



Enzyme assisted degradation of potential soy protein allergens with special emphasis on the technofunctionality and the avoidance of a bitter taste formation



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ABSTRACT

Soy protein hydrolysates are often rejected by consumers due to their unpleasant bitter taste. In the present study, soy protein isolate (SPI) was hydrolyzed using five proteases and combinations thereof to prevent bitterness. Hydrolysis was performed as one- and two-step process and the extent of hydrolysis was evaluated by SDS-PAGE and degree of hydrolysis analyses. Enzymatic hydrolysis performed as both, one- and two-step process, increased the degree of hydrolysis from initially 2.1% up to 30%. Most peptides generated were < 20 kDa and major soybean allergens (glycinin, β -conglycinin) were effectively degraded as shown by SDS-PAGE and liquid chromatography mass spectrometry (LC-MS/MS) analyses. The bitterness of SPI hydrolysates was evaluated by a sensory panel on a 10-cm continuous scale, ranging from 0 (no perception) to 10 (strong perception). Bitterness of the resulting hydrolysates was remarkably reduced to a minimum of 1.3 compared to unhydrolyzed SPI with a bitterness intensity of 2.8. Hydrolysates showed increased protein solubility at both pH 4.0 and 7.0, emulsifying capacity up to 810 mL g⁻¹ and foaming activity up to 2706%. The study shows that enzyme combinations are an effective approach to produce hypoallergenic soy hydrolysates that combine low bitterness and superior techno-functional properties.

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1. Introduction

Soybean (*Glycine max* (L.) MERR.) is the most important valuable crop that provides the largest source of vegetable protein to human diet. Particularly, soy protein isolates are often favoured as functional and nutritional ingredients due to the high protein content (ca. 90%).

However, consumption of soy-containing food products can cause severe and even fatal allergic reactions such as anaphylactic shock. The Food and Agriculture Organization of the United Nations (FAO) listed soybean as one of the eight priority allergens ("big 8") which comprises those foods that cause 90% of all Immunoglobulin E (IgE)-mediated allergies (FDA, 2004). At least 16 IgE-binding soy proteins have been characterized, but only the two storage proteins Gly m5 (β -conglycinin) and Gly m6 (glycinin) have been suggested

to be the major fractions containing allergens (Amnuaycheewa & de Mejia, 2010; FARRP, 2015; Holzhauser et al., 2009).

In recent years, the need to control soybean allergy by methods other than avoidance has spurred the development of new technologies, including genetic modifications as well as thermal and non-thermal treatments (Shriver & Yang, 2011; Verhoeckx et al., 2015). An effective approach to reduce the allergenicity of soybean is the enzymatic hydrolysis, which has already been proven in numerous studies using selective proteases from animal, plant or microbial origin (Wilson, Blaschek, & de Mejia, 2005; Yamanishi et al., 1996). Generally, enzymatic hydrolysis is widely applied to upgrade the functional features, including solubility, foaming and emulsifying properties (Ortiz & Wagner, 2002; Tsumura et al., 2005).

However, protein hydrolysates are accompanied by the formation of bitter peptides, which impedes their utilization as food ingredients. The bitterness of hydrolysates is due to the release of low molecular weight peptides composed of mainly hydrophobic amino acid residues (Ishibashi et al., 1988; Ney, 1979; Seo, Lee, &

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Baek, 2008). Thus, the prevention, reduction, or elimination of bitter taste is essential to make the hydrolysates acceptable to consumers.

Effective debittering methods, however, have not yet been successfully developed. Numerous options have been investigated in the debittering of food protein hydrolysates including selective separation or adsorption of bitter peptides such as treatment with activated carbon, extraction with alcohol, isoelectric precipitation, and chromatography (Saha & Hayashi, 2001). The bitterness in hydrolysates was also masked via the addition of various agents such as polyphosphates, specific amino acids and through the admixture with intact proteins (FitzGerald & O'Cuinn, 2006; Sujith & Hymavathi, 2010; Sun, 2011). Debittering via transpeptidation of specific amino acids by T-plastein reaction in addition to cross-linking using microbial transglutaminase as well as the modification of taste signaling have also been conducted (Sun, 2011).

Much effort has been made in peptidase-mediated debittering of protein hydrolysates that means an enzymatic hydrolysis of bitter peptides with, particularly exo-peptidases such as aminopeptidases, alkaline/neutral proteases and carboxypeptidases. A reduction in bitterness has been observed in proteinase hydrolysis of food proteins during concomitant or subsequent incubation with exopeptidase-rich enzyme preparations which cleave adjacent to hydrophobic amino acid residues (Chae, In, & Kim, 1998; FitzGerald & O'Cuinn, 2006).

Although many studies have been applied in either the reduction of the allergenicity or the debittering of hydrolysates, literature on an effective enzyme combination for sufficiently reducing both allergenicity and bitterness, while improving the techno-functional properties, is not available.

The present study was conducted to investigate (1) the effectiveness of different enzyme combinations on the degradation of the major soybean allergens (glycinin, β -conglycinin), (2) their effects on the bitter taste, and (3) the techno-functional properties of the resulting hydrolysates. Protein degradation was evaluated by determining the degree of hydrolysis as well as electrophoresis (SDS-PAGE) and mass spectrometry (LC-MS/MS) analyses. A specific emphasis has been given to the debittering effect of enzyme combinations. The effects on the techno-functional properties (protein solubility, emulsifying capacity, foaming activity, density, and stability) were also studied.

2. Materials and methods

2.1. Raw materials and chemicals

Soybeans (*G. max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany). Alcalase® 2.4 L FG (2.4 AU-A/g, endoprotease from *Bacillus licheniformis*), Flavourzyme® 1000 L (1000 LAPU/g, endo- and exoprotease from *Aspergillus oryzae*), and Neutrase® 0.8 L (0.8 AU-N/g, endoprotease from *Bacillus amyloliquefaciens*) were kindly provided by Novozymes A/S (Bagsvaerd, Denmark). Papain (cysteine-protease from *papaya latex*) (≥ 10 units/mg, E.C. 3.4.22.2, Sigma no P4762) was purchased from Sigma-Aldrich Inc. (St. Louis, U.S.A) and Corolase® 7089 (850 UHb/g, endopeptidase from *Bacillus subtilis*) was kindly provided by AB Enzymes GmbH (Darmstadt, Germany).

All chemicals used were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) if not stated separately.

2.2. Preparation of soy protein isolates (SPI)

Soy protein isolate (SPI) was prepared from soybean seeds (*G. max* (L.) MERR.) as previously described by Meinschmidt,

Sussmann, Schweiggert-Weisz, and Eisner (2016).

2.3. Enzymatic hydrolysis of SPI

For the experiments, five different commercially available food-grade enzymes were used. These protease preparations are commonly used in the food industry for the production of protein hydrolysates. Reaction conditions (50 °C and pH 8.0) were chosen according to producers' application sheet. The dosage of 0.5%, 0.05% and 1% for endo- and exoproteases has been applied according to unpublished preliminary experiments.

