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Enzymatic Hydrolysis of Pigeon Pea Sprout Protein and its Potential to Generate Savory Taste

Ketut Ratnayani[1]*, Putu Ajeng Agustini[1], Ni Wayan Wisaniyasa[2], Ni Made Puspawati[1] and I Nengah Wirajana[1]

[1]*Chemistry Study Program, Faculty of Mathematic and Natural Sciences, Udayana University, UNUD Campus Street, Jimbaran, Bali - 80361, Indonesia*
[2]*Food Science and Technology Study Program, Faculty of Agricultural Technology, Udayana University, UNUD Campus Street, Jimbaran, Bali - 80361, Indonesia*

**Corresponding author*

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A B S T R A C T

The germination process is one way to improve the quality of legume protein, which will be used as a substrate in the production of protein hydrolysate. This study aims to hydrolyze pigeon pea (*Cajanus cajan* (L.) Millsp.) sprout protein concentrate by using alcalase enzymes to obtain protein hydrolysate which has the potential to generate a savory or umami taste. The research began with total protein extraction to generate pigeon pea sprout protein concentrate which was then used as a substrate in the hydrolysis process treated with variations ratio of the Enzyme to the Substrate (E/S ratio). Each protein hydrolysate obtained was characterized based on free α-amino content, soluble protein content, and sensory evaluation of the savory taste level. Protein hydrolysis results in the range of E/S ratio 0.1% - 1.5% showed that the E/S ratio 1.5% was able to produce the highest content of free α-amino (2.95±0.08 mg/mL) and soluble protein content (13.69±0.11 mg/mL). Besides that, the highest sensory evaluation score was obtained in the E/S ratio of 1.0%. This result shows that the protein hydrolysate of pigeon pea sprouts can be used as a natural flavor.

Introduction

During the germination period, hydrolysis of the storage protein in the seed occurs to form a group of short peptides to be translocated to the growing embryo for nutrient supply (Mandal *et al.*, 2008). This degradation process begins when there is contact between the endogenous protease in the bean seed with water so that it penetrates the bean seed cell (imbibition process). Germination of seeds which will be used as a source of protein substrate in the hydrolysis process is one of the factors that can

affect the results of enzymatic hydrolysis of protein from legumes. So when pea sprout protein is hydrolyzed, the resulting peptide chain will be shorter, compared to protein hydrolysate resulting from pea hydrolysis which does not go through the germination process (Bau *et al.,* 2000). Thus, pigeon pea that has been germinated has more potential if used as a protein source for hydrolysis, considering that it has a higher soluble protein content and a shorter size compared to those without germination.

Several researchers have previously studied pigeon pea sprouts, but the study mainly focused on the nutritional and bioactivity aspects, and there is only a little research regarding the utilization of pigeon pea sprout protein. In a similar study on pigeonpea, hydrolysate of pigeonpea protein (non-germinated) produced using alcalase, pancreatin and pepsin were found to be rich in antioxidant bioactive peptides by Olagunju *et al.,* (2018). Ratnayani *et al.,* (2019) found that the duration of germination had a significant effect on the total peptide and free amino acid content of germinated pigeon pea extract, which peaked at 72 hours after germination. Ohanenye *et al.,* (2021) found that the germination process can naturally increase the digestibility and bioactivity of pigeon pea protein. In general, protein properties can be improved by enzymatic hydrolysis. However, the degree of improvement varies between proteases (Zhao *et al.,* 2011). Enzymatic protein hydrolysis produces protein hydrolysate which has a savory taste due to the formation of free amino acids and simple peptides. Hydrolysis of ingredients that contain high protein using protease enzymes can produce short peptides that have a salty, savory, and sweet taste so they have the potential to be safe flavoring agents (Maehashi and Arai, 2002). Amino acids in legume protein hydrolysate such as glutamic acid, glycine, aspartic acid, and alanine are known to provide a salty, savory, and sweet taste that can be used as a natural flavor ingredient.

