International Journal of Agro Nutrifood Practices
Vol-4, Issue-1, March 2024
e-ISSN: 2583-066X

Effect of Enoki Mushroom Fibrous Protein on Plant Protein Formulation Digestibility

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Abstract

This study investigates the impact of fibrous proteins from enoki mushrooms on the digestibility of a plant-based formulation (2B), measured by the degree of hydrolysis (DH). The fibrous protein (E) was derived from Flammulina velutipes (enoki mushroom), while the bio-ink (B), a plant-protein formulation, consisted of pea protein (10%), soy protein (10%), and cornmeal (5%) in sodium alginate (0.5%) and water (74.5%). The plant proteins were combined to provide a complete profile of essential amino acids. Three samples with different ratios were studied: (i) sample with the highest fibrous protein content (2B:2E), (ii) sample with lower fibrous protein content (2B:1E), and (iii) sample without any fibrous protein (2B). These samples were heated at 60°C for five minutes and afterward subjected to in-vitro digestion (IVD), whereby free amino acids were measured using the ortho-phthalaldehyde (OPA) assay. The DH of the samples was calculated by dividing the free amino acid groups after digestion, by those before digestion. Significant differences were observed in the digestibility of the samples, based on their DH which were: 65.1% ($P < 0.05$), 54.1% ($p = 0.000343$), and 45.8% ($p = 0.000239$), for 2B, 2B:1E, and 2B:2E, respectively. Thus, the 2B:2E sample exhibited the lowest digestibility, followed by 2B:1E and 2B ($P < 0.05$). Likely the formation of more S-S bonds in the samples after heating made 2B:2E and 2B:1E more resistant to enzymatic action. We concluded that the 2B:1E ratio was optimal, as it balances the benefits of enoki mushrooms without greatly reducing digestibility.

Keywords

Amino Acids, Degree of Hydrolysis, Enoki Mushrooms, Digestibility.

BACKGROUND

The paradigm shift towards plant-based diets is reshaping the landscape of the food industry, driven by a growing awareness of environmental sustainability, ethical considerations, and the desire for healthier lifestyles[1]. In the pursuit of creating plant-based foods that not only meet but exceed consumers' expectations, the selection of ingredients plays a pivotal role. Among these ingredients, enoki mushrooms (*Flammulina velutipes*) stand out as a novel and promising addition to plant-based food formulation, by enhancing digestion when introduced in a suitable amount. This results in more amino acids available after digestion of the food, improving the nutritional value in them.

MOTIVATION

On a compositional basis, 100 grams of enoki mushrooms contain approximately 88.3 grams of water, 7.81 grams of fat, and 2.7 grams of dietary fiber, with the remainder comprising sugars, mineral ions, and vitamins[2]. Therefore, enoki mushrooms are low in calories, making them a suitable addition to plant-based formulations for individuals looking to manage their caloric intake. Additionally, enoki mushrooms are a good source of dietary fiber, particularly beta-glucans[3] which offers several benefits. Dietary fiber is crucial in digestive health, promoting regular bowel movements and supporting overall gut health. Enoki mushrooms also boast various vitamins and minerals, including B vitamins such as niacin and riboflavin, potassium, and copper[4]. These nutrients contribute to the overall nutritional content of plant-based products, should they be incorporated. From a medicinal food perspective, enoki mushrooms have medicinal properties as they contain antioxidants, including ergothioneine and polyphenols, which help combat oxidative stress in the body[5]. Palatability-wise, enoki mushrooms have a mild, delicate flavour and a unique, slender appearance, making them versatile ingredients in plant-based cooking. They can be easily incorporated into a variety of dishes, adding texture and visual appeal.

