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Thesis for the Degree of Master of Engineering

Optimization of Protease Production by *Bacillus licheniformis* SF5-1 and Bioactive Activity of Its Hydrolysate from Soybean Meal

by

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Department of Food Science and Technology

The Graduate School

Pukyong National University

February, 2024

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(*Bacillus licheniformis* SF5-1 에 의한 단백질 분해효소 생산 최적화 및 대두박 추출 가수분해물의 생리활성)

Advisor : Prof. Ji-Young Yang

by
Yikang Sim

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Engineering

in Department of Food Science and Technology,
The Graduate School, Pukyong National University

February, 2024

Optimization of Protease Production by *Bacillus licheniformis* SF5-1 and Bioactive Activity of Its Hydrolysate from Soybean Meal

A dissertation

by

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PUKYONG NATIONAL UNIVERSITY 부경대학교

Contents

PUKYONG NATIONAL UNIVERSITY 부경대학교

Bacillus licheniformis SF5-1 에 의한 단백질 분해효소 생산 최적화 및 대두박에서 추출한 가수분해물의 생리활성

심 이 강

부경대학교 대학원 식품공학과

요 약

대두박은 단백질 함량이 매우 높지만, 인체 내 소화율이 낮아 동물 사료로 주로 사용되고 있다. 따라서 대두박의 인체 소화율을 높이고 발효를 통해 생리활성 활성을 높이기 위해 이번 연구를 진행하였다. 천연 단백질 분해 효소 생산균으로 *Bacillus licheniformis* SF5-1을 선택하였다. *B.licheniformis* SF5-1이 단백질 분해 효소를 최대한 생산할 수 있는 최적의 조건을 예측하기 위해 반응 표면 분석법을 이용하였다. 반응 표면 방법론의 실험적 디자인은 Box-Behnken design을 채택하였다. 수용성 전분 2.02%, 탈지 대두박 13.74%, 염화칼슘 1.08%의 농도에서 생성된 단백질 분해 효소의 활성은 0.09 μmol/g·min이었다.

SDS-PAGE를 통하여 180 kDa 이상의 단백질 분자가 63 kDa 이하의 단백질 또는 펩타이드로 발효되는 것을 확인하였다. 이는 발효 후 대두박에서 결합 아미노산 함량의 증가와 유리 아미노산 함량의 전체적인 감소를 근거로 뒷받침되었다. 이로 인해 단백질의 소화 흡수율은 29.24%에서 39.52%로 10.28% 증가하였다. 발효대두박 추출물의 총 페놀 함량은 169.31 mg/mL에서 308.73 mg/mL로 증가하였다. 발효대두박 추출물의 항산화능 실험 결과, DPPH 라디칼 소거능이 11.76%에서 14.02%로 증가, 그리고 ABTS 라디칼 소거능이 12.27%에서 17.49%로 증가하여 발효대두박 추출물의 항산화능 증가를 확인하였다. 추출물의 항당뇨 활성은 50 mg/mL농도의 추출물에서 α-amylase의 저해능이 2.51%에서 6.76%, 그리고 5 mg/mL농도의 추출물에서 α-glucosidase의 저해능이 22.8%에서 79.19%로 증가하는 것을 확인하였다.

I. Introduction

Soybean was consumed for a long time as a great nutrition provider such as proteins, lipids and dietary fibers. It contains abundant protein and its protein nutritional profile is similar to that of animals except for sulfur amino acid (Zheng et al., 2022). Soybean and its products are consumed all around the world, but especially more used in East Asia than any others (Rizzo & Baroni, 2018). Soybean is usually used as fermented products because of their taste, flavor, and digestibility enhancement. Each product varies by fermentation conditions, such as temperature, moisture, type of strain and additional nutrients, therefore products have as many variations as the countries which consumed soybean (Kwon et al., 2010). In a case of lipid, soybean oil accounts for 28% of edible oil, and is second only to palm oil in global market (Kim et al., 2015; Foster et al., 2009). Soybean oil is extracted during solvent extraction from bean pod and major components of fatty acid is linoleic acid (Towa et al., 2011). Soybean meal is a by-product of soybean oil production, which is defatted and

contains abundant protein (Dozier & Hess., 2011). Furthermore, it is much more economical and environmental than using other protein sources. It is one of the reasons that soybean meal is utilized as a material that can enhance protein content of feeds (Storebakken et al., 1998; Burr et al., 2008; Yuan et al., 2016). Despite of their protein content, some legumes contain trypsin inhibitors, which reduce protein digestibility. Therefore, soybean meal requires a fermentation process to use it as a material for foods and feeds (Dunsford et al., 1989). Furthermore, fermentation made protein smaller and it resulted in an improvement of absorption so that could help people who have low digestibility (Chen et al., 2010; Zheng et al., 2022).

Bacillus licheniformis is one of the bacteria which contribute to soybean fermentation (Han et al., 2021; Jeong et al., 2014; Jeong et al, 2017). This study utilized a strain, *Bacillus licheniformis* SF5-1, a strain patented as having high proteolytic activity. In previous studies, fermentation of soybean meal performed to reduce the molecular size of protein for enhancing the digestive absorption rate and prevent digestive disease, for example, diarrhea (Chu et al., 2019; Hung et al., 2019).

Furthermore, there is plenty of traction about plant-based proteins in the protein market nowadays (Tonsor et al., 2021). These results are expected to have benefits for people with protein digestive disorders or poor digestive absorption.

This study aimed to get an advantage by making soybean meal protein low-molecular using *B.licheniformis* SF5-1 as a natural protease source. To find out the optimal condition of producing protease, Box-Behnken design was utilized as a model of response surface method. The concentration of carbon, nitrogen, and calcium chloride of medium were selected as variables. *B.licheniformis* SF5-1 was inoculated in soybean meal to detect the increased low molecular of soybean meal protein after fermentation of *B.licheniformis* SF5-1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ehtylbenzthizaoline-6-sulphonic acid (ABTS) radical scavenging activity, total phenol content, *in vitro* protein digestibility, and antidiabetic activity were measured to confirm the enhancement of bioactive activity after fermentation.

