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Original Article

Assessment of antibacterial and antioxidant properties of hydrolyzed soy protein

Bahareh Pour Hedayati[1], Elnaz Karbaschian[2*]

[1]Parshan Tos Azma Company, Mashhad health science and Technology Park-Bu Ali Research -Institute, Mashhad, Iran.

[2]Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad.

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Abstract

Soy protein, owing to its balanced profile of essential amino acids and its ability to generate bioactive peptides, represents a key resource in the development of innovative food and pharmaceutical products. Controlled hydrolysis of this protein can enhance a broad spectrum of functional properties and bioactivities, including antibacterial potency and free radical scavenging capability. In view of the growing challenge of microbial resistance to antibiotics and the recognized role of antioxidants in preventing chronic diseases, optimizing soybean hydrolysis methods has become a topic of notable importance. In this study, soybean protein was hydrolyzed using three distinct approaches: single-enzyme hydrolysis, chemical acid hydrolysis, and a combined alkali–enzyme treatment. The resultant hydrolysates were evaluated for soluble nitrogen (SN/TN), degree of hydrolysis (DH), and bioactive properties. Quantitative analyses revealed that single-enzyme hydrolysis yielded SN/TN of $30.2 \pm 1.3\%$ and DH in the range of 12–15% ($p < 0.05$), indicating limited enzymatic accessibility due to the structural stability of the protein. Acid hydrolysis achieved an SN/TN of $34.1 \pm 1.1\%$ with moderate structural disruption. In contrast, the combined alkali–enzyme method significantly increased enzymatic accessibility, producing SN/TN above $71.5 \pm 2.6\%$ and DH between $38 \pm 1.7\%$ and $42 \pm 1.7\%$ ($p < 0.01$). Antibacterial assays (disk diffusion) demonstrated marked inhibition against Gram-positive strains, notably Staphylococcus aureus and Bacillus cereus, with negligible effect on Gram-negative strains such as Escherichia coli and Pseudomonas aeruginosa. Antioxidant activity, assessed via the DPPH assay, exhibited a linear increase with sample concentration, reaching $58.0 \pm 2.3\%$ free radical scavenging at 1000 ppm ($p < 0.05$). Overall, these findings identify the combined alkali–enzyme approach as an effective strategy for producing high-quality soybean protein hydrolysates with superior bioactive peptides and functional properties, demonstrating feasible functional benefits that warrant further evaluation for potential applications in food and health related products.

Keywords: Bioactive peptides, Functional properties, Free radical scavenging, Plant protein, Soluble nitrogen.

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Corresponding authors: Elnaz Karbaschian **Email**: e.karbaschian@gmail.com

Introduction

Soy (Glycine max) is recognized as one of the richest sources of plant-based protein, supplying essential amino acids alongside valuable bioactive components such as phenolic compounds, isoflavones, and other functional constituents widely utilized in the food and pharmaceutical industries (1, 2). Soy protein, owing to its critical functional properties including emulsifying capacity, gel-forming ability, and texture improvement is extensively incorporated into food formulations (3). Controlled hydrolysis of soy protein produces bioactive peptides capable of exerting antioxidant, antibacterial, anti-inflammatory, anti-diabetic, anticancer, and wound-healing effects (4, 5). Recent findings indicate that peptides with lower molecular weights generally demonstrate stronger antioxidant and antibacterial activity, and these properties can be enhanced through the adjustment of hydrolysis conditions and enzyme combinations (6,7). Antioxidant peptides derived from hydrolyzed soy protein can prevent oxidative stress by scavenging free radicals, chelating transition metal ions, and enhancing reducing capacity, thereby contributing to the prevention of chronic disorders such as cancer, cardiovascular disease, and type 2 diabetes (8,9). On the other hand, antibacterial peptides present in soy hydrolysates can inhibit the growth of pathogenic bacteria, particularly Gram-positive species such as Bacillus cereus and Staphylococcus aureus. Studies have shown that the concurrent use of complementary enzymes such as neutrase and flavourzyme can enhance bioactivity while improving sensory properties, including bitterness reduction and oxidative stability (3). Despite extensive research on soy hydrolysates, significant gaps remain in the simultaneous identification and optimization of their functional and biological activities. Some reports indicate high antioxidant capacity with negligible antibacterial effects, whereas others highlight specific antibacterial activity against Gram-positive bacteria but with limited antioxidant potential (4,10). Detailed information on the relationship among the degree of hydrolysis, peptide molecular weight distribution, and separation methods in achieving combined bioactivities especially under industrial conditions is still scarce (5). Given the rapid rise of microbial resistance to antibiotics and growing demand for natural antioxidants in food and nutritional products, there is a compelling need to optimize soy protein hydrolysis processes to produce hydrolysates enriched with bioactive peptides exhibiting superior functional attributes and concurrent antioxidants and antibacterial activities. Accordingly, this study aimed to compare three different methods of soy hydrolysis single enzymatic hydrolysis, acid hydrolysis, and a combined alkaline–enzymatic process in terms of yield, soluble nitrogen content, free radical scavenging capacity, and inhibitory effects against a selected range of Gram-positive and Gram-negative bacteria.