Enzymatic hydrolysis was performed with various enzyme combinations (Table 1) in thermostatically controlled reactors. Therefore, SPI was dispersed in deionized water at a protein concentration of 5% (w/w) and adjusted to 50 °C and pH 8.0 with 1 M NaOH prior to enzyme addition. A total volume of 1000 mL was used for the proteolysis reaction. Hydrolysis was performed either as one-step or two-step process and 10 enzyme combinations were carried out using various endo- and exoproteases (Table 1). The enzyme concentrations used were 500 LAP-U/g for Flavourzyme, 0.6 AU-A/g for Alcalase, 212.5 UHb/g for Corolase 7089, 0.2 AU-N/g for Neutrase and ≥ 250 units/g for Papain, which correspond to an enzyme to substrate ratio (E/S) of 1% (v/w), 0.5% (v/w), 0.5% (v/w), 0.5% (v/w) and 0.05% (w/w) of the total protein, respectively.

For the one-step process, enzymes were simultaneously added to the vessel and SPI was digested for 4 h, while aliquots were taken after 30, 120, and 240 min. During the two-step process, an endoprotease was applied in the first hydrolysis stage for pre-digestion (see Table 1). After 1 h incubation, Flavourzyme (1% v/w) was added for subsequent debittering by additional hydrolysis for another 2 h and aliquots were taken after 30, 120, and 240 min. During hydrolysis, the mixture was stirred, while the pH-value and temperature were maintained constantly.

Hydrolysis was stopped by heat treatment at 90 °C for 20 min. Control dispersions (no enzyme addition) were prepared under the same incubation conditions and heat inactivation treatment. All samples were frozen at –50 °C and lyophilized (BETA 1–8, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Experiments were performed in duplicate.

2.4. Determination of protein degradation due to enzymatic hydrolysis

2.4.1. Degree of hydrolysis (DH) using the o-phthaldialdehyde (OPA) method

The DH of all samples was calculated in triplicate ($n = 3$) by determining the free α -amino groups with o-phthaldialdehyde (OPA) using serine as standard according to Nielsen, Petersen, and Dambmann (2001).

2.4.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution of the samples was determined according to Laemmli (1970) using SDS-PAGE under reducing conditions. The sample preparation and gel running conditions were chosen as described elsewhere (Meinschmidt et al., 2016). Protein visualization was performed by Criterion Stain-Free Gel Doc™ EZ Imager (Bio-Rad).

2.5. Protein identification by liquid chromatography tandem mass spectrometry (LC-MS/MS)

2.5.1. Sample preparation for LC-MS/MS analysis

Protein separation was performed using SDS polyacrylamide gel electrophoresis (SDS-PAGE). The soy protein isolate was mixed with

Table 1

Combination of protease preparations for SPI hydrolysis.

Process	System	Endoprotease (E/S %)				Exoprotease (E/S %)
		Alcalase	Corolase 7089	Neutrase	Papain	Flavourzyme
One-step	S1	0.5	—	—	—	1
	S2	—	0.5	—	—	1
	S3	0.5	0.5	—	—	1
	S4	0.5	0.5	0.5	—	1
	S5	0.5	—	0.5	—	1
	S6	—	0.5	0.5	—	1
	S7	—	—	—	0.05	1
Two-step	S8 ^a	0.5	—	—	—	1
	S9 ^a	—	0.5	—	—	1
	S10 ^a	—	—	—	0.05	1

^a SPI was hydrolyzed for 1 h with the endoprotease and after 1 h the exoprotease Flavourzyme was added and hydrolysis was continued.

1× Laemmli Buffer and incubated for 5 min at 95 °C and proteins were alkylated by adding acrylamide and incubation at room temperature (RT) for 30 min. An aliquot of 10 µL (≈ 50 µg protein) was loaded onto a 4–20% precast gel (Mini-PROTEAN® TGX™ Gels 4–20%, 10-well comb, Bio-Rad Laboratories) and the separation was performed at 200 V for 35 min using a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories). After electrophoresis the proteins were visualized by staining with Coomassie Brilliant Blue (PageBlue™ Protein Staining Solution, Thermo Fisher Scientific). The gel lane was manually cut into 20 slices and each slice was subsequently cut into 1 mm³ gel pieces. Gel pieces were destained twice with 100 µL 50% (v/v) acetonitrile (ACN, Merck KGaA), 20 mM ammonium bicarbonate (ABC, Sigma–Aldrich Chemie GmbH) at 37 °C for 30 min and dehydrated with 100 µL 100% ACN at RT for 10 min. Excess solvent was removed using a vacuum centrifuge (SAVANT DNA120 SpeedVac Concentrator, Thermo Fisher Scientific) for 30 min. The dried gel pieces were rehydrated in 20 µL 10 ng/µL trypsin solution (Trypsin NB Sequencing Grade, SERVA Electrophoresis GmbH) for 1 h on ice and 50 µL 10% (v/v) ACN, 20 mM ABC were added. The digestion was performed over night at 37 °C by gently mixing (350 rpm) and stopped by addition of 5 µL 10% (v/v) ACN, 0.5% (v/v) trifluoroacetic acid (TFA, J.T. Baker) for 30 min at RT. The supernatants were transferred into fresh reaction tubes (Safe-Lock Tubes, Eppendorf AG) and peptides were extracted twice using 20 µL 10% (v/v) ACN, 0.2% (v/v) TFA for 5 min at RT. The extracts were combined with the supernatants and dried in a vacuum centrifuge. The dried peptide extracts were redissolved in 30 µL 2% (v/v) ACN, 1% (v/v) TFA by shaking at 600 rpm for 30 min. After centrifugation at 13,000 × g for 10 min (Centrifuge 5417 C, Eppendorf AG) 20 µL of each sample were transferred into HPLC-vials prior to MS-analysis.

2.5.2. LC-MS/MS analysis

Nano-LC-ESI-MS/MS analysis was performed as described recently (Schröder, Rohrbeck, Just, & Pich, 2015; Zeiser, Gerhard, Just, & Pich, 2013) using a nano-flow ultra-high pressure liquid chromatography system (RSLC, Thermo Fisher Scientific) coupled online to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). An adequate amount of the proteolytic digests of the protein samples were injected and preconcentrated on a reversed phase trapping column (Acclaim PepMap®, C18 particle 3 µm size, 2 cm length, 75 µm i.d., pore size 100 Å, Thermo Fisher Scientific) at a flow rate of 6 µL/min with 0.1% (v/v) TFA in 5 min, followed by a separation on a 50 cm reversed phase separating column (Acclaim PepMap® RSLC nanoViper, C18 particle 2.4 µm size, 50 cm length, 75 µm i.d., pore size 100 Å, Thermo Fisher Scientific). The peptides were eluted with a multi-step binary gradient (solvent A: 0.1% (v/v) formic acid (FA, Merck KGaA), solvent B: 0.1% (v/v) FA 80% (v/v) ACN) at a flow rate of 250 nL/min.