II. Materials and Methods

1. Materials

This study utilized *B.licheniformis* SF5-1 stored in laboratory with 70% glycerol solution at -80°C. Strain was cultured in TSB media at 45ºC, 48 hours to activate. Soybean meal was purchased from Jeungan-ri herbal village cooperation (Yang-pyeong, Korea). For a fermentation, soybean meal, potato starch (Shinyo pure chemicals Co., Ltd, Seoul, Korea), and calcium chloride (Samchun chemical Co., Ltd, Seoul, Korea) were adopted as nitrogen source, carbon source, and mineral source respectively. All solvents and chemicals utilized in this study were pure grade.

2. Protease activity measurement of soybean meal

fermented by *B.licheniformis* SF5-1

Protease activity was measured using casein as a substrate according to Sigma's non-specific protease activity assay (Cupp-Enyard, 2008). Fermented soybean meal samples (1 ± 0.01 g) were centrifuged for 10 min (4℃, 9,600 ×g). The supernatant was freeze-dried and stored. Freeze-dried sample was diluted with 1 mL of 10 mM sodium acetate buffer and utilized as crude enzyme solution. 100 uL of the solution was reacted with 500 μL of 0.65% casein at 37ºC for 10 minutes and then deactivated by adding 500 μL of 110 mM trichloroacetic acid (TCA) solution. The solution was incubated at 37 ºC, 30 minutes. The reactant was filtered using an syringe filter (0.45 μm PTFE syringe filter, Adventec, Tokyo, Japan). The filtered solution was reacted with reagent (Folin & Ciocalteu reagent (Sigma-Aldrich Co., Saint Louis, Missouri, United States) : calcium chloride : sample; 1 : 5 : 2; v/v) at 37ºC for 30 minutes. 1 unit of protease activity is defined as the amount of the protease resulting in the release of 1 μg tyrosine per minute at 37ºC under the standard assay conditions.

3. Design of experiment for Box-Behnken design in optimizing the medium composition for protease production by B.licheniformis SF5-1

Response surface methodology with three independent factors viz X (concentration of soluble starch (%; w/v)), Y (concentration of soybean meal (%; w/v)), and Z (concentration of calcium chloride (%; w/v)) was performed randomly and independently to obtain accuracy. This study utilized Box-Behnken design as an experimental design. Soluble starch (0.5 ~ 3.5%; w/v), soybean meal (10 ~ 20%; w/v), and sodium chloride (0.4 ~ 1.4%; w/v) were encoded in three levels (-1, 0, 1) of independent variables as shown in Table 1. Second-order polynomial equation for renponse surface analysis shows in equation (1):

$$Y = a_0 + a_1x + a_2x + a_3x + a_{12}xy + a_{13}xz + a_{23}yz + a_{11}x^2 + a_{22}y^2 + a_{33}z^2 \qquad (1)$$

Table 1. Coded and actual levels of independent variables for experimental design of response surface method

(unit : %)

Variables	Symbol	Range of levels		
		Low (-1)	Center (0)	High (+1)
Soluble starch (%; w/v)	*x*	0.5	2	3.5
Soybean meal (%; w/v)	*y*	10	15	20
Calcium chloride (%; w/v)	*z*	0.4	0.9	1.4

4. Protein molecular size measurement of soybean meal fermented by *B.licheniformis* SF5-1

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation was performed to modified the former research (Singh, Shepherd, & Cornish., 1991) to compare the size of protein molecular before and after fermentation. This study adopted 10% acrylamide gel as a resolving gel and 5% acrylamide gel as a stacking gel as shown in Table 2. Staining solution prepared with 0.1% of Coomassie brilliant blue R-250 (Sigma-Aldrich Co., Missouri, USA), 40% of methanol, and 10% of glacial acetic acid with diluted water was consumed to visualize proteins resolved in SDS-PAGE. Destaining solution with 40% (v/v) of methanol and 10% (w/v) of glacial acetic acid with diluted water was prepared to remove unnecessary staining solution from gel.

5. Bound and free amino acid content measurement of

Table 2. Composition of gel utilized in SDS-PAGE

	Resolving gel (12%)	**Stacking gel (5%)**
Water	3.2 mL	5.86 mL
30% Acrylamide	4.0 mL	1.34 mL
1.5 M Tris-HCl, pH 8.8	2.6 mL	-
0.5 M Tris-HCl, pH 6.8	-	2.6 mL
10% SDS	100 μL	100 μL
10% Ammonium persulfate	100 μL	100 μL
TEMED[1)]	10 μL	10 μL
Total	10.01 mL	10.01 mL

[1)]N,N,N,N’,N’-Tetremethylenediamin

soybean meal fermented by *B.licheniformis* SF5-1

Content of bound and free amino acid was analyzed in accordance with the guidelines set forth by the 2023 Food Code (Food Code, 2023). To analyze bound amino acids, 10 mg of sample was hydrolyzed with 10 mL of 6 N hydrochloric acid at 110ºC for 24 hours. After hydrolysis, hydrochloric acid was removed by utilizing rotary vacuum evaporator at 40ºC. Remained sample was diluted with 0.02 N hydrochloric acid to make test solution. To analyze free amino acids, 3 g of samples were shaken for 15 minutes with 3 mL of 16% trichloroacetic acid solution. After shaking, sample was centrifuged at 3,000 ×g for 15 minutes and utilized the supernatant as a test solution. The test solution was diluted with 0.02 N hydrochloric acid. Buffer for biological fluid (Kanto Chemical Holdings, INC., Tokyo, Japan) and amino acids mixture standard solution (FUJIFILM wako pure chemical, Co., Osaka, Japan) was utilized as a buffer and standard solution. Amino acid analyzer (L-8900, Hitachi, Tokyo, Japan) was utilized for analysis.