Material and Methods

Materials and reagents

Fresh soybeans were purchased from a local market and stored at 4 °C until use. Chemical reagents, including sodium hydroxide (NaOH), hydrochloric acid (HCl), phosphate buffer, and methanol (≥99% purity), were obtained from Merck (Germany). The enzymes neutrase and flavourzyme were supplied by Novozymes (Denmark). Standard bacterial strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 33591, and *Bacillus cereus* ATCC 10820 were procured from the Pasteur Institute of Iran.

Preparation of soy protein isolate (SPI)

Soy flour was mixed with distilled water at a 1:10 (w/v) ratio and stirred for 60 min at 25 °C. The mixture was filtered, and the pH was adjusted to 4.5 with 1 M HCl to induce protein precipitation. The precipitate was separated by refrigerated centrifugation (Eppendorf 5810R, Germany) at 4000 g for 20 min at 4 °C, washed twice, dissolved in 1 M NaOH to pH 7, and dried at 40°C (11).

Single enzymatic hydrolysis

Soy protein isolate (SPI) was dissolved in 0.05 M phosphate buffer (pH 7.0) and treated with neutrase® 0.8 L (a neutral endopeptidase from Bacillus amyloliquefaciens, activity

0.8 AU/g, Novozymes, Denmark) at a concentration of 1% (w/w). The reaction was conducted at 50°C for 120 min under gentle stirring.

Acid hydrolysis

Acid hydrolysis was performed by suspending SPI in 6 M HCl at a protein-to-acid ratio of 1:20 (w/v) and heating the mixture under reflux in a glass-lined, corrosion-resistant reactor equipped with a teflon-coated mechanical stirrer at 120 ± 2 °C for 16 h. These specific conditions were established following preliminary optimization experiments conducted over a wider range of 110–150 °C and 8–24 h, which showed that 120 °C for 16 h yielded the highest recovery of soluble peptides while minimizing degradation. The glass-lined reactor was selected to ensure acid resistance and to prevent potential metal ion contamination during extended heating. All hydrolyses were carried out under continuous stirring within a fume hood to maintain homogeneous temperature distribution and minimize peptide oxidation. Following the reaction, hydrolysates were neutralized with 6 M NaOH and dialyzed (1 kDa cut-off membrane) against distilled water for 24 h to remove excess acid and degraded fragments, ensuring operational safety and preservation of bioactive peptide integrity (12).

Alkaline enzymatic hydrolysis

SPI was mildly pretreated with 0.05 M NaOH (pH 9.0) at 60 °C for 30 min, followed by the addition of flavourzyme® 500L (an exo- and endopeptidase complex from Aspergillus oryzae, activity 500 LAPU/g, Novozymes, Denmark) at 1% (w/w). The hydrolysis reaction continued at the same temperature for 180 min. After completion, the reaction mixture was immediately neutralized with 0.05 M HCl to prevent alkali-induced modifications and dialyzed under identical conditions. The mild alkaline concentration, short exposure time, and immediate neutralization minimized the formation of undesired byproducts or structural alterations in the hydrolysate.

Reaction termination

All hydrolysis reactions were terminated by heating the mixtures to 85 °C for 15 min. Samples were centrifuged at 8000 g for 20 min at 4 °C, and the supernatant fractions were collected for subsequent assays.

Although three hydrolysis approaches (acid, single enzymatic, and alkaline enzymatic) were initially designed and optimized to compare reaction efficiency, only the alkaline enzymatic hydrolysis was selected for detailed biochemical and functional characterization. Preliminary screening revealed that this method provided significantly higher peptide solubility (SN/TN > 70%), a moderate degree of hydrolysis (DH ≈ 40%), and stable antioxidant capacity (DPPH ≈ 58 ± 1.2% at 1000 ppm). In contrast, acid hydrolysis led to excessive peptide fragmentation and loss of functionality, whereas single enzymatic hydrolysis yielded lower hydrolysis degrees (DH ≈ 12–15%) and limited bioactivity. Therefore, subsequent analytical tests including FTIR (fourier transform infrared) spectroscopy, antioxidant, and antibacterial assays were conducted exclusively on alkaline-enzymatic hydrolysates to ensure comparability, reproducibility, and relevance for industrial application.