MS survey scans were acquired in a mass range of m/z

300–1600 at a resolution of 60,000 at 400 m/z in the orbitrap analyzer. The 10 most intense ions were selected for CID fragmentation (MS/MS) with a normalized collision energy of 38 in the ion trap, taking into account a minimum intensity of 2000 counts and a dynamic exclusion of 10 s.

Raw MS data were processed using the software PEAKS Studio (Version 7.0, Ma et al. 2003) and searched against *G. max* UniProtKB/Swiss-Prot database. Proteins were stated identified by a false discovery rate of 0.01 on protein and peptide level and a minimum number of five unique peptides.

2.6. Determination of the chemical composition and techno-functional properties

2.6.1. Chemical composition

The protein and dry matter content was determined as described by AOAC methods using a protein calculation factor of $N \times 6.25$ (AOAC, 2005a, 2005b).

2.6.2. Techno-functional properties

2.6.2.1. Protein solubility. Protein solubility of the samples was determined at pH 4.0 and 7.0 following the method of Morr et al. (1985).

2.6.2.2. Emulsifying capacity. The emulsifying capacity (EC) was determined in duplicate as suggested by Wang and Johnson (2001).

2.6.2.3. Foaming properties. Foaming activity was determined according to Phillips, Haque, and Kinsella (1987). The foaming density was measured by weighing a specified quantity of foam volume. The ratio of foam volume to foam weight was defined as foaming density in g L⁻¹. The foaming stability was estimated as the percent loss of foam volume after 60 min.

2.7. Bitterness evaluation of SPI hydrolysates

Bitterness of all samples was quantified as previously described in Meinlschmidt et al. (2016). A ten-member sensory panel (7 female, 3 male) had been screened and trained with caffeine solutions over two months (1 h per session, twice a week) according to DIN 10959. An Alcalase hydrolysate (E/S 0.5% v/w, production conditions: pH 8.0, 60 °C, 180 min without pH adjustment) at different solution concentrations was chosen to assess bitterness (Meinlschmidt et al., 2016). A 10-cm continuous scale anchored from 0 (no perception) to 10 (strong perception) was taken for bitterness evaluation. For scale-calibration, Alcalase hydrolysates with concentrations of 1.0 and 2.5 g L⁻¹ (w/w) were estimated by the panel to correspond to bitter intensities of 5 and 10, respectively.

For sample evaluation, SPI hydrolysates were mixed with tap

water to a protein equivalent of 2.5 g L⁻¹ (w/w) and the pH was adjusted to pH 7.0 with 1 M NaOH. The samples (10 mL) were randomly presented to the panel in small plastic cups, which were coded by arbitrary numbers (three-digits). At each session (performed in 10 sessions, 1 h each) the panel was firstly supplied with the reference solutions containing 1.0 and 2.5% Alcalase hydrolysate. Panellists were instructed to assign bitterness scores to the samples (eight samples per session) on the 10-cm continuous scale. Water and plain crackers were served for palate cleansing in between. Each panellist did a monadic evaluation of the samples at individual speed. Sensory evaluation was at least performed in duplicate (n = 2).

2.8. Statistical analysis

All data are expressed as means ± standard deviation from at least two independent experiments. Chemical data were analyzed using one-way Analysis of variances (ANOVA) and means were generated and adjusted with Bonferroni post hoc test. Sensory data were also subjected to ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test. Statistical analysis was performed with SPSS 20.0 (SPSS for windows: SPSS Inc. Chicago, IL, USA) and probability *p* < 0.05 indicated statistically significant differences.

3. Results and discussion

The experiments were divided into two parts, first various enzyme combination systems S1–S10 (Table 1) were screened to find a proper combination for the hydrolysis of SPI (dry matter 94.4%; protein content 94.6%), while producing a low bitter taste. Based on these results, individual hydrolysates were analyzed in more detail, including the techno-functional properties.

3.1. Screening of different enzyme combination systems

3.1.1. Effect on protein degradation

3.1.1.1. Degree of hydrolysis (DH). The DH can give an initial indication for the reduction of allergenic compounds as enzymatic hydrolysis involves the cleavage of peptide bonds, resulting in a breakdown of the intact protein into peptides (Sujith & Hymavathi, 2010).

The DH was monitored during hydrolysis of SPI and the results are shown in Table 2 and Fig. 1. Native SPI had a DH value of 2.1%, whereas the DH significantly (*p* < 0.05) increased in the course of hydrolysis.

Table 2
Degree of Hydrolysis (DH) (%) of hydrolyzed SPI by different protease combinations obtained by the screening trials.

Process	System	Degree of hydrolysis (%)							
		Time of hydrolysis (min)							
		0	30	60	90	120	180	240	300
One-step	S1	2.1 ± 0.0 ^a	11.6 ± 0.3 ^b	—	—	21.5 ± 1.7 ^c	—	23.6 ± 0.2 ^c	—
	S2	2.1 ± 0.0 ^a	10.1 ± 1.6 ^b	—	—	13.5 ± 1.6 ^{b,c}	—	16.8 ± 1.6 ^{c,d}	—
	S3	2.1 ± 0.0 ^a	15.5 ± 1.3 ^b	—	—	24.7 ± 1.3 ^c	—	28.9 ± 0.8 ^d	—
	S4	2.1 ± 0.0 ^a	15.2 ± 0.4 ^b	—	—	22.3 ± 0.8 ^c	—	27.3 ± 0.8 ^d	—
	S5	2.1 ± 0.0 ^a	22.2 ± 1.1 ^b	—	—	26.9 ± 1.6 ^c	—	29.9 ± 0.2 ^c	—
	S6	2.1 ± 0.0 ^a	10.0 ± 0.5 ^b	—	—	14.1 ± 0.5 ^c	—	16.0 ± 0.5 ^d	—
	S7	2.1 ± 0.0 ^a	9.0 ± 0.3 ^b	—	—	12.0 ± 0.1 ^c	—	14.0 ± 0.3 ^d	—
Two-step	S8	2.1 ± 0.0 ^a	—	11.1 ± 0.3 ^b	19.1 ± 0.6 ^c	—	25.0 ± 0.7 ^d	—	30.0 ± 0.2 ^e
	S9	2.1 ± 0.0 ^a	—	4.1 ± 0.1 ^b	10.6 ± 0.4 ^c	—	14.4 ± 0.2 ^d	—	16.7 ± 0.1 ^e
	S10	2.1 ± 0.0 ^a	—	5.2 ± 0.1 ^b	11.2 ± 0.2 ^c	—	13.5 ± 0.1 ^d	—	14.2 ± 0.9 ^d

Results are expressed as means ± standard deviation. Means followed with different superscript letters (a,b,c,d,e) within each line are significantly different (*p* < 0.05) relating to one enzyme combination system (one-way ANOVA, Bonferroni).