6. *In vitro* protein digestibility of soybean meal fermented by *B.licheniformis* SF5-1

Protein *in vitro* digestion was performed modified a previous research (Savoie and Gauthier, 1986). To compose a digestive environment in the stomach, 1 g of samples, both fermented and unfermented soybean meal were dissolved with 10 mL of diluted water in a flask. 10 mL of pepsin (25000 unit/mL, pH 2.0) was injected as a protease of stomach digest and stirred at 100 rpm, 30 minutes. After the reaction, pH was adjusted to 8 with 0.1 N NaOH to stop the primary enzymatic reaction. Secondary digestion which compose the digestive environment in the small intestine was performed by adjusting 10 mL of 800 units/mL pancreatin (7 mg/mL in phosphate buffer) at 100 rpm for 8 hours. After the second digestion, the enzyme activity was inactivated with the same volumes of 20% TCA with total volume. The first and second phase digestion solution were mixed with the same volumes of ethanol and kept at 4ºC, for 12 hours to precipitate undigested proteins. Samples were centrifuged for 20 minutes (4ºC, 10,000 ×g). Precipitate

was diluted in 5 mL of methanol to extract the undigested proteins. The solutions were centrifuged for 5 minutes (4ºC, 600 ×g). Protein content of supernatant was detected by utilizing the Lowry nitrogen quantification assay (Lowry et al., 1951).

7. Total phenol content of soybean meal fermented by *B.licheniformis* SF5-1

To measure the antioxidant activity, fermented and unfermented soybean meal was extracted with methanol. 1 g of each freeze-dried samples was extracted in 20 mL of methanol for 2 hours and filtered through no. 6 filter paper (Adventec). The filtrates were dried using a speed vacuum concentrator (HyperVac, Gyrozen Co., Ltd, Gimpo, Korea) and dried filtrates were stored at -80ºC for further analysis. The dried filtrates were reconstituted at 80% methanol in distilled water and filtered through a 0.45 μm PTFE syringe filter (Adventec). 0.25 mL of extract was mixed with 4 mL of distilled water and 0.25 mL of Folin-Ciocalteu reagent

(Sigma-Aldrich Co.), vortexed for 30 seconds, and left in the dark room for 5 minutes. The mixture was added 0.5 mL of saturated sodium carbonate and left in a dark room for 30 minutes, at 37ºC. Absorbance was measured before and after 30 minutes at 725nm using a microplate reader (Apoch 2, BioTek Co., Vermont, United States). Results were presented by gallic acid equivalents through the calibration curve.

8. Antioxidant activity analysis of soybean meal fermented by *B.licheniformis* SF5-1

Antioxidant activity was measured by utilizing DPPH and ABTS radical scavenging activity. DPPH radical scavenging activity was analyzed to determine antioxidant activity of soybean meal extract (Samruan, Oonsivilai, & Oonsivilai., 2012). The filtrates dried above were diluted in 1 mL of methanol and utilized as extracts. DPPH was dissolved at a concentration of 0.5 mmol/L in methanol. 3.75 mL of DPPH solution was mixed with 0.25 mL of the extract and reacted for 30 minutes at 37ºC

in dark room. Methanol was utilized instead of extract as a negative control group. Before and after the reaction, absorbance was measured at 517 nm with microplate reader (Apoch 2, BioTek Co.). The radical scavenging activity was calculated with following equation (2):

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{As_{517nm}}{Ac_{517nm}}\right) \times 100 \qquad (2)$$

where As_{517nm}, absorbance of sample at 30 minutes; Ac_{517nm}, absorbance of control at 30 minutes.

ABTS radical scavenging activity was analyzed following another research (Ksouri, R et al., 2009). The filtrates dried above were diluted in 1 mL of methanol and utilized as extracts. ABTS solution was produced by mixing the same volume of 14mM ABTS reagent and 4.9 mM potassium sulfate solution, then stored in a dark room for 24 hours. Before analysis, the ABTS solution got a 700 ± 0.020 absorbance at 734 nm by diluting with ethanol. 950 μL ABTS solution was mixed with 50 μL of the extract with various concentrations. Ethanol was utilized instead of extract as a negative control group. Absorbance was detected after the reaction for 6 minutes at 37ºC. The radical scavenging activity was calculated with

the following equation (3):

$$\text{ABTS radical scavenging activity (\%)} = \left(1 - \frac{As_{734nm}}{Ac_{734nm}}\right) \times 100 \qquad (3)$$

where As_{734nm}, absorbance of sample at 6 minutes; Ac_{734nm}, absorbance of control at 6 minutes.

9. Antidiabetic activity analysis of soybean meal fermented by *B.licheniformis* SF5-1

α-Amylase and α-glucosidase inhibitory activity was measured by the modified method of former research (Telagari, M & Hullatti, K, 2015). The filtrates dried above were diluted in 1 mL of distilled water and utilized as extracts. In the α-amylase inhibitory activity method, 100 μL phosphate buffer (100 mM, pH = 6.8), 20 μL α-amylase (10 unit/mL), and 40 μL of the extract with various concentrations were mixed and consumed as a reaction reagent. The reaction reagent was preincubated for 20 minutes at 37ºC to stabilize the enzyme. Then, 40 μL of 1% soluble starch was mixed as a substrate and incubated for 30 minutes at 37ºC.

After incubating, 200 μL 3,5-dinitrosalicylic acid reagent was treated in each tube and boiled for 10 minutes. Absorbance was detected at 540 nm using a microplate reader (Apoch 2, BioTek Co.). A reaction reagent without extract was utilized as a negative control group. The inhibitory activity was calculated with following equation (4):

$$\text{α-Amylase inhibitory activity (\%)} = \left(1 - \frac{As_{540nm}}{Ac_{540nm}}\right) \times 100 \qquad (4)$$

where As^{540nm}, absorbance of test sample at 30 minutes; Ac^{540nm}, absorbance of control at 30 minutes.

In the α-glucosidase inhibitory activity method, 100 μL of phosphate buffer (100 mM, pH = 6.8), 20 μL α-glucosidase (1 unit/mL), and 40 μL of extract with various concentrations was mixed and consumed as a reaction reagent. The reaction reagent was preincubated for 20 minutes at 37ºC to stabilize the enzyme. Then, 40 μL ρ-nitrophenyl glucopyranoside (ρ -NPG) (5 mM) was mixed as a substrate and incubated for 20 minutes at 37ºC. The enzyme reaction was stopped by treating 100 μL Na_2CO_3 (0.1 M). The absorbance of p-nitrophenyl, which is released through the decomposition of ρ-NPG, was measured at 405 nm using a microplate

reader (Apoch 2, BioTek Co.). The reaction reagent without extract was utilized as a negative control group. The inhibitory activity could be calculated with the following equation (5):

$$\alpha\text{-Glucosidase inhibitory activity (\%)} = \left(1 - \frac{As_{405nm}}{Ac_{405nm}}\right) \times 100 \quad (5)$$

where As_{405nm}, absorbance of test sample at 20 minutes; Ac_{405nm}, absorbance of control at 20 minutes.

