

**EFFECT OF ENZYMOLYSIS ON THE ANTIOXIDANT ACTIVITY AND
FUNCTIONAL PROPERTIES OF THE SOLUBLE SOY PROTEINS**

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Abstract

Since multiple functionality of the protein derivatives is widely needed, identifying tools for modulating it is highly desired. The aim of the present study was to test the influence of three different endopeptidases, namely bromelain, Neutrase and trypsin, on the antioxidant and functional properties of the soy proteins. The soluble proteins content of the obtained hydrolysates varied with the hydrolysis degree, which ranged between 1.85% (in case of trypsin) and 10.27% (in case of bromelain). The resulting peptide mixtures exhibited higher antioxidant activity values compared to the native soluble proteins. Both DPPH and ABTS-based methods indicated that the sample hydrolysed with Neutrase was the most bioactive, whereas the sample prepared with trypsin exhibited the lowest antioxidant activity. The bioinformatics tools revealed that mainly the hydrophobic di- and tripeptides were responsible for the increased antioxidant activity of the hydrolysates. The impact of enzyme assisted hydrolysis on the functional properties was estimated by determining the foaming and emulsion forming properties of the hydrolysates. The best foaming properties were registered for the hydrolysate obtained with Neutrase (the overrun was by 25-50% higher compared to the native proteins). All tested emulsions exhibited predominant viscous like behaviour. The results indicated that soy proteins hydrolysis with bromelain, Neutrase and trypsin could be exploited for the knowledge-based improvement of the antioxidant activity or for modulating the functional properties of the protein derivatives.

Keywords: soy proteins, enzyme assisted hydrolysis, antioxidant activity, functional properties, rheological behavior

Introduction

Soy is one of the most widely cultivated plant around the world, representing an essential source of high-quality protein as it contains all the indispensable amino acids necessary for human body (Haile Ma *et al.*, 2023). On the other hand, the complexity of soy proteins structure limits their applications for food formulations. The compact globular structure of soy proteins leads to thermal instability, molecular inflexibility and reduced functional properties (Haile Ma *et al.*, 2023). Enzymatic hydrolysis is used frequently to improve the physicochemical, nutritional, functional and sensorial properties of proteins. Most of the physiological and functional properties of proteins are associated to biologically active peptides encrypted in the parental protein sequence (Coscueta *et al.*, 2019). Performed under controlled conditions, enzymatic hydrolysis promotes the production of peptide hydrolysates with specific bioactivity, or peptides with multifunctional properties. Many reports suggested the positive influence of enzymatic hydrolysis on generating compounds with health promoting benefits from different protein sources (Thamnarathip *et al.*, 2016). The soy protein hydrolysates were found to exert positive effects on health like antioxidant, antihypertensive, hypocholesterolemic properties, prevention of osteoporosis or reduction of the incidence of stomach, colorectal and breast cancer (Thamnarathip *et al.*, 2016; Coscueta *et al.*, 2019). These properties are closely connected with the degree of hydrolysis, amino acid composition and molecular size distribution. The antioxidant capacity represents one of the most important bioactivities, due to peptides potential of eliminating the free radicals, making them very attractive to be used as natural antioxidants. Previous studies showed that protein hydrolysates containing peptides with molecular weight lower than 10 kDa exhibited increased antioxidant activity and increased solubility compared to the native protein hydrolysate (Halim *et al.*, 2018).

An important element to be considered when aiming to obtain superior functionality is the source of enzymes used for hydrolysis. The protein hydrolysate preparation can be performed with enzymes of animal (trypsin, pepsin, chymotrypsin etc), vegetal (papain, bromelain, ficin) and microbial (Alcalase, Neutrase, Chymosin, Flavourzyme, Protamex) origin (Du and Li, 2022). Depending on the type of the enzyme used, the hydrolysis of soy proteins could increase the number of ionizable groups and the exposure of hydrophobic groups, reduce the molecular size and improve the emulsification capability and emulsion-stabilizing ability, water absorption, foaming, solubility and gelation (He *et al.*, 2023; Islam *et al.*, 2023). Limited enzymatic hydrolysis by proteases improves the foaming capacity of soy proteins but reduces foam stability, while a high level of degree of hydrolysis increases the solubility, but reduces the foam ability (Sun *et al.*, 2011). Recently, Knežević-Jugović *et al.* (2023) tested the hydrolysis capacity of several commercial food-grade proteases (Alcalase, Neutrase, papain, Everlase, Umamizyme, Flavourzyme) on soy proteins concentrate, with the aim to obtain ingredients with improved functionality. The authors showed that the functionality was dependent on enzyme type, the hydrolysates obtained by using a combination of Neutrase and Flavourzyme presented the highest antioxidant activity and essential amino acid

content. In this context, the aim of the study was to modulate the bioactivity and functional properties of the soluble soybean proteins by means of limited hydrolysis assisted by different endopeptidases.

Materials and methods

Materials

The soy protein isolate (Supro 430IP, 84.69±0.35% protein and 9.1% moisture) was supplied by KUK (Romania). Three different enzymes were used to prepare the soy protein hydrolysates: bromelain (Carl Roth, Karlsruhe, Germany), Neutrase 5.0 BG (Novo Nordisk, Bagsværd, Denmark) and trypsin (Merck, Darmstadt, Germany). Sunflower oil (Spornic, Prutul SA, Galati, Romania) was used to prepare the emulsions.

The reagents dithiothreitol (DTT), L-serine, o-phthalaldehyde (OPA), sodium dodecyl sulphate (SDS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were supplied by Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). All other chemical and solvents were of analytical grade.

Chemical characterization of the protein and hydrolysate samples

The crude protein content of the soy protein isolate subjected to enzyme assisted hydrolysis was assessed using the Dumas method and a conversion factor of 6.25. The content of soluble proteins was determined by Lowry method (Lowry *et al.*, 1951). The moisture content was determined through the air-oven method (AACC 44-15.02 method, 1999).

Obtaining the protein hydrolysate

Protein suspensions of 12% (w/v) concentration were first prepared by well mixing over 4 h at room temperature of 22±1°C on a magnetic stirrer, such as to ensure the complete hydration. The protein hydrolysis was initiated by adding the following enzymes: bromelain (0.5 g/100g proteins), Neutrase (1 g/100 g proteins) and trypsin (1 g/100 g proteins). The hydrolysis was carried out at 50°C while continuously shaking the samples at 100 rpm, by using an orbital shaker (SI300R; Jeio Tech, Oxfordshire, UK). After 68h of hydrolysis the pH was adjusted to 7.0, and afterwards the enzyme reaction was immediately stopped by heat treatment at 90°C for 5 minutes. Upon cooling to room temperature, the soluble peptides were separated through centrifugation for 10 min at 14,000 rpm, and the supernatants were subjected to freeze-drying (CHRIST Alpha 1–4 LD plus, Osterode am Harz, Germany). The soluble peptide fractions were further used for characterization. The soy protein hydrolysates prepared with bromelain, Neutrase and trypsin were coded SPB, SPN and SPT, respectively. The Control sample was prepared following the same procedure, except for the enzyme addition step.

