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Hemp Seed Protein Hydrolysate Enriched with γ-Aminobutyric Acid and Peptides by Microbial Bioconversion

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Abstract: Hemp seed protein (HSP), a by-product of hemp oil processing, was converted into a functional protein ingredient enriched with γ-aminobutyric acid (GABA) and peptides through a two-step microbial fermentation process. To enhance peptide and free amino acid production from HSP, it was hydrolyzed using alkaline protease produced by *Bacillus subtilis* HA. The HSP was hydrolyzed at a degree of 40% at 55 °C for 24 h, yielding a pH of 6.55, an acidity of 1.22%, and 205.45 mg% tyrosine equivalents. This process resulted in the production of low molecular-weight peptides. (<5000 Da) The total amino acid content and branched-chain amino acids (leucine, isoleucine, and valine) were 6.78 mg/g and 1.47 mg/g. Subsequently, the production of γ-aminobutyric acid (GABA) in the HSP hydrolysate was optimized through co-fermentation with lactic acid bacteria in the presence of 5% MSG at 30 °C for 5 days. The serial co-fermented HSP hydrolysate exhibited a GABA content of 33.98 mg/g and a viable bacterial count of 9.51 log CFU/mL for *Lb. plantarum* KS2020. This serial co-fermentation process, combining proteolysis and lactic acid fermentation, not only increased the peptide content but also promoted GABA accumulation, positioning HSP hydrolysate as a promising candidate for functional foods with potential health benefits.

Keywords: hemp seed protein; γ-aminobutyric acid; *Bacillus subtilis*; *Lactiplantibacillus plantarum*

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1. Introduction

Proteins are essential macronutrients for the human body, playing a crucial role in growth and maintenance [1]. Dietary protein can be sourced from meat, dairy products, nuts, certain vegetables, grains, and beans. Notably, plant-based proteins are gaining increasing attention for their ethical, personal (vegan), and environmental benefits [2]. Additionally, plant-based proteins offer numerous health advantages due to the presence of bioactive compounds and properties that have been associated with various health benefits in numerous studies [3]. The exploration of new sustainable protein sources, particularly through the utilization of by-products as raw materials for plant-based proteins, has garnered significant critical attention [4].

Cannabis sativa L. (Hemp), a plant from the family *Cannabaceae*, has been cultivated for various purposes, including as a source of fiber, edible oil, food, and medicine, as well as for recreational and spiritual uses. With the commercialization of industrial hemp, its cultivation has spread worldwide, yielding outputs comparable to those of soybeans [5]. The by-product of hemp seed remaining after oil extraction is predominantly composed of protein, which holds significant potential as a raw material for protein production. Hemp seed protein (HSP) boasts a digestibility rate of 84.1–86.2% and a higher Protein Digestibility Corrected Amino Acid Score compared to certain grains, nuts, and some legumes [6]. The superior nutritional profile of hemp seed protein could drive advancements in the development of plant-based protein foods. However, the main protein in HSP, edestin, is not easily soluble in neutral or acidic conditions, which limits its use in food processing

applications [7]. Several studies have demonstrated that using enzymes such as alcalase and flavourzyme for hydrolysis, followed by the evaluation of antioxidant activity, can improve protein quality [8–10]. Furthermore, fermentation, an economical and valuable biotechnological method, has the potential to enhance the quality of HSP even further [11]. However, there is a scarcity of empirical studies focused on increasing the value of HSP through fermentation.

Bacillus subtilis possesses enzymes that efficiently break down proteins to produce peptides and functional compounds [12]. Fermentation using *Bacillus subtilis* offers the potential to produce a variety of fermentation products alongside protein decomposition, making it more versatile and valuable than commercial enzymes like alcalase or flavourzyme [13]. This versatility could lead to the development of higher value-added materials in biotechnology and functional foods. Additionally, *B. subtilis* helps maintain an optimal pH and low acidity, creating a favorable environment for the growth of lactic acid bacteria (LAB) [14]. It also synthesizes pyridoxal-5′-phosphate, the active form of vitamin B_6, which acts as a coenzyme for glutamic acid decarboxylase, the key enzyme in γ-aminobutyric acid (GABA) biosynthesis [15,16]. As a result, alkaline fermentation using *B. subtilis* is applied prior to LAB fermentation to support GABA production. GABA is an inhibitory neurotransmitter known to promote muscle growth by increasing serum growth hormone levels and enhancing protein synthesis [17]. While GABA naturally occurs in foods such as germinated rice, it is present only in trace amounts. Therefore, microbial fermentation or chemical synthesis is often employed to produce higher concentrations of GABA [18–20]. Microbial synthesis of GABA is particularly promising due to its environmental friendliness and cost-effectiveness compared to chemical synthesis [21]. Previous research has shown that LAB, fungi, and yeast are capable of synthesizing GABA [22,23]. *Lactiplantibacillus plantarum* (LAB) is widely used in industrial food fermentation, particularly for its ability to produce large quantities of GABA [24,25].

In this study, we aimed to enhance the peptide content of hemp seed protein (HSP) through hydrolysis using *B. subtilis*, which also provides co-enzymes that promote GABA production. Additionally, we aimed to further increase GABA content by utilizing *Lb. plantarum* in a sequential fermentation process, ultimately developing a multi-functional ingredient enriched with both peptides and GABA.