10. Statistical analysis

All analyses were performed in triplicates. Statistical processing was carried out using Minitab software program (Minitab 19, Minitab LLC., State College, Pennsylvania, U.S.) and significant difference were determined with 95% confidence through one way analysis of variance (ANOVA) Tukey analysis.

III. Results and Discussion

1. Optimisation of fermentation conditions in protease production by *B.licheniformis* SF5-1 according to concentration of soluble starch, soybean meal and calcium chloride

Composition of media is one of the most important factors for growth and enzyme production. Protease production of *B.licheniformis* SF5-1 in varying content of carbon, nitrogen, and mineral sources was analyzed in this study. For optimization the production of protease, Box-Behnken experimental design of the response surface method was utilized. Result of response surface method suggested that *B.licheniformis* SF5-1 has an optimal protease activity in media which has a component of 2.0152 % starch, 13.7374 % soybean meal, and 1.0768% calcium chloride. Under optimal conditions, the protease produced by *B.licheniformis* SF5-1 was predicted to produce 0.0859 μmol/g·min. On the optimum condition, the

experiment was performed for confirming the variation of protease production. Verification value of response surface methodology was 0.0882, which is shown very close to predicted value. The relationship between the three factors and protease production was shown in equation (2):

$$Y = 0.0119 + 0.0035\,x + 0.0094\,y + 0.0113\,z + 0.000083\,xy + 0.000041\,xz + 0.008930\,yz + 0.000029\,x^2 + 0.003920\,y^2 + 0.001155\,z^2 \quad (2)$$

Table 3 shows the result of ANOVA test for the design model. The significance of the model is highly significant within 1% through *p*-value ($p<0.001$). Lack of fit test also can establish the significance of the model through its *p*-value ($p>0.05$). The R^2 value of 0.9774 indicated that Eq.2 has an accuracy of 97.74% in optaining the predicted results under experimental conditions. Response surface plots and the contour plots of the response surface method showing the effect of two independent variables as shown in figure 1, 2, and 3. The other variable was fixed at

Table 3. ANOVA for Box-Behnken design model for optimizing protease activity of *B.licheniformis* SF5-1

Source	Sum of squares	df	Mean of square	F value	*p*-value	Significance
Model	$5.2 \cdot 10^{-5}$	9	$0.6 \cdot 10^{-5}$	24	0	S[1)]
A	$5.2 \cdot 10^{-4}$	1	$0.2 \cdot 10^{-5}$	6.23	0.06	NS[2)]
B	$5.2 \cdot 10^{-3}$	1	$0.2 \cdot 10^{-5}$	80.6	0	S
C	$5.2 \cdot 10^{-5}$	1	$0.3 \cdot 10^{-5}$	11.1	0.02	S
A^2	$5.2 \cdot 10^{-5}$	1	$0.1 \cdot 10^{-5}$	2.84	0.15	NS
B^2	$5.2 \cdot 10^{-5}$	1	$2.3 \cdot 10^{-5}$	94	0	S
C^2	$5.2 \cdot 10^{-5}$	1	$0.1 \cdot 10^{-5}$	5.12	0.07	NS
AB	$5.2 \cdot 10^{-5}$	1	$0.1 \cdot 10^{-5}$	3.86	0.11	NS
AC	$5.2 \cdot 10^{-5}$	1	$0.2 \cdot 10^{-5}$	8.65	0.03	S
BC	$5.2 \cdot 10^{-5}$	1	$0.2 \cdot 10^{-5}$	8.36	0.03	S
Residual	$5.2 \cdot 10^{-5}$	5	0			
Lack of fit	$5.2 \cdot 10^{-5}$	3	0	1.49	0.43	NS
Pure error	0	2	0			
Cor. Total	$5.2 \cdot 10^{-5}$	14				

R^2, 0.9774; Adj-R^2, 0.9366; Pred-R^2, 0.7339

[1)] S: significant

[2)] NS: not significant

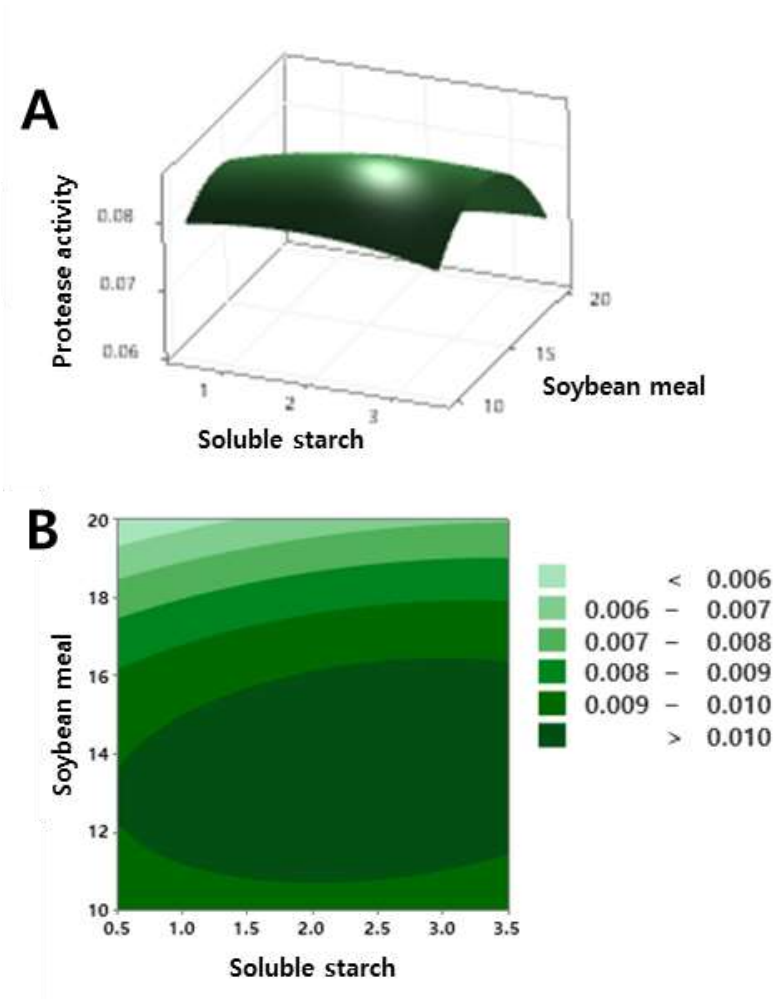

Figure 1. Plots of protease production versus concentration of soluble starch and soybean meal with calcium chloride fixed at the mean value of the test range
A, response surface plot; B, contour plot

