



Soy protein hydrolysates fermentation: Effect of debittering and degradation of major soy allergens



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ABSTRACT

The debittering effect of induced liquid state fermentation (*Lactobacillus perolens*, *Rhizopus oryzae*, and *Actinomyces elegans*) on different soy protein hydrolysates has been investigated. The hydrolytic action was monitored by SDS-PAGE and degree of hydrolysis analyses. Sensory perception using quantitative descriptive analysis (QDA), employing multivariate statistical principal component analysis (PCA), techno-functional properties, and the microbial competitiveness (MALDI-TOF-MS) have been evaluated. SDS-PAGE profiles evidenced that the enzyme preparations degraded most of major soy allergens (β -conglycinin, glycinin), while subsequent fermentation did not further change the profiles. All strains investigated effectively reduced bitterness to a minimum of 0.7 on a 10-cm continuous scale (0 = no perception; 10 = strong perception) compared to non-fermented hydrolysates (2.8–8.0) and untreated soy protein isolate (2.8). Protein solubility, emulsifying and oil-binding capacity as well as foaming activity and gelation behaviour were enhanced depending on the protease used; subsequent fermentation further improved foaming stability and gelation concentration. PCA of descriptive sensory data revealed that fermentation apparently upgrade the organoleptic perception by effectively decreasing the bitter taste, simultaneously reducing the *beany* off-flavour of soy. Consequently, enzymatic hydrolysis combined with subsequent fermentation represents a promising method for the production of hypoallergenic soy hydrolysates with pleasant taste and great technofunctionality.

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1. Introduction

Food allergy is a common disease and affects approximately 220–250 million people worldwide (WAO, 2013). Both the number of incriminated foods and the frequency of severe reactions appear to be rising dramatically. Food allergens are naturally occurring proteins of the allergenic foods, and small regions, called epitopes, are responsible for the immunoglobulin E (IgE)-mediated allergic response by acting as an antigen (Taylor & Hefle, 2001).

Soybean (*Glycine max* (L.) Merr.) is one of the most important valuable crops in the world due to its considerable amount of high quality proteins and excellent functional properties. However, soy ranks among the “big 8” allergens which comprises those foods that causes over 90% of all documented food allergies in the U.S (FDA, 2004; Taylor & Hefle, 2001). Although 16 IgE-reactive allergenic proteins have been identified, just the two storage proteins glycinin (Gly m6) and β -conglycinin (Gly m5) are considered as

major soybean allergens (Amnuaycheewa & de Mejia, 2010; FARRP, 2015; Holzhauser et al., 2009).

The only method currently available to avoid any allergic reaction is complete dietary exclusion of the offending foods. As food intolerances emerged considerably in the last years, science and food industry are searching for new technologies, including thermal and non-thermal technologies to control soybean allergy other than avoidance (Shriver & Yang, 2011; Verhoeckx et al., 2015). The technological approach hitherto to decrease allergenicity has largely been empirical due to the lack of detailed knowledge on integral epitope structure. There are very few practical applications of food processing that effectively reduce allergenicity, including enzymatic hydrolysis, which has proven to be efficient in destroying epitopes (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016; Wilson, Blaschek, & de Mejia, 2005; Yamanishi et al., 1996). However, a serious quality concern facing the manufacturer of hydrolysates is the formation of bitter taste, often restricting the commercial exploitation as food ingredient. Bitterness is often attributed to the release of low molecular weight peptides containing hydrophobic amino acid residues, particularly leucine,

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proline, phenylalanine, and tyrosine (Ishibashi et al., 1988), although the exact cause is not fully understood and conflicting research exists in the literature. The hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of peptide have been studied as possible influences for bitterness (Kim, Kawamura, Kim, & Lee, 2008).

Many attempts have been made to prevent, reduce, eliminate or even mask the bitterness of food hydrolysates. These include selective separation or adsorption of bitter peptides such as treatment with activated carbon, extraction with alcohol, iso-electric precipitation, chromatography on silica gel, hydrophobic interaction chromatography (Saha & Hayashi, 2001). The bitterness in hydrolysates was also masked via the addition of various agents, and through the admixture with intact proteins (FitzGerald & O'Cuinn, 2006; Sujith & Hymavathi, 2010; Sun, 2011). Debitting via transpeptidation, cross-linking as well as the modification of taste signalling have also been conducted (Sun, 2011). These methods, however, are commonly cost-effective and can cause a significant loss of essential amino acids (Pedersen, 1994).

Considerable attention has been paid to peptidase-mediated debittering of protein hydrolysates. That means an enzymatic hydrolysis of bitter peptides with, particularly exo-peptidases from different sources (FitzGerald & O'Cuinn, 2006; Ishibashi et al., 1988; Meinlschmidt, Schweiggert-Weisz, Brode, & Eisner, 2016). Exopeptidases cleave adjacent to hydrophobic amino acid residue at the C- or N-terminus of the proteins/peptides. Based on specificity for hydrolysis of synthetic substrates, they are classified as aminopeptidases, alkaline/neutral proteases and carboxypeptidases (Ishibashi et al., 1988). Microorganisms are an important source of exopeptidases, which are expressed in the media to hydrolyse proteins as nutrient source. Microbial strains such as the genus *Lactobacillus* are extensively used as a debittering starter adjunct for the production of protein hydrolysates lacking bitter taste (Raksakulthai & Haard, 2003; Saha & Hayashi, 2001). Similar effects have been observed during hydrolysis with fungal protease preparations and microbial aminopeptidases hydrolyzing bitter peptides and liberating aromatic amino acids, which are important precursors of aroma compounds (Izawa, Tokuyasu, & Hayashi, 1997). Commonly, L-leucine aminopeptidases (LAP) are commonly used for debittering as they are efficient in removing single or pairs of hydrophobic amino acids. Some of the commercial aminopeptidases that are used to reduce bitterness in foods are also from lactic acid bacteria (LAB) and fungal enzyme preparations such as *Rhizopus oryzae*, *Aspergillus oryzae* and *Aspergillus sojae* (Nampoothiri, Nagy, Kovacs, Szakacs, & Pandey, 2005). But also the carboxypeptidases from *Actinomyces elegans* are well known to reduce bitterness (Fu, Li, & Yang, 2011; Li et al., 2008).

The objective of this study was to investigate the degradation of major allergens (glycinin and β -conglycinin) of soy protein isolates (SPI) using different microorganisms as well as their ability to diminish the bitter taste. SPI hydrolysates prepared with Alcalase, papain, or an enzyme combination (papain + Flavourzyme, two-step process) were inoculated with the LAB *Lactobacillus* (*L. perolens*) as well as the two mold strains *Actinomyces* (*A. elegans*) and *Rhizopus* (*R. oryzae*). Their effect on major soybean allergens (glycinin, β -conglycinin) by means of degree of hydrolysis and SDS-PAGE has been investigated. Sensory characteristics (smell, taste, and mouthfeeling) were analyzed using principal component analysis (PCA), along with quantitative descriptive analysis (QDA). In addition, the techno-functional properties (protein solubility, emulsifying, foaming, water- and oil-binding, gelation) were also regarded as the final protein preparation should be applied as food ingredient.

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*) and Flavourzyme® 1000 L (1000 LAPU/g, endo- and exoprotease from *A. oryzae*) 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.). *Lactobacillus perolens* DSM 12744, *A. elegans* DSM 1174, and *R. oryzae* DSM 2200 were purchased from the Germany Resource Center (DSMZ, Braunschweig, Germany).