2. Materials and Methods

2.1. Materials

Hemp seed protein (HSP) was purchased from JHemp Co., Republic of Korea (GyeongBuk, Republic of Korea). *Dendropanax morbiferus* extract, used for enzyme production, was purchased from O'kannae Co., Ltd. (ChungBuk, Republic of Korea), and skim milk was bought from Seoul Milk Co. (Seoul, Republic of Korea). Yeast extract (YE) was procured from Choheung Co., Ltd. (Gyeonggi, Republic of Korea). Glucose and L-monosodium glutamate (MSG) were supplied by Daehan Jedang Co. (KS H-2003, Incheon, Republic of Korea) and CJ Jeil Jedang Co. (Seoul, Republic of Korea), respectively.

2.2. Preparation of Starter Cultures

Bacillus subtilis HA (KCCM 10775P), isolated from Cheonggukjang, was used for the initial fermentation. A colony of *B. subtilis* HA, cultured on an MRS agar medium, was inoculated into 5% skim-milk broth and shaken at 160 rpm for 24 h at 42 °C. The resulting *B. subtilis* HA culture was then used as a starter. *Lactiplantibacillus plantarum* KS2020 (KCCM 12782P) was selected for its high GABA production capability, having been isolated from kimchi. The strain was cultivated on an MRS agar plate at 30 °C for 48 h. It was then inoculated into 100 mL of MRS broth and incubated at 30 °C for 24 h.

2.3. Production of Alkaline Protease and Hydrolysate

The protein-hydrolyzing enzyme from *Bacillus subtilis* HA was used to produce hemp seed protein (HSP) hydrolysate. An alkaline protease was prepared using *Dendropanax*

morbiferus extract, following An's modified method [26]. The *Dendropanax morbiferus* extract (20%, *v*/*v*) was sterilized at 121 °C for 15 min, after which 2% glucose (*w*/*v*) and 1% skim milk were added. *B. subtilis* HA, previously cultured in 5% skim-milk broth, was then inoculated into the mixture at a 5% level and incubated at 42 °C with shaking at 160 rpm for 24 h. The protease activity was assayed using the Folin-phenol reagent. To hydrolyze high concentrations of HSP, alkaline protease, prepared for various HSP concentrations (20–40%), was mixed with HSP and the reaction was conducted at 55 °C with shaking at 160 rpm for 24 h.

2.4. Production of GABA

For the production of high concentrations of GABA, *Lactiplantibacillus plantarum* KS2020 fermentation was carried out by varying the concentration of the substrate, monosodium glutamate (MSG), from 5–10%. In total, 60% of the HSP hydrolysate was mixed with sterilized 2% glucose (*w*/*v*), 5–10% MSG (*w*/*v*), and 0.5% yeast extract (*w*/*v*). Following this, *Lb. plantarum* KS2020, previously cultured in MRS broth, was inoculated into the mixture at a 1% (*v*/*v*) level (9.10 log CFU/mL ± 0.02). The co-fermentation of the HSP hydrolysate was then conducted at 30 °C for 5 days under static conditions.

2.5. Viable Bacterial Counts

Viable bacterial counts for *Lb. plantarum* KS2020 and *B. subtillis* HA were determined using the standard plate count method. The procedure involved serial dilution and selective MRS agar plates with a pH of 6.5 ± 0.2. The fermented culture was serially diluted and spread-plated on a selective medium. Incubation took place at 30 °C for 48 h, and the viable bacterial count was expressed as log CFU/mL.

2.6. pH and Titratable Acidity

The pH was measured with a pH meter (SevenEasy pH, Mettler-Toledo AG, Schwerzenbach, Switzerland). Titratable acidity was determined by diluting 1 mL of the sample with 9 mL of distilled water and titrating with 0.1 normality NaOH until a pH of 8.3 was reached. The equivalent weights of lactic acid and acetic acid were then used to calculate the total titratable acidity (%, *v*/*v*).

2.7. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To confirm the degree of protein hydrolysis of the HSP by the alkaline protease, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using equipment from Hofer Scientific Instrument, (San Francisco, CA, USA) [27]. The HSP hydrolysate was first centrifuged at 15,000 rpm for 15 min, after which the supernatant was dissolved in an SDS-sample buffer (5×, 0.15 M Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol). The mixture was then heated at 100 °C for 5 min. Subsequently, 10 µL of the sample was loaded onto a gradient SDS-PAGE gel (5–13%, O-RAD, Seoul, Republic of Korea) and subjected to electrophoresis. The gel was stained using the Instant Blue solution (Expedeon Ltd., Cambridgeshire, UK). A protein marker (Thermo Scientific, Waltham, MA, USA) and 0.5% skim milk were used as a standard protein solution.