Not many studies have investigated the effect of variations in the enzyme-substrate ratio on protein hydrolysate from legumes, particularly pigeon pea. The enzyme-substrate ratio is the ratio of the amount of enzyme-substrate used during protein hydrolysis, for example, if the ratio of enzyme-substrate used is 1% (w/w), then the ratio is 1 gram of enzyme in 100 grams of substrate. In this study, pigeon pea sprout was used as a protein source to be hydrolyzed using alcalase. The resulting protein hydrolysate has different characteristics (peptide and free amino acids composition) when the pigeon pea sprout protein substrate is used in the protein hydrolysis, considering the molecular size of the protein substrate is different from non-germinated pigeon pea. The differences in peptides and free amino acid composition in the protein hydrolysate can affect its potency as a natural flavor. Therefore, this research aims to hydrolyze pigeon pea sprout protein concentrate by using alcalase to obtain protein hydrolysate which has the potential as a natural flavor.

To achieve this goal, a series of research stages were carried out, starting with the germination process, and flouring to obtain pigeon pea sprout flour. Furthermore, the hydrolysis process of pigeon pea sprout protein was carried out on a protein concentrate substrate by varying the ratio of enzyme to substrate (E/S) treatment. Each protein hydrolysate obtained was characterized based on several parameters, namely: soluble protein content, free α-amino content, and sensory evaluation of the savory taste level. In addition, the most potential protein hydrolysate producing the savory taste was analyzed for its amino acid composition.

Materials and Methods

The pigeon peas were bought at a local market in the Buleleng district of Bali. The chemicals used include NaOH, HCl, CuSO4, ninhydrin reagent, biuret reagent, leucine, and alcalase enzyme.

Sample Preparation of Pigeon Pea Sprouts

Pigeonpea seeds were soaked for 30 minutes with 0.07% NaClO in a 1:5 ratio. After 30 minutes, the pigeon peas were rinsed three times with tap water. The next step is the imbibition process, in which the

pigeon peas are soaked using warm water (50°C), at a ratio of 1:3. They are then left to soak for 12 hours at room temperature. After the imbibition process, the pigeon peas were germinated at room temperature for 72 hours. The resulting pigeon pea sprouts were stored in the freezer at -20°C for 24 hours to terminate the germination process. The dried pigeonpea sprouts were then hulled, mixed and sieved through a 60 mesh sieve to produce pigeonpea sprout flour.

Pigeon Pea Sprout Protein Extraction (Bamdad *et al.*, (2009) with modifications)

As much as 20 g of pigeon pea sprout flour was put into a beaker glass. Subsequently, it was extracted by adding 200 mL of 0.01% NaOH and the mixture was stirred using a shaker rotator for 60 minutes. Then, the mixture was centrifuged for 15 minutes at 4^{o}C at 5000 rpm. The supernatant resulting from centrifugation was accommodated in a beaker glass, while the pellet was extracted again with half of the initial volume, namely 100 mL of 0.01% NaOH.

The total supernatant was then collected and 2N HCl was added until a pH of 4.5 was reached and centrifugation was carried out again. The pellets from the centrifugation results were washed with 100 mL of distilled water, then the pH was adjusted to 4.5 with the addition of 2N HCl and centrifuged again. The resulting pellet was collected. It was called pigeon pea sprout protein concentrate. This protein concentrate was then analyzed for its total protein content using the Kjeldahl method.

Hydrolysis of Pigeon Pea Protein Sprout Concentrate with Alcalase Enzyme (E/S Ratio Variation)

The protein substrate solution of pigeon pea sprouts protein concentrate (4.5% in phosphate buffer pH 8.0) was hydrolyzed with alcalase enzyme under the incubation conditions as follows: the E/S ratio variation 0.1; 0.5; 1.0, and 1.5%, using 3 hours hydrolysis time. The hydrolysis process was conducted on a water-bath shaker at 50^{o}C. After hydrolysis, the enzyme inactivation process was carried out by heating the solution at 100^{o}C for 10 minutes. Then, each solution was centrifuged for 10 minutes at 4000 rpm. The supernatant obtained is a protein hydrolysate sample which is then stored in a freezer at a temperature of -20^{o}C before further analysis.

Analysis of soluble protein content in protein hydrolysate of pigeon pea sprouts

Soluble protein was analyzed spectrophotometrically using the Biuret method with BSA standard solution (Layne, 1957).

