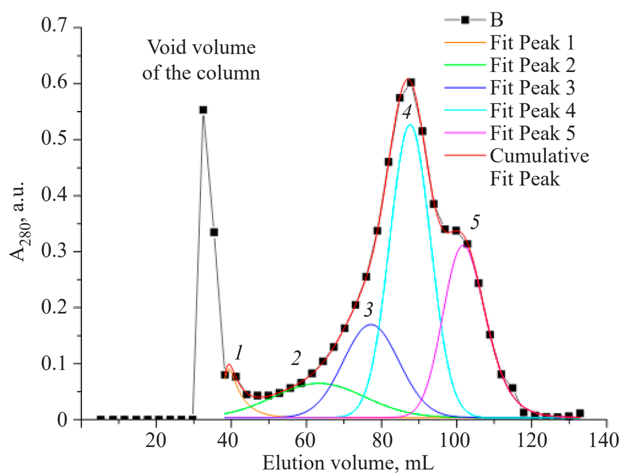

Fig. 6. Decomposition of peaks by Gaussian curves in the elution profile of peptide fractions

Table 3. Characterization of fractions in soy hydrolysate

Name of peak	Peak area	Elution volume, mL	Molecular weight of fraction, kDa	Fraction content, %
Void volume of the column	2.7	32.5	>32.0	13.6
1	0.5	38.0	31.7	2.5
2	1.9	61.5	6.1	9.6
3	3.1	75.6	2.3	15.7
4	7.1	87.5	1.0	35.9
5	4.5	98.8	0.5	22.7

CONCLUSIONS

The conducted research established that the application of enzymatic deglycosylation as a pretreatment of soy flour can be used to significantly increase the degree of protein hydrolysis. It is likely that the cleavage of the

carbohydrate moiety of β-conglycinin leads to a loss of conformational stability in the protein molecule to increase the accessibility of peptide bonds to the action of the proteolytic enzyme. Optimal conditions for the hydrolysis of soy proteins using a neutral protease-based enzyme preparation were determined. The developed approach of preliminary deglycosylation of β-conglycinin can be used to increase the yield of low-molecular-weight peptides in the production of soy hydrolysates.

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Authors' contributions

V.N. Leontiev — planning experiments, discussing results, writing the text of the manuscript.

O.I. Lazovskaya — carrying out experiments, processing and discussing results, writing the text of the manuscript.

The authors declare no conflicts of interest.

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