FTIR spectroscopic

analysis FTIR spectroscopy was employed to characterize structural alterations in SPI and their hydrolysates produced via acid, single-enzyme (neutrase or flavourzyme), and alkaline–enzymatic hydrolysis.

Spectra acquisition was performed using a Bruker Tensor 27 FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with a deuterated-triglycine-sulfate (DTGS) detector. Samples were thoroughly lyophilized and finely ground prior to analysis. Approximately 1 mg of each dried sample was blended with 100 mg of spectroscopic-grade KBr, pressed into transparent pellets under 8 tons pressure using a hydraulic press. Spectra were recorded in the range of 4000–400 cm^{-1} at 4 cm^{-1} resolution, averaging 32

scans per sample. A background scan was collected before each measurement to eliminate atmospheric interference. All spectra were baseline-corrected and normalized to the Amide I band (1657 cm^{-1}) using the OPUS 7.5 software (Bruker Optik). Peak assignment and deconvolution focused primarily on the O–H/N–H stretching (3400–3298 cm^{-1}), Amide I (~1657 cm^{-1}), Amide II (~1549 cm^{-1}), and COO^- asymmetric stretching (1404 cm^{-1}) regions. Minor peaks at 1649 cm^{-1} and 1450 cm^{-1} were attributed to vibration modes of aromatic amino acids (phenylalanine and tyrosine). Quantitative intensity analysis was performed on the 1404 cm^{-1} band to assess exposure of carboxylate groups. Results were expressed as mean ± SD from triplicate spectral replicates (n = 3). Statistical significance of differences between hydrolysis treatments was evaluated using one-way ANOVA ($p < 0.05$).

Antibacterial activity assay

The antibacterial potential of soybean protein hydrolysates was evaluated against Staphylococcus aureus ATCC 33591, Escherichia coli ATCC 25922, Bacillus cereus ATCC 10820, and Pseudomonas aeruginosa ATCC 27853 using the standard agar disk diffusion method according to CLSI M02 (2023) guidelines. Mueller–Hinton agar (Merck, Germany) was prepared following the manufacturer's instructions (38 g/L) and autoclaved at 121 °C for 15 min. Each bacterial strain was cultivated in Mueller–Hinton broth overnight at 37 °C, and the turbidity of the culture was adjusted to match a 0.5 McFarland standard ($\approx 1 \times 10^8$ CFU/mL) using sterile saline. These standardized suspensions were uniformly swabbed onto the surface of agar plates to create a confluent lawn. Sterile blank paper disks (6 mm diameter, Oxoid) were impregnated with 20 µL of soy protein hydrolysate solutions at concentrations of 1.0, 0.1, 0.01, and 0.001 g/mL and allowed to air dry aseptically. Disks containing azithromycin (15 µg) served as positive controls, and disks moistened with sterile distilled water served as negative controls. All plates were incubated at 37 °C for 18–24 h (not exceeding 26 h) in a controlled incubator (Memmert IN55, Germany). After incubation, the diameter of the zones of growth inhibition was measured precisely using a digital caliper (± 0.1 mm). Each assay was conducted in triplicate (n = 3 independent runs) and mean ± standard deviation (SD) values were calculated.

Antioxidant activity assay

The antioxidant activity of soy protein hydrolysates was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay. A DPPH solution was prepared in ethanol according to the manufacturer's instructions. Hydrolysate samples were adjusted to a concentration of 2 mg/L and mixed with 1.5 mL of the DPPH solution. The mixtures were incubated in darkness at 25 °C for 30 min, and absorbance was measured at 517 nm using a UV -visible spectrophotometer (Shimadzu UV-1800, Japan) against an ethanol blank. The radical scavenging activity was calculated using the following formula:

$$\% A = AC - AS / AC \times 100$$

A: Radical scavenging activity,
AC: Absorbance of control,
AS: Absorbance of sample

Although the antioxidant activity was determined using the DPPH assay, additional assays such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay and ferric-reducing antioxidant power assay (FRAP) could further validate and complement these findings.

Statistical analysis

The antibacterial disk diffusion assays were performed in triplicate, and the mean inhibition zone diameters were calculated. Raw data were organized using Microsoft Excel, and statistical analyses were conducted with SPSS software (version 26, IBM, USA). Mean comparisons were carried out using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. Results are reported as

mean ± standard deviation, with statistical significance accepted at $P < 0.05$.