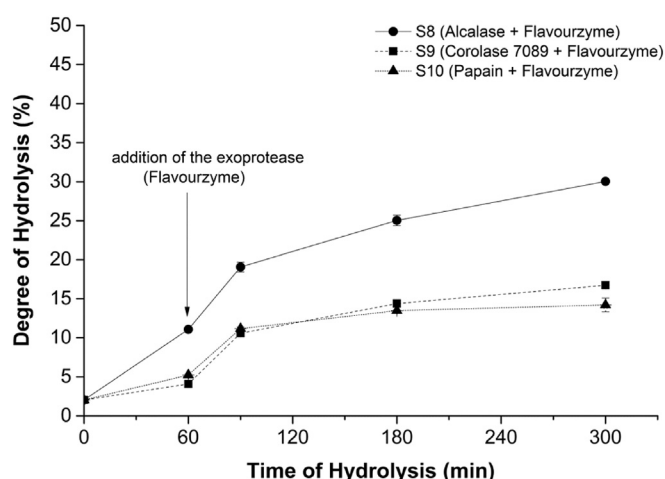


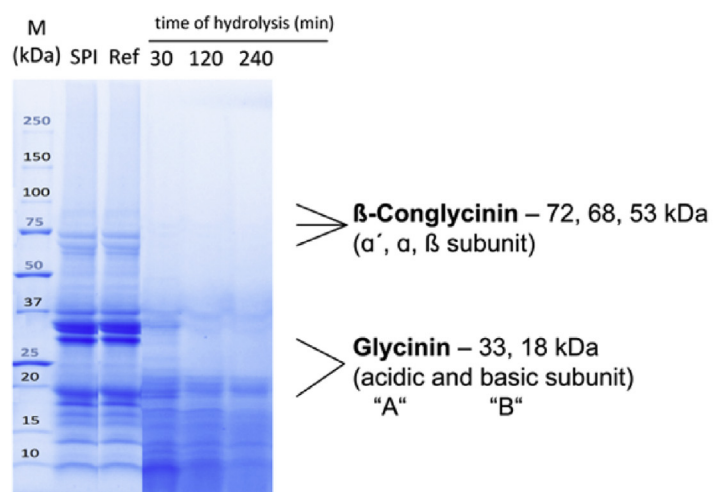
Fig. 1. Hydrolysis curves of the two-step process enzyme combination systems S8 (Alcalase + Flavourzyme), S9 (Corolase 7089 + Flavourzyme), and S10 (papain + Flavourzyme). Results are expressed as means ± standard deviation.

Among the one-step process hydrolysates, S5 reached the highest DH value of about 29.9% after 4 h, followed by S3, S4, and S1 with DH values of 28.9%, 27.3%, and 23.6%, respectively. These hydrolysates were prepared with enzyme combination systems containing Alcalase. Meinlschmidt et al. (2016) hydrolyzed SPI with single enzyme, but the same reaction conditions and a DH value of about 13% was reached after 2 h of hydrolysis with Alcalase. In contrast, hydrolysis for 2 h with a combination containing Alcalase increased the DH up to 26.9% (S6). The lowest DH values of 16.8%, 16.0%, and 14.0% after digestion for 4 h were found for S2, S6, and S7, respectively.

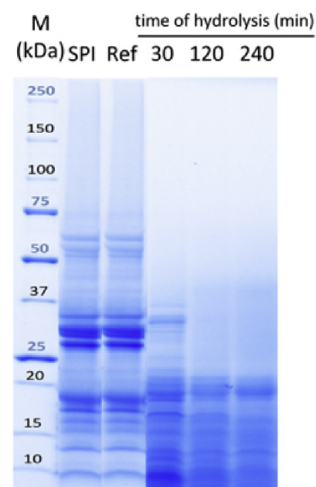
The two-step enzyme combinations S8–S10 resulted in a low initial hydrolyzation rate during the first hour of pre-digestion as evidenced by low DH values (Table 2 and Fig. 1). After the addition of Flavourzyme, the hydrolyzation rate increased rapidly during the initial stage. The cleavage of peptide bonds by the endopeptidases increases the number of end peptide sites for the action of the exoprotease Flavourzyme. S8, a combination of Alcalase and Flavourzyme, results in the highest DH value of 30% after 5 h, while S9 and S10 had lower DH values of about 16.7% and 14.2%, respectively.

3.1.1.2. Electrophoretic (SDS-PAGE) and mass spectrometry (LC-MS/MS) analyses. SDS-PAGE profiles of native SPI, references and selected SPI hydrolysates are exemplarily shown in Fig. 2. As shown