Evaluation of the degree of hydrolysis

The method proposed by Nielsen et al. (2001) was used to determine the soy proteins degree of hydrolysis. An UV-Vis spectrophotometer (Libra S22, Biochrom, Cambridge, UK) was used to measure the absorbance, at wavelength of 340 nm, of the coloured compounds resulting from the specific reaction occurring between the primary amino groups of the amino acids and OPA reagent. L-serine was used as standard and the blank sample was prepared with deionized water. The absorbance values of the samples (A_{sample}), standard (A_{standard}), and blank (A_{blank}) were registered exactly after 2 min of reaction with OPA reagent. The degree of hydrolysis (DH) was calculated as:

$$\text{DH (\%)} = h/h_{\text{tot}} \cdot 100 \quad (1)$$

where h and h_{tot} represent the hydrolysed bonds and the total number of peptide bonds, respectively, per protein equivalent. The value of h_{tot} depends on protein composition and for soy proteins is 7.8 (Adler-Nissen, 1986; Nielsen *et al.*, 2001).

For each protein hydrolysate obtained in the study, the number of hydrolysate bonds was determined as indicated by Nielsen *et al.* (2001):

$$h = [(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{standard}} - A_{\text{blank}}) \cdot 0.9516 \cdot d \cdot 100/x \cdot p - \beta]/\alpha \quad (2)$$

where d is dilution, x is the amount of sample used for analysis (g), p is the protein concentration (%), while the specific values of α and β for soy protein are 0.970 and 0.342, respectively.

Antioxidant activity

The methods described by Dumitrașcu *et al.* (2022) were used to assess the DPPH and ABTS⁺ radicals-scavenging activity (DPPH-RSA and ABTS-RSA, respectively) of the soy protein hydrolysates. In case of both methods the results were reported as:

$$\text{Inhibition (\%)} = (1 - A/A_0) \cdot 100 \quad (3)$$

where A is the absorbance of the sample and A_0 is the absorbance of the blank prepared with methanol 80%, instead of protein hydrolysate.

Assessment of the bioactive peptides with antioxidant activity

The bioinformatics tools were further employed to identify the peptides encrypted in the main soy proteins, which exhibit antioxidant properties. The UniProt database (The UniProt Consortium, 2023) was first interrogated to identify the full records on primary structure of the main soy proteins. The following UniProt codes were selected from the UniProt database: P04776 - glycinin G1 (GY1), P04405 - glycinin

G2 (GY2), P11828 - glycinin G3 (GY3), P02858 - glycinin G4 (GY4), P04347 - glycinin G5 (GY5), P0DO16 - β -conglycinin alpha subunit (CG-3), P0DO15 - β -conglycinin alpha subunit (CG-2), P11827 - β -conglycinin alpha' subunit (CG-1), P25974 - β -conglycinin beta subunit1 (CG-4) and F7J077 - β -conglycinin beta subunit2 (CG-4). The primary sequences of these proteins were then used to check, against the content of the BIOPEP-UWM database (Minkiewicz et al., 2019), for the existence of bioactive peptides with experimentally proved antioxidant activity.

Color properties

The CIELab color parameters of the soy protein hydrolysates were measured on the supernatant obtained after samples centrifugation, using the Chroma Meter CR-410 (Konica Minolta Sensing Americas Inc., Ramsey, NJ, USA). A volume of 15 ml of each sample was used for measurements. The lightness (L^* ranges between 0 corresponding to black/dark and 100 corresponding to white/bright) of the samples was registered together with the chromatic colours (a^* - provides indication on the presence of green (-) or red (+) shades, and b^* - provides indication on the presence of blue (-) or yellow (+) shades).

The color differences (ΔE) induced by the enzyme assisted hydrolysis in respect to the Control were calculated with equation 4. The color saturation was determined by calculating chroma (C^*) with equation 5.

$$\Delta E = ((L^*_{\text{sample}} - L^*_{\text{control}})^2 + (a^*_{\text{sample}} - a^*_{\text{control}})^2 + (b^*_{\text{sample}} - b^*_{\text{control}})^2)^{1/2} \quad (4)$$

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (5)$$

Foaming properties

The soy protein hydrolysates were used to prepare solutions of 6% (w/v) concentration, which were foamed for two minutes at three different speeds (5000, 7000 and 9000 rpm) using the Ultra Turax® IKA T18 basic homogenizer (KA-Werke GmbH and Co. KG, Staufen, Germany).

The volume of the peptides solution (V_0) and the volume of the foam (V_f), registered after the vigorous mixing, were used to determine the foaming capacity (FC) using equation 6:

$$FC (\%) = (V_f - V_0)/V_0 \times 100 \quad (6)$$

The volume of the foams (V_{15}) was measured after storing for 15 minutes the foamed samples at room temperature of samples. The foam stability (FS) was determined as follows:

$$FS (\%) = (V_{15} - V_0)/(V_f - V_0) \times 100 \quad (7)$$

Emulsions preparation and characterization

The freeze-dried hydrolysate samples were first used for preparing suspensions of 6% (v/w). Emulsions were further formulated with sunflower oil fraction (ϕ) of 0.5 and the mixtures were homogenized for 5 min at 15,000 rpm, using the Ultra Turrax.

Emulsifying activity

The emulsifying activity index (EAI) was determined as described by Zhang et al. (2014), using the method of Pearce and Kinsella (1978). The emulsions were vigorously mixed with 0.1 % (w/v) SDS such as to get a dilution factor (F) of 100, and the absorption (A) at wavelength of 500 nm was read every 2 min, over a total period of 10 min. The EAI was calculated using equation 8.

$$\text{EAI (m}^2/\text{g)} = (2 \times T \times A \times F \times 0.0001)/(\phi \times L \times C) \quad (8)$$

where T = 2.303, L = path length of the cuvette (1 cm), and C = concentration of the soy protein hydrolysate (g/mL).

Rheological behavior

Fundamental rheological measurements were carried out at 20°C on the obtained emulsions using the controlled-stress rheometer (AR2000ex, TA Instruments Ltd, New Castle, DE, USA). A cone–plate geometry (cone angle of 2°) and a closing gap of 1000 μm were selected for all tests.