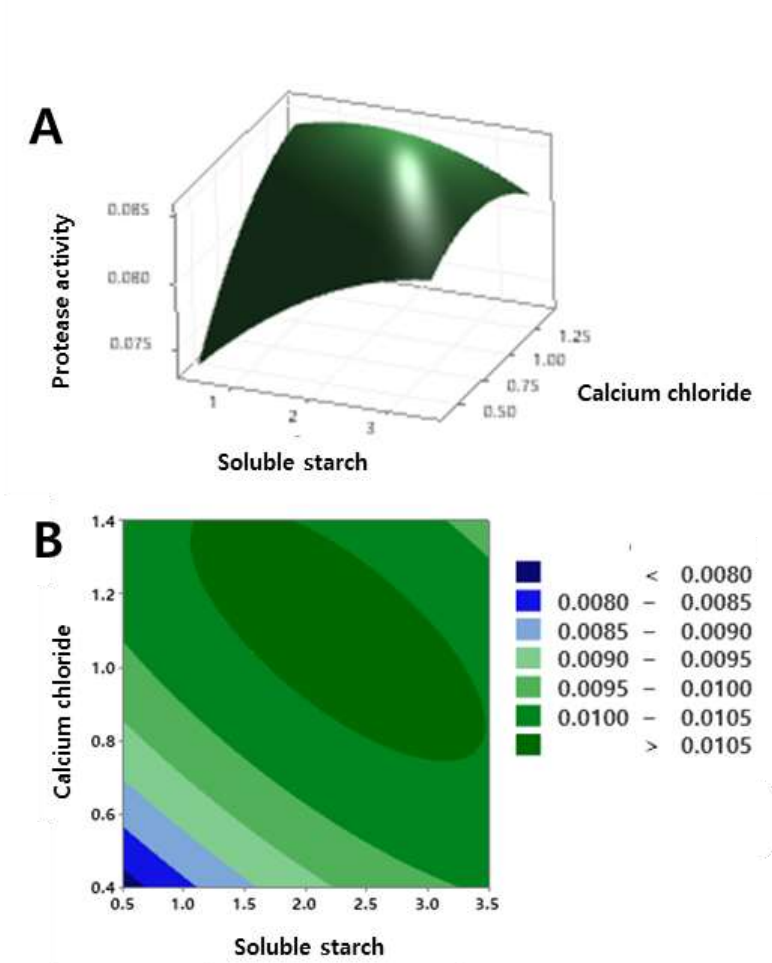

Figure 2. Plots of protease production versus concentration of soluble starch and calcium chloride with soybean meal fixed at the mean value of the test range

A, response surface plot; B, contour plot

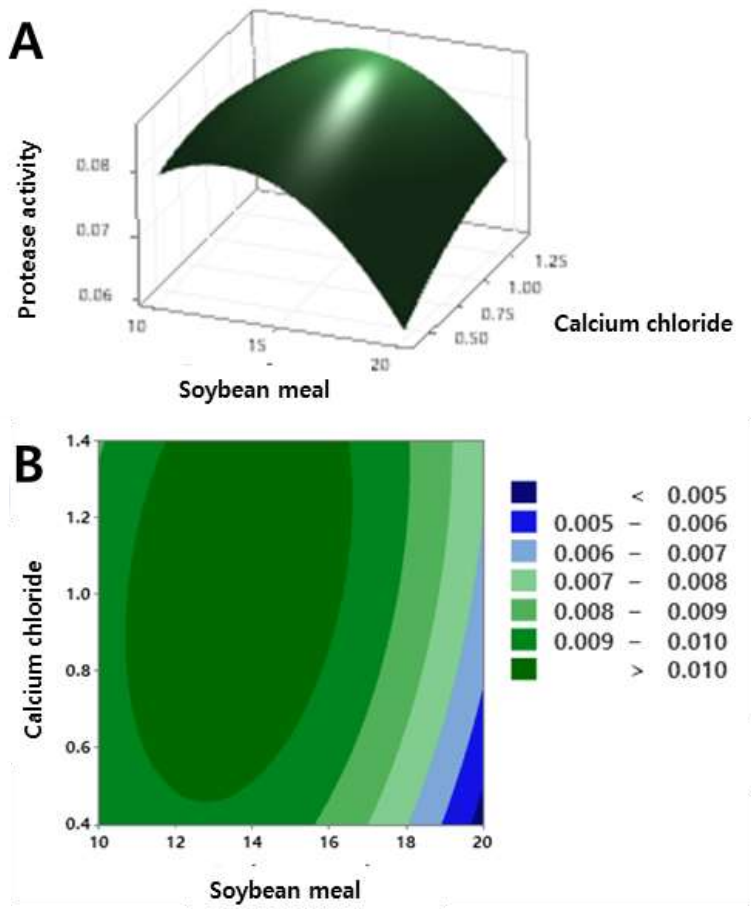

Figure 3. Plots of protease production versus concentration of soybean meal and calcium chloride with soluble starch fixed at the mean value of the test range

A, response surface plot; B, contour plot

the mean value of the test range.

2. Protein molecular size measurement of soybean meal fermented in optimal protease production condition of *B.licheniformis* SF5-1 using SDS-PAGE

Protein size of fermented soybean meal was measured through SDS-PAGE (Liu et al., 2007), and unfermented soybean was utilized as a negative control group. Box (a) of figure 4 shows that the protein bands at the top of line A are not visible at the top of line B. These bands indicate that the proteins larger than 180 kDa have been digested by the fermentation of *B.licheniformis* SF5-1. And line B has more thicker bands than line A. These suggest that the proteins above 180 kDa have been digested into proteins or peptides below 63 kDa.