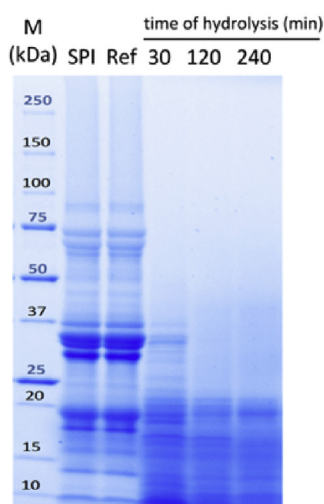
A. S1



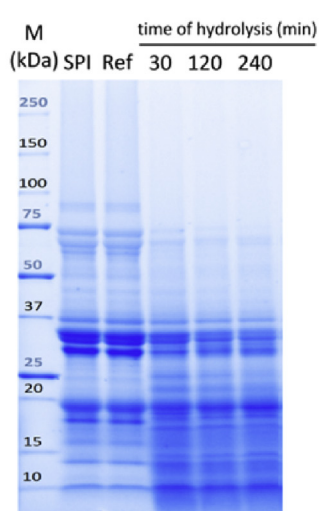
B. S3



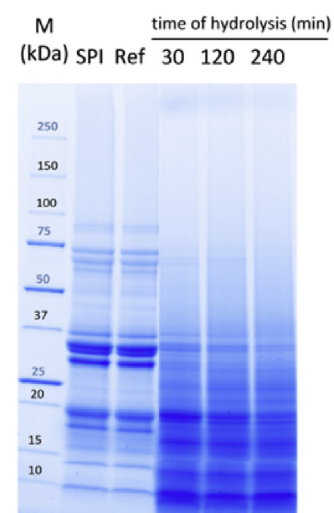
C. S5



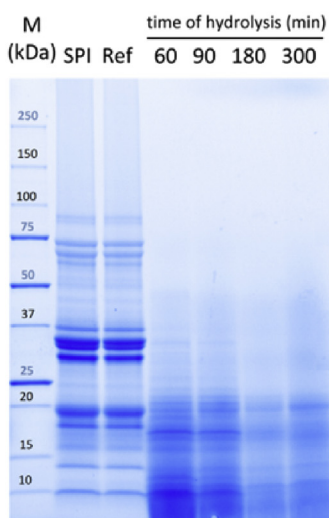
D. S6



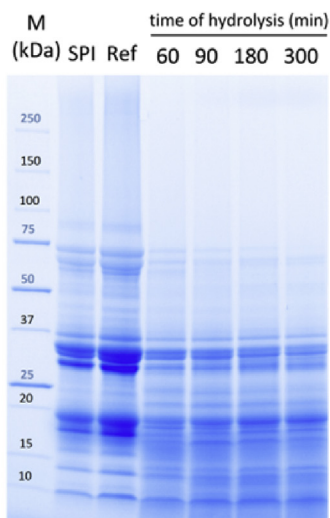
E. S7



F. S8



G. S9



H. S10

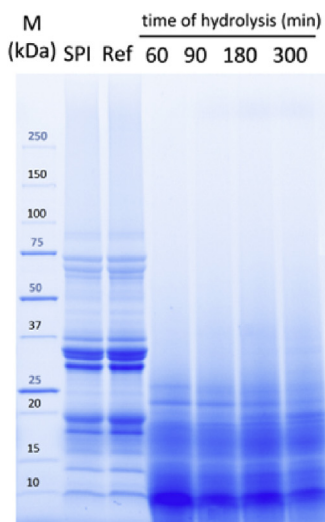


Fig. 2. SDS-PAGE patterns of SPI hydrolysates prepared with commercial available proteases in combination (screening). M- Molecular weight standard indicated in kilo Dalton (kDa); SPI- soy protein isolate; Ref-reference of each protease (no enzyme addition); Electrophoresis was carried out with 4–20% polyacrylamide gradient gels. α', α- and β-subunits of β-conglycinin; "A" and "B": acidic and basic subunit of glycinin.

in Fig. 2A, native SPI and the reference presented characteristic electrophoretic patterns for soy proteins: The first three bands are α' (~72 kDa), α (~68 kDa), and β (~53 kDa) subunits of β -conglycinin (Gly m5), followed by glycinin (Gly m6), which is composed of the acidic ("A", ~29–33 kDa) and basic subunit ("B", 18–22 kDa) (Amnuaycheewa & de Mejia, 2010).

Gel-based LC-MS/MS analyses of native SPI were additionally performed to further verify the identity and presence of the two major soy allergens β -conglycinin and glycinin, including their subunits (Fig. 3 and Table 3). Therefore, a total of 20 protein slices, consisting of both abundant and less abundant proteins were selected, digested with trypsin and analyzed. Data listed in Table 3 include selected slices and the corresponding subunits of major soy allergens, the accession number of the proteins as in *G. max* UniProtKB/Swiss-Prot database, protein confidence score (–10 lgP), number of high-confidence supporting peptides, number of high-confidence supporting peptides that are uniquely mapped to the protein as well as the sequence coverage. Although a few additional proteins have been identified, the subunits of β -conglycinin and glycinin represent the proteins with the highest score and number of supporting peptides within the assigned gel slices (see Fig. 3,

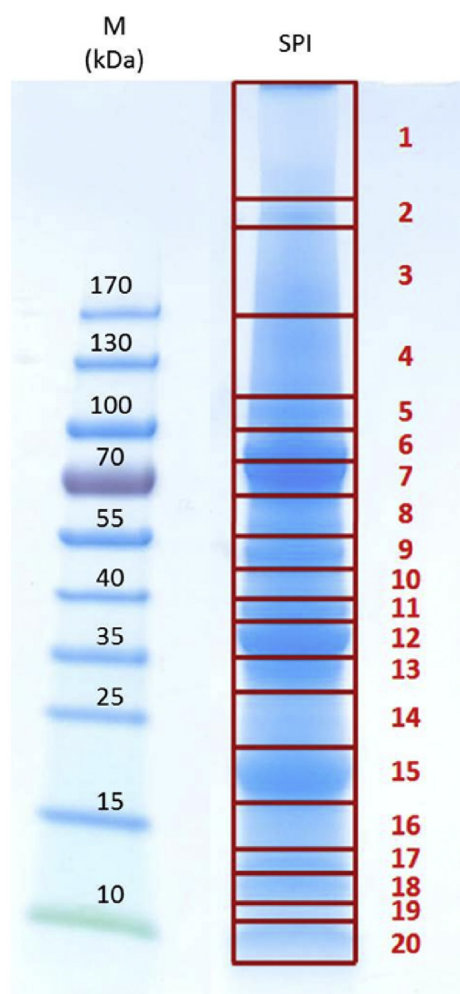


Fig. 3. SDS-PAGE pattern of native SPI. Excised protein bands used for LC-MS/MS identification of the subunits of β -conglycinin and glycinin. M – Molecular weight standard indicated in kilo Dalton (kDa); SPI – soy protein isolate; the gel lane was cut into 20 slices as indicated by red rectangles. Each slice was analyzed separately in order to determine the location of the proteins of interest.

slices 6, 7, 9, 11, 12, 15).

LC-MS/MS analysis of trypsin-digested 72, 68, and 53 kDa proteins indicated that these proteins were the α' (alphaprime)-, α (alpha)-, and β (beta)-subunits of β -conglycinin, having a high sequence coverage of 74, 62, and 84%, respectively. Furthermore, the high score values ranging from 402.64 to 463.36 gives an extremely high level of confidence that this identification is correct. The acidic and basic subunits of glycinin are represented as full-length proteins within the database. Therefore, differentiation of the location of the acidic and basic polypeptide chains in the SDS-PAGE was performed based on the respective sequence coverage and number of supporting peptides and confirmed by the high score –10 lgP values (Table 3). Hence, the acidic polypeptide chains (A1–A5) were identified with higher sequence coverage (see Table 3, sequence coverage 71–91%) and a larger number of high confident peptides in slices 11 and 12, whereas the basic polypeptide chains (G1–G5) were identified with high confidence in slice 15, corresponding to a molecular mass of 29–33 and 18–22 kDa, respectively.

Already after 30 min of hydrolysis with S1, β -conglycinin was completely decomposed and small amounts of glycinin remained within 4 h. Glycinin "A" was partially hydrolyzed, whereas glycinin "B" was more resistant. S3 (Fig. 2B), S4 (data not shown), and S5 (Fig. 2C) showed similar profiles than S1, but were more effective as glycinin "A" was instantly hydrolyzed after 2 h. Profiles of S2 (data not shown), S6 (Fig. 2D), and S9 (Fig. 2G) were merely the same and exhibit a low activity against Gly m6. Gly m5 diminished within the first 30 min, and almost completely vanished after 3 h (S9) and 4 h (S2 and S6).

The most effective combinations were the two-step systems S8 and S10, hydrolyzing all allergens to a greater degree and most of the peptides obtained after 60 min of pre-digestion were <20 kDa. After the addition of Flavourzyme, the SDS-PAGE only slightly changed. Peptides released after pre-hydrolysis were further hydrolyzed by the exo-proteases of Flavourzyme, which cleave small peptides at the end of proteins, leading to no visible effect on the electrophoretic profile.