2.8. Tyrosine Equivalent Peptide

To indirectly measure the peptide content in the HSP hydrolysate, the Folin-phenol reagent was used to determine the tyrosine content in the peptides of the HSP hydrolysate [28]. An equal volume of 0.44 M TCA solution was added to the HSP hydrolysate and incubated at 37 °C for 30 min. After the proteins were precipitated by centrifugation at 15,000 rpm for 10 min, 1 mL of the supernatant was mixed with 2.5 mL of 0.55 M Na_2CO_3 and 0.5 mL of 3-fold diluted phenol reagent. The mixture was then allowed to react at 37 °C for 30 min. The absorbance was measured at 660 nm using a spectrophotometer (Amersham Biosciences, Amersham, UK). L-tyrosine was used as a standard.

2.9. Quantitative Analysis of Free Amino Acids

The analysis of free amino acids before and after hydrolysis of HSP was performed using HPLC. For the derivatization of amino acids in the sample, 70 μL of borate buffer and 20 μL of AccuQ-fluor reagent were sequentially added to 10 μL of the test solution according to the ACCQ test method, followed by heating in an drying oven (Bnf Korea Co., Ltd., Gyeonggi, Republic of Korea) at 55 °C for 10 min. Fluorescence detection (λex: 250 nm, λem: 395 nm) was employed for analysis. The mobile phase consisted of Waters AccQ Taq eluent A (Phase A) and 60% acetonitrile (Phase B), with a flow rate of 1.0 mL/min. The concentration of the measured sample was then calculated using the formula provided below.

$$\text{Amino acid quantification (mg/100 g)} = (S \times M \times 50\ \text{mL} \times 100 \times D)/(W \times 1{,}000{,}000)$$

S: Sample (μg/mL), M: Molecular weight (g/mol), D: Dilution factor, W: Specimen collection weight (g).

2.10. Qualitative Analysis of GABA

Monosodium glutamate (MSG) consumption and GABA production in co-fermented HSP hydrolysate were assessed using thin layer chromatography (TLC) on a silica gel 60 F254 plate (Merck KGaA, Darmstadt, Germany). A 2 μL sample of each supernatant was spotted onto the plate and subsequently eluted using a developing solvent consisting of an organic layer made up of n-butyl alcohol, glacial acetic acid, and distilled water in a 3:1:1 ratio. After drying, the silica gel plate was treated with a coloring reagent (0.2% ninhydrin) and then dried at 100 °C. This method enabled the visualization and quantification of MSG consumption and GABA production in the samples. The intensities of the spots on the TLC plate were measured using Image-J (Fiji).

$$\text{GABA spot intensity} = \text{GABA intensity}/\text{Standard intensity}$$

$$\text{MSG spot intensity} = \text{MSG intensity}/\text{Standard intensity}$$

2.11. Quantitative Analysis of the Free Amino Acids

GABA and the free amino acids in the co-fermented HSP hydrolysate were analyzed using high-performance liquid chromatography (HPLC) with a Waters 2475 instrument (Waters, Milford, MA, USA). The dried sample underwent derivatization at room temperature for 30 min, after which it was mixed with solvent A (140 mM NaHAc, 0.15% TEA, 0.03% EDTA, 6% CH_3CN, pH 6.1). The mixture was then filtered through a 0.45 μm syringe filter. The sample was injected using an HPLC auto-sampler (1100 series; Hewlett Packard, Palo Alto, CA, USA) connected to a C18 column (Nova-Pak 4 μm; Waters, Milford, MA, USA) [29]. The mobile phase consisted of Waters AccQ Taq eluent (Phase A), 60% acetonitrile (Phase B), and tertiary distilled water (Phase C), applied over 25 min at a flow rate of 1 mL/min. The amino acid content was determined by measuring the absorbance at 250 nm using a UV detector (Waters, Milford, MA, USA).

2.12. Statistical Analysis

With the exception of SDS-PAGE and the qualitative analysis of GABA and free amino acids, all experiments were conducted in triplicate, and the resulting data were presented as the mean value ± standard deviation. Statistical analysis was performed using a two-way analysis of variance (ANOVA) with SPSS version 27.0 (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test ($p < 0.05$) was applied to identify significant differences among the samples.

3. Results and Discussions

3.1. Preparation of Hemp Seed Protein (HSP) Hydrolysate

The alkaline protease was efficiently produced by *Bacillus subtilis* HA over 24 h, resulting in an enzyme activity of 443 U/mL. *Bacillus subtillis* is known for its ability to produce high yields of alkaline protease. The protease activity of *B. subtillis* BS was recorded at 400 U/ mL after fermenting for 80 h in a 50 L fermenter [30].

To assess the degradation of HSP, the hydrolysate of HSP (10–40%, *w*/*v*) was analyzed using SDS-PAGE, as shown in Figure 1A. In the SDS-PAGE patterns, the control HSP sample, which was not treated with the enzyme, displayed intact protein bands with no significant changes over 24 h.

