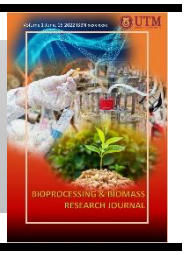
Bioprocessing and Biomass Technology

Journal homepage: https://bioprocessing.utm.my



Research Article

In Vitro Digestibility and Kinetic Modelling of Pea and Brown Rice Protein Powder Blends at Different Mixing Ratios

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ARTICLE INFO

Article History:
Received 7 August 2025
Received in revised form 25 August 2025
Accepted 25 August 2025
Available online 30 December 2025

Keywords:
Bioavailability,
Digestibility,
Plant protein,
Pea protein,
Brown rice protein

ABSTRACT

Global trend suggests a dietary shift from animal-based to plant-based diets, indicating a growing demand in related markets. As such, plant-based protein sources have raised scientific interest for their health benefits and sustainability. Plant-based protein powders are nutritional supplements derived from protein-rich plants and serve as an alternative of protein source for vegan, athletes and consumers with dietary restrictions. However, plant-based proteins often lack one or more essential amino acids and exhibit lower digestibility, thus requiring combination with other proteins to ensure nutritional adequacy. This study investigates the digestibility aspect of commercialized plant-based protein isolates. To overcome the imbalanced amino acid profile and low digestibility of plant-based protein powder, a blend of pea and brown rice protein isolate was formulated. The effects of mixing different blending ratios on digestibility were studied using *in vitro* digestibility test and FTIR spectroscopy. Protein release kinetics of digested protein samples were modelled using zero order, first order, Higuchi and Hixson-Crowell models. The results show that blending pea with brown rice protein isolate improved digestibility compared to pure protein isolates. Pea to brown rice ratio of 4:1 recorded the highest digestibility (75.14%), justified by the FTIR analysis result that indicated fewer functional groups and bonds. The optimal ratio was determined to be 7:3 pea to brown rice protein isolate. Majority of samples can best be described by Higuchi model suggesting a diffusion-controlled release mechanism.

INTRODUCTION

Global trend indicates a gradual shift towards a plant-based diet due to increased health benefits, environmental impact and concerns on ethical issues. Compared with meat-heavy diets, a plant-based diet reduces greenhouse gas emission emissions by 75%, land use by 75%, water consumption by 54%, and biodiversity impact by 65% (Scarborough et al., 2023). A survey had also found that 61% of Malaysian are consuming more plant-based foods and reducing meat intake (Murugesan, 2021), whereas European consumers are also reducing animal product consumption (Guyomard et al., 2021