While enoki mushrooms are not a significant source of soluble protein when added to plant protein formulation, they may indirectly impact plant protein digestibility in the following way. As enoki mushrooms contain insoluble fibrous proteins, when added to plant proteins, they may prevent the enzymes from digesting the plant proteins, decreasing the digestibility of plant protein formulation. Furthermore, in the context of cooking, subjection to heat induces the formation of disulfide (S-S) bonds from thiol (S-H) groups in fibrous proteins[6]. This thermal treatment enhances the cross-linking of protein chains, thereby augmenting the fibrous network structure. The resultant increase in disulfide bonding contributes to a more rigid and robust protein matrix, which in turn increases the protein's resistance to enzymatic degradation. Therefore, this study aims to determine the optimal ratio of plant protein formulation and enoki mushrooms after cooking to maximize digestibility, enhancing amino acid yield and overall nutritional value.

The plant proteins chosen are pea protein isolate, soy protein concentrate, and yellow cornmeal powder. These three plant proteins complement each other to give a complete essential amino acids profile, which is a dietary requirement for human beings[7]. Pea and soy protein lack cysteine and methionine[8], both of which are sulfur-containing amino acids, which can be complemented by adding a yellow corn meal that contains some amounts of cysteine and methionine[9]. These plant proteins will be mixed and fortified with enoki mushrooms in differing ratios to study their impact on digestibility in this experiment.

OBJECTIVES

The protein, S-H bonds, and S-S bonds quantification in the selected plant-based formulation samples were carried out to establish relationships and draw correlations between the samples and digestibility results later. The methods used for such quantification include the BCA (Bicinchoninic Acid) assay for protein determination, Ellman's assay to gauge S-H bond concentration, and the spectroscopic method to quantify S-S bonds. These quantification methods play a pivotal role in understanding plant-based foods' total protein and fibrous protein content and how their differing quantities affect digestibility. After the quantification of the different samples, the samples were heated at 60°C to mimic cooking, and IVD was carried out on the food samples. Afterward, the DH of the samples was determined, whereby the DH (%) tells which sample has the greatest digestibility, indicated by the greatest DH value (%). The sample with the greatest DH is the best for potential consumption as it can release the most amino acids during the digestion process. Consequently, the sample exhibiting the greatest extent of protein digestibility would be considered the most nutritionally advantageous among the samples.

MATERIALS AND METHODS

Plant-protein formulation

The plant-based formulation consists of plant proteins that contain essential amino acids. The pea, soy, and corn meal proteins complement each other to provide complete essential amino acids. The plant-based formulation is termed Bioink (B) in this research paper.

Table 1. Bioink composition (% by mass in comparison with water).

Ingredient	Composition (%)
Pea protein	10
Soy protein Isolate	10
Corn meal flour	5
Sodium Alginate	0.5
Water	74.5

Table 2. Ratio of Bioink and Enoki mushroom ratio.

Bioink (ratio)	Enoki mushroom (ratio)
2	2
2	1
2	0
0	1
0	2

BCA test for proteins in samples

The samples were prepared in 20mg/mL concentration in DI water. The BCA working reagent was prepared by mixing the BCA reagent (containing Bicinchoninic Acid), copper sulfate, and a stabilizing reagent. The BCA reagent was mixed with the protein samples and the mixture was incubated at an elevated temperature (usually 37°C) for a specific period. During the incubation, the BCA reagent reacted with the proteins, resulting in the formation of a purple-colored complex. Following incubation, the samples were measured at 562 nm using a spectrophotometer. A calibration curve was prepared using known protein concentrations to quantify the samples' protein concentration to establish a linear relationship between absorbance and protein concentration.[10]

The Ellman's reagent (4mg/mL) was prepared in a reaction buffer (0.1 M Sodium Phosphate, 1mM EDTA). Standard solutions containing cysteine monochloride dihydrate ranging from 1.5mM to 0.25mM were prepared with 50µL of Ellman's reagent each. For sample preparation, the sample was dissolved to a concentration of 40g/L, and 50µL of Ellman's reagent was mixed with 250µL of the sample in a centrifuge tube containing 2.5mL of the buffer. The plate setup involved pipetting 200µL of the prepared sample into a well of a 96-well plate, followed by recording the absorbance at 412nm using UV-Vis spectroscopy.