Determination of Free α-Amino Content

Measurement of free α-amino content was carried out spectrophotometrically using ninhydrin reagent and leucine as a standard amino acid. The first step was to construct a leucine standard curve with various concentrations, namely 0; 0.1; 0.2; 0.3; and 0.4 mg/mL.

Add 0.6 mL of ninhydrin reagent to 1 mL of sample. Then the mixture was heated at 85^{o}C for 8 minutes. After cold, the absorbance of the solution was read at a wavelength of 570 nm. The calibration curve was obtained by plotting the absorbance value vs leucine concentration.

Sensory Evaluation

Sensory evaluation was carried out by testing the level of savory taste using 15 untrained panelists by tasting the protein hydrolysate solution and giving a score of the savory taste level in an assessment form.

Amino Acid Composition Analysis Using HPLC Methods

The total amino acid composition analysis of the sample was carried out according to the procedure described by Waters Corporation (2012). In the first step, the sample was hydrolyzed with 6 N HCl.

After hydrolysis, samples were then derivatized using the AccQ Fluorine Reagent Kit. Two mobile phases (AccQ-Tag ultraeluent and aquabides), a C18 column, and a PDA (photodiode array) detector were used in the UPLC instrument applied column temperature of 49°C and flow rate of 1.05 mL/min.

Results and Discussion

Substrate Preparation

In the enzymatic hydrolysis stage of pigeon pea sprout protein, a substrate was used in the form of pigeon pea sprout protein concentrate obtained from the extraction of pigeon pea sprout flour using the alkaline extraction-isoelectric precipitation method.

The results of the analysis of the total protein content of the protein concentrate with the Kjeldahl method were obtained at 85.13%, so it is a good material to be used as a substrate in protein hydrolysis.

Hydrolysis of Pigeon Pea Sprout Protein Concentrate Using Alcalase Enzyme

Hydrolysis of pea sprout protein concentrate using the alcalase enzyme was carried out at its optimum working conditions, namely pH 8 and temperature 50^{o}C. The alcalase enzyme is an alkaline protease enzyme produced by *Bacillus lichiformis* and has been used extensively in hydrolyzing food proteins.

Alcalase is stable at moderately alkaline pH values, but it is not as stable at other pH conditions. Its optimal activity can be found at pH 10, with maximum activity at a temperature of 70ºC, and the enzyme maintains full activity at room temperature in the pH range of 5 to 11 (Tacias-Pascacio *et al.*, 2018).

The Effect of E/S Ratio Variations on Free α-Amino Content of Protein Hydrolysate

Free α-amino content is a parameter indicating the amount of free amine group carrier compound (-NH_2) released due to the hydrolysis of peptide bonds from protein substrates (Bougatef *et al.*, 2012).

Data in Figure.1 shows that the free α-amino content of protein hydrolysate increased with the increase in the E/S ratio used in the hydrolysis process. In the E/S ratio range of 0.1 - 1.5%, the highest free α-amino levels were obtained, reaching 2.95±0.08 mg/mL.

The free α-amino content is directly proportional to the number of broken peptide bonds, which when divided by the N_{total} content of the initial substrate will be calculated as the degree of hydrolysis (Rutherford, 2010).

The high catalytic power of the enzyme alcalase has also been demonstrated in research by Liu *et al.*, (2022) who hydrolyzed mung bean protein using different commercial proteases namely Neutrase, Flavourzyme, and Papain. The alcalase was able to produce the highest degree of hydrolysis compared to other enzymes and the lowest molecular weight (<10 kDa).

The Effect of E/S Ratio Variations on Soluble Protein Content of Protein Hydrolysate

The soluble protein content is a parameter that indicates the amount of protein that decomposes and undergoes conversion from insoluble proteins to more soluble short-chain peptides as a result of hydrolysis (Aluko and Monu, 2003). The resulting short peptides have a lower molecular weight, making them easier to dissolve.