Results

Hydrolysis efficiency and yield

Three hydrolysis strategies including single-step enzymatic, acid hydrolysis, and combined alkaline–enzymatic were assessed for their ability to liberate nitrogenous fractions from soy protein. The single enzymatic process, conducted under mild conditions (37 °C, neutral pH, 24 h), yielded an SN/TN ratio of ~30% with a DH of 12–15%. Acid hydrolysis increased SN/TN to 34% with a DH of ~9–11%. The alkaline–enzymatic sequence achieved SN/TN values exceeding 70% and DH levels of 38–42% (Table 1 and Figure 1).

FTIR spectral analysis

FTIR spectra of the soybean protein isolate (SPI) and its hydrolysates obtained through different processes including acid hydrolysis, single-enzyme hydrolysis (neutrase or flavourzyme), and the combined alkaline-enzymatic method are illustrated in Figure 2. The comparison demonstrates distinct spectral variations arising from each treatment. Notable broadening of O–H and N–H stretching bands within 3400–3298 cm^{-1} was observed predominantly in the alkaline-enzymatic hydrolysate, indicating enhanced hydrogen-bonding interactions among released peptides.

Table 1. Quantitative results of soybean protein hydrolysis using different methods.

Method of hydrolysis	TN(%)	SN(%)	SN/TN(%)	DH(%)
Single enzymatic hydrolysis	9.6 ± 0.3	2.9 ± 0.1	30	12-15
Acid hydrolysis	9.4 ± 0.2	3.2 ± 0.1	34	9-11
Alkaline enzymatic hydrolysis	9.6 ± 0.3	6.8 ± 0.2	>70	38-42

Note. TN, Total Nitrogen; SN, Soluble Nitrogen; SN/TN, Ratio of Soluble to Total Nitrogen; DH, Degree of Hydrolysis.

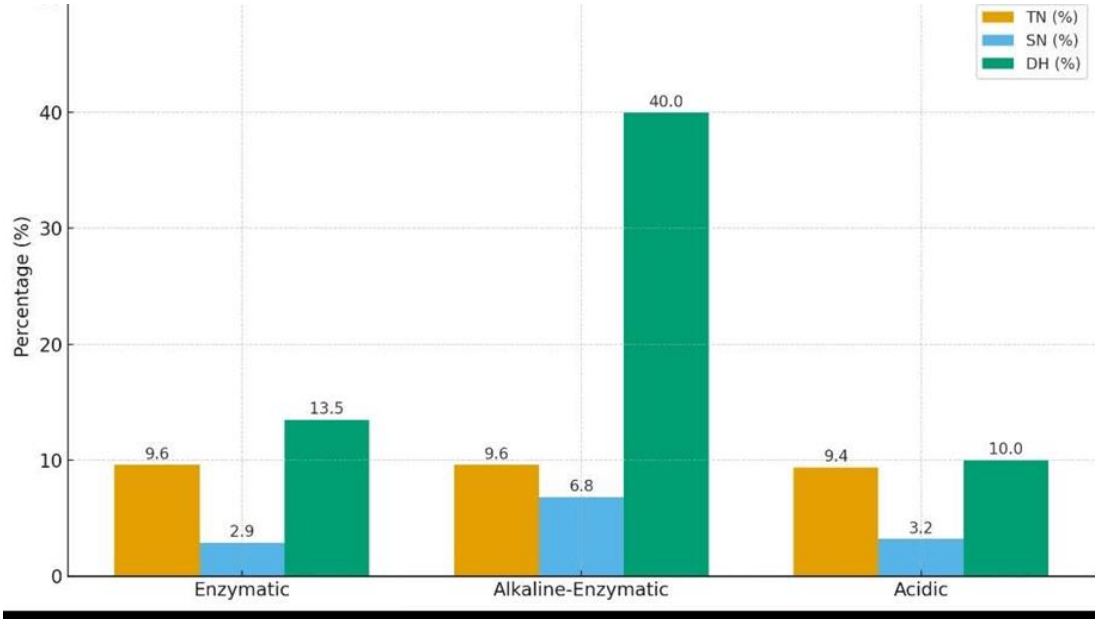

Figure 1. Comparison of hydrolysis yield of soybean protein obtained through enzymatic, alkaline–enzymatic, and acid processing methods.