S-S bonds of proteins in samples

The determination of disulfide bond concentration in protein samples was achieved through a method involving the measurement of absorbance at 283nm and the use of Beer Lambert's Law. To begin, 40g/L of the sample containing disulfide bonds is dissolved in a 10-3 M EDTA buffer with a pH of 8. Dithiothreitol (DTT) was then added to break the disulfide bonds, and the resulting solution was homogenized. Subsequently, 250µL of the prepared solution is pipetted into a well of a 96-well plate for UV-Vis spectroscopy. Absorbance at 283 nm was recorded for triplicate samples. The disulfide bond concentration was calculated for each sample using the Beer Lambert's Law equation ($C=\varepsilon lA$), with a molar extinction coefficient of 273 based on the methodology of Iyer and Klee (1972).[11]

In-vitro digestion

In-vitro digestion was carried out using the protocol as described by Menikus and his coworkers.[12] The samples were heated at 60°C to mimic cooking, and subjected to IVD as stated in the steps below.

Oral Phase

A 0.5 g food sample was minced with approximately 20 mL of water in a 250 mL beaker for 2-5 minutes. The resulting liquid mixture was transferred into a centrifuge tube and spun at 10,000 rpm for 10 minutes. Pepsin was taken out of the fridge to reach room temperature. After removing the supernatant, a 0.1-0.5-gram solid sample was weighed and diluted with 5 mL of water in a 50 mL centrifuge tube. This sample was then incubated in a 37°C oven. To prepare the enzyme solution, 53.57 mg of amylase was mixed with 4 mL of Simulated Saliva Fluid (SSF), 975 µL of water, and 25 µL of 0.3 M CaCl2 in a 5 mL Eppendorf tube and incubated in a 37°C oven. Once the sample and enzyme reached equilibrium at 37°C, the mixture was poured and pipetted into a 50 mL centrifuge tube. A 10 mL portion of the sample mixture was fixed on a rotary incubator set at a 90-degree angle for 2 minutes.

Gastric Phase

While the oral phase samples were incubating, 10 mg of pepsin was dissolved in 8 mL of cold Simulated Gastric Fluid (SGF) with 5 µL of 0.3 M CaCl2. The solution was vortexed only when necessary. The pH was adjusted to 3 using 40 µL of 6 M HCl, and an additional 1 M HCl was added in 10 µL increments. The enzyme solution was then added to the oral bolus, with further pH adjustments made by adding 1 M HCl in 10 µL increments until reaching pH 3. The total volume of acid added was recorded as x. To complete the volume to 20 mL, (1995 – x) µL of water was added. The 20 mL sample mixture was fixed on a rotary incubator set at a 90-degree angle for two hours.

Intestinal Phase

A mixture of 102.8 mg of pancreatin, 164 mg of bile extract, and 40 µL of 0.3 M CaCl2 was added to 16 mL of Simulated Intestinal Fluid (SIF). The mixtures were combined, and the pH was adjusted by adding 1 M NaOH in 10 µL increments until reaching pH 7. The total volume of base added was recorded as y. To complete the volume to 40 mL, (3960 – y) µL of water was added. The 40 mL sample mixture was fixed on a hula mixer in a 37°C oven, set at a 90-degree angle, for two hours.

Degree of hydrolysis using the OPA method

OPA reacts with the primary amine group to determine the amount of free amino acids group, and therefore degree of hydrolysis (DH). The OPA and sample solution were mixed in a 1: 1 ratio for 2 mins. Immediately after, a UV-Vis spectrophotometer measured the solution mixture at 340nm. The degree of hydrolysis was calculated using this equation

$$\mathrm{DH} = \frac{H}{H_{tot}} \times 100\% \qquad (1)$$

Where H refers to the amount of free amino acids in the digested sample, and H_{tot} is deduced by the number of free amino acids in the digested food samples, both using the OPA assay. H_{tot} was obtained by adding 6N of HCl to the samples at 118°C overnight, while H was obtained using the in-vitro digestion of the food samples[12].