Based on the data in Figure 2, shows that the value of soluble protein content of alcalase hydrolysate increased with increasing E/S ratio or the number of enzymes used in protein hydrolysis. The highest value of soluble protein content, reaching 13.69±0.11 mg/mL, can be achieved by using an E/S ratio of 1.5%. The results obtained follow the statement of Wardi *et al.*, (2019), that is, as the E/S ratio used increased, the soluble protein content also increased.

Table.1 Data of Amino Acid Composition Analysis of Protein Hydrolysate Prepared by 1.0% E/S Ratio Treatment.

Amino Acid Type	g/100g
L-Glutamic Acid*	0.336
L-Phenylalanine	0.279
L-Leucine	0.214
L-Arginine	0.179
L-Aspartic Acid*	0.174
L-Serine	0.156
L-LycineHCl	0.131
L-Proline	0.104
L-Valine	0.102
Glycine	0.101
L-Isoleucine	0.099
L-Threonine	0.098
L-Histidine	0.091
L-Tyrosine	0.086
L-Alanine	0.082

*This value describes the total number of aspartic acids including Asparagine and the total number of glutamic acids include glutamine

Fig.1 Effect of E/S Ratio Variation on Free α-amino Contents of Protein Hydrolysate

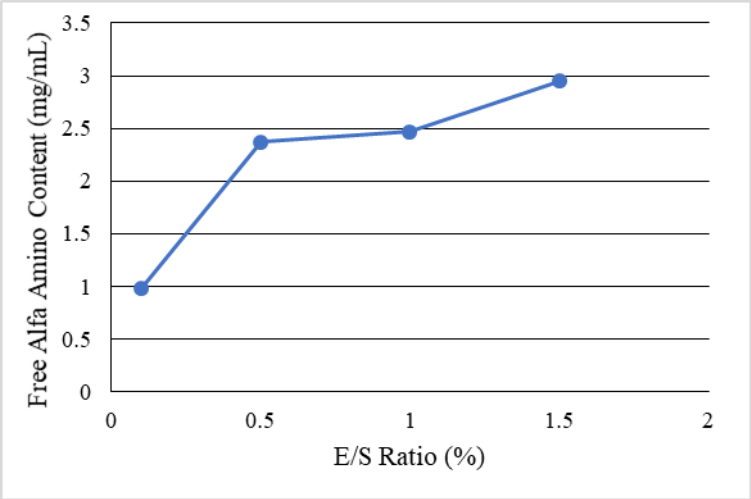


Fig.2 Effect of E/S Ratio Variation on Soluble Protein Content of Protein Hydrolysate

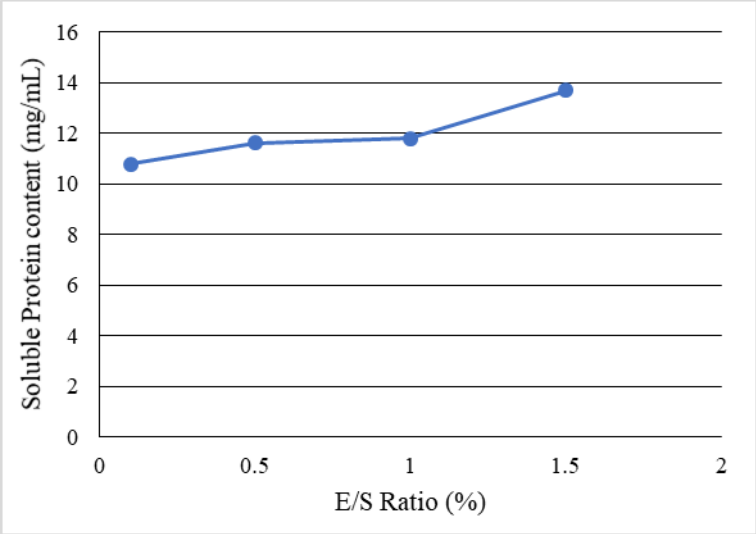
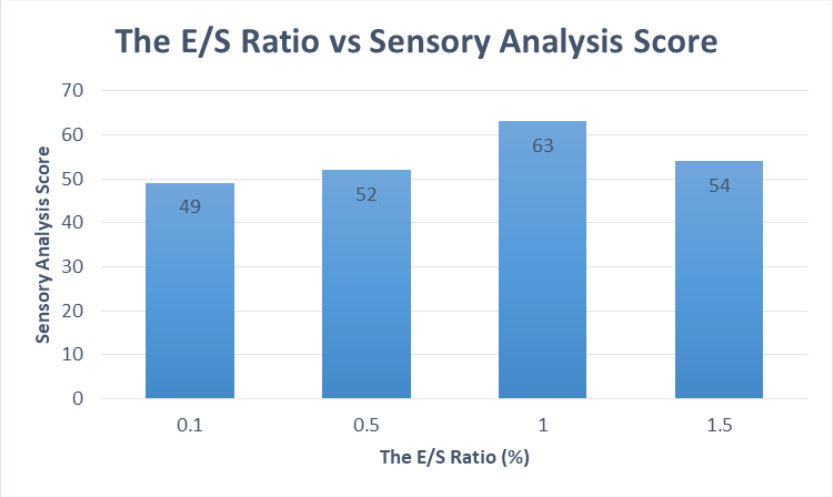