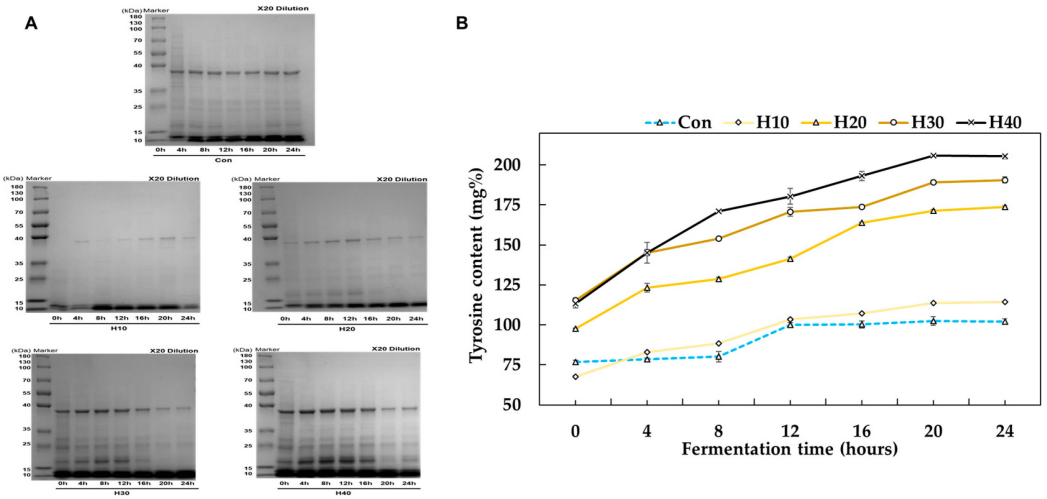

Figure 1. Degree of hydrolysis and SDS-PAGE profile according to differences in HSP content. (**A**) Effects of alkaline protease to HSP hydrolysate on SDS-PAGE profile. (**B**) Tyrosine content of HSP hydrolysate according to differences in hemp seed concentration. Con: 20% hemp + water, H10: 10% hemp + alkaline protease, H20: 20% hemp + alkaline protease, H30: 30% hemp + alkaline protease, H40: 40% hemp + alkaline protease, HSP: hemp seed protein. The error bars indicate standard deviations. Statistically significant differences were observed between the groups ($p < 0.05$). $n = 3$ for each group.

In contrast, when alkaline protease was added to HSP concentrations of 10% and 40% (H10, H40), the protein bands between 15–40 kDa initially darkened until around 12 h and then gradually lightened. In the H10 condition, large molecular weight proteins were decomposed to less than 10 kDa after 8 h, with further hydrolysis continuing thereafter. In the H20-H40 conditions, most proteins in the 15–20 kDa and 25–40 kDa ranges were almost completely degraded within 24 h. HSP is a seed storage protein composed of 60–80% protein, primarily a globular protein called edestin. Edestin's two subunits have molecular weights of approximately 35 kDa and 20 kDa [31]. This suggests that the protein bands observed in the SDS-PAGE are likely edestin, which appears to have been effectively degraded by the alkaline protease from *B. subtilis* HA over the course of 24 h. A similar trend was observed in the tyrosine equivalent content of the HSP hydrolysate, which gradually increased over the 24-h period (Figure 1B).

Under conditions where alkaline protease was not added, the tyrosine content was measured at 76.68 mg% at the start of hydrolysis, increasing to 102.25 mg% after 12 h, and then stabilizing. The increase in tyrosine content in the control condition is likely due to the exposure of internal protein structures caused by heat denaturation. When HSP was heated above 80 °C for 10 min, significant protein denaturation was observed, although this effect was less pronounced at 40 °C [32]. This suggests that continuous heat denaturation occurred even when hydrolysis was performed at 55 °C for 24 h. In the case of HSP hydrolyzed with the alkaline protease, the tyrosine content surged during the first

20 h and then remained relatively stable. For the H40 condition, the tyrosine content on the first day of hydrolysis was 205.45 mg%, while in the H20 condition, it was 173.64 mg%. This indicates that alkaline protease effectively decomposes HSP.

The initial total amino acid content of the control HSP and H20 was 547.50 mg/100 g and 584.33 mg/100 g, respectively. While H20 had a slightly higher amino acid content than the control HSP, the difference was not statistically significant (Table 1). After 24 h of hydrolysis, the total amino acid content of H20 increased to 678.50 mg/100 g, whereas the control HSP decreased to 372.83 mg/100 g. The main amino acid in HSP, arginine, showed a significant increase after hydrolysis, with the highest increases occurring in the order of arginine > tyrosine > serine. Arginine is known to enhance skeletal muscle growth and reduce fat [33]. Additionally, essential amino acids like leucine, isoleucine, and valine [34], which are critical for protein synthesis, rapidly decreased in the control HSP after 24 h. However, in the H20 condition, these amino acids remained stable throughout the hydrolysis process. In conclusion, hydrolyzed HSP is expected to be more beneficial for absorption and muscle building compared to raw HSP.

Table 1. Amino acid composition of HSP hydrolyzed by alkaline protease.