4.6 A paired t-test was performed to determine the level of significance between the samples, for the various assays.

RESULTS AND DISCUSSIONS

Protein concentration

The total protein content of the samples was measured using the BCA assay at a concentration of 20 mg/mL. The results were then compared to the calibration graph using Bovine Serum Albumin (BSA) to determine the concentration.

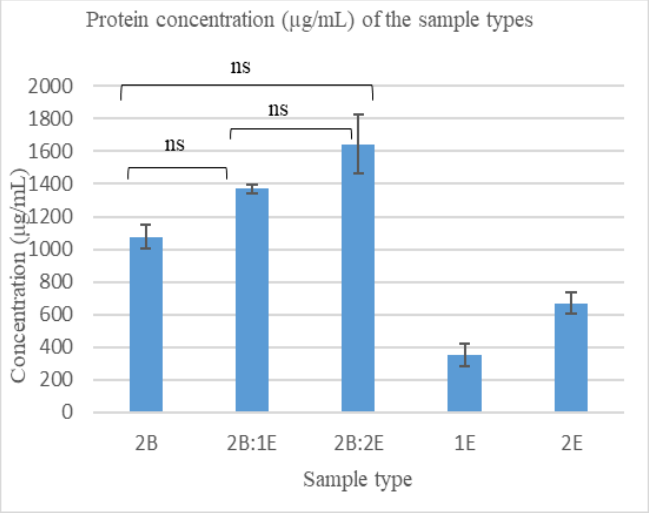

Figure 1. Protein concentration (µg/mL) of the sample types, whereby ns refers to $P > 0.05$. 2B Bioink; 2B:1E Bio-ink and Enoki mushroom in 2:1 ratio; 2B:2E Bio-ink and Enoki mushroom in a 2:2 ratio.

In Figure 1, the results revealed a slight variation in protein content among the three sample groups. The sample with the highest enoki mushroom content (2B:2E) exhibited the highest protein content, followed by the samples with lower enoki mushroom content, which showed a moderately lower protein concentration (2B:1E). Notably, the samples without enoki mushrooms (2B) displayed the lowest protein content.

In **Figure 1**, upon doing t – test, the values were all $P > 0.05$, indicating that the proteins in Enoki mushroom did not contribute to any significant differences between the protein content of the samples. This was beneficial for the study as similar protein concentrations between samples allowed for a fair study of the effect of fibrous protein in Enoki mushrooms, on protein digestion, as the level of amino acids present after digestion were less likely to be affected by different concentrations of proteins at the start.

Concentration of S-H bonds

The concentration (mmol) of S-H bonds in a food sample provided insights into its fibrous content, and higher concentrations indicated a greater fibrous texture. The S-H bond concentration of the samples was shown in Figure 2.

International Journal of Agro Nutrifood Practices
Vol-4, Issue-1, March 2024
e-ISSN: 2583-066X