SDS-PAGE profile of S7 (Fig. 2E) showed great changes in the molecular weight distribution, but being less effective than S10 (Fig. 2H). It is noteworthy that SDS-PAGE results of S7 (one-step) and S10 (two-step), both combinations of papain and Flavourzyme, showed visible differences in the electrophoretic profiles. These findings suggest that enzymes might compete and negatively affect each other, when hydrolyzing concomitantly. The same trend can be found for S1 and S8, where the two-step process seems to be more effective in hydrolyzing soybean allergens.

3.1.2. Effects on the bitterness

One of the attributes that reduces the consumer acceptance of protein hydrolysates is the presence of a strong bitter taste due to the presence of low molecular weight peptides composed of mainly hydrophobic amino acids (Ney, 1979; Saha & Hayashi, 2001). Bitterness was evaluated by a sensory panel on the basis of the 10-cm continuous scale (Table 4).

Native SPI showed a bitterness score of 2.6. With an exception of the hydrolysates prepared with enzyme combinations containing Alcalase (S1, S3, S4, S5, and S8), the bitterness of all hydrolysates remained widely unchanged within the first hour of hydrolysis compared to native SPI, but clearly tend to decrease at the end of hydrolysis.

All hydrolysates generated with combination systems containing Alcalase (S1, S3, S4, S5, and S8) reached higher bitterness scores up to 7.9 (S8). These results may be expected when the DH values of Alcalase containing hydrolysates were considered. It has previously been shown that hydrolysate bitterness increases with increasing

Table 3Identification of the subunits of β -conglycinin and glycinin in native SPI using LC-MS/MS analysis.

Slice	Protein (subunit)	Accession#	Score –10 lgP	# Peptides	# Unique peptides	Sequence coverage (%)
6	α' subunit β -conglycinin	P11827	463.36	188	169	74
7	α subunit β -conglycinin	P13916	402.64	56	47	62
9	β subunit β -conglycinin	P25974	412.70	123	109	84
11	acidic subunit (A3) glycinin G5	P04347	368.98	84	72	79
12	acidic subunit (A1) glycinin G1	P04776	423.36	125	90	91
	acidic subunit (A2) glycinin G2	P04405	400.02	95	51	92
	acidic subunit (A) glycinin G3	P11828	360.06	69	34	90
	acidic subunit (A4,5) glycinin G4	P02858	395.39	66	58	71
15	basic subunit glycinin G1	P04776	508.83	129	87	85
	basic subunit glycinin G2	P04405	471.94	113	78	88
	basic subunit glycinin G3	P11828	431.35	67	35	71
	basic subunit glycinin G4	P02858	522.81	116	85	73
	basic subunit glycinin G5	P04347	436.32	71	40	73

Accession: The accession number of the protein as in database.**Score (–10lgP):** The protein confidence score; p: probability.**#Peptides:** The number of high-confidence supporting peptides.**#Unique peptides:** The number of high-confidence supporting peptides that are uniquely mapped to the protein. Unique peptides with the same sequence but different modifications are only counted once in this number.**Sequence coverage:** The percentage of the protein sequence covered by supporting peptides. → Explanations given by the Software PEAKS (User Manual)**Table 4**

Sensory perception (bitterness) of hydrolyzed SPI by different proteases of the screening trials.

Process	System	Bitterness intensity							
		Time of hydrolysis (min)							
		0	30	60	90	120	180	240	300
One-step	S1	2.6 ± 0.9 ^a	4.1 ± 1.3 ^{a,b}	–	–	4.6 ± 1.5 ^b	–	4.5 ± 1.5 ^b	–
	S2	2.6 ± 0.9 ^a	3.0 ± 1.6 ^a	–	–	3.7 ± 1.5 ^a	–	2.6 ± 2.2 ^a	–
	S3	2.6 ± 0.9 ^a	3.8 ± 1.3 ^{a,b}	–	–	5.7 ± 1.0 ^c	–	4.9 ± 1.6 ^{b,c}	–
	S4	2.6 ± 0.9 ^a	4.6 ± 1.8 ^b	–	–	3.8 ± 1.4 ^{a,b}	–	6.4 ± 1.6 ^c	–
	S5	2.6 ± 0.9 ^a	5.4 ± 1.4 ^b	–	–	4.6 ± 1.4 ^b	–	4.0 ± 1.4 ^{a,b}	–
	S6	2.6 ± 0.9 ^a	2.1 ± 1.6 ^a	–	–	2.5 ± 1.6 ^a	–	2.5 ± 1.7 ^a	–
	S7	2.6 ± 0.9 ^a	3.4 ± 1.6 ^a	–	–	2.4 ± 1.2 ^a	–	2.3 ± 1.5 ^a	–
Two-step	S8	2.6 ± 0.9 ^a	–	7.9 ± 1.8 ^b	6.3 ± 1.2 ^{b,c}	–	4.9 ± 1.5 ^{c,d}	–	3.4 ± 1.5 ^{a,d}
	S9	2.6 ± 0.9 ^a	–	4.4 ± 1.6 ^a	2.0 ± 1.5 ^a	–	3.2 ± 1.5 ^a	–	1.9 ± 1.6 ^a
	S10	2.6 ± 0.9 ^a	–	2.4 ± 1.5 ^a	2.6 ± 1.5 ^a	–	1.9 ± 1.5 ^a	–	2.1 ± 0.8 ^a

Results are expressed as means ± standard deviation. Means followed with different superscript letters (a,b,c,d) within each line are significantly different ($p < 0.05$) relating to one enzyme combination system (one-way ANOVA, Tukey's HSD).

DH (Barry, O'Cuinn, Harrington, O'Callaghan, & FitzGerald, 2000). According to the findings, this hypothesis cannot be entirely confirmed as the bitterness score of hydrolysate S8 decreased in the course of hydrolysis to a minimum of 3.4 (5 h), while the DH increased from 11.1% to 30%. However, the DH values were the highest among all combinations tested, which means that bitterness can be expected to be high at high DH values.

Hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of peptides have been studied as possible influences in bitter taste of hydrolysates (Kim, Kawamura, Kim, & Lee, 2008). Several research groups found that Alcalase has the tendency to hydrolyze at hydrophobic amino acid residues, which cause a high bitterness (Adler-Nissen, 1986; Ishibashi et al., 1988). Reduction in bitterness might be due to the fact that Flavourzyme is a mixture of endo- and in particular exo-peptidase activities, which selectively release N-terminal amino acid residues, degrading bitter peptides.

3.2. Effects of selected enzyme combinations on the potential allergenicity, technofunctionality and sensory properties

Enzymatic hydrolysis with enzyme combination systems S3, S4, S5, S6, S7, S9, and S10 were repeated with respect to a less bitter taste and an effective degradation of the major soy allergens. The time of hydrolysis for each combination was chosen individually to

find a proper reaction condition and enzyme combination for hydrolyzing SPI (Table 5). In addition, the techno-functional properties in terms of protein solubility, emulsifying capacity, foaming activity as well as foaming density, and stability were examined.