All chemicals used in this study 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)

SPI was prepared from soybean seeds using the technique as previously described by Meinlschmidt, Schweiggert-Weisz, et al. (2016). Briefly, soybeans were de-hulled, flaked, and defatted with *n*-hexane. SPI was prepared by acidic pre-extraction (pH 4.5, 1:8 w/v flakes to water ratio, 1 h) of soybean flakes. After stirring for 1 h at room temperature, the suspension was separated using a decanter (3250 $\times$ g, 60 min). Subsequently, alkaline protein-extraction (pH 8.0, 1:8 w/v, 1 h) of flakes residue was performed and the suspension was separated (3250 $\times$ g, 60 min). The supernatant was adjusted to pH 4.5 for protein precipitation, followed by centrifugation (5600 $\times$ g, 130 min). The obtained SPI was neutralized, pasteurized (70 °C, 10 min) and spray-dried.

2.3. Enzymatic hydrolysis of SPI

The enzymatic hydrolyses using Alcalase, papain, and the enzyme combination prepared with papain and Flavourzyme were performed as previously described Meinlschmidt, Schweiggert-Weisz, et al. (2016) and Meinlschmidt, Sussmann, et al. (2016). Briefly, for each hydrolysis, dispersions of SPI in sterile deionized water (5 g/100 g) were prepared and transferred into thermostatically-controlled reactors.

The hydrolysis using Alcalase (E/S: 0.5 mL/100 g protein) was conducted at 50 °C and pH 8.0 for 2 h, while the papain (E/S: 0.05 g/100 g protein) hydrolysate was prepared at 80 °C and pH 7.0 for 30 min (Meinlschmidt, Sussmann, et al., 2016). The enzyme combination was performed as two-step process, where SPI was pre-digested with papain (E/S: 0.05 g/100 g protein) for 1 h at 50 °C and pH 8.0 and subsequently incubated with Flavourzyme (E/S: 1 mL/100 g protein) for 30 min (Meinlschmidt, Schweiggert-Weisz, et al., 2016). During hydrolysis, the mixture was stirred, while pH and temperature were kept constant. To terminate the hydrolysis, samples were boiled at 90 °C for 20 min. After cooling, the hydrolysates were kept at 4 °C prior to fermentation (Fig. 1). Each hydrolysis was performed in duplicate.

2.4. Fermentation of SPI hydrolysates

2.4.1. Strains, media, growth conditions, and preparation of inocula

L. perolens, *A. elegans*, and *R. oryzae* were cultivated overnight under their respective optimal growth temperature in deMan Rogosa Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA), yeast phosphate soluble starch (YPSS) medium (15 g L⁻¹ starch, 4.0 g L⁻¹ yeast extract, 1.0 g L⁻¹ K₂HPO₄, and 0.5 g L⁻¹ MgSO₄·7H₂O) and potato dextrose (PD) broth (Carlo-Roth GmbH, Karlsruhe,

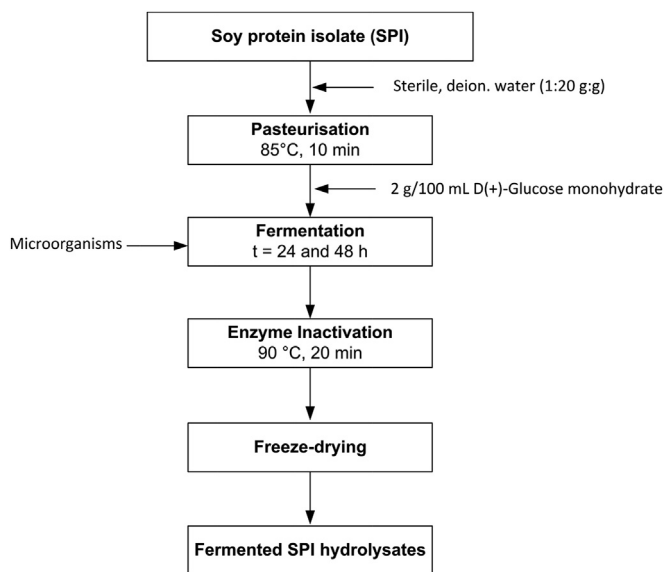


Fig. 1. Process scheme for the enzymatic hydrolysis and subsequent fermentation of SPI.

Germany), respectively (see Table 1).

For solid media, 15 g L⁻¹ agar was added to the respective broth. After cell enumeration, a calculated aliquot of the preculture was centrifuged for 10 min at 9000 × g, the pelleted cells were washed twice with Ringer solution (Merck KGaA, Darmstadt, Germany) and were resuspended in 1 mL sterile distilled water, which was used as inocula.

2.4.2. Induced liquid-state fermentation of SPI

Prior to induced liquid-state fermentation, 2 g/100 mL of glucose (Sigma–Aldrich Inc., St. Louis, USA) was dispensed into the hydrolysates to favour the growth of the inoculated strains. Three liquid-state fermentations for each SPI hydrolysate were performed, including induced fermentations with *L. perolens*, *A. elegans*, and *R. oryzae* (Fig. 1). The hydrolysates were inoculated with a start inoculation concentration of either 1 × 10⁸ colony forming units (CFU) per milliliter of *L. perolens* or 10 mL/100 mL of *A. elegans* or 20 mL/100 mL of *R. oryzae* from precultures (based on preliminary studies, data not shown).

The fermentation process was carried out aerobically at 30 °C for 6 h in conical flasks without agitation. Samples were collected after 6 h for pH value and microbial measurement and fermentation was terminated by heat treatment at 90 °C for 20 min. The pH value of the samples was measured initially and after fermentation using a disinfected pH electrode (WTW, Weilheim, Germany) calibrated at pH 7.0 and 4.0. After inactivation, the fermented samples were frozen at –50 °C and lyophilized utilizing a lyophilisator (BETA 1–8, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). All fermentations were performed in two independent replicates.

Table 1

List of employed strains including abbreviations, and growth/culture conditions.

Strain	No.	Abbreviation	Type	Growth/Culture conditions
<i>Actinomucor elegans</i>	DSM 1174	<i>A. elegans</i>	Mold	24 °C, aerob, YPSS
<i>Lactobacillus perolens</i>	DSM 12744	<i>L. perolens</i>	Lactic acid bacteria	28 °C, aerob, MRS
<i>Rhizopus oryzae</i>	DSM 2200	<i>R. oryzae</i>	Mold	30 °C, aerob, PD

DSM = Deutsche Sammlung von Mikroorganismen; MRS = DeMan, Rogosa and Sharpe; PD = potato dextrose; YPSS = yeast phosphate soluble starch.

2.5. Microbiological analyses

2.5.1. Determination of the total viable counts by measuring the colony forming units (CFU)

At time intervals of 0 h (directly after inoculation) and 6 h, the fermentation process was monitored by determining the total viable counts by measuring the number of colony forming units. Aliquots (100 µL) of fermented SPI hydrolysates were in triplicate serially diluted with Ringer solution and 100 µL of certain dilutions was spread homogeneously with a Drigalski spatula in duplicate on the surface of pre-dried plate count agar-plates (Merck KGaA, Darmstadt, Germany) for measuring the total viable counts. The hydrolysates, which were fermented with *L. perolens*, were also plated and counted on MRS agar to quantify and favour the growth of this microorganism. All dishes were incubated aerobically at 30 °C for 48 ± 2 h. The colonies were counted and expressed as colony forming units per milliliter (CFU mL⁻¹).