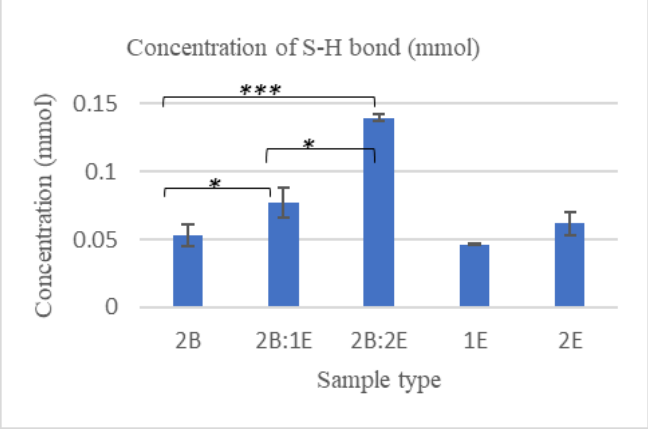

Figure 2. Concentration of S-H bonds (mmol) of the sample types, whereby *P < 0.05, **P < 0.005, and ***P < 0.0005. 2B Bio-ink; 2B:1E Bio-ink and Enoki mushroom in 2:1 ratio; 2B:2E Bio-ink and Enoki mushroom in a 2:2 ratio.

In Figure 2, the concentration of S-H bonds was highest in the 2B:2E sample, followed by the 2B:1E sample, and lowest in the 2B sample, with the level of significance indicated in the figure legend. The elevated concentration of S-H bonds observed in the samples containing Enoki mushrooms is likely attributed to the fibrous proteins present in these mushrooms. Specifically, the higher S-H bond concentration in the 2B:2E sample can be ascribed to the increased ratio of Enoki mushrooms, which contain fibrous proteins with cysteine residues[13]. These residues contribute to a higher concentration of S-H bonds in the bioink formulations with greater Enoki mushroom content. Quantification of the S-H bonds was essential as S-H promoted S-S bond formation when a sample was subjected to heat before IVD, thereby creating a more fibrous texture. These phenomena would have decreased the digestibility of the bioink, such as in 2B:1E and 2B:2E. As an increased concentration of S-H bonds showed extensive cross-linking of the fibrous protein through the formation of S-S bonds after heating of samples, it possibly made 2B:1E and 2B:2E more resistant to enzyme degradation during in-vitro digestion, as seen from the DH of the samples in reverse order: 2B > 2B:1E > 2B:2E.

Concentration of S-S bonds

Similar to 5.2, the concentration (mmol) of S-S bonds indicates the quantity of fibrous protein content in a sample, with higher concentrations representing increased protein fibrous content. The protein fibrous content of the samples is illustrated in Figure 3.

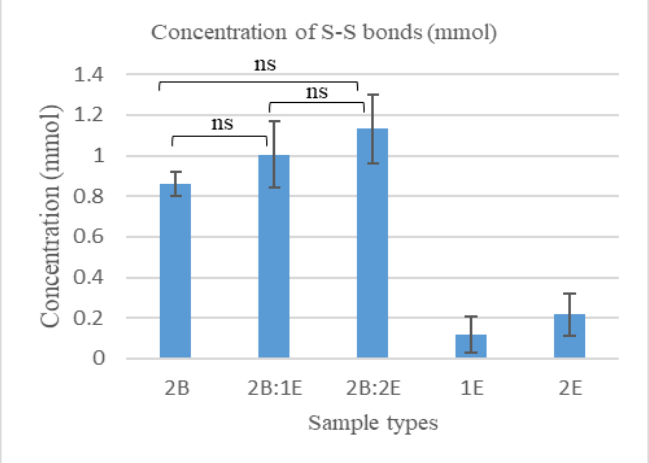

Figure 3. Concentration of S-S bonds (mmol) of the sample types, whereby ns refers to P > 0.05. 2B Bio-ink; 2B:1E Bio-ink and Enoki mushroom in 2:1 ratio; 2B:2E Bio-ink and Enoki mushroom in a 2:2 ratio.

In Figure 4, a trend was observed of the S-S bond levels being highest in the 2B:2E sample, lower in 2B:1E, and lowest in pure bio-ink, although the differences were not significant ($p>0.05$) Enoki mushroom fibrous protein structures likely contributed to the higher S-S bonds in the 2B:1E and 2B:2E samples. Higher Enoki ratios correlate with more S-S bonds, indicating increased fibrous protein content and protein coiling[14].