Amino Acid (mg/100 g)	Con 0 h	H20 0 h	Con 24 h	H20 24 h
Asp	2.01 ± 0.00 [1)]	28.33 ± 0.29	5.83 ± 0.58	26.33 ± 0.29
Glu	87.83 ± 1.53	77.67 ± 1.12	6.00 ± 0.00	29.33 ± 1.16
His	38.24 ± 3.61	43.33 ± 0.29	12.00 ± 0.50	27.354 ± 0.29
Arg	3.00 ± 0.00	3.50 ± 0.00	39.02 ± 1.80	66.50 ± 0.29
Lys	13.14 ± 1.00	6.00 ± 0.00	28.00 ± 0.00	36.50 ± 0.00
Phe	30.17 ± 1.61	34.83 ± 0.76	14.83 ± 2.47	32.17 ± 1.53
Tyr	6.17 ± 0.58	2.83 ± 0.29	32.83 ± 1.04	37.00 ± 0.00
Ala	46.50 ± 0.00	46.03 ± 0.00	41.84 ± 0.29	45.00 ± 1.73
Ile	32.83 ± 0.76	35.83 ± 0.06	14.00 ± 0.00	37.00 ± 0.00
Leu	50.17 ± 0.29	58.83 ± 0.29	22.83 ± 1.26	68.17 ± 2.93
Met	18.00 ± 0.00	19.60 ± 0.29	12.17 ± 0.29	20.33 ± 0.29
Val	41.83 ± 0.76	45.50 ± 0.17	22.00 ± 0.00	42.67 ± 0.29
Thr	28.17 ± 0.29	19.50 ± 1.32	10.17 ± 1.61	22.6 + 7 ± 0.76
Ser	2.04 ± 0.00	9.67 ± 0.23	15.01 ± 2.18	42.33 ± 0.58
Cys	20.83 ± 1.76	0.50 ± 0.00	14.00 ± 0.00	3.00 ± 0.00
Gly	36.02 ± 0.00	28.67 ± 0.76	16.12 ± 1.32	17.67 ± 0.76
Pro	91.00 ± 1.80	123.7 ± 0.12	63.33 ± 0.17	84.50 ± 0.00
Total	547.50 ± 1.60	584.33 ± 1.62	372.83 ± 3.02	678.50 ± 1.48

[1)] All values are mean ± standard deviation of three replicates (n = 3). Con: 20% hemp + water, H20: 20% hemp + alkaline protease.

3.2. Physicochemical Properties of HSP Hydrolysate

The physical characteristics of HSP hydrolysate were analyzed during enzymatic hydrolysis. The initial bacterial count before hydrolysis was measured at 7.72 log CFU/mL and decreased thereafter (Figure 2A). After 24 h of hydrolysis at 55 °C, the viable bacterial counts for H10, H20, H30, and H40 decreased to 4.67, 5.12, 5.12, and 5.22 log CFU/mL, respectively. As the HSP content decreased, the viable bacterial count of *B. subtilis* HA correspondingly declined.

The pH gradually decreased as hydrolysis progressed (Figure 2B). The initial pH for H10 and H20 ranged from 7.84 to 8.21, dropping to 6.63 and 6.84 within 8 h, after which it remained stable. For H30 and H40, the pH decreased to 6.87 and 7.15 by 16 h of hydrolysis and then stabilized. The titratable acidity of the HSP hydrolysate also varied depending on the HSP content (Figure 2C). The higher the HSP content, the higher the titratable acidity; for H40, it started to increase after 12 h of hydrolysis, reaching 1.22% acidity. This suggests that the longer lag time for increasing acidity is due to the buffering capacity provided by the higher HSP content. Conversely, the lower the HSP content, the faster the acidity rose during the early stages of hydrolysis. In all conditions with varying HSP content, the

acidity increased to a certain level and then decreased again. The control H20 (HSP 20%) without the addition of the alkaline protease showed a slight increase in acidity to 0.17%, indicating lower acidity compared to the H20 treated with the alkaline protease.