2.5.2. Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was performed to verify that the inoculated strains were dominant in the samples during fermentation. Fresh colony material (a single colony) was smeared on a polished steel MSP 96 target (Bruker Daltonik), spotted with 1 µL of a saturated α-cyano-4-hydroxy-cinnamic acid matrix solution in –50 mL dL acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonik) and air dried at room temperature. The analysis (n = 3) of the mass spectra was performed by a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI Biotyper software package (version 3.0). For external mass calibration, a bacterial standard (Bruker Daltonics) was used.

2.6. Determination of protein degradation due to enzymatic hydrolysis and fermentation

2.6.1. Degree of hydrolysis (DH) using the o-phthalaldehyde (OPA) method

The DH of all samples was calculated in triplicate by the determination of free α-amino groups with o-phthalaldehyde (OPA) using serine as a standard according to Nielsen, Petersen, and Dambmann (2001) and as described in Meinlschmidt, Sussmann, et al. (2016).

2.6.2. 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. Sample preparation and running conditions were chosen according to Meinlschmidt, Sussmann, et al. (2016) using 4–20% midi Criterion™ TGX Stain-Free™ precast gels. Protein visualization was performed by Criterion Stain-Free Gel Doc™ EZ Imager (Bio- Rad).

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

2.7.1. Chemical composition

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

2.7.2. Techno-functional properties

2.7.2.1. Protein solubility. Protein solubility of the samples was determined at pH 4.0 and 7.0 following the method of Morr et al. (1985). Briefly, 1 g of the sample was suspended in 50 mL 0.1 mol L⁻¹ sodium chloride solution, which was adjusted to pH 7.0 and 4.0 using –0.1 mol L NaOH or 0–1 mol L HCl, respectively. Non-dissolved fractions of the samples were separated by centrifugation at 20,000 × g for 15 min and the protein content of the supernatant was determined according to AOAC (2005b).

2.7.2.2. Emulsifying capacity. The emulsifying capacity was determined in duplicate as suggested by Wang and Johnson (2001). Briefly, protein solutions of 1 g/100 g were prepared using an Ultraturrax® (IKA-Werke GmbH & Co. KG, Staufen, Germany). Rapeseed oil was added at a constant rate of 10 mL min⁻¹ until phase inversion of the emulsion was observed, accomplished by continuous determination of the emulsion's conductivity (conductivity meter LF 521 with electrode KLE 1/T, Wissenschaftlich-technische Werkstätten GmbH, Weilheim, Germany). The volume of oil needed for phase inversion was used to calculate the EC (mL oil per g sample).

2.7.2.3. Water and oil binding capacity. Water binding capacity was analyzed according to the AACC 56-20 official method (AACC, 2000). Oil binding capacity was determined using the method described by Ludwig, Ludwig, and Pingel (1989).

2.7.2.4. Foaming properties. Foaming activity was determined according to Phillips, Haque, and Kinsella (1987) using a whipping machine (Hobart 50-N, Hobart GmbH, Offenburg, Germany). Briefly, a 5 g/100 g protein solution was whipped for 8 min. The relation of the foam volume before and after whipping of the sample was utilized for the calculation of the foaming activity. The foaming density and stability were analyzed as described elsewhere (Meinlschmidt, Sussmann, et al., 2016). In brief, the foaming density was measured by weighing a specified quantity of foam volume, where 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.

2.7.2.5. Gelation. The least gelation concentration of all samples was determined according to Sathe, Deshpande, and Salunkhe (1982). Briefly, protein samples were dispersed in 5 mL distilled water in concentrations between 2 and 20 g/100 mL. The test tubes containing the dispersions were boiled for 1 h followed by cooling in an ice bath (4 °C) for 2 h. The least gelation concentration was determined by inversion of the test tubes.

2.8. Sensory evaluation

2.8.1. Quantitative descriptive analysis (QDA)

QDA is based on the ability of panellists to reproducibly measure specific attributes of a sample to yield a comprehensive quantitative product description amenable to statistical analysis (Stone & Sidel, 1985). A ten-member sensory panel (7 female, 3 male) was selected and trained over two months (1 h per session, twice a week) according to the principles of QDA (Stone & Sidel, 1985). The panel

generated a broad list of attributes characteristic for the samples in terms of smell, taste, and mouthfeeling during several sessions (1 month, 2-times per week). The attributes and definitions are listed in Table 2.

A specific emphasis has been given to the evaluation of the bitter taste and the bitterness was quantified as previously described (Meinlschmidt, Sussmann, et al., 2016). The sensory panel had been screened and trained with caffeine solutions according to DIN 10959 to evaluate bitterness. Alcalase hydrolysates (E/S 0.5 mL/100 g, production conditions: pH 8.0, 60 °C, 180 min without pH adjustment) with concentrations of 1.0 and 2.5 g L⁻¹ were estimated by the panel to correspond to bitter intensities of 5 and 10, respectively. Each panellist did a monadic evaluation of the samples at individual speed. Sensory evaluation was at least performed in duplicate.

2.8.2. Sample preparation and sensory evaluation

For sample evaluation (performed in eight sessions, 1 h each), samples were mixed with tap water to a protein equivalent of 2.5 g/100 mL and neutralized (pH 7.0) with –1 mol L NaOH. The samples (10 mL) were randomly presented to the panel at room temperature in plastic cups, which were coded by arbitrary numbers (three-digits). To every session, the reference solutions containing 1.0 g/100 mL and 2.5 g/100 mL Alcalase hydrolysate were given to the panellists. Water and plain crackers were provided for palate cleansing in between.

The panel was instructed to evaluate the attributes (Table 2) of four samples at each session on a 10-cm continuous scale, ranging from 0 (= no perception) to 10 (= strong perception).

2.8.3. Multivariate statistical data analysis – principal component analysis (PCA)

PCA is a widely used multivariate analytical statistical technique that can be applied to QDA data to reduce the set of dependent variables to a smaller set of underlying variables based on patterns of correlation among the original variables. PCA attempts to identify underlying variables or factors that explain the pattern of correlations within a set of observed variables. It is a powerful method to identify factors that do have impact on the sensory profile. Therefore, the results of QDA were processed by means of PCA using the R-based PanelCheck software (Nofima, Ås, Norway; version 1.4.0.).

2.9. Statistical analysis

Statistical analysis of chemical data was performed using SPSS 20.0 (SPSS v 20.0 SPSS Inc. Chicago, IL, USA). All data were analyzed using one-way analysis of variances (ANOVA) and means were generated and adjusted with the Bonferroni post hoc test. Descriptive sensory data were also subjected to one-way ANOVA with the use of Tukey's honestly significant difference (HSD) post hoc test. Probability $p < 0.05$ indicated statistically significant differences.

3. Results and discussion

3.1. Microbial growth during fermentation

In a preliminary study (data not shown), 25 different microorganisms from lactobacilli, molds and yeasts were screened in different inoculation concentrations. Beyond the LAB group, *L. perolens* showed the most promising results for bitterness reduction of SPI. This microorganism was therefore included in this study, although it is known to produce off-flavours in soft drinks (Back et al., 1999) and is therefore commonly not tolerated in foods.

Table 2

Sensory attributes and definitions used in the profile analysis of SPI, SPI hydrolysates, and the fermented SPI hydrolysates.