The linear viscoelastic region (LVR) was first determined for all emulsions upon running a strain sweep test, during which the oscillating strain was increased from 0.1 to 100%, while keeping fixed the frequency of 1 Hz. Frequency sweep tests were carried out in the LVR by controlling frequency in the 0.1 - 100 Hz range. The storage modulus (G') and loss modulus (G'') of the samples were monitored over the oscillatory tests.

The viscosity of the emulsions was registered while running shear-rate-controlled rotational tests at increasing shear rate values in the 0.1 - 100 s^{-1} domain.

The results of the triplicate measurements were recorded and analysed using the TA Rheology Advantage Data Analysis Software V 4.8.3. (TA Instruments, New Castle, DE, USA).

Statistical analysis

The experimental measurements were performed at least in duplicate and mean \pm standard deviation values were reported. The one-way ANOVA and Tukey test were applied to identify significant differences between samples using Minitab 19 (Minitab LLC, State College, PA, USA) software.

Results and discussion

Influence of enzyme on the soy protein hydrolysis

In addition to the nutritional values, the use of different protein derivatives as ingredient in different food formulation is decided based on the bioactivity and

functional properties. These properties of the proteins mainly depend on the molecular size and architecture, as well as on the intra- and intermolecular interactions, which further influence the protein behaviour within the food matrix. In the present study, three different enzymes were selected to cleave specific peptide bonds, such as to ensure various DH of the soy proteins. No important differences in terms of DH values were registered between the SPB and SPN samples, whereas a significantly lower DH of 1.85% was observed in the case of SPT (Table 1). The specificity of the endopeptidases used for proteins hydrolysis influenced the DH. Bromelain (E.C. 3.4.22.4.) is a cysteine protease of plant origin, which preferentially cleaves the peptides bonds involving Lys, Arg, Phe and Tyr residues, while Neutrased is a neutral metallo-proteinase (3.4.24.28.) which has affinity for the peptide bonds involving the Leu, Phe, and other hydrophobic amino acids (Kunst, 2002). As indicated by Hughes *et al.* (2011), the soy protein isolates have rather high amounts of these particular amino acids (Lys 6.2 g/100g protein, Arg 7.9 g/100 g protein, Phe 5.1 g/100 g protein, Tyr 3.8 g/100 g protein, Leu 8.0 g/100 g protein). Therefore, both bromelain and Neutrased are able to recognize and break many peptide bonds of the soy proteins, explaining the results obtained in the present study (Table 1).

Table 1. The degree of hydrolysis (DH) and soluble protein content (SPC) of the soy protein hydrolysates obtained with bromelain (SPB), Neutrased (SPN) and trypsin (SPT).

Sample	DH, %	SPC, g/100 g
Control	0.75±0.14 ^b	15.44±0.35 ^c
SPB	10.27±0.55 ^a	47.47±2.81 ^a
SPN	9.70±0.47 ^a	40.02±2.81 ^a
SPT	1.85±0.06 ^b	29.84±1.76 ^b

* Different superscript lowercase letters (a, b, c) are assigned to mean values from a column which are statistically significant at $p < 0.05$.

On the other hand, trypsin (3.4.21.4.) is a serine protease which has high specificity for the peptide bonds which involve the carboxyl group of Arg and Lys residues (Kunst, 2002; Zhao and Hou, 2009; Ding *et al.*, 2021). Moreover, the whole soybeans contain important amounts of trypsin inhibitors (trypsin inhibitory activity of 16-27 mg/g sample), which can affect protein digestibility (Vagadia *et al.*, 2017). It should be noted that, although the trypsin inhibitors are considered heat labile, as reviewed by Vagadia *et al.* (2017) the residual activity in the soy proteins isolates can be rather high (trypsin inhibitory activity of 1-30 mg/g sample). The presence of trypsin inhibitors in the soy protein isolate used in the present study might explain the limited DH ensured by trypsin (Table 1).

As expected, the soy proteins hydrolysis resulted in significant improvement of the soluble proteins content, in respect to the control, for all tested enzymes. In agreement with the DH values, the highest SPC was obtained in case of SPB, followed by SPN (Table 1). Our observations are in agreement with the results reported by Islam *et al.* (2022), who attributed the greater solubility of the soy

proteins digested with Alcalase and Protamex to the low molecular weights peptides resulted at high DH.

Antioxidant activity of the soy protein hydrolysates

Proteins hydrolysis results in the release of various peptides and free amino acids, which might exert different biological activities, depending on the substrate subjected to transformation, enzyme specificity and the DH. Different bioactive peptides, released through enzymatic hydrolysis of the proteins of plant, animal or microbial origin, might play various physiological roles because of the antioxidant, antimicrobial and immunomodulatory activities, hypocholesterolemic and antihypertensive effects, minerals binding ability etc. (Sarmadi and Ismail, 2010).

Unlike other antioxidant compounds, the peptides with antioxidant activity provide several advantages: they are safe, can be obtained through rather low-cost processes, have good absorption, and deliver additional nutritional and potential health benefits to the host (Sarmadi and Ismail, 2010).

Although many studies were focused on the antioxidant activity of the peptides, the exact mechanism standing behind this property is not well known. As reviewed by Sarmadi and Ismail (2010), various peptides might scavenge the free radicals, inhibit lipid peroxidation, and/or chelate the metal ions. Two different methods were used in the present study for estimating the influence of the enzymes on the antioxidant activity of the resulting soy protein hydrolysates. The DPPH radical scavenging activity method which is based on the electron transfer, and the ABTS radical scavenging method which is based on hydrogen atom transfer (Shalaby and Shanab, 2013) were selected, such as to ensure an efficient scanning of the antioxidant activity of the obtained soy protein hydrolysates.

Analysing the results presented in Figure 1, one can see that both methods indicated the significant increase of the antioxidant activity upon enzyme assisted hydrolysis of the soy proteins ($p < 0.05$). These results indicate that enzymes ensured the release of different bioactive peptides which are inactive when encrypted within the packed three-dimensional structure of the parent proteins.