Fig.3 Effect of E/S Ratio Variation on Sensory Evaluation Score of Protein Hydrolysate



Effect of E/S Ratio Variation on Sensory Evaluation Score of Protein Hydrolysate

Based on data shown in Figure3., shows that protein hydrolysate produced using an E/S ratio of 1.0% treatment was able to generate the highest score for the level of savory taste (in the E/S ratio variation range of 0.1% -1.5 %).

This means that the 1.0% E/S ratio treatment has the most potential to generate a savory taste which is possibly since the protein hydrolysate can produces the most peptides and certain free amino acids which produce a savory and umami taste.

Umami is one of the basic tastes that can be detected by humans and could be attributed to the free amino acids (aspartic acid and glutamic acid). It is also can be attributed to the short peptides (with molecular weight less than 3 kDa containing one or more of these kinds of two amino acids) resulting through protein digestion. Amin *et al.,* (2020) found a novel umami peptide GK-15 from the water extract of tempeh (a traditional fermented soybean product).

According to Zhang *et al.*, (2017), 52 peptides were reported to demonstrate umami flavour, and various new umami peptides. Peptides contributing to umami flavour have been continuously reported. The presence of umami peptides in moromi, which was produced by the fermentation of yellow soybeans with *Aspergillus oryzae*, was discovered by Lioe *et al.*, (2004).

The results of sensory evaluation of the presence of other tastes (besides the savory taste) produced from protein hydrolysate show that the use of an E/S ratio above 1.0% tends produce a bitter taste. This is probably due to the hydrolysis process which is too strong to produce short peptides with certain amino acids which give rise to a bitter taste. This is in accordance with the statement of Maehashi and Arai (2002) that protein hydrolysate with proteases can give a bitter taste because of the existence of hydrophobic amino acids in the polypeptide chain and short peptides.

Amino Acid Composition of The Protein Hydrolysate

Several amino acids have the potential to provide a savory and umami taste. Amino acids require a hydrophilic medium structure in their L-enantiomer for umami taste, for example, amino acids such as L-glutamic acid, L-aspartic acid, L-lysine, and L-proline (Kawai *et al.*, 2012). In addition, leucine and glutamine are also amino acids that have a savory taste (Haefeli and Glasser, 1990). As shown in Table 2, the protein hydrolysate produced using a 1.0% E/S ratio treatment was rich in some amino acids that potentially exhibit a savory taste i.e. glutamic acid, aspartic acid, leucine, glutamine, lysine, and proline. The glutamic acid content of the protein hydrolysate in Table 2 describes the total number of glutamic acids including glutamine.

Increasing the E/S ratio used in the hydrolysis of pigeonpea sprout protein concentrate can raise the free α-amino content and the soluble protein content of the obtained protein hydrolysate. The highest soluble protein content was achieved at an E/S ratio of 1.5% with a soluble protein content of 13.69±0.11 mg/mL and the highest free α-amino content was 2.95±0.08 mg/mL. Meanwhile, the 1.0% E/S ratio treatment was able to exhibit the highest score for the level of savory taste in the sensory evaluation step which is rich in amino acids which the potential to generate savory taste.

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