Attribute group	Attribute	Description
Smell	Buttery	Smell associated with diacetyl and cream cheese
	Beany	Smell of raw soybeans/legumes; dusty/musty
Taste	Buttery	Taste associated with diacetyl and cream cheese
	Bitter	Fundamental taste sensation elicited by caffeine, quinine
	Beany	Legume- or soybean-like, green; dusty/musty; aroma, flavour and aftertaste associated with raw soybeans
Mouthfeeling	Mouthcoating	Degree of coating inside the mouth after swallowing
	Astringent	Trigeminal sensation and aftertaste elicited by pure cranberry juice

The yeast *Saccharomyces cerevisiae* was not included in this study as the sensory characteristics of the SPI hydrolysate after fermentation with the yeast was not acceptable. Beyond the mold group, *A. elegans* and *R. oryzae* - their debittering potential was already described in Li et al. (2008) and Chien et al. (2002)- showed also the most promising results in the preliminary study.

The microbial growth was monitored by the pH changes during fermentation, the total viable counts as well as MALDI-TOF-MS analysis. Untreated SPI, containing a dry matter content of 94.4 g/100 g, a protein content of 94.6 g/100 g, and an ash content of 4.6 g/100 g, had an initial pH value of 6.7. As a representative of LAB, *L. perolens* convert glucose into mainly $\iota(+)$ -lactic acid. Therefore, the pH of all SPI hydrolysates decreased within the first 6 h from 8.0 (hydrolysate from Alcalase and the enzyme combination) and 7.0 (hydrolysate from papain) to 7.5 and 6.1, respectively. This acidification – characteristic for LAB – led to an inhibition of the growth of other competing or pathogen microorganisms (Song et al. 2008). This ability of *L. perolens* to dominate fermentation could be monitored by the determination of the total viable counts and MALDI-TOF-MS analysis of fermented samples. A slight growth of *L. perolens* was determined with 1.5×10^8 CFU mL⁻¹. MALDI-TOF-MS analyses reported a reliable identification of the inoculated strain with log(score) values of ≥ 2.3 (highly probable species identification) and confirmed the absence of other competitive strains and the dominance of *L. perolens* in the fermented samples.

Similar results have been obtained for *A. elegans* and *R. oryzae*. The pH value decreased from 8.0 (Alcalase and the enzyme combination) and 7.0 (hydrolysate from papain) to approximately 7.6 and 6.9, respectively. MALDI-TOF-MS analysis could not be conducted as the characteristic wooly texture of the mycelia made a MALDI-TOF-MS analysis impossible. Nevertheless, the visual colonies on the surface of strain-specific plates confirmed the ability of these strains to dominate the fermentation, which was also observed by Song et al. (2008).

3.2. Degradation of soy proteins due to enzymatic hydrolysis and subsequent fermentation

3.2.1. Degree of hydrolysis (DH)

The DH value gives a first indication for the reduction of allergenic compounds as the enzymatic digestion of proteins results in a breakdown of the intact protein structure into smaller peptides and amino acids due to the cleavage of peptide bonds (Nagodawithana, Nelles, & Trivedi, 2010; Sujith & Hymavathi, 2010). Untreated SPI had an average DH value of 2.1%, whereas the DH value significantly ($p < 0.05$) increased in the course of enzymatic hydrolysis. The highest DH value of 12.2% was achieved after hydrolysis with Alcalase followed by DH values of 9.7% and 4.0% by applying the enzyme combination and papain, respectively. These findings are in great accordance with previous results (Meinschmidt, Schweiggert-Weisz, et al., 2016; Meinschmidt, Sussmann, et al., 2016). However, fermentation did not led to a change in the DH

value (data not shown), which means that the proteins were not further hydrolyzed by microbial strains.

3.2.2. Electrophoretic analysis (SDS-PAGE)

Fig. 2 displays the densitograms corresponding to the bands obtained by SDS-PAGE analysis of the standard marker, untreated SPI and the SPI hydrolysates. In addition, individual bands glycinin and β -conglycinin units were quantified by Image Lab™ Software and their relative hydrolyzation in relation to untreated SPI fractions was calculated.

Untreated SPI (Fig. 2B) presented typical electrophoretic patterns for native soy proteins, showing high intensity protein bands corresponding to β -conglycinin subunits α' (~72 kDa), α (~68 kDa), and β (~53 kDa), followed by the two subunits of glycinin, the acidic subunit (“A”) at approximately 29–33 kDa and the basic subunit (“B”) at around 18–22 kDa (Amnuaycheewa & de Mejia, 2010). These bands have previously been identified and characterized using LC-MS/MS analysis (Meinschmidt, Schweiggert-Weisz, et al., 2016).

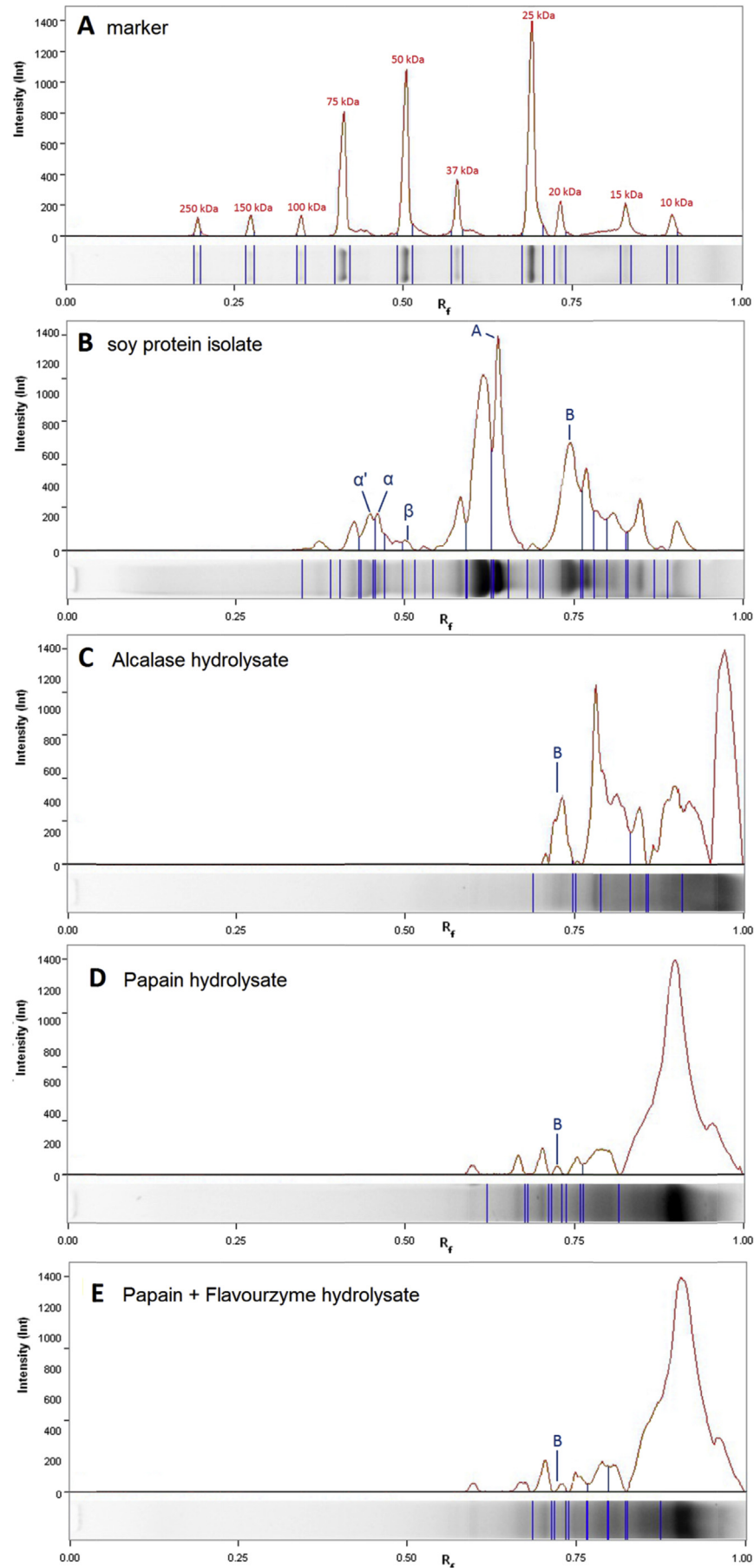
Fig. 2C–E showed that the β -conglycinin and the acidic subunit of glycinin completely disappeared after enzymatic hydrolysis applying Alcalase, papain, or the enzyme combination. However, the basic subunit of glycinin was more resistant to enzymatic hydrolysis as this subunit was not completely hydrolyzed. The subunit “B” was reduced by 97.7%, 97.6%, and 90.8% after enzymatic treatment with papain, the enzyme combination, and Alcalase, respectively. In addition, most of the peptides were <20 kDa (Fig. 2C–E), considering the resulting hydrolysates as less allergenic or even hypoallergenic (Nagodawithana et al., 2010).