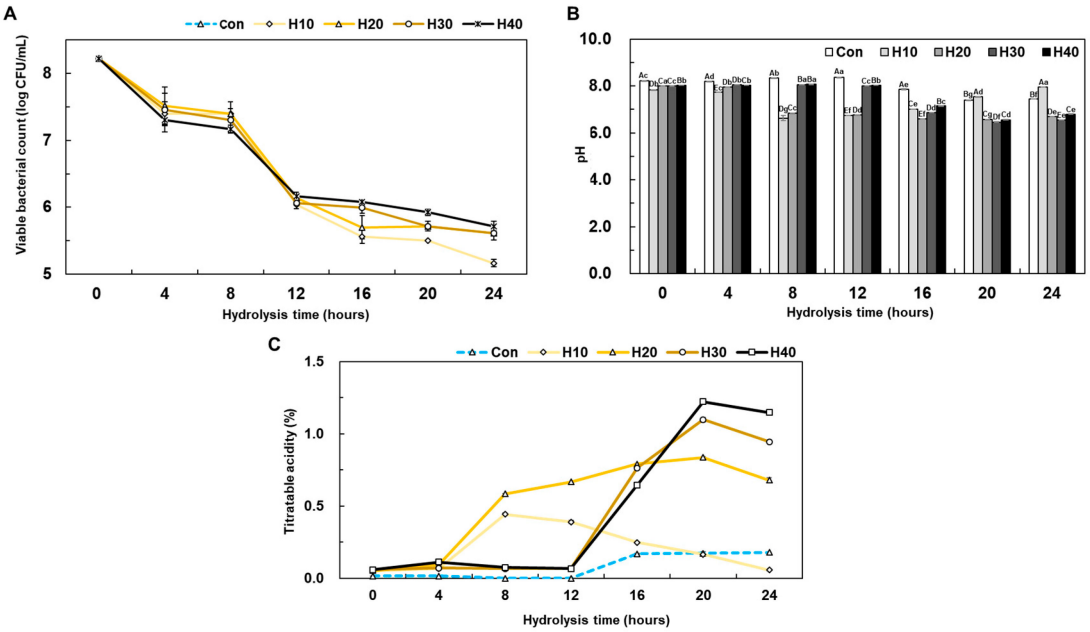

Figure 2. Viable bacterial count, pH, and acidity according to differences in HSP content. (**A**) Viable bacterial count of *B. subtilis* HA in the hydrolysis process. (**B**) pH of the hydrolysis process. (**C**) Acidity of the hydrolysis process. Con: 20% hemp + water, H10: 10% hemp + alkaline protease, H20: 20% hemp + alkaline protease, H30: 30% hemp + alkaline protease, H40: 40% hemp + alkaline protease, HSP: hemp seed protein. The error bars indicate standard deviations. Statistically significant differences were observed between the groups ($p < 0.05$). $n = 3$ for each group.

On the other hand, the increased acidity during the hydrolysis of HSP is likely due to the production of lactic acid by *B. subtilis* HA [35]. When *B. subtilis* HA is provided with nitrogen supplements but has a limited carbon supply, lactic acid production can occur [36]. During HSP hydrolysis, the combination of nitrogen supplementation, high temperature (55 °C), and limited carbon availability for *B. subtilis* HA growth, led to the production of acid. The lactic acid produced can then be utilized by microorganisms as a carbon source [37]. Consequently, the acidity may decrease during hydrolysis as *B. subtilis* HA utilizes the organic acids as a carbon source.

In this study, we observed that, in addition to peptides generated through the hydrolysis by *Bacillus subtilis* HA, various metabolites were produced. Further studies are needed to accurately assess the impact of these compounds on HSP hydrolysates.

3.3. Analysis of Co-Fermented HSP Hydrolysate

Co-fermentation using lactic acid bacteria (LAB) was performed in HSP hydrolysate (H20), where most proteins had been hydrolyzed over 24 h. The initial viable bacterial count of *B. subtilis* HA was 5.58 log CFU/mL, which decreased as LAB fermentation progressed, reaching 4.49–4.65 log CFU/mL by the 5th day of fermentation (Figure 3A). For LAB, the initial viable bacterial count was 7.10 log CFU/mL (Figure 3B). The number of viable bacteria increased on the 1st day of co-fermentation but dropped proportionally with the MSG content. Under conditions of HSP hydrolysate with 5% MSG, LAB counts significantly increased to 9.74 log CFU/mL on the 3rd day of co-fermentation, followed by a slight decrease. In the cases of 8% MSG and 10% MSG, the viable bacterial counts increased on the 1st day but were lower compared to the HSP hydrolysate with 5% MSG, and subsequently declined as co-fermentation progressed. Previous research has found that the growth

rate of *Lactiplantibacillus plantarum* is negatively affected by sodium concentration, with inhibition occurring when NaCl content exceeds 8% [38]. The higher sodium content in HSP hydrolysate, due to the higher MSG content, likely contributed to the reduced viable LAB counts.