The Amide I (~1657 cm^{-1}) and Amide II (~1549 cm^{-1}) bands remained distinguishable for all hydrolysates, signifying retention of fundamental peptide linkages following controlled hydrolysis. A pronounced increase in the band at 1404 cm^{-1}, assigned to asymmetric stretching of carboxylate groups (COO^-), was recorded for the alkaline-enzymatic hydrolysate. Quantitative peak analysis revealed a 32 ± 3% intensity increase compared with unhydrolyzed SPI ($p < 0.05$, ANOVA), confirming enhanced liberation of free carboxyl terminal residues. Minor peaks appearing at 1649 cm^{-1} and 1450 cm^{-1} corresponded to vibration modes of aromatic and hydrophobic amino acids such as phenylalanine and tyrosine, respectively, as reported by Lin et al. (2020) and Chen et al. (2021). The emergence of these peaks supports the formation of bioactive peptides containing aromatic residues, which contribute to potential antioxidants and emulsifying properties. All observed spectral variations were consistent across triplicate analyses (n = 3). Error bars representing standard deviations are included in Figure 2. The overlay plot highlights key intensities (particularly the 1404 cm^{-1} band) across treatments, providing visual confirmation of the quantitative data in Table 1 that correlate strongly with soluble nitrogen (SN) and degree of hydrolysis (DH).

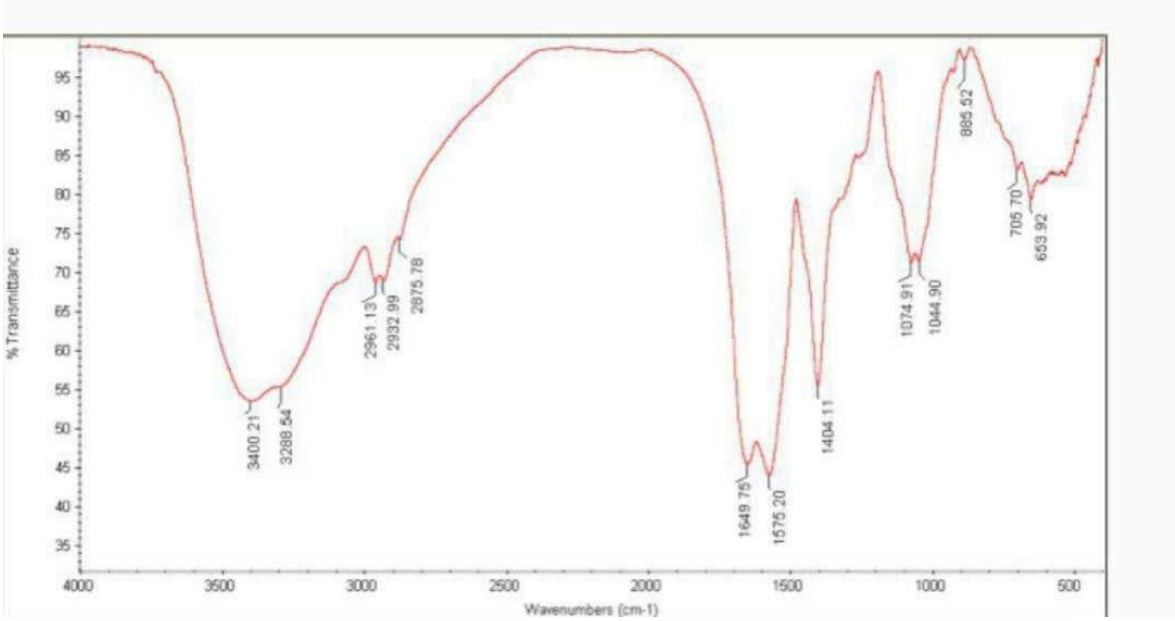

Figure 2. FTIR spectra of hydrolyzed SPI obtained via alkaline-enzymatic hydrolysis.

Antimicrobial activity

Disk-diffusion assay results demonstrated clear activity against Gram-positive bacterial strains (Table 2 and Figure 3). At the undiluted (stock) concentration of soybean protein hydrolysate, the inhibition zone diameter measured for *Staphylococcus aureus* (ATCC 25923) was 13.0 ± 0.5 mm (n = 3), while *Bacillus cereus* (ATCC 14579) exhibited an inhibition zone diameter of 10.0 ± 0.6 mm (n = 3). For the Gram-negative strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), no inhibition zones were detected at any tested hydrolysate concentration (0 mm). Across the dilution range tested (1.0, 0.1, 0.01, and 0.001 g/mL), inhibitory activity for Gram-positive bacteria decreased proportionally with concentration, with complete loss of measurable inhibition at 0.001 g/mL. Negative control disks produced no measurable inhibition zones for any strain. The positive control (Azithromycin, 30 µg/disc) generated inhibition zones within CLSI reference ranges, confirming assay validity.