However, electrophoretic profiles of the fermented hydrolysates did not show visually changes (data not shown).

3.3. Effects of enzymatic hydrolysis and subsequent fermentation on the sensory perception of SPI

3.3.1. Bitterness intensities

Fig. 3 shows the bitterness scores of all samples. Untreated SPI had a bitter intensity of 2.8 and the hydrolysates generated with papain and the enzyme combination resulted in comparable bitterness scores of 3.0 and 2.8, respectively. The bitterness of the Alcalase hydrolysate showed a significantly ($p < 0.05$) higher value of 8.0. Subsequent fermentation with all strains led to significantly ($p < 0.05$) reduced bitterness scores. After fermentation, the bitterness values of the papain hydrolysate (3.0) and the hydrolysates prepared with the enzyme combination (2.8) were 4-times lower (0.7–1.3) compared to non-fermented samples. The Alcalase hydrolysate with an initial bitterness value of 8.0 was nearly halved by fermentation with the strains to scores of 3.9, 5.1, and 5.4, respectively. Alcalase is a serine endoprotease and has the tendency to hydrolyze at hydrophobic amino acid residues, thereby, nonpolar amino acid residues at the C-terminus of the resulting peptides remain and cause a relatively high bitterness (Ishibashi et al., 1988; Saha & Hayashi, 2001; Sun, 2011). Fu et al., (2011) and Li et al. (2008)



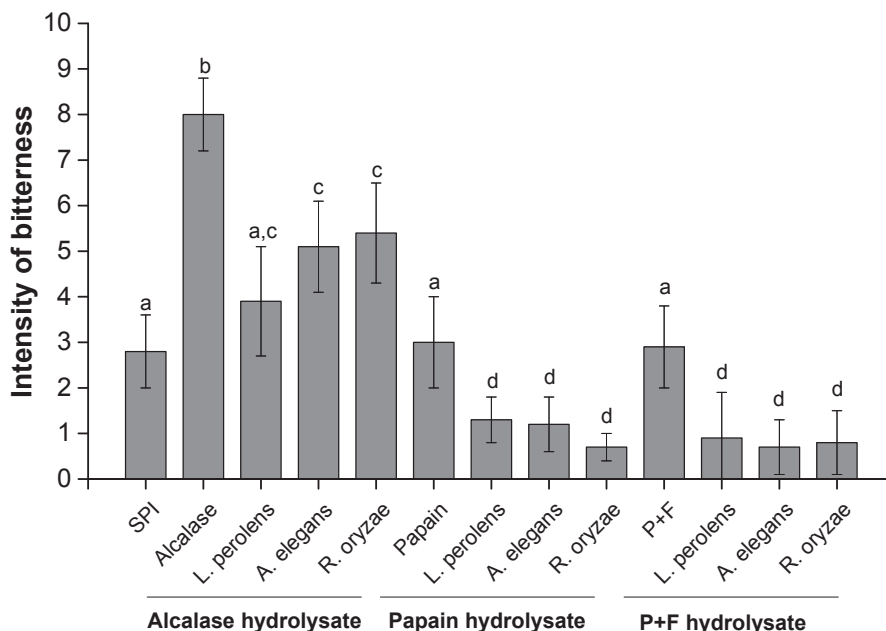


Fig. 3. Bitterness intensities of SPI, SPI hydrolysates, and fermented SPI hydrolysates. Each value is expressed as means $\pm$ standard deviation scored on the 10-cm continuous scale (0 = no perception; 10 = strong perception) by ten panellists. Means followed with different superscript letters (a,b,c,d) are significantly different ($p < 0.05$) following one-way ANOVA (Tukey's HSD).

reported that the bitterness of soybean hydrolysates produced with Alcalase decreased when the hydrolysate was treated concomitant or subsequent with an *A. elegans* extract. They concluded that the exopeptidases, particular the dominant carboxypeptidases, of *A. elegans* were responsible for the debittering effect by selectively releasing hydrophobic amino acids from the C-terminus of proteins or peptides. The exoproteolytic LAP of *R. oryzae* is able to mainly remove leucine from the N-terminus (Rahulan, Dhar, Nampoothiri, & Pandey, 2011). However, the LAP activity of *R. oryzae* required a significantly higher dose rate (20 mL/100 mL) to generate a similar reduction in bitterness than the *A. elegans* carboxypeptidases (10 mL/100 mL). This might be explained by the fact that bitterness

increases with the number of hydrophobic amino acids such as proline, leucine, tyrosine, and the carboxypeptidases of *A. elegans* is able to hydrolyze most of these residues, whereas the LAP of *R. oryzae* mainly cleaves α -leucine residues from the N-terminus. Fermentation with *L. perolens* was equally effective in debittering SPI hydrolysates than the two mold strains.

3.3.2. Descriptive sensory analysis (QDA): sensory profile

The effect of fermentation on the sensory profile (sensory attributes, see Table 2) using QDA by rating on the 10-cm continuous scale (0 = no perception; 10 = strong perception) has been determined and the results are shown in Table 3.

Table 3

Sensory evaluation (QDA) results of each sensory attribute (smell, taste, mouthfeeling) for SPI, SPI hydrolysates, and the fermented SPI hydrolysates.

	Smell		Taste			Mouthfeeling	
	Buttery	Beany	Buttery	Bitter	Beany	Mouthcoating	Astringent
SPI	2.9 $\pm$ 0.8 ^a	4.6 $\pm$ 1.1 ^a	1.3 $\pm$ 1.2 ^a	2.8 $\pm$ 0.8 ^a	6.4 $\pm$ 0.6 ^a	4.6 $\pm$ 1.3 ^a	3.3 $\pm$ 1.8 ^a
Alcalase hydrolysate							
<i>L. perolens</i>	3.3 $\pm$ 0.9 ^a	4.5 $\pm$ 0.9 ^{a,b}	0.7 $\pm$ 0.9 ^a	8.0 $\pm$ 0.8 ^b	2.7 $\pm$ 1.4 ^b	3.3 $\pm$ 1.5 ^{a,b}	5.1 $\pm$ 1.0 ^a
<i>A. elegans</i>	6.4 $\pm$ 1.2 ^b	1.3 $\pm$ 1.1 ^c	5.3 $\pm$ 1.1 ^b	3.9 $\pm$ 1.2 ^{a,c}	1.6 $\pm$ 1.0 ^c	2.4 $\pm$ 1.2 ^b	3.4 $\pm$ 1.9 ^a
<i>R. oryzae</i>	1.2 $\pm$ 0.8 ^c	3.1 $\pm$ 1.2 ^{a,c}	0.8 $\pm$ 0.8 ^a	5.1 $\pm$ 1.0 ^c	3.3 $\pm$ 1.4 ^{b,c}	2.3 $\pm$ 1.6 ^b	2.4 $\pm$ 1.0 ^{a,b}
	2.3 $\pm$ 1.6 ^{a,c}	3.3 $\pm$ 1.3 ^{a,c}	2.0 $\pm$ 1.3 ^a	5.4 $\pm$ 1.1 ^c	3.3 $\pm$ 0.9 ^{b,c}	2.4 $\pm$ 1.2 ^b	2.4 $\pm$ 1.1 ^{a,b}
Papain hydrolysate							
<i>L. perolens</i>	3.8 $\pm$ 1.3 ^a	3.6 $\pm$ 1.9 ^a	0.8 $\pm$ 1.0 ^a	3.0 $\pm$ 1.0 ^a	3.8 $\pm$ 1.8 ^{b,c}	4.4 $\pm$ 1.7 ^a	3.3 $\pm$ 1.0 ^a
<i>A. elegans</i>	6.4 $\pm$ 1.4 ^b	1.1 $\pm$ 1.2 ^c	6.2 $\pm$ 1.5 ^b	1.3 $\pm$ 0.5 ^d	1.5 $\pm$ 0.9 ^{b,c}	3.1 $\pm$ 1.0 ^a	1.6 $\pm$ 1.4 ^{a,b}
<i>R. oryzae</i>	2.2 $\pm$ 1.0 ^{a,c}	3.6 $\pm$ 1.8 ^a	1.1 $\pm$ 0.7 ^a	1.2 $\pm$ 0.6 ^d	3.2 $\pm$ 1.4 ^{b,c}	2.7 $\pm$ 1.2 ^b	2.7 $\pm$ 1.2 ^{a,b}
	4.2 $\pm$ 1.4 ^a	2.9 $\pm$ 1.4 ^{a,c}	1.8 $\pm$ 1.2 ^a	0.7 $\pm$ 0.3 ^d	2.9 $\pm$ 1.6 ^{b,c}	2.7 $\pm$ 1.5 ^b	2.2 $\pm$ 1.4 ^{a,b}
P + F hydrolysate							
<i>L. perolens</i>	3.1 $\pm$ 1.5 ^a	2.5 $\pm$ 1.6 ^{a,c}	2.3 $\pm$ 1.4 ^a	2.9 $\pm$ 0.9 ^a	2.9 $\pm$ 1.2 ^{b,c}	3.3 $\pm$ 1.3 ^{a,b}	4.1 $\pm$ 1.1 ^a
<i>A. elegans</i>	5.2 $\pm$ 1.1 ^b	1.8 $\pm$ 1.3 ^c	5.3 $\pm$ 1.2 ^b	0.9 $\pm$ 1.0 ^d	2.1 $\pm$ 1.1 ^{b,c}	2.1 $\pm$ 1.1 ^b	1.6 $\pm$ 1.1 ^{a,b}
<i>R. oryzae</i>	3.1 $\pm$ 1.5 ^a	3.3 $\pm$ 1.5 ^{a,c}	2.4 $\pm$ 1.5 ^a	0.7 $\pm$ 0.6 ^d	2.4 $\pm$ 1.1 ^{b,c}	1.9 $\pm$ 1.3 ^b	1.2 $\pm$ 1.0 ^b
	3.7 $\pm$ 1.5 ^a	2.4 $\pm$ 1.1 ^b	2.5 $\pm$ 1.5 ^a	0.8 $\pm$ 0.7 ^d	2.8 $\pm$ 1.4 ^{b,c}	2.6 $\pm$ 1.2 ^b	1.7 $\pm$ 1.5 ^{a,b}

Results are expressed as means $\pm$ standard deviation. Means followed with different superscript letters (a,b,c,d) within each column are significantly different ($p < 0.05$, 10 panellists) following one-way ANOVA (Tukey's HSD). For the sensory evaluation the 10-cm continuous scale was used (0 = no perception; 10 = strong perception).

Fig. 2. Electropherograms and the corresponding electrophoretic mobility maps obtained by SDS-PAGE of (A) molecular weight standard (marker) in kilo Dalton (kDa), (B) SPI, (C) Alcalase hydrolysate, (D) papain hydrolysate, and (E) the enzyme combination (papain + Flavourzyme, two-step process). Electrophoresis was carried out with 4–20% polyacrylamide gradient gels. R_f is the relative front/mobility; α' , α , β are the subunits of β -conglycinin; "A" and "B" are the acidic and basic subunit of glycinin.

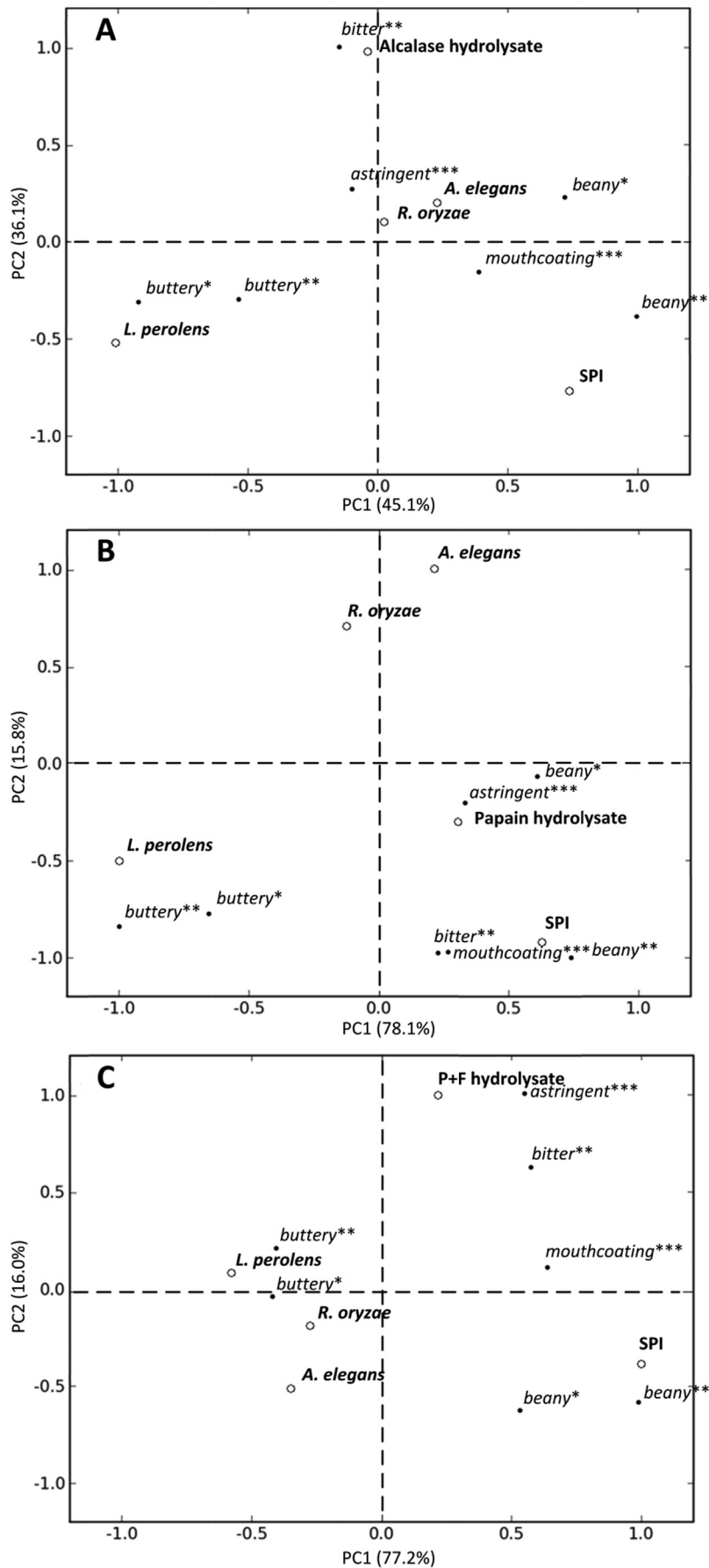


Fig. 4. Bi-plots obtained from PCA of sensory attributes (smell, taste, and mouthfeeling) for SPI, SPI hydrolyzed with Alcalase (A), papain (B), and the enzyme combination (papain + Flavourzyme, two-step process) (C), and the fermented SPI hydrolysates. * smell; ** taste; ***mouthfeeling.

Table 4

Principal component (PC) scaled loadings for SPI, SPI hydrolysates, and the fermented SPI hydrolysates obtained by principal component analysis (PCA).

Scaled loadings	Attributes	PC1	PC2
Alcalase hydrolysate	<i>Buttery</i> *	−0.795	0.077
	<i>Beany</i> *	0.796	−0.103
	<i>Buttery</i> **	−1.000	0.235
	<i>Bitter</i> **	0.195	−1.000
	<i>Beany</i> **	0.764	0.625
	<i>Mouthcoating</i> ***	0.429	0.286
	<i>Astringent</i> ***	0.063	−0.408
Papain hydrolysate	<i>Buttery</i> *	−0.678	−0.759
	<i>Beany</i> *	0.611	−0.073
	<i>Buttery</i> **	−1.000	−0.853
	<i>Bitter</i> **	0.273	−0.977
	<i>Beany</i> **	0.742	−1.000
	<i>Mouthcoating</i> ***	0.250	−0.981
	<i>Astringent</i> ***	0.338	−0.205
P + F hydrolysate	<i>Buttery</i> *	−0.453	−0.527
	<i>Beany</i> *	0.572	0.361
	<i>Buttery</i> **	−0.773	−1.000
	<i>Bitter</i> **	0.538	−0.554
	<i>Beany</i> **	1.000	−0.303
	<i>Mouthcoating</i> ***	0.615	−0.435
	<i>Astringent</i> ***	0.508	−0.688

Loadings with an absolute letter greater 0.550 are shown in bold type. * smell; ** taste; *** mouthfeeling.

Evaluation of untreated SPI resulted in the following smell-scaling: *buttery* (2.9) and *beany* (4.6); taste-scaling: *buttery* (1.3) and *beany* (6.4); mouthfeeling-scaling: *mouthcoating* (4.6) and *astringent* (3.3). Soy proteins are often not recognized due to their unpleasant *beany* off-flavour, which is due to lipid oxidation, polar lipids, and lipoxygenase catalyzed oxidation of soybean oil to volatile compounds such as isopentanol, *n*-hexanal and hexanol (Shogren, Mohamed, & Carriere, 2003). After enzymatic hydrolysis and fermentation, the *beany* taste was significantly ($p < 0.05$) reduced from initially 6.4 to a minimum of 1.5 (Table 3). This was also observed by Pinthong, Macrae, and Rothwell (1980), who found that fermented soy milk contained less *n*-hexanal, which was converted into *n*-hexanoic acid, reducing the *beany* off-flavour. After fermentation with *L. perolens*, the fresh *buttery* smell and taste of all hydrolysates were significantly ($p < 0.05$) higher compared to untreated SPI and SPI hydrolysates, increasing from initially 2.9 and

1.3 to 6.4 and 6.2, respectively, as observed for the papain hydrolysate. *L. perolens* belongs to the heterofermentative LAB family, converting the available sugars into lactic acid, acetic acid, ethanol and CO₂ via pyruvate to produce energy (Leroy & De Vuyst, 2004). Pyruvate can be converted to diacetyl, which is well known to produce a *buttery* flavour, might also be responsible for a lower bitterness, acting as masking agent.

3.3.3. Multivariate statistical analysis (PCA)

PCA was applied on QDA data to analyze the interrelationships among the samples with respect to the sensory attributes. The resulting bi-plots of the uncorrelated principal components (PCs) 1 and 2 of each hydrolysate, including untreated SPI and fermented hydrolysates, and the scaled loadings are represented in Fig. 4A–C and Table 4.

PCA on sensory data of SPI, the Alcalase hydrolysate and the fermented Alcalase hydrolysates (Fig. 4A) showed that PC1 and PC2 explained 45.1% and 36.1%, respectively, of the observed variation (81.2% in total). The major components contributing to the PC1 dimension (Table 4, factor loadings > 0.550) were *buttery* (−0.795/−1.000) as well as *beany* (0.796/0.764) smell and taste, representing a strong contrast between these two attributes. In contrast, PC2 is mainly described by the attributes *bitter* (−1.000) and *beany* (0.625) taste. The *bitter* taste is loaded on the Alcalase hydrolysate, whereas the hydrolysate fermented with *L. perolens* was well characterized by the attribute *buttery*. Untreated SPI and the hydrolysates fermented with the two mold strains were correlated with the attributes *beany*, *mouthcoating*, and *astringent*. According to the scaled loadings in Table 4, the attributes *beany* smell and taste highly loaded on PC1, dominating SPI and hydrolysates fermented with *A. elegans* and *R. oryzae*.

Fig. 4B presented the PCA results of untreated SPI, the papain hydrolysate and the fermented samples. PC1 and PC2 accounted for 93.9% of the total data variance (PC1 = 78.1%; PC2 = 15.8%). The *beany* smell and taste (scaled loadings: 0.611/0.742) positively correlate with the PC1 dimension, where untreated SPI and the papain hydrolysate are loaded. PC2 was negatively correlated to all sensory attributes; consequently, the samples fermented with the two mold strains *A. elegans* and *R. oryzae*, which are located in proximity with high intensity in the positive side of PC2, were nearly “neutral” tasting. Here, again, samples following fermentation with *L. perolens* are highly explained by *buttery* smell and taste (−0.678/−1.000).

Table 5

Techno-functional properties, including emulsifying capacity, water- and oil-binding capacity, and foaming properties (activity, stability, and density), of SPI, SPI hydrolysates, and fermented SPI hydrolysates.

Sample	Emulsifying capacity (mL g ^{−1})	Water-binding capacity (mL g ^{−1})	Oil-binding capacity (mL g ^{−1})	Foaming activity (%)	Foaming stability (%)	Foaming density (g L ^{−1})
SPI	660 ± 5 ^a	2.6 ± 0.1 ^a	0.0 ± 0.0 ^a	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
Alcalase hydrolysate	438 ± 8 ^b	0.2 ± 0.2 ^b	2.1 ± 0.1 ^b	2766 ± 0 ^b	0 ± 0 ^b	32 ± 0 ^{b,c}
<i>L. perolens</i>	508 ± 18 ^b	0.2 ± 0.1 ^b	1.7 ± 0.1 ^c	2442 ± 16 ^{c,d}	0 ± 0 ^b	37 ± 1 ^{b,c}
<i>A. elegans</i>	512.5 ± 4 ^b	0.3 ± 0.1 ^b	2.3 ± 0.2 ^b	2316 ± 16 ^{d,e,f}	4 ± 0 ^{b,c}	37 ± 4 ^{b,c}
<i>R. oryzae</i>	513 ± 4 ^b	0.0 ± 0.0 ^c	2.3 ± 0.0 ^b	2363 ± 32 ^e	2 ± 0 ^b	39 ± 2 ^{b,c}
Papain hydrolysate	705 ± 0 ^{a,c}	3.9 ± 0.2 ^d	3.3 ± 0.0 ^d	2583 ± 0 ^c	78 ± 0 ^{d,e,f}	37 ± 0 ^b
<i>L. perolens</i>	795 ± 10 ^{d,e}	3.8 ± 0.1 ^d	2.1 ± 0.0 ^b	2551 ± 63 ^c	70 ± 6 ^{d,e,f}	36 ± 1 ^{b,c}
<i>A. elegans</i>	795 ± 0 ^{d,e}	0.0 ± 0.0 ^c	2.1 ± 0.0 ^b	2268 ± 0 ^{d,e,f}	78 ± 2 ^{d,e,f}	41 ± 2 ^{b,c}
<i>R. oryzae</i>	798 ± 3 ^{d,e}	0.0 ± 0.0 ^c	2.1 ± 0.0 ^b	2300 ± 32 ^{d,e,f}	70 ± 2 ^{e,f}	45 ± 2 ^{b,c}
P + F hydrolysate	810 ± 5 ^{d,e}	0.0 ± 0.0 ^c	1.8 ± 0.1 ^c	2173 ± 0 ^f	88 ± 0 ^{a,d}	41 ± 0 ^{b,c}
<i>L. perolens</i>	818 ± 8 ^{d,e}	0.0 ± 0.0 ^c	2.2 ± 0.1 ^b	1171 ± 40 ^g	94 ± 0 ^a	88 ± 2 ^d
<i>A. elegans</i>	848 ± 53 ^d	0.0 ± 0.0 ^c	2.0 ± 0.1 ^b	1924 ± 0 ^h	14 ± 0 ^c	41 ± 3 ^{b,c}
<i>R. oryzae</i>	763 ± 4 ^{c,e}	0.0 ± 0.0 ^c	2.3 ± 0.0 ^b	1970 ± 16 ^h	4 ± 0 ^{b,c}	47 ± 1 ^c

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).

The PCA results of untreated SPI and the samples prepared with the enzyme combination (papain + Flavourzyme, two-step), including the fermented hydrolysates, are visualized in Fig. 4C and Table 4, where PC1 (16.0%) and PC2 (77.2%) explained 93.2% of the total variance. PC1, where untreated SPI is located, is highly correlated to the *beany* smell and taste (0.572/1.000) and *mouthcoating* mouthfeeling (0.615), but negatively linked to *buttery* taste (−0.773). The hydrolysate is located on the PC2 dimension, which is not described by the attribute *bitter* (−1.000). Untreated SPI and the hydrolysate prepared with the enzyme combination are described by the terms *beany*, *bitter*, *mouthcoating* and *astringent*, whereas fermentation led to a loss of these sensory attributes often negative associated with native soybeans. Fermentation with *L. perolens*, again, led to the formation of a *buttery* taste (−0.773), whereas samples fermented with the two mold strains were less explained by all sensory attributes, suggesting nearly “neutral” tasting products.

3.4. Effects of enzymatic hydrolysis and subsequent fermentation of SPI on the techno-functional properties

Functional properties of proteins connote the physicochemical characteristics, which govern the behaviour of proteins in the food matrix. Enzymatic hydrolysis is a powerful tool in the modification of technofunctionality (Ortiz & Wagner, 2002).

Untreated SPI exhibited a protein solubility at pH 4.0 and 7.0 of 5.0% and 44.0%, respectively. Enzymatic hydrolysis significantly ($p < 0.05$) increased the protein solubility at both pH 4.0 and 7.0 to a maximum of 77.4% and 82.9% (Alcalase), respectively, as previously described in Meinschmidt, Schweiggert-Weisz, et al. (2016) and Meinschmidt, Sussmann, et al. (2016). Irrespective of the applied strain, subsequent fermentation did not lead to further changes. The same trend can be found for the EC (Table 5), WBC and OBC, where subsequent fermentation did not lead to further changes compared to SPI hydrolysates (Meinschmidt, Schweiggert-Weisz, et al., 2016; Meinschmidt, Sussmann, et al., 2016).

Enzymatic hydrolysis applying Alcalase, papain, or the enzyme combination greatly enhanced the foaming activity from initially 552% up to 2766%, 2583%, and 2173%, respectively, whereas fermentation did not further changed the foaming activity (Table 5). However, fermentation of the hydrolysate prepared with the enzyme combination resulted in a significantly ($p < 0.05$) reduced foaming stability from initially 90%–14% (*A. elegans*) and 4% (*R. oryzae*), whereas fermentation with *L. perolens* increased the stability to 94%. As might be expected, the foaming density of all hydrolyzed and fermented samples significantly ($p < 0.05$) decreased from initially 215 g L^{−1} to a minimum of 40 g L^{−1}. The least gelation concentration was determined as well. Untreated SPI was able to form a gel at a protein concentration of 10 g/100 mL. Hydrolysis of SPI with Alcalase yielded in a deteriorated gelation concentrations of 12 g/100 mL, which was further negatively affected by fermentation (gelation concentration 16–18 g/100 mL). In contrast, enzymatic treatment using papain or the enzyme combination increased the gelation concentration to 8 g/100 mL, while further fermentation – except fermentation with *L. perolens* – did not further change the gelation concentration. The improved gelation concentration of 4 g/100 mL following fermentation with *L. perolens* of the hydrolysate prepared with the enzyme combination might be due to synthesized exopolysaccharides (EPS). These EPS can form an adherent cohesive layer and interact with proteins to form a gel network, which might also be the reason for increased foaming stability (94%).

4. Conclusions

The debittering of different soy hydrolysates could be achieved

to a large extent by fermentation using *L. perolens*, *A. elegans* and *R. oryzae*. However, a complete debittering of the hydrolysates was not reached, because neither carboxy- nor aminopeptidases are able to hydrolyze proline residues, which are responsible for a strong bitter taste. In order to provide non-bitter protein base as food ingredient, other microorganisms containing prolyl dipeptidyl aminopeptidase activities such as *Lactococcus lactis* might provide a powerful method to completely debitter protein hydrolysates as they can hydrolyze dipeptides with the sequence X-proline, releasing proline residues (Zevaco, Monnet, & Gripon, 1990). In addition, elucidation of the mechanisms for bitter taste perception of peptides would aid in evaluating the sensory quality of the fermented hydrolysates and for further research on general food acceptability. Identification of the resulting bitter peptides and free amino acids might also be worth further research activities in order to gain deeper insight into peptide structure and correlation between bitter peptides and taste perception.

Further research on the evaluation of the residual immunoreactivity and allergenicity using *in vivo* and *in vitro* test methods such as immunoassays including sandwich ELISA and western blot as well as clinical trials comprising oral challenge tests are indispensable future research activities. Those studies are in particular essential prior to the utilization of these fermented hydrolysates as low-allergen or hypoallergenic food ingredient with pleasant taste and superior techno-functional properties as we have shown in the current study.

Conflict of interest

The authors have declared no conflicts of interest.

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