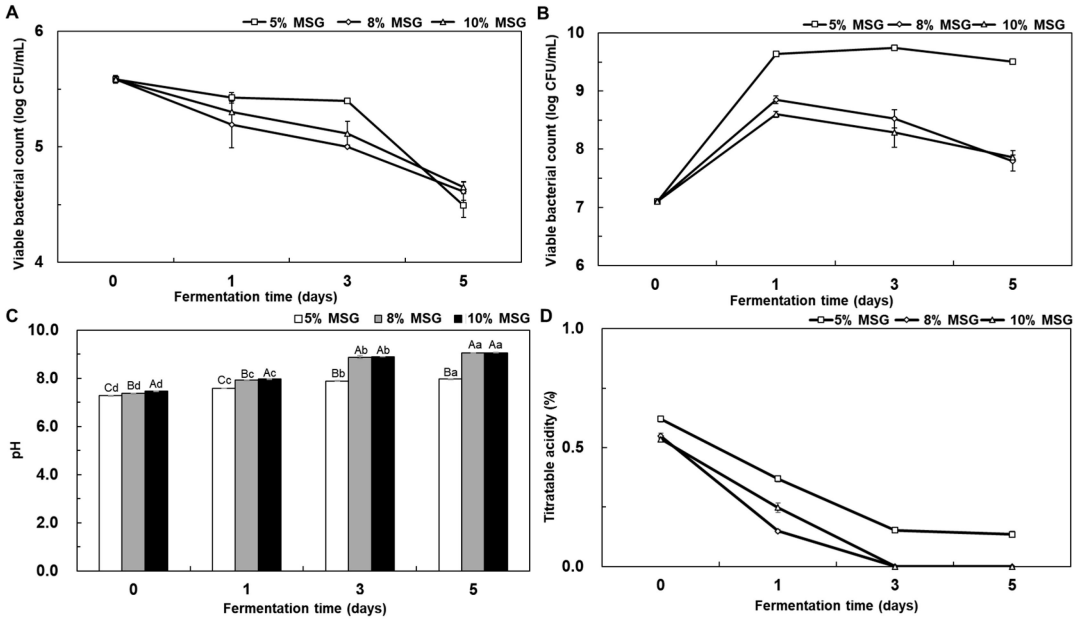

Figure 3. Viable bacterial count, pH, and acidity of HSP hydrolysate co-fermented by *Lb. plantarum* KS2020. (**A**) Viable bacterial count of *B. subtilis* HA in lactic acid bacteria fermentation. (**B**) Viable bacterial count of *Lb. plantarum* KS2020 in lactic acid bacteria fermentation. (**C**) pH of lactic acid bacteria fermentation. (**D**) Acidity of lactic acid bacteria fermentation; 5% MSG: HSP: HSP hydrolysate added 5% MSG; 8% MSG: HSP hydrolysate added 8% MSG; 10% MSG: HSP hydrolysate added 10% MSG; MSG: monosodium L-glutamate; HSP: hemp seed protein. HSP hydrolysate was prepared with HSP 20% (H20). The error bars indicate standard deviations. Statistically significant differences were observed between the groups ($p < 0.05$). $n = 3$ for each group.

During the co-fermentation of HSP hydrolysate, the pH continued to rise until the 5th day of fermentation, with the increase being greater for 8% and 10% MSG compared to 5% MSG (Figure 3C). Additionally, the acidity of all HSP hydrolysate samples decreased during LAB co-fermentation, eventually reaching nearly 0% titratable acidity (Figure 3D). The glutamic acid decarboxylase enzyme in LAB plays a role in the bioconversion of glutamic acid to GABA, consuming protons in the process, which leads to an increase in pH [39]. If the pH exceeds the allowable buffering range during the conversion of MSG, it could negatively impact enzyme activity and, consequently, GABA production [40]. Overall, the increase in pH and decrease in titratable acidity during LAB fermentation suggest that GABA production is ongoing. However, the significant pH changes observed in HSP hydrolysate with 8% MSG may inhibit the activity of the glutamic acid decarboxylase enzyme in LAB, thereby reducing GABA production.

Contents of GABA using TLC showed similar results (Figure 4A). With 5% MSG, most of the MSG was converted to GABA by the 3rd day of co-fermentation, while in the other conditions (8% and 10% MSG), some MSG remained unconverted until the end of co-fermentation. The glutamate decarboxylase (GAD) enzyme, which is responsible for converting glutamate into GABA, reaches peak activity during the stationary phase. However, as the process enters the death phase, GAD activity decreases, leading to a slowdown in GABA production [41]. This decline is further influenced by the reduction in viable bacterial numbers and the depletion of MSG. In LAB fermentation, a noticeable drop in viable bacterial count and MSG consumption was observed after the 3rd day, particularly under conditions with 8% and 10% MSG. This suggests that extending fermen-

tation beyond 5 days may be less active in GABA conversion, likely due to the limiting activity of GAD (Figure 4B,C). Therefore, it is concluded that the optimal MSG content for GABA conversion during LAB co-fermentation of HSP hydrolysate is 5%. The GABA and glutamic acid content under the optimized conditions were measured at 33.98 mg/g and 14.05 mg/g, respectively (Table 2). A 3.5% GABA is the highest value reported for GABA production by microbial process. In summary, HSP, a by-product of oil processing, was efficiently hydrolyzed using an alkaline protease prepared by *B. subtilis* HA. The serial co-fermentation by *Lb. plantarum* KS2020 resulted in a higher GABA-enriched HSP hydrolysate that also contains peptides. The GABA conversion rate using HSP was 67.96%, significantly higher than that of general commercial LAB, which ranges from 9.83% to 26.2%, and surpasses current industrial GABA production through microbial fermentation, measured at 5.83–23.01 mg/g [42–45]. The recommended intake of GABA for muscle mass and strength improvement is 100 mg [46], suggesting that consumption of GABA-enriched HSP hydrolysate (3.0 mL) may be beneficial.