Degree of hydrolysis of the samples

The degree of hydrolysis was calculated using the formula from DH $= \frac{H}{HH_{tot}} \times 100\%$ (1) The concentration of amino acid (µg/mL) in the digested samples was measured using the OPA assay, and protein digestibility was assessed based on the degree of hydrolysis after digestion.

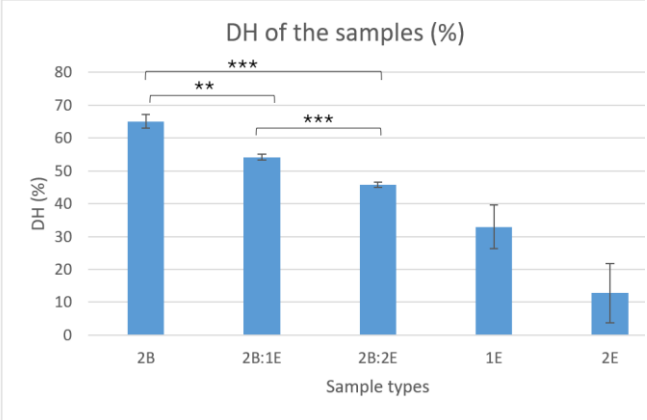

Figure 4. Degree of hydrolysis of the different samples. 2B Bio-ink; 2B:1E Bio-ink and Enoki mushroom in 2:1 ratio; 2B:2E Bio-ink and Enoki mushroom in a 2:2 ratio. Also, *P < 0.05, **P < 0.005, and ***P < 0.0005.

From **Figure 4Figure 4**, the DH was the greatest in 2B, lesser in 2B:1E, and the least in 2B:2E, with the level of significance indicated in the figure legend. The DH was

respectively 65.1%, 54.1%, and 45.8%. The results might be attributed to the presence of a higher concentration of fibrous proteins, possibly from the formation of additional disulfide (S-S) bonds following sample heating, as discussed in Section 5.2. This increase in fibrous protein content could explain the greater resistance to enzymatic degradation observed in the 2B:1E and 2B:2E samples, [15] as compared to 2B.

The extent of digestion followed the sequence: 2B > 2B:1E > 2B:2E, with the level of significance indicated in the figure legend. The greatest digestibility was observed in the 2B sample without enoki fortification, and digestibility decreases as the ratio of enoki mushroom increases. Significant differences in digestion could be attributed to the influence of fibrous proteins from enoki mushrooms, which potentially results in a more highly crosslinked matrix that resists enzymatic activity, thus reducing digestibility.

CONCLUSION

This research demonstrated that incorporating fibrous proteins from Enoki mushrooms into the 2B formulation significantly reduced digestibility after cooking. A more moderate decrease in digestibility was observed in the 2B:1E ratio, while a greater reduction was noted in the 2B:2E ratio. Despite the observed trend, it remains beneficial to include enoki mushrooms in plant-based food formulations due to their numerous health advantages. The 2B:1E ratio was preferable to 2B:2E, as it resulted in a higher availability of amino acids after digestion, potentially leading to better absorption by the gut. This study provided valuable insights into enhancing plant-based formulations with fibrous proteins from mushroom sources. Future research will explore the effects of fibrous proteins derived from plants, fruits, or other fungi on selected plant-based formulations.

Acknowledgment

The authors gratefully acknowledge the financial support and research facilities provided by the Singapore Institute of Food and Biotechnology Innovation (SIFBI). Additionally, we would like to thank the Lim Boon Heng fund from the Newcastle Research and Innovation Centre in Singapore (NewRIIS) for their generosity in and funding HXL for this conference.

REFERENCES

[1] D. McClements and L. Großmann, “The Rise of Plant-Based Foods,” 2022, pp. 1–21. doi: 10.1007/978-3-030-96764-2_1.

[2] FoodData Central, “Mushrooms, enoki, raw.”