3. *In vitro* protein digestibility of soybean meal

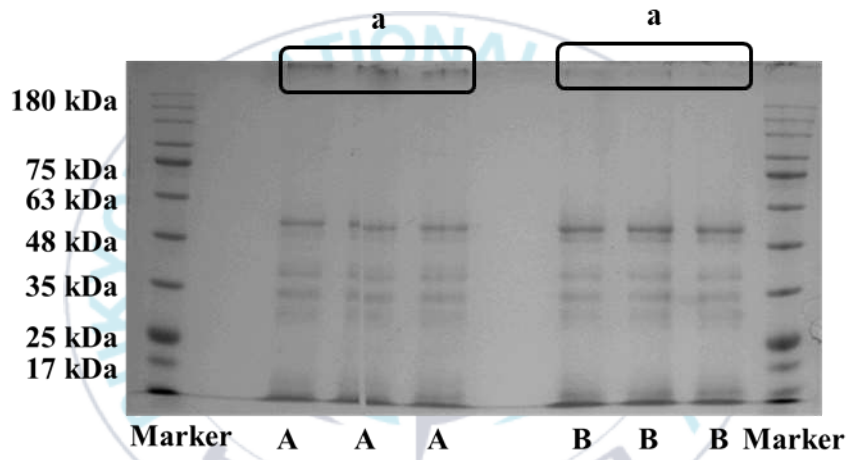

Figure 4. SDS-PAGE gel of unfermented and fermented soybean meal

A, unfermented soybean meal; B, fermented soybean meal; a, protein bands larger than 180 kDa

fermented in optimal protease production condition of *B.licheniformis* SF5-1

In vitro protein digestibility was performed in an environment of the stomach and small intestine with pepsin and pancreatin enzymes each (Aguirre et al., 2022). *In vitro* protein digestibility was determined using bovine serum albumin as a standard. Table 4 shows about 10.28% enhancement of *in vitro* protein digestibility by fermentation. This result suggested that fermentation of *B.licheniformis* SF5-1 can make soybean proteins or peptides easier to digest in the human digestion system.

4. Bound and free amino acids content of soybean meal fermented in optimal protease production condition of *B.licheniformis* SF5-1

The difference between bound and free amino acids of soybean meal fermented by *B.licheniformis* SF5-1 is the presence of bonds. Bound amino acids exist in the form of proteins or peptides, whereas free amino

Table 4. *In vitro* protein digestibility of soybean meal fermented in optimal condition of *B.licheniformis* SF5-1

(unit : %)

	***In vitro* digestibility**
SBM[1]	29.24 ± 2.54[3)a4)]
FSBM[2]	39.52 ± 4.06[b]

[1)] SBM: unfermented soybean meal
[2)] FSBM: fermented soybean meal
[3)] Values are Mean ± SD. Values are the mean of triplicates.
[4)] The same letters in the line are not significantly different at the 5% level ($p < 0.05$).

acids exist as a single amino acid. Content of bound amino acids in sample was analyzed. Table 5 suggests the overall reduction trend in the level of bound amino acid content after fermentation. This result indicated that amino acids that are bound as proteins or peptides are broken down in the bond by fermentation, resulting in a reduction in content. Furthermore, table 5 shows that the content of essential amino acids among the free amino acids has an increasing trend after fermentation, except for isoleucine as shown in table 6. This result supported the finding that fermentation of *B.licheniformis* SF5-1 breaks down protein and peptide molecules, reducing the content of bound amino acids. And this result suggested an increase in the digestibility and absorption of protein.

5. Total phenol content of soybean fermented in optimal protease production condition of *B.licheniformis* SF5-1

Total phenol content is an indicator to determine the amount of

Table 5. Content of bound amino acids of soybean meal fermented in optimal condition of *B.licheniformis* SF5-1

(unit : mg/100g)

Type	Amino acids	SBM[1)]	FSBM[2)]
Essential amino acids	Threonine	608.95	496.75
	Valine	226.90	185.50
	Methionine	276.65	228.75
	Isoleucine	960.05	795.35
	Leucine	221.90	184.45
	Phenylalanine	240.65	195.35
	Lysine	121.30	106.25
	Histidine	249.00	194.90
Nonessential amino acid	Aspartic acid	77.15	55.25
	Serine	233.20	181.80
	Glutamic acid	396.00	326.65
	Glycine	190.10	160.15
	Alanine	281.15	239.80
	Cystine	371.55	293.20
	Tyrosine	150.85	123.85
	Arginine	383.60	282.95
	Proline	258.05	238.80

[1)] SBM: unfermented soybean meal

[2)] FSBM: soybean meal fermented in optimal condition of *B.licheniformis* SF5-1

Table 6. Content of essential amino acids among free amino acids in soybean meal

(unit : mg/100g)

Amino acids	SBM[1)]	FSBM[2)]
Threonine	0.35	0.42
Valine	1.98	0.42
Methionine	0.07	0.28
Leucine	0.85	2.55
Phenylalanine	0.64	3.11
Lysine	2.76	1.48
Histidine	0.78	1.70
Isoleucine	1.05	1.05

[1)]SBM: unfermented soybean meal
[2)]FSBM: soybean meal fermented in optimal condition of *B.licheniformis* SF5-1

compounds contained in the plants are generally treated as natural antioxidants (Yang et al., 2019). Total phenol content was detected in fermented soybean meal and unfermented soybean meal samples as shown in table 7. In fermented sample, total phenol content was 308.73 mg of GAE/mL, twice as high as in unfermented one, 169.31 mg of GAE/mL. Another research utilizing *Bacillus amyloliquefaciens* SWJS22 showed that the content of total phenolic compounds approximately doubled (Yang et al., 2019), a trend similar to that found in this study.