3.2.1. Effect on the protein degradation

3.2.1.1. Degree of hydrolysis (DH). The results of the DH values were in accordance with the screening trials (see 3.1). The highest DH values of about 31.0%, 22.0%, and 21.6%, respectively, could be obtained by S3, S4, and S5. Lower DH values of 15.8%, 14.7%, 13.0%, and 11.9% were achieved after incubation with S9, S7, S6, and S10, respectively.

3.2.1.2. Electrophoretic analysis (SDS-PAGE). In addition to the SDS-PAGE profiles (Fig. 2), individual bands of glycinin and β -conglycinin were quantified by Image Lab™ software. SPI hydrolysis was expressed as percentage of disappearance of the protein fraction bands with respect to native SPI fractions (Table 5).

Enzyme combinations S3, S4, S5, S7, and S10 were most effective in the overall degradation with proteolytic activities of 95.8%, 98.1%, 97.6%, 97.9%, and 99.5%, respectively. These combinations completely hydrolyzed β -conglycinin subunits and glycinin "A", whereas glycinin "B" was affected with varying degrees (Fig. 2 and Table 5). The hydrolysate generated with S6 showed a less effective overall proteolytic activity of about 83.8%, evidenced by SDS-PAGE

Table 5
Degradation of SPI main allergen fractions by different proteases. Results are expressed as means $\pm$ standard deviation. Means followed with different superscript letters (a,b,c) within each column are significantly different ($p < 0.05$) following one-way ANOVA (Bonferroni).

Process	System	Time of hydrolysis (min)	Soy protein isolate (% hydrolyzed) ^a					Total average
			β-Conglycinin			Glycinin		
			α′	α	β	A	B	
One-step	S3	30	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	79.2 ± 0.8 ^a	95.8 ± 0.4 ^a
	S4	120	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	90.5 ± 1.0 ^b	98.1 ± 0.4 ^b
	S5	240	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	88.0 ± 1.3 ^b	97.6 ± 0.6 ^b
	S6	120	79.6 ± 1.0 ^b	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	39.3 ± 0.9 ^c	83.8 ± 0.4 ^c
	S7	240	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	89.7 ± 0.9 ^b	97.9 ± 0.4 ^b
Two-step	S9	300	67.6 ± 0.7 ^c	100.0 ± 0.0 ^a	27.9 ± 0.1 ^b	60.7 ± 0.3 ^b	25.8 ± 0.2 ^d	56.4 ± 0.1 ^d
	S10	90	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	97.6 ± 0.4 ^e	99.5 ± 0.2 ^b

α , α' , β : main fractions of β -conglycinin.

A: acidic fraction of glycinin.

B: basic fraction of glycinin.

^a: % of hydrolysis of each main fraction of soy protein isolate treated with different enzyme combination systems with respect to those of SPI without treatment.

analysis (see Fig. 2G). The α and β subunit of β -conglycinin and glycinin "A" were completely degraded, whereas the α' subunit and glycinin "B" were only reduced by 79.6% and 39.3%, respectively. S9 showed the smallest changes as the overall proteolytic activity was 56.4%.

3.2.2. Effects on the bitterness

The panel was asked again to evaluate the bitter taste of the hydrolysates on the basis of the 10-cm continuous scale and the results are presented in Fig. 4.

Native SPI was judged with a bitterness intensity of 2.8. The bitter taste, bitterness score 4.0, of S3 was not significantly ($p < 0.05$) higher than that of native SPI, whereas the hydrolysates generated with S4 and S5 resulted in significantly ($p < 0.05$) higher bitterness of 4.3 and 4.3, respectively. Instead, enzyme combinations containing no Alcalase preparation led to significantly lower bitterness intensities of 1.3 (S6 and S7), 1.6 (S10) and 2.2 (S9). As previously outlined, incubation of SPI with an enzyme combination containing Alcalase resulted in the highest bitterness. This fact was expected, because Alcalase was known to produce a strong bitter taste due to the release of bitter peptides, which are mainly composed of hydrophobic amino acid residues (Seo et al., 2008). Meinschmidt et al. (2016) found a bitterness score of 8.2 for a

hydrolysate obtained only by Alcalase, whereas an enzyme combination containing Alcalase facilitated a bitter intensity of 4.0 (S3).

In addition, the panel judged all protein hydrolysates as having good organoleptic properties in terms of less intense *beany* and *green* smell and taste, which are commonly negative attributes associated with soybean products (profile analysis, data not shown).

3.2.3. Effects on the technofunctionality

Soy protein hydrolysates are used in foods as functional and nutritional ingredients, and enzymatic hydrolysis is one of the most efficient methods for modifying the functional properties (Ortiz & Wagner, 2002).

3.2.3.1. Protein solubility. Protein solubility is considered a key functional property since good solubility is a prerequisite for the formation of foams and emulsions. The results are represented graphically as a function of pH 4.0 and 7.0 in Fig. 5.

The minimum solubility of 5.0% of native SPI was detected at pH 4.0, near the isoelectric point of soybean proteins, but was significantly ($p < 0.05$) higher after hydrolysis with all enzyme combination systems. Enzyme combinations containing Alcalase (S3, S4 and S5) had the highest solubility at pH 4.0 of 70.3%, 79.9% and 87.0%, respectively, whereas the other systems had a solubility

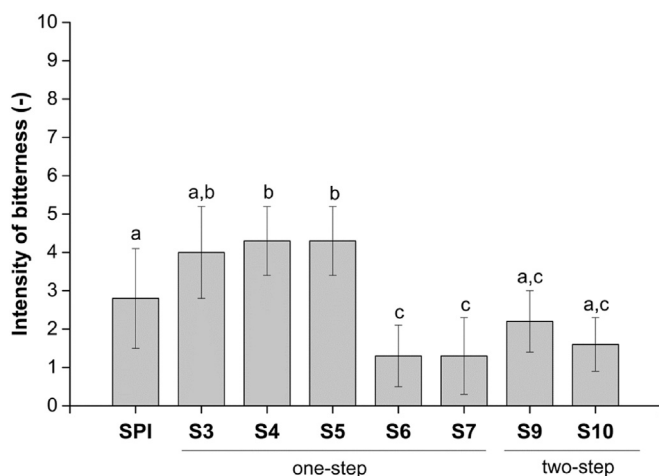


Fig. 4. Bitterness intensities of SPI and SPI hydrolysates. Each value is expressed as means $\pm$ standard deviation scored on a 10-cm line scale, ranging from 0 (no perception) to 10 (strong perception), by ten panellists. Means followed with different superscript letters (a,b,c) are significantly different ($p < 0.05$, 10 panellists) following one-way ANOVA (Tukey's HSD).