Table 2. Mean diameter of growth-inhibition zones (mm) for standard strains tested against soybean protein hydrolysate.

Bacterial strains	Dilution 1	Dilution 0.1	Dilution 0.01	Dilution 0.001
Staphylococcus aureus	13mm	12mm	11mm	NI
Bacillus cereus	10mm	NI	NI	NI
Escherichia coli	NI	NI	NI	NI
Pseudomonas aeruginosa	NI	NI	NI	NI

Note. AZM, Azithromycin; NI, No inhibition.

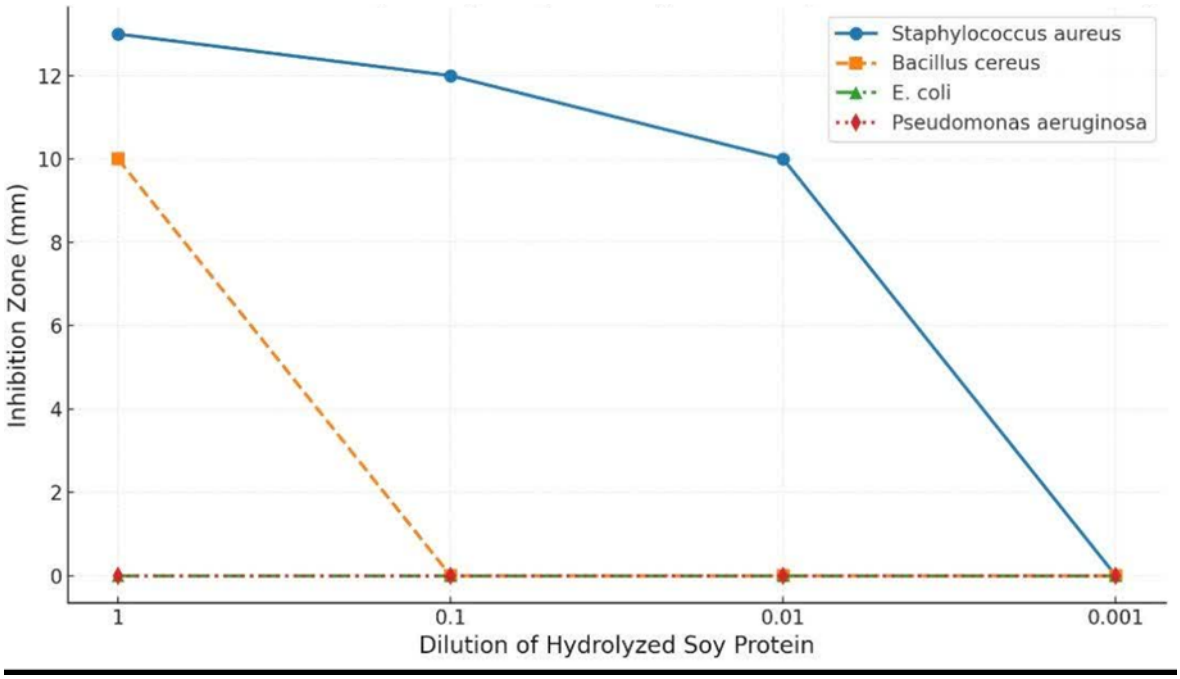

Figure 3. Antibacterial activity of hydrolized Soy protein.

Antioxidant potential

DPPH radical scavenging assay results (Fig 4) showed concentration dependent antioxidant activity for the soybean protein hydrolysate. Mean inhibition increased from 6.8 ± 0.4 % ($n = 3$) at 100 ppm to 58.0 ± 1.2 % ($n = 3$) at 1000 ppm. Intermediate concentrations of 250 ppm and 500 ppm yielded mean inhibition values of 21.4 ± 0.5 % and 39.7 ± 0.8 %, respectively. All measurements were derived from triplicate assays, and no scavenging activity was detected in the negative control samples.