6. Antioxidant activity of soybean fermented in optimal protease production condition of *B.licheniformis* SF5-1

DPPH and ABTS radical scavenging activity are antioxidant measurement analyses to measure the ability to interrupt the oxidation of free radicals (Baliyan et al., 2022). DPPH and ABTS radical scavenging activity of fermented soybean meal extract, unfermented soybean meal extract, and

Table 7. Total phenol content of soybean meal extract

(unit : mg of GAE/g)[1)]

Sample	Total phenol content
FSBM[2)] extract	308.73 ± 3.01[4)a5)]
SBM[3)] extract	169.31 ± 3.24[b]

[1)] Total phenol content was presented by milligrams of gallic acid equivalents per gram of sample.
[2)] FSBM: soybean meal fermented in optimal condition of *B.licheniformis* SF5-1
[3)] SBM: unfermented soybean meal extract
[4)] Values are Mean ± SD. Values are the mean of triplicates.
[5)] The same letters in the line are not significantly different at the 5% level ($p < 0.05$)

gallic acid was determined in various concentrations as shown in table 8. DPPH radical scavenging activity was increased from 11.76% to 14.02% after fermentation. Also, ABTS radical scavenging activity tended to increase from 12.27% to 17.49%. Fermented soybean meal extract has a DPPH radical scavenging activity equivalent to approximately 40 μg/mL of gallic acid and ABTS radical scavenging activity equivalent to approximately 2.5 μg/mL of gallic acid. Other research has shown that fermentation increased DPPH and ABTS radical scavenging activity in soybeans (Sumruan et al., 2012). Isoflavones and phenolic compounds were the two main antioxidant compounds in soybeans (Yoon & Park., 2014; Pabich, Marciniak, & Kontek., 2021), so the increased antioxidant activity was supported by the result of total phenol content above.

7. Antidiabetic activity of soybean meal fermented in optimal protease productivity condition of *B.licheniformis* SF5-1

Table 8. DPPH and ABTS radical scavenging activities of extracts and gallic acid

(Unit : %)

Sample	DPPH radical scavenging activity	ABTS radical scavenging activity
40 ug/mL Gallic acid	15.42 ± 1.16[3)a4)]	-
2.5 ug/mL Gallic acid	-	12.65 ± 3.53[d]
FSBM[1)] extract	14.02 ± 1.07[ab]	17.49 ± 0.66[d]
SBM[2)] extract	11.76 ± 1.39[b]	12.27 ± 0.64[d]

[1)] FSBM: soybean meal fermented in optimal condition of *B.licheniformis* SF5-1
[2)] SBM: unfermented soybean meal
[3)] Values are Mean ± SD. Values are the mean of triplicates.
[4)] The same letters in the line are not significantly different at the 5% level ($p < 0.05$)

α-Amylase and α-glucosidase are the main enzymes that are conscious of human carbohydrate digestion (Ali, H., Houghton, P. J., & Soumyanath, A., 2006). Each of the enzymes performs digestion mainly in the mouth and small intestine. The inhibition activity of α-amylase and α-glucosidase was measured as concentration of fermented soybean meal extracts as shown in table 9 and table 10. Both inhibitory activities increased in proportion to extract concentration, suggesting that soybean meal extract has antidiabetic activity and that fermented soybean meal extract is more inhibitory than unfermented. Comparing the experimental results of the two enzymes, α-glucosidase was inhibited by 32.1% at a concentration of 5 mg/mL extracts, whereas α-amylase was inhibited by 6.76% at 50 mg/mL, suggesting that fermented soybean meal extracts have more inhibition activity on α-glucosidase than on α-amylase. In previous research, there was a result that specific peptides of fermented soybean inhibit α-amylase and α-glucosidase (Tang et al., 2023). These results suggest peptides capable of inhibiting α-glucosidase are produced during the fermentation of *B.licheniformis* SF5-1.

Table 9. α-Amylase inhibition activity by concentration of soybean meal extract

(Unit : %)

	Concentration of extract (mg/mL)	
	25	**50**
FSBM[3] extract	3.11±0.35[1)a2)]	6.76±0.26[c]
SBM[4] extract	0.91±0.23[b]	2.51±1.21[ab]

[1] Values are Mean ± SD. Values are the mean of triplicates.
[2] The same letters in the line are not significantly different at the 5% level ($p < 0.05$)
[3] FSBM: soybean meal fermented in optimal condition of *B.licheniformis* SF5-1
[4] SBM: unfermented soybean meal

Table 10. α-Glucosidase inhibition activity by concentration of soybean meal extract

(Unit : %)

	Concentration of extract (mg/mL)	
	5	**10**
FSBM[2)]	32.1±0.45[1)a2)]	79.19±0.22[b]
SBM[3)]	3.51±0.52[c]	22.8±0.8[d]

[1)] Values are Mean ± SD. Values are the mean of triplicates.
[2)] The same letters in the line are not significantly different at the 5% level ($p < 0.05$)
[2)] FSBM: soybean meal fermented in optimal condition of *B.licheniformis* SF5-1
[3)] SBM: unfermented soybean meal

VI. Summary

Soybean meal is very high in protein, but it is used in animal feed mainly because of its poor digestibility in the human body. Therefore, this study was conducted to increase the digestibility of soybean meal in the human body and the bioactive activity through fermentation by utilizing *B.licheniformis* SF 5-1. *B.licheniformis* SF5-1 was selected as the natural protease producer. Response surface methodology was used to determine the optimal conditions for *B.licheniformis* SF5-1 to get maximum protease activity. The Box-Behnken design was adopted as the experimental design. At 2.02% soluble starch, 13.74% soybean meal, and 1.08% calcium chloride, the predicted protease activity was 0.09 μmol/g·min. The digestion of proteins above 180 kDa to proteins or peptides below 63 kDa was verified by the result of SDS-PAGE. This resulted in a 6.33% increase in protein digestibility from 33.19% to 39.52%. The antioxidant capacity of the fermented soybean meal extract increased from 169.31 mg/mL to 308.73 mg/mL, the DPPH radical scavenging capacity increased from 11.76% to 14.02%, and the ABTS radical scavenging capacity increased

from 12.27% to 17.49%, confirming the increased antioxidant capacity of the fermented soybean meal extract. The antidiabetic activity of the extract increased from 2.51% to 6.76% inhibition of α-amylase at 50 mg/mL concentration and from 22.8% to 79.19% inhibition of α-glucosidase at 5 mg/mL concentration.