[3] S. Rezaeian, H. R. Pourianfar, and S. A. Dowom, “Comparative assessment of β-glucan composition and structural characterization in wild and commercial Enoki mushrooms, Flammulina velutipes,” *Journal of Food Composition and Analysis*, vol. 120, p. 105286, 2023, doi: https://doi.org/10.1016/j.jfca.2023.105286.

[4] M. E. Valverde, T. Hernández-Pérez, and O. Paredes-López, “Edible mushrooms: Improving human health and promoting quality life,” Jan. 20, 2015, *Hindawi Publishing Corporation*. doi: 10.1155/2015/376387.

[5] H. Nguyen Duy Bao, H. Ushio, and T. Ohshima, “Antioxidative Activity and Antidiscoloration Efficacy of Ergothioneine in Mushroom (Flammulina velutipes) Extract Added to Beef and Fish Meats,” *J Agric Food Chem*, vol. 56, pp. 10032–10040, Oct. 2008, doi: 10.1021/jf8017063.

[6] N. Ooms, K. J. A. Jansens, B. Pareyt, S. Reyniers, K. Brijs, and J. A. Delcour, “The impact of disulfide bond dynamics in wheat gluten protein on the development of fermented pastry crumb,” *Food Chem*, vol. 242, pp. 68–74, 2018, doi: https://doi.org/10.1016/j.foodchem.2017.09.007.

[7] F. Blachier *et al.*, “Tolerable amounts of amino acids for human supplementation: summary and lessons from published peer-reviewed studies,” *Amino Acids*, vol. 53, no. 9, pp. 1313–1328, 2021, doi: 10.1007/s00726-021-03054-z.

[8] P. Qin, T. Wang, and Y. Luo, “A review on plant-based proteins from soybean: Health benefits and soy product development,” *J Agric Food Res*, vol. 7, p. 100265, 2022, doi: https://doi.org/10.1016/j.jafr.2021.100265.

[9] D. D. Loy and E. L. Lundy, “Chapter 23 - Nutritional Properties and Feeding Value of Corn and Its Coproducts,” in *Corn (Third Edition)*, S. O. Serna-Saldivar, Ed., Oxford: AACC International Press, 2019, pp. 633–659. doi: https://doi.org/10.1016/B978-0-12-811971-6.00023-1.

[10] J. M. Walker, “The Bicinchoninic Acid (BCA) Assay for Protein Quantitation,” in *Basic Protein and Peptide Protocols*, J. M. Walker, Ed., Totowa, NJ: Humana Press, 1994, pp. 5–8. doi: 10.1385/0-89603-268-X:5.

[11] K. S. Iyer and W. A. Klee, “Direct Spectrophotometric Measurement of the Rate of Reduction of Disulfide Bonds,” *Journal of Biological Chemistry*, vol. 248, no. 2, pp. 707–710, Jan. 1973, doi: 10.1016/s0021-9258(19)44430-x.

[12] M. Minekus *et al.*, “A standardised static in vitro digestion method suitable for food-an international consensus,” *Food Funct*, vol. 5, no. 6, pp. 1113–1124, 2014, doi: 10.1039/c3fo60702j.

[13] H. L. R. Gomez, J. P. Peralta, L. A. Tejano, and Y. W. Chang, “In silico and in vitro assessment of portuguese oyster (Crassostrea angulata) proteins as precursor of bioactive peptides,” *Int J Mol Sci*, vol. 20, no. 20, Oct. 2019, doi: 10.3390/ijms20205191.

[14] K. Shimada and J. C. Cheftel, “Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate,” *J Agric Food Chem*, vol. 36, no. 1, pp. 147–153, Jan. 1988, doi: 10.1021/jf00079a038.

[15] N. Ajomiwe, M. Boland, S. Phongthai, M. Bagiyal, J. Singh, and L. Kaur, “Protein Nutrition: Understanding Structure, Digestibility, and Bioavailability for Optimal Health,” Jun. 01, 2024, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/foods13111771.