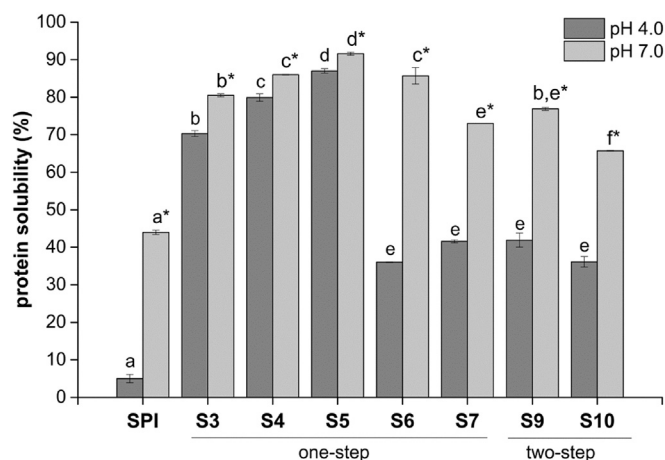


Fig. 5. Solubility of SPI and SPI hydrolysates at pH 4.0 and pH 7.0. Results are expressed as means $\pm$ standard deviation. Means followed with different superscript letters (a,b,c,d,e,f) are significantly different ($p < 0.05$) following ANOVA (Bonferroni). *indicates the solubility at pH 7.0.

between 36.0% and 41.9%. At pH 7.0 a generally higher solubility could be measured. Native SPI had a solubility of 44.0%. Applying S3, S4, S5 and S6 caused a solubility of 80.5%, 85.7%, 91.6% and 86.0%, respectively. These enzyme combinations were most effective in the reduction of the molecular weight thereby creating smaller peptides. Adler-Nissen (1986) and Ortiz and Wagner (2002) introduced that the release of smaller peptides and the corresponding increase in ionizable groups such as amino and carboxyl groups, which can interact with water molecules, are responsible for enhanced solubility.

3.2.3.2. Emulsifying properties. Native SPI had an EC of 660 mL g⁻¹, while all hydrolysates, except hydrolysates generated with S3, S4 and S5, showed a significantly ($p < 0.05$) enhanced EC. The EC increased to 810 mL g⁻¹, 750 mL g⁻¹, 700 mL g⁻¹, and 693 mL g⁻¹ after hydrolysis with S10, S6, S7, and S9, respectively. Enzymatic hydrolysis has already been shown by several studies to improve the emulsifying properties (Wu, Hettiarachchy, & Qi, 1998). The increased EC may be due to the degradation of large protein molecules, exposure of hydrophobic groups and enhanced protein solubility, wherefore protein surface activity was improved, resulting in a better emulsifying activity (Wu et al., 1998). S3 showed an EC of about 645 mL g⁻¹, which is comparable to native SPI. However, the EC of hydrolysates prepared with S4 and S5 decreased to 560 mL g⁻¹ and 480 mL g⁻¹, respectively, which might be due to a high content of small peptides, negatively affecting protein film formation since a minimum peptide length is desirable for an effective protein–protein interaction.

3.2.3.3. Foaming properties. Table 6 summarizes the foaming properties. Foaming activity of all hydrolysates was remarkably improved up to 2706% (S3), which is nearly 4-times higher than that of native SPI (552%). Due to hydrolysis, more flexible and low-molecular weight aggregates were formed. As a consequence, peptides could transfer to the air–water interface more rapidly and the protein–protein interaction improved the strength of the viscoelastic cohesive film, dropping the surface tension efficiently (Tsumura et al., 2005). The compact structure of native SPI molecules led to a lower transferring-speed of proteins to the air–water interface, lowering the foaming activity.

S6 and S9 had the lowest foaming activity of about 1522% and 1576%, respectively. There is an evidence of a trend towards increased foamability when β -conglycinin faded and glycinin became dominant (Tsumura et al., 2005). This might explain the lower foamability of S6 and S9, which were less effective in degrading β -conglycinin as evidenced by SDS-PAGE analysis (Fig. 2 and Table 5).

The results of the foaming stability and density indicated no

improvement compared to native SPI (Table 6). The most stable foam was achieved with S10 (88%), which is near to that of native SPI (90%), followed by S6, S7, S9, and S3 with 80%, 80%, 66%, and 16%, respectively. The hydrolysates prepared with S4 and S5 exhibited no foaming stability (foam decomposes directly after whipping), which might be due to excessive hydrolysis of proteins, leading to a reduced ability of polypeptides to interact at the air–water interface so that less viscoelastic films will cause decreased foaming stability (Martinez, Sanchez, Ruiz-Henestrosa, Patino, & Pilosof, 2007). Some larger protein components were essential for stable foams, which are indeed not present in S4 and S5 due to excessive hydrolysis (SDS-PAGE, Fig. 2 and Table 5).

4. Conclusions

The study was conducted to investigate the effects of enzymatic hydrolysis with various enzyme combinations on the potential allergenicity, technofunctionality, and sensory properties of SPI. The results revealed that hydrolysis with enzyme combinations turned out to be a great approach for enhancing the DH, thereby degrading the major soybean allergens, while improving the techno-functional properties. However, SDS-PAGE and the DH are indirect methods to investigate the reduction of the allergic potential. Therefore, further research should be conducted to get detailed knowledge of the allergen structure. Epitope-specific antibodies against soy allergens should be developed to assess the residual immunoreactivity using enzyme-linked immunosorbent assays (ELISA). In addition, sera from soy allergy suffering people could be useful in different immunoblots to get a deep insight in soy protein allergy. A specific emphasis has been given to the utility of enzyme combinations for debittering SPI hydrolysates, which was indeed successfully achieved, decreasing the bitter taste to a minimum of 1.3 (S6 and S7, see Fig. 4). According to the results, the two-step combination system S10 (papain + Flavourzyme) turned out to be the most appropriate enzyme combination to address a low level of intact allergenic proteins, low bitter taste, and superior techno-functional properties. Besides the organoleptic tests, the analysis of bitter peptides – their characterization and quantification – might be of particular importance to get a deeper insight in the enzyme assisted hydrolytic reactions.

Conflict of interest

The authors have declared no conflicts of interest.

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Table 6

Foaming properties (activity, density, and stability) of SPI and SPI hydrolysates.

Process	System	Foaming activity (%)	Foaming stability (%)	Foaming density (g L ⁻¹)
–	SPI	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
One-step	S3	2706 ± 0 ^b	16 ± 0 ^b	34 ± 0 ^b
	S4	2395 ± 0 ^c	0 ± 0 ^c	30 ± 0 ^c
	S5	2173 ± 0 ^d	0 ± 0 ^c	29 ± 0 ^d
	S6	1522 ± 2 ^e	80 ± 0 ^d	65 ± 0 ^e
	S7	2016 ± 0 ^f	80 ± 0 ^d	45 ± 0 ^f
Two-step	S9	1576 ± 0 ^g	66 ± 0 ^e	59 ± 0 ^g
	S10	2173 ± 5 ^d	88 ± 0 ^f	41 ± 0 ^h

Results are expressed as means ± standard deviation. Means followed with different superscript letters (a,b,c,d,e,f,g,h) within each column are significantly different ($p < 0.05$) following one-way ANOVA (Bonferroni).

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