V. References

Aguirre, L., Cámara, L., Smith, A., Arroyo, J. J., de Juan, A. F., Fondevila, G., & Mateos, G. G. (2022). Chemical composition, protein quality indicators and *in vitro* protein digestibility of commercial soybean meals from different origins for use in poultry feeding. Animal Feed Science and Technology, 293, 115473.

Ali, H., Houghton, P. J., & Soumyanath, A. (2006). α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. Journal of Ethnopharmacology, 107(3), 449-455.

Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R. P., & Chang, C. M. (2022). Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules, 27(4), 1326.

Cupp-Enyard, C. (2008). Sigma's non-specific protease activity assay-casein as a substrate. Journal of Visualized Experiments, (19), e899.

de Oliveira Silva, F., & Perrone, D. (2015). Characterization and stability of bioactive compounds from soybean meal. LWT-Food Science and Technology, 63(2), 992-1000.

Han, D. M., Chun, B. H., Kim, H. M., & Jeon, C. O. (2021). Characterization and correlation of microbial communities and metabolite and volatile compounds in doenjang fermentation. Food Research International, 148, 110645.

Hung, D. Y., Cheng, Y. H., Chen, W. J., Hua, K. F., Pietruszka, A., Dybus, A., Lin, C. S., & Yu, Y. H. (2019). *Bacillus licheniformis*-fermented products reduce diarrhea incidence and alter the fecal microbiota community in weaning piglets. Animals, 9(12), 1145.

Jeong, D. W., Heo, S., Lee, B., Lee, H., Jeong, K., Her, J. Y., Lee, K., &

Lee, J. H. (2017). Effects of the predominant bacteria from meju and doenjang on the production of volatile compounds during soybean fermentation. International Journal of Food Microbiology, 262, 8-13

Jeong, D. W., Kim, H. R., Jung, G., Han, S., Kim, C. T., & Lee, J. H. (2014). Bacterial community migration in the ripening of doenjang, a traditional Korean fermented soybean food. Journal of Microbiology and Biotechnology, 24(5), 648-660.

Kim, H. J., Ha, B. K., Ha, K. S., Chae, J. H., Park, J. H., Kim, M. S., Sovetgul, A., Grover, S., Son, C. K., & Lee, J. D. (2015). Comparison of a high oleic acid soybean line to cultivated cultivars for seed yield, protein and oil concentrations. Euphytica, 201, 285-292.

Kwon, D. Y., Daily III, J. W., Kim, H. J., & Park, S. (2010). Antidiabetic effects of fermented soybean products on type 2 diabetes. Nutrition Research, 30(1), 1-13.

Liu, S., Zhou, R., Tian, S., & Gai, J. (2007). A study on subunit groups of soybean protein extracts under SDS-PAGE. Journal of the American Oil Chemists' Society, 84, 793-801.

Ministry of Food and Drug Safety (MFDS). (2023). Food Code. Retrived from https://www.mfds.go.kr/eng. Accessed on Nov. 7, 2023.

Pabich, M., Marciniak, B., & Kontek, R. (2021). Phenolic compound composition and biological activities of fractionated soybean pod extract. Applied Sciences, 11(21), 10233.

Rizzo, G., & Baroni, L. (2018). Soy, soy foods and their role in vegetarian diets. Nutrients, 10(1), 43.

Samruan, W., Oonsivilai, A., & Oonsivilai, R. (2012). Soybean and fermented soybean extract antioxidant activities. International Journal of Nutrition and Food Engineering, 6(12), 1134-1137.

Savoie, L., & Gauthier, S. F. (1986). Dialysis cell for the *in vitro* measurement of protein digestibility. Journal of Food Science, 51(2), 494-498.

Singh, N., Shepherd, K., & Cornish, G. (1991). A simplified SDS-PAGE procedure for separating. Journal of Cereal Science, 14, 203-208.

Storebakken, T., Kvien, I. S., Shearer, K. D., Grisdale-Helland, B., Helland, S. J., & Berge, G. M. (1998). The apparent digestibility of diets containing fish meal, soybean meal or bacterial meal fed to Atlantic salmon (*Salmo salar*): evaluation of different faecal collection methods. Aquaculture, 169(3-4), 195-210.

Tang, X., Chen, X., Wang, H., Yang, J., Li, L., Zhu, J., & Liu, Y. (2023). Virtual Screening Technology for Two Novel Peptides in Soybean as Inhibitors of α-Amylase and α-Glucosidase. Foods, *12*(24), 4387.

Telagari, M., & Hullatti, K. (2015). *In-vitro* α-amylase and α-glucosidase inhibitory activity of Adiantum caudatum Linn. and Celosia argentea Linn. extracts and fractions. Indian Journal of Pharmacology, *47*(4), 425.

Tonsor, G. T., Lusk, J. L., & Schroeder, T. C. (2023). Market potential of new plant-based protein alternatives: insights from four US consumer experiments. Applied Economic Perspectives and Policy, 45(1), 164-181.

Towa, L. T., Kapchie, V. N., Wang, G., Hauck, C., Wang, T., & Murphy, P. A. (2011). Quantity and quality of free oil recovered from enzymatically disrupted soybean oleosomes. Journal of the American Oil Chemists' Society, 88, 1581-1591.

Yang, J., Wu, X. B., Chen, H. L., Sun-Waterhouse, D., Zhong, H. B., & Cui, C. (2019). A value-added approach to improve the nutritional quality of soybean meal byproduct: Enhancing its antioxidant

activity through fermentation by *Bacillus amyloliquefaciens* SWJS22. Food Chemistry, 272, 396-403.

Yoon, G. A., & Park, S. (2014). Antioxidant action of soy isoflavones on oxidative stress and antioxidant enzyme activities in exercised rats. Nutrition Research and Practice, 8(6), 618-624.

Yuan, L., Chang, J., Yin, Q., Lu, M., Di, Y., Wang, P., Wang, Z., Wang, E., & Lu, F. (2017). Fermented soybean meal improves the growth performance, nutrient digestibility, and microbial flora in piglets. Animal Nutrition, 3(1), 19-24.

Zheng, L., Regenstein, J. M., Zhou, L., & Wang, Z. (2022). Soy protein isolates: A review of their composition, aggregation, and gelation. Comprehensive Reviews in Food Science and Food Safety, 21(2), 1940-1957.