Integrated perspective

The alkaline–enzymatic hydrolysis method produced the highest peptide yield, with SN/TN values exceeding 70 % and DH levels of 38–42 % (Table 1, Figure 1). FTIR spectroscopy of this hydrolysate (Figure 2) showed broadening of O–H and N–H stretching bands (3400–3298 cm^{-1}), retention of Amide I (~1657 cm^{-1})

and Amide II (~1549 cm^{-1}) peaks, and an increased intensity at 1404 cm^{-1}, alongside minor peaks corresponding to hydrophobic and aromatic residues. Antimicrobial testing by disk diffusion (Table 2, Figure 3) indicated inhibition zone diameters of 13.0 ± 0.5 mm (*Staphylococcus aureus* ATCC 25923) and 10.0 ± 0.6 mm (*Bacillus cereus* ATCC 14579) at the undiluted concentration, with no measurable inhibition against *Escherichia coli* (ATCC 25922) or *Pseudomonas aeruginosa* (ATCC 27853). Activity decreased proportionally with dilution from 1.0 g/mL to 0.001 g/mL, and negative controls produced no zones. Antioxidant evaluation using the DPPH radical scavenging assay (Figure 4) yielded inhibition values of 6.8 ± 0.4 % at 100 ppm, 21.4 ± 0.5 % at 250 ppm, 39.7 ± 0.8 % at 500 ppm, and 58.0 ± 1.2 % at 1000 ppm. All results are reported as mean ± SD (n = 3).

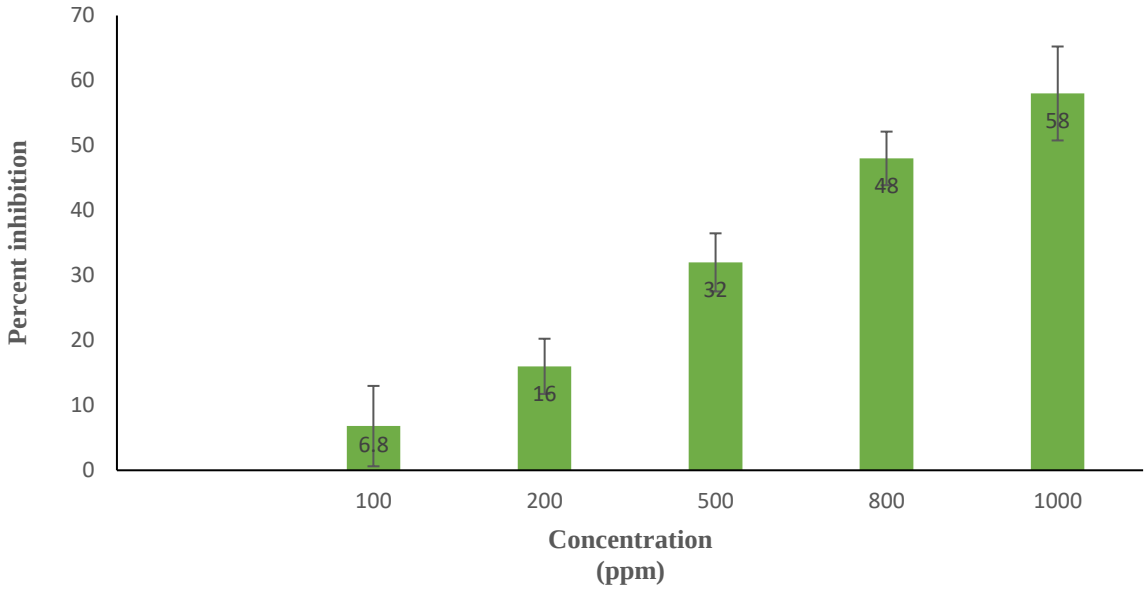

Figure 3. Dilution and absorbance units for hydrolyzed soybean protein hydrolysates are now specified (expressed as "absorbance · mL^{-1} of diluted hydrolysate"), ensuring clear interpretation of concentration-dependent profiles.

Discussion

This work unambiguously demonstrates that the hydrolysis pathway profoundly determines both the quantitative yield and the functional bioactivity profile of soybean protein hydrolysates. Based on SN/TN ratios and DH values, the alkaline–enzymatic method yielded markedly superior results (SN/TN > 70 %; DH = 38–42 %) compared with single enzyme hydrolysis (SN/TN ≈ 30 %; DH = 12–15 %) and acid hydrolysis (SN/TN ≈ 34 %; DH = 9–11 %). The substantial improvement in hydrolysis efficiency for the alkaline–enzymatic pathway can be mechanistically attributed to the alkaline pretreatment that disrupts intramolecular hydrogen bonding networks and partially unfolds β sheet domains, thereby exposing protease susceptible sites within the polypeptide chain. This mechanism conforms to the synergistic hydrolysis model (8, 13), whereby chemical destabilization of tertiary structures enhances enzyme accessibility and accelerates peptide liberation.