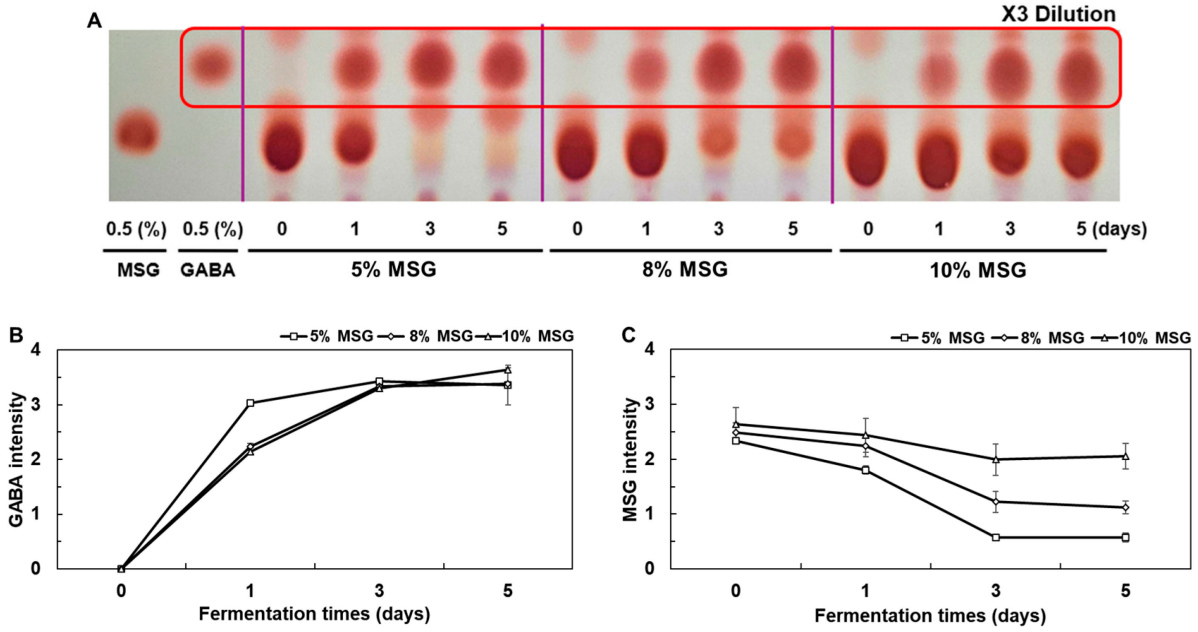

Figure 4. GABA and MSG intensity of HSP hydrolysate co-fermented by *Lb. plantarum* KS2020. (**A**) TLC of lactic acid bacteria fermentation. (**B**) GABA intensity of lactic acid bacteria fermentation. (**C**) MSG intensity of lactic acid bacteria fermentation; 5% MSG: HSP hydrolysate added 5% MSG; 8% MSG: HSP hydrolysate added 8% MSG; 10% MSG: HSP hydrolysate added 10% MSG; GABA: γ-aminobutyric-acid; MSG: monosodium L-glutamate; HSP: hemp seed protein. HSP hydrolysate was prepared with HSP 20% (H20). The error bars indicate standard deviations. Statistically significant differences were observed between the groups ($p < 0.05$). $n = 3$ for each group.

Table 2. Content of GABA and glutamic acid in HSP hydrolysate co-fermented by *Lb. plantarum* KS2020.

Co-Fermentation	GABA (mg/g)	Glutamic Acid (mg/g)
HSP hydrolysate added MSG 5%	33.98 ± 1.24 [1)]	14.05 ± 0.82

[1)] All values are mean ± standard deviation of three replicates ($n = 3$). HSP hydrolysate was prepared with HSP 20% (H20) (HSP: hemp seed protein).

4. Conclusions

This study optimized conditions for converting hemp seed protein (HSP), a by-product, into a high-value protein material through fermentation. Protein hydrolysis was conducted using alkaline protease from *Bacillus subtilis* HA, with hydrolysis efficiency influenced by HSP concentration, and further enhanced through serial co-fermentation with *Lactiplantibacillus plantarum* KS2020 for GABA production. Using alkaline protease from *B. subtilis*

HA (443 U/mL), the degradation of 20% HSP was effectively achieved within 16–20 h, resulting in the production of 173.64 mg% tyrosine content.

Following the hydrolysis process, analysis of free amino acid content revealed approximately double the total amino acid content compared to the control group (20% HSP). Additionally, arginine and branched-chain amino acid (BCAAs: leucine, isoleucine, and valine) levels significantly increased after hydrolysis compared to the pre-hydrolysis control. The viable bacterial count of *B. subtilis* HA during HSP hydrolysis consistently decreased across all HSP concentrations. Furthermore, a notable increase in acidity was observed during HSP hydrolysis, with titratable acidity levels rising proportionally to the HSP concentration (H10: 0.44%, H40: 1.22%).

Although the degradation rate of alkaline protease fermented from *B. subtilis* HA was slower than that of commercial enzymes such as flavourzyme, microbial fermentation provided advantages beyond protein hydrolysis. The process contributed to the production of metabolites, including lactic acid. These findings suggest the need for further investigation into the biochemical and functional properties of HSP hydrolysates.

In the co-fermentation process with lactic acid bacteria, physicochemical properties such as pH, acidity, and GABA content varied based on MSG concentration as a precursor. During co-fermentation with *Lb. plantarum* KS2020 using 20% HSP hydrolysate supplemented with 5% MSG, the viable bacterial count reached 9.51 log CFU/mL after the 5th day. A significant portion of MSG was completely converted, resulting in a 3.39% GABA yield. Specifically, the pH and titratable acidity were measured at pH 7.96 and 0.14%, respectively. However, when the MSG concentration exceeded 5%, a decline in viable bacterial count and a reduction in GABA conversion efficiency were observed.

These findings demonstrate that GABA-enriched HSP hydrolysates can be successfully developed through microbial bioconversion using *B. subtilis* HA and *Lb. plantarum* KS2020. This approach offers a promising strategy for producing multifunctional bioactive materials with potential applications in the food, nutraceutical, and pharmaceutical industries.

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