Different studies available in the literature indicate higher antioxidant activity of the protein hydrolysates with increasing the DH. For instance, Lee *et al.* (2008) reported higher levels of antioxidant activity with increasing the DH of SPI hydrolysed with papain, Neutrase, bromelain and Flavourzyme. Yoo and Chang (2016) also reported superior antioxidant activity of soy protein hydrolysates with high DH value. The factors influencing the proteins hydrolysis efficiency (enzyme, hydrolysis duration, pH, temperature, etc.) are prone to modulate the antioxidant profile of the resulting peptides (Lee *et al.*, 2008; Wang *et al.*, 2022). Taking into account that the enzymes used in the present study for preparing the soy protein hydrolysates have different cleavage specificity, the antioxidant capacity was not correlated with DH values. The SPN sample exhibited the highest DPPH- and ABTS-RSA values. Although trypsin ensured the lowest DH of 1.85 ± 0.06 % ($p < 0.05$) of the soy proteins, the SPT sample presented higher levels of DPPH-RSA ($p < 0.05$) compared to sample hydrolysed with bromelain (Figure 1a). No significant differences were observed between SPT

and SPB samples in terms of ABTS-RSA (Figure 1b). For all investigated samples, the radical scavenging activity was higher in case of the ABTS- compared to DPPH-based method (Figure 1). Similar findings were previously reported by Dumitrașcu *et al.* (2023) when investigating the antioxidant properties of the peptides released by bromelain, Neutrase and trypsin from the spent brewer's yeast proteins. They highlighted the importance of the higher stability of the DPPH compared to the ABTS⁺ radicals, as well of the differences in the reaction mechanisms of the two methods (Dumitrașcu *et al.*, 2023).

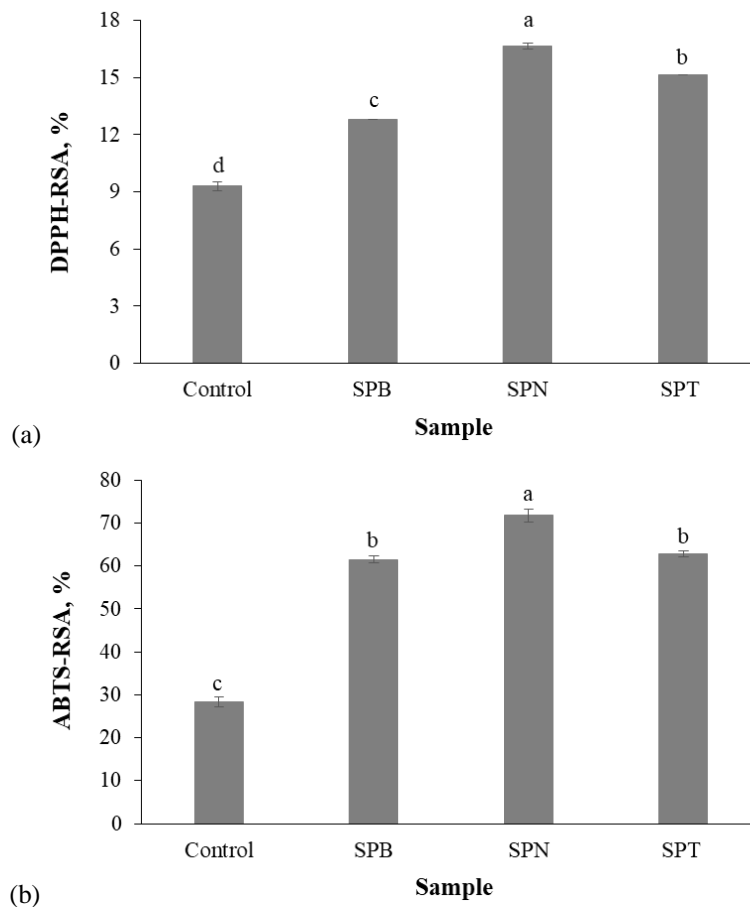


Figure 1. DPPH radical scavenging (DPPH-RSA) activity (a) and ABTS radical scavenging (ABTS-RSA) activity (b) of the soluble fraction of soy proteins (Control) after hydrolysis with bromelain (SPB), Neutrase (SPN) and trypsin (SPT). Mean antioxidant activity values marked with different lowercase letters (a, b, c, d) are significantly different ($p < 0.05$).

The bioactive peptides usually have molecular weights lower than 6 kDa, and consists of 2-20 amino acids (Sarmadi and Ismail, 2010). The antioxidant activity of the peptides is influenced by the composition, presence of hydrophobic amino acids

and their positioning in the peptide chain. It has been reported that the unpurified soy protein hydrolysates exert better antioxidant properties compared to the purified peptides obtained from β -conglycinin (Chen *et al.*, 1995; Sarmadi and Ismail, 2010). Depending on the enzyme used for the cleavage of the peptide bonds and on the final DH, in addition to the bioactive peptides the protein hydrolysates might include free amino acids, such as Tyr, Trp, Met, Lys, Cys, and His, which can contribute to the overall antioxidant activity of the mixture. The mechanisms through which different amino acids exhibit their antioxidant properties are various: the aromatic amino acids are able to scavenge the electron deficient radicals through donating protons; the imidazole group of the His might donate hydrogen, trap the lipid peroxy radical and/or chelate the metal ions; Cys can directly participate to interaction with the radicals, through the -SH group (Sarmadi and Ismail, 2010).

Depending on the sedimentation coefficients from ultracentrifugation, the water-soluble soy proteins can be classified as 2S, 7S, 11S and 15S globulins. Considering that 7S (β -conglycinin) and 11S (glycinin) represent over 80% of soy proteins (Nishinari *et al.*, 2014), the primary sequences of these proteins were selected from the UniProt database and used for checking the existence of encrypted bioactive peptides, by means of the bioinformatics tools. According to the information deposited in the BIOPEP-UWM, the oligomers of both β -conglycinin and glycinin include multiple copies of various bioactive dipeptides and tripeptides with demonstrated antioxidant activity (Table 2). Part of the antioxidant activity measured experimentally on the soy protein hydrolysates might be also due to the eight different tetrapeptides or to the peptides with 5-16 amino acids, originating from glycinin (FVPH, YQEP, EFLQ, VVLY, PYPQ, YLAGNQEQE, DEQIPSHPPR, VNPESQQGSPR, GKHQEEENEGGSI, NALEPDHRVESEGG) or β -conglycinin (PAGY, TTYT, LPHH, VIPAGYP, VNPHDHQN, LVNPHDHQN, LLPHADADY, SLVNNDDRDS, IGINAENNQRN, FVDAQPQQKEEG, HEQKEEHEWHRKEE, LQSGDALRVPSGTTYT).

Table 2. Peptides with antioxidant activity encrypted in the main soy proteins, namely glycinin and β -conglycinin. The information was collected by interrogating the UniProt for getting the primary structure of the proteins, and the BIOPEP-UWM for identifying the potential bioactive peptides.

UniProt entry - Protein name (gene name)	Bioactive peptides with demonstrated antioxidant activity [location]
P04776-1 - glycinin G1 (GY1-1)	<i>Dipeptides:</i> IY [91-92],[103-104],[133-134],[333-334],[382-383]; EL [405-406]; MY [152-153]; KD [293-294]; LK [39-40],[193-194],[466-467]; KP [40-41],[60-61],[266-267],[287-288]; TW [54-55]; AW [149-150]; HC [296-297] <i>Tripeptides:</i> PHY [372-374]; RHN [322-324]; RHQ [129-131]; LKP [39-41]; TFE [110-112]; YNL [374-376]; LSW [352-354] <i>Tetrapeptides:</i> FVPH [370-373] <i>Peptides with more than 5 amino acids:</i> YLAGNQEQE [183-191]; GKHQEEENEGGSI [207-220]

P04776-1 - glycinin G1 (GY1-2)	<i>Dipeptides:</i> IY [91-92],[103-104],[133-134],[333-334]; MY [152-153]; KD [293-294]; LK [39-40],[193-194]; KP [40-41],[60-61],[266-267],[287-288]; TW [54-55]; AW [149-150]; HC [296-297] <i>Tripeptides:</i> RHN [322-324]; RHQ [129-131]; EAK [370-372]; LKP [39-41]; TFE [110-112]; LSW [352-354] <i>Peptides with more than 5 amino acids:</i> YLAGNQEQE [183-191] GKHQEEENEKSI [207-220]
P04405 - glycinin G2 (GY2)	<i>Dipeptides:</i> IY [88-89],[323-324],[372-373]; EL [395-396]; MY [149-150]; LK [36-37],[190-191],[226-227],[345-346],[456-457]; KP [37-38],[57-58],[268-269]; TY [107-108]; TW [51-52]; AW [146-147]; LW [342-343] <i>Tripeptides:</i> PHY [362-364]; RHQ [126-128]; LKP [36-38] <i>Tetrapeptides:</i> YQEP [108-111]; FVPH [360-363] <i>Peptides with more than 5 amino acids:</i> YLAGNQEQE [180-188]; MRKPQEEDDDDE [266-278]
P11828 - glycinin G3 (GY3-1; GY3-2)	<i>Dipeptides:</i> AY [146-147]; IY [91-92],[130-131],[368-369]; EL [391-392]; MY [149-150]; KD [286-287]; LK [39-40],[341-342]; KP [40-41],[60-61],[280-281]; TW [54-55]; HC [289-290] <i>Tripeptides:</i> PHY [358-360]; RHN [308-310]; RHQ [126-128]; LKP [39-41]; TFE [110-112]; YNL [360-362]; LSW [338-340] <i>Tetrapeptides:</i> FVPH [356-359]; EFLQ [188-191] <i>Peptides with more than 5 amino acids:</i> YLAGNQEQE [180-188]
P02858 - glycinin G4 (GY4-1; GY4-2)	<i>Dipeptides:</i> LH [80-81],[389-390]; HL [81-82]; HH [505-506]; LY [432-433]; IY [437-438],[450-451]; AH [526-527]; EL [62-63],[473-474],[538-539]; KD [514-515]; IR [138-139]; LK [63-64],[387-388],[513-514],[539-540]; KP [3-4],[320-321]; TY [157-158]; TW [55-56]; WG [545-546] <i>Tripeptides:</i> ADF [399-401]; THH [504-506]; LHE [389-391]; LHL [80-82]; PHW [440-442]; RHF [139-141]; VKV [560-562]; PEL [61-63]; TFE [111-113]; SVL [228-230]; YNL [529-531] <i>Tetrapeptides:</i> VVLY [430-433] <i>Peptides with more than 5 amino acids:</i> ALEPDHR [39-45]; DEQIPSHPPR [292-301]; VNPESQQGSPR [549-559]; NALEPDHRVESEGG [38-51]
P04347 - glycinin G5 (GY5-1; GY5-2)	<i>Dipeptides:</i> LH [355-356]; HL [82-83]; HH [470-471]; LY [398-399]; IY [403-404]; EL [63-64],[438-439]; KD [479-480]; IR [138-139]; LK [504-505]; KP [3-4],[115-116]; TY [157-158]; TW [56-57] <i>Tripeptides:</i> ADF [365-367]; THH [469-471]; LHE [355-357]; RHF [139-141]; GGE [457-459]; PEL [62-64]; TFE [112-114]; SVL [227-229]; GSH [80-82]; YNL [494-496]; YPQ [89-91] <i>Tetrapeptides:</i> PYPQ [88-91]; VVLY [396-399] <i>Peptides with more than 5 amino acids:</i> ALEPDHR [40-46]; NALEPDHRVESEGG [39-52]
P0DO16 - β - conglycinin alpha subunit (CG-3)	<i>Dipeptides:</i> AY [26-27]; IY [414-415]; EL [186-187],[473-474],[499-500],[556-557]; IR [214-215],[384-385]; LK [61-62],[477-478],[570-571]; KP [241-242],[315-316],[404-405]; TY [292-293]; MM [1-2] <i>Tripeptides:</i> YYV [293-295]; TYY [292-294]; PHF [453-455]; PHQ [159-161]; RHK [189-191]; KAI [458-460]; KCL [37-39]; NEN [301-303]; RDY [231-233]; VFL [14-16]; LAF [557-559]

	<p><i>Tetrapeptides:</i> LLPH [451-454]; PAGY [509-512]; TTY Y [291-294] <i>Peptides with more than 5 amino acids:</i> VIPAGYP [507-513]; LQSGDALRVPSGTTY Y [279-294]; SLVNNDDRDS [267-276]; IGINAENNQRN [527-537]</p>
P0DO15 - β -conglycinin alpha subunit (CG-2)	<p><i>Dipeptides:</i> AY [26-27]; IY [414-415]; EL [186-187],[473-474],[499-500],[556-557]; IR [214-215],[384-385]; LK [61-62],[477-478],[570-571]; KP [241-242],[315-316],[404-405]; TY [292-293]; MM [1-2] <i>Tripeptides:</i> YYV [293-295]; TYY [292-294]; PHF [453-455]; PHQ [159-161]; RHK [189-191]; KAI [458-460]; KCL [37-39]; NEN [301-303]; RDY [231-233]; VFL [14-16]; LAF [557-559] <i>Tetrapeptides:</i> LLPH [451-454]; PAGY [509-512]; TTY Y [291-294] <i>Peptides with more than 5 amino acids:</i> VIPAGYP [507-513]; LQSGDALRVPSGTTY Y [279-294]; SLVNNDDRDS [267-276]; IGINAENNQRN [527-537]</p>
P11827 - β -conglycinin alpha' subunit (CG-1)	<p><i>Dipeptides:</i> HH [265-266]; AY [26-27]; IY [430-431]; EL [402-403],[489-490],[515-516],[572-573]; KD [46-47],[559-560],[580-581]; IR [400-401]; LK [61-62]; KP [257-258],[331-332],[420-421]; TY [308-309]; MM [1-2] <i>Tripeptides:</i> PHH [264-266]; YYV [309-311]; TYY [308-310]; PHF [469-471]; PHQ [111-113],[149-151],[152-154]; RHK [205-207]; KAI [474-476]; KCL [37-39]; RDY [247-249]; YNL [293-295]; VFL [14-16],[454-456]; LAF [573-575] <i>Tetrapeptides:</i> LLPH [262-265]; LPHH [263-266]; TTY Y [307-310]; PAGY [525-528] <i>Peptides with more than 5 amino acids:</i> LLPHH [262-266]; LLPHHADADY [262-271]; VIPAGYP [523-529]; FVDAQPQQKEEG [594-605]; HEQKEEHEWHRKEE [117-130]</p>
P25974 - β -conglycinin beta subunit1 (CG-4)	<p><i>Dipeptides:</i> LH [118-119]; HH [88-89]; AY [345-346]; LY [438-439]; IY [248-249]; EL [212-213],[307-308],[333-334],[390-391]; KD [377-378]; IR [53-54],[218-219]; LK [24-25],[143-144],[404-405]; KP [80-81],[154-155]; TY [131-132]; MM [1-2]; RY [157-158],[329-330] <i>Tripeptides:</i> PHH [87-89]; ADF [92-94]; HDH [138-140]; YYL [132-134]; TYY [131-133]; PHD [137-139]; PHF [287-289]; IKL [146-148]; KAI [292-294]; RDY [70-72]; YNL [116-118] <i>Tetrapeptides:</i> LLPH [85-88],[285-288]; LPHH [86-89]; TTY Y [130-133] <i>Peptides with more than 5 amino acids:</i> LLPHH [85-89]; VNP HDHQ N [135-142]; LVNPHD HQ N [134-142]; FVDAQPQQKEEG [412-423]; YPFVVN [346-351]</p>
F7J077 - β -conglycinin beta subunit2 (CG-4)	<p><i>Dipeptides:</i> LH [118-119]; HH [88-89]; AY [345-346]; LY [438-439]; IY [248-249]; EL [212-213],[307-308],[333-334],[390-391]; KD [377-378]; IR [53-54],[218-219]; LK [24-25],[143-144],[404-405]; KP [80-81],[154-155]; TY [131-132]; MM [1-2]; RY [157-158],[329-330] <i>Tripeptides:</i> PHH [87-89]; ADF [92-94]; HDH [138-140]; YYL [132-134]; TYY [131-133]; PHD [137-139]; PHF [287-289]; IKL [146-148]; KAI [292-294]; RDY [70-72]; YNL [116-118] <i>Tetrapeptides:</i> LLPH [85-88],[285-288]; LPHH [86-89]; TTY Y [130-133]</p>

Peptides with more than 5 amino acids: LLPHH [85-89];
 VNPHDHQN [135-142]; LVNPHDHQN [134-142];
 FVDAQPQQKEEG [412-423]; YPFVVN [346-351]

Color properties of the soy protein hydrolysates

The enzyme used for preparing the soy protein hydrolysates influenced in different manner the color properties measured on the liquid samples (Table 3).

When compared to the Control, a significant decrease of the luminosity coordinate was observed for all sample prepared with enzyme addition ($p < 0.05$). The lowest values of L^* were measured in the SPN (67.96 ± 0.34) and SPB (68.59 ± 0.11) samples, suggesting that the soy protein hydrolysates prepared with Neutrase and bromelain presented significantly darker color compared to the SPT and Control samples (Table 3). These results are in agreement with the observations of Dumitrașcu et al. (2023), showing a negative correlation between the soluble protein content of the spent brewer's yeast hydrolysates and the luminosity of the samples. Analyzing the results presented in Table 1, one can see that the lowest soluble protein content was measured on the Control sample (15.44 ± 0.35 g/100 g), whereas the highest values were registered for SPN (40.02 ± 2.81 g/100g) and SPB (47.47 ± 2.81 g/100 g) hydrolysates.

Table 3. Color coordinates of the soluble fraction of soy proteins prior (Control) and after hydrolysis with bromelain (SPB), Neutrase (SPN) and trypsin (SPT).

Samples	L*	a*	b*	C*	ΔE
Control	70.51 ± 0.61^a	1.27 ± 0.23^b	0.21 ± 0.09^d	1.24	-
SPB	68.59 ± 0.11^c	1.98 ± 0.04^a	1.49 ± 0.31^c	2.48	2.00
SPN	67.96 ± 0.34^c	$1.13 \pm 0.03^{b,c}$	5.03 ± 0.59^a	5.28	6.88
SPT	69.52 ± 0.28^b	0.92 ± 0.06^c	2.88 ± 0.57^b	3.34	4.41

Mean values from the same column marked with different lowercase letters (a, b, c) are significantly different ($p < 0.05$), based on Tukey test.

ΔE – color difference was calculated with equation (4) using the mean L^* , a^* and b^* values. C^* - chroma was calculated with equation (5) using the mean a^* and b^* values.

Regardless of the enzyme used for hydrolysis, the positive a^* and b^* indicated the prevalence of red and yellow shades in the color of the samples subjected to measurements. The highest and lowest intensity of the red shades were observed in case of the SPB (a^* of 1.98 ± 0.04) and SPT (a^* of 0.92 ± 0.06) samples, whereas the highest and lowest intensity of the yellow shades were observed in case of the SPN (b^* of 5.03 ± 0.59) and Control (b^* of 0.21 ± 0.09) samples, respectively (Table 3). The Control presented the lightest color among all tested samples (C^* of 1.24), whereas the samples prepared with Neutrase exhibited the highest saturation (C^* of 5.28). Based on the color differences in respect to the Control (ΔE), it can be finally stated that the color of the hydrolysates is highly influenced by the enzymes used for the hydrolysis step; the highest ΔE of 6.88 was calculated for the sample prepared

with Neutrase, whereas the lowest ΔE of 2.00 was registered for the sample prepared with bromelain (Table 3).

Functional properties of the soy protein hydrolysates

Foaming properties, emulsifying activity and rheological behaviour of the emulsions prepared with soluble soy proteins prior and after digestion with bromelain, Neutrase and trypsin were tested.

Foaming properties

Foaming properties of the soy protein hydrolysates were evaluated at increasing homogenization speed, in terms of foaming capacity and foam stability, and the results were presented in Figure 2. As indicated in Figure 2(a), the increase in the foaming speed determined the significant improvement of the foaming capacity of the soy protein-based samples. Our results agree with Dumitrașcu *et al.* (2023) who reported the significant improvement of the foaming ability of the yeast protein hydrolysates when raising the homogenization speed from 5000 to 9000 rpm.

For all tested foaming speeds, the highest values of the foaming capacity were registered for the soy protein hydrolysates prepared with trypsin. Thus, the SPT sample foamed for 2 min at 9000 rpm exhibited the highest overrun of $720.00 \pm 28.28\%$ (Figure 2a). When looking at the enzyme influence, one can observe no important difference between the Control and the SPN sample when foamed at 7000 rpm, or between SPB and SPN samples when foamed at 5000 rpm. These results suggest that the limited hydrolysis ensured by trypsin impacted to the most extent the foaming ability of the soluble soy proteins.

Regarding the stability of the foams, it is shown in Figure 2(b) that the samples digested with trypsin provided the most stable foams, and the foams generated upon hydrolysing the samples with bromelain and Neutrase displayed noticeably lower ($p < 0.05$) levels of stability. The effect of the foaming speed on the stability of the foams over 15 min of storage at room temperature varied with the enzyme used for preparing the protein hydrolysate (Figure 2b). In case of the control and SPN samples the highest foam stability values of $84.77 \pm 2.03\%$ and $26.24 \pm 0.61\%$, respectively, were registered when mixing at 7000 rpm, whereas in case of the SPB sample, the highest stability was registered when the foam was generated at 5000 rpm. The only exception concerns the soy protein sample hydrolyzed with trypsin, in which case the foam stability was independent of the foaming speed (Figure 2b).

Many investigations have been done on the potential of soy proteins to develop and stabilize foams. Liang *et al.* (2020) indicated that the desirable foaming capability of the soy protein hydrolysate dispersions was dependent on the quick development of a cohesive air-water interfacial film after the addition of air to the protein liquid dispersion. The native soy proteins are more difficult to convert into a continuous film surrounding the gas phase dispersed into the liquid. As a result of releasing higher amounts of peptides with lower molecular weight and more flexible structure compared to the native proteins, limited proteins hydrolysis was reported to improve the functional properties of the proteins. In particular this process results in enhancing the amphiphilic properties of the peptides, which are therefore faster

absorbed at the gas-water interface, improving the foaming properties (Dumitrașcu *et al.*, 2023). For instance, the limited hydrolysis assisted by Alcalase ensured better functional properties of the soy proteins, in particular better foaming capacity and foam stability. The enzymatic treatment significantly increases the foam capacity, which is dependent on the rate of protein diffusion to the interface. At long-term adsorption, foam stability often correlates with surface pressure and, to a lesser extent, with surface dilatational modulus (Ruiz-Henestrosa *et al.*, 2009)

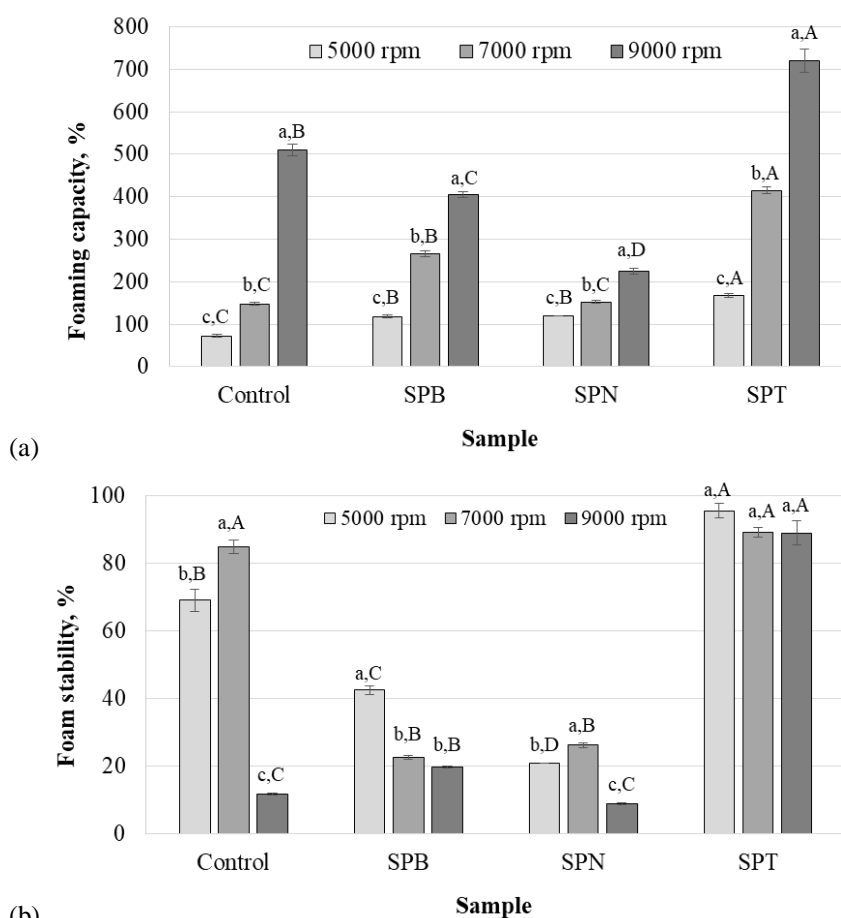


Figure 2. Foaming capacity (a) and foam stability (b) of the soy protein isolate, prior to (Control) and after hydrolysis with bromelain (SPB), Neutrase (SPN) and trypsin (SPT). For the same enzyme treatment, mean values which do not share a lowercase letter (a, b, c) are statistically significant at $p < 0.05$. For the same foaming speed, mean values which do not share an uppercase letter (A, B, C, D) are statistically significant at $p < 0.05$.

Emulsifying activity

Soy protein derivatives are currently used as emulsifying agents in various food formulations produced at industrial scale. The good emulsifying properties are

related to the amphiphilic nature of the soy protein fractions. Because of these properties, the soy proteins are able to decrease the interfacial tension upon interaction with the oil droplets, which are well dispersed into the protein solution.

In order to test the influence of the enzyme assisted hydrolysis on the emulsifying properties of the soluble soy proteins, the hydrolysates were further used to prepare emulsions using sunflower oil fraction of 0.5. The EAI of the samples was determined over a 10 min period and the results indicated that the emulsion were rather stable over the entire tested period (Figure 3). The soy proteins hydrolysis resulted in important decrease ($p < 0.05$) of the EAI values, the lowest values being recorded for the SPT sample (Figure 3). Shortening of the polypeptide chains upon enzyme assisted hydrolysis most probably affected their ability to organize into stable layers, which were essential for preventing the coalescence phenomenon. Unlike the trend observed in the present experiment, Zhang et al. (2014) previously reported higher EAI values when performing limited soy proteins hydrolysis with Neutrase. Moreover, they showed that hydrolysates glycosylation with maltodextrin was an efficient way of improving the emulsifying properties

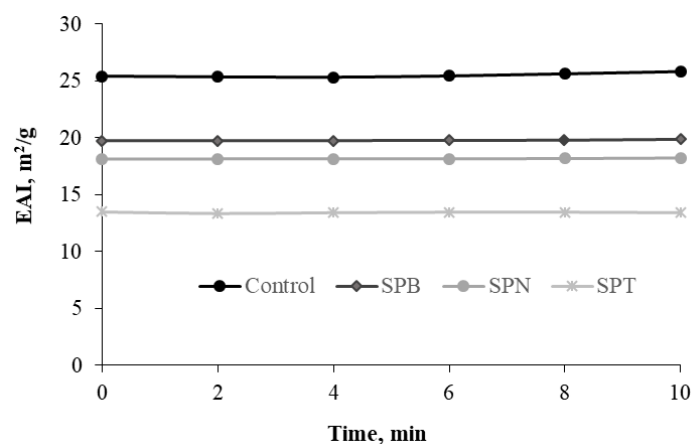


Figure 3. Evolution of the emulsifying activity index (EAI) of the soy proteins (Control) after hydrolysis with bromelain (SPB), Neutrase (SPN) and trypsin (SPT).

Rheological behavior of the emulsions

The rheological properties of the obtained emulsions were evaluated by running oscillatory scanning and steady shear measurements.

Analyzing the relative magnitude of the elastic and viscous moduli measured during the oscillatory rheological tests, it was observed that all tested emulsions presented predominantly viscous behavior, with G'' prevailing over G' (Table 4). This rheological behavior of the tested emulsions might be due to the low relaxation phenomenon associated to the oil droplets deformations (Zhu *et al.*, 2020). Helgeson *et al.* (2007) explained that when emulsions were subjected to a stress, the nonlinear viscoelastic behavior might be expected because of the progressive disruption of

their specific structure. Indeed, the complex modulus G^* , which is a measure of the overall emulsion structure resistance to deformation, was determined upon running frequency sweep measurements, by considering both the elastic and viscous moduli, as recoverable and non-recoverable components, respectively (Dumitrașcu *et al.*, 2023). Analyzing the results presented in Table 4 one can observe that all the tested samples presented frequency dependent G^* values. In agreement with the observations of Dumitrașcu *et al.* (2023), our study indicated that the rigidity of the emulsions, when exposed to lower stress values compared to the yield stress, varied with the flow motion. The rheological parameters measured at low-frequency provide information regarding the behavior of the emulsions over the slow-motion flow for a long period. As expected, the use of enzymes for obtaining soy protein hydrolysates resulted in weaker emulsions characterized by slightly lower G^* values compared to the Control. The SPB sample which had the highest DH of 10.27% was characterized by the lowest G^* of 0.175 ± 0.015 Pa (Table 4). On the other hand, the G^* values determined at higher frequency of 10 Hz varied significantly with the enzyme ($p < 0.05$): SPN emulsion presented the strongest structure (G^* of 14.319 ± 1.148), being followed by SPB (G^* of 9.303 ± 0.509), SPT (G^* of 4.656 ± 0.572) and Control (G^* of 4.208 ± 0.188). These values provide an indication regarding emulsions behavior at fast motion for short timescales.

For all tested emulsions, the stepped flow tests indicated the shear stress increase (Figure 4) and the apparent viscosity decrease (Table 4), when raising the shear rate values from 0.1 to 100 s^{-1} . The higher apparent viscosity values measured at low shear rate suggest that the tested emulsions are able to retain their shape during storage, while the significantly lower apparent viscosity values registered at high shear rate indicate the ability to flow when poured out of a beaker (Zhu *et al.*, 2020).

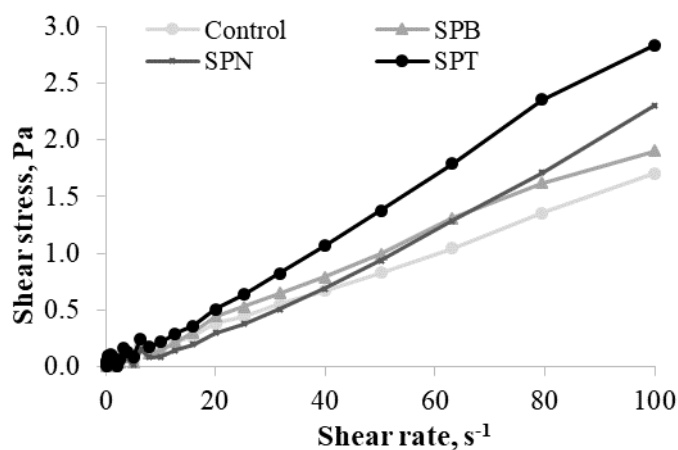


Figure 4. Rheograms showing the evolution of the shear stress with the shear rate for the emulsions prepared with soluble fraction of soy proteins prior (Control) and after hydrolysis with bromelain (SPB), Neutrase (SPN) and trypsin (SPT)/

Table 4. Rheological parameters collected during the oscillatory and stepped flow tests performed on emulsions prepared with soluble fraction of soy proteins prior (Control) and after hydrolysis with bromelain (SPB), Neutrase (SPN) and trypsin (SPT).

Samples	Strain sweep test	Frequency sweep test		Stepped Flow Test	
		G* (Pa) at 1 Hz	G* (Pa) at 10 Hz	Viscosity (Pa·s) at shear rate of 1 s ⁻¹	Viscosity (Pa·s) at shear rate of 10 s ⁻¹
Control	G' > G''	0.214±0.003 ^a	4.208±0.188 ^c	0.094±0.001 ^a	0.015±0.001 ^b
SPB	G' > G''	0.175±0.015 ^b	9.303±0.509 ^b	0.077±0.004 ^b	0.014±0.001 ^b
SPN	G' > G''	0.205±0.016 ^{ab}	14.319±1.148 ^a	0.071±0.003 ^b	0.009±0.001 ^c
SPT	G' > G''	0.184±0.018 ^{a,b}	4.656±0.572 ^c	0.095±0.005 ^a	0.022±0.001 ^a

Mean values from the same column marked with different lowercase letters (a, b, c) are significantly different ($p < 0.05$), based on Tukey test.

Conclusions

The influence of the different endopeptidase on the functional properties and antioxidant capacity of the soluble soy proteins was tested. The limited soy proteins hydrolysis assisted by bromelain, neutral proteinase and trypsin, resulted in soluble peptide mixtures with improved properties. The soy protein hydrolysates prepared with trypsin are suitable for obtaining stable emulsions with the highest viscosity among all tested variants, and displayed the best foaming properties. The peptide mixture obtained with Neutrase presented the highest antioxidant activity levels, being followed by soy protein hydrolysates prepared with bromelain and trypsin. The bioinformatics tools indicated that the di- and tripeptides encrypted in the soy proteins are mainly responsible for the antioxidant properties of the hydrolyzed samples.

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