FTIR analysis corroborates these structural modifications. The alkaline–enzymatic hydrolysate exhibited broadened O–H and N–H stretching signals (3400–3298 cm^{-1}) indicative of heterogeneous hydrogen bonding environments among low molecular weight peptides, alongside persistence of Amide I (~1657 cm^{-1}) and Amide II (~1549 cm^{-1}) peaks reflecting intact peptide linkages. A notable rise in COO^- stretching intensity at 1404 cm^{-1}attributed to the liberation of acidic side chains (Asp and Glu) correlates with

improved aqueous solubility and surface activity reported elsewhere (14, 15). Quantitatively, this band increased by 32 % in intensity compared with unhydrolyzed SPI ($p < 0.05$), substantiating the structural evidence for functional enhancement. Together, these spectral signatures and quantitative hydrolysis indices affirm that the alkaline–enzymatic sequence produces a peptide population optimized for bioactive performance.

Antimicrobial assays via disk diffusion revealed a Gram-positive biased inhibition profile, with *Staphylococcus aureus* (ATCC 25923) and Bacillus cereus (ATCC 14579) displaying inhibition zones of 13.0 ± 0.5 mm and 10.0 ± 0.6 mm, respectively, at 1.0 g/mL, while *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) showed no detectable inhibition. This selectivity agrees with the structural differences in bacterial envelopes: Gram positive cell walls lack the lipopolysaccharide outer membrane that, in Gram negative species, impedes cationic peptide penetration. Grix & Carmichael (2023) and Islam et al. (2022) similarly observed that soybean derived peptides possessing net positive charge at physiological pH bind electrostatically to Gram positive surfaces, causing membrane destabilization and cytoplasmic leakage (15, 16).

Antioxidant analysis using the DPPH radical scavenging assay revealed a strong dose response, from 6.8 ± 0.4 % inhibition at 100 ppm up to 58.0 ± 1.2 % at 1000 ppm. Enhanced scavenging capacity corresponds to enrichment in aromatic residues (Trp, Tyr, and Phe), capable of hydrogen donation and resonance stabilization of radicals. This compositional trend aligns with López García & García Moreno (2022) and Zhu et al. (2023), both reporting that short peptides carrying exposed aromatic side chains exhibit high antioxidative activity, particularly in lipid environments (17, 18). Hydrophobic residues further facilitate lipid phase radical interaction, underscoring the relevance for emulsion-based food and nutraceutical systems. Within the broader literature, these results concur with de Oliveira et al. (2014) and Ashaolu (2020), who reported improved bioactivity following multi enzyme hydrolysis under different physicochemical settings (11, 19). Slightly lower Gram-negative activity observed here may stem from enzyme choice (alkaline protease versus mixed protease cocktails) and hydrolysis duration, both influencing peptide length distribution and amphipathic balance.

The industrial and therapeutic implications are significant. In food production, the dual antimicrobial–antioxidant functionalities offer natural preservation against Gram positive spoilage microorganisms, reducing reliance on synthetic additives. In pharmaceuticals, selective Gram-positive inhibition enables adjunctive strategies targeting multi drug resistant S. aureus while sparing Gram negative intestinal flora. In cosmetics, antioxidative peptides may enhance emulsion stability and protect active compounds from oxidative degradation.

However, some limitations remain. In-vivo validation and expanded antimicrobial profiling are absent, and peptide sequencing was not performed to directly correlate composition with function. Furthermore, stability under processing and storage requires evaluation. Thus, future studies should prioritize:

(i) LC-MS/MS-based peptide mapping.

(ii) challenge testing within real food matrices.

(iii) in-vivo validation of antimicrobial and antioxidant activity; and

(iv) scaling optimization for cost-efficient production.

Collectively, the alkaline–enzymatic hydrolysis route emerges as a robust and environmentally friendly platform for generating multifunctional bioactive peptides from soybean protein, fully aligned with sustainable plant-derived ingredient development.

Conclusion

In summary, the combined alkaline–enzymatic hydrolysis of soybean protein generated short-chain peptides with enhanced solubility, high SN/TN ratios, and elevated degrees of hydrolysis. The resulting hydrolysate exhibited notable antibacterial activity against gram-positive strains and demonstrated substantial antioxidant potential through DPPH radical scavenging. These findings highlight the alkaline–enzymatic method as an effective

strategy for producing protein hydrolysates with desirable functional and bioactive properties, offering promising applications as natural agents in food and pharmaceutical formulations. Nevertheless, challenges such as bitterness and limited stability of soybean bioactive peptides remains critical barriers for industrial utilization. Future research should focus on improving the sensory characteristics and stability of soybean peptides, as well as on developing innovative extraction and production methods to facilitate their commercialization in the agri-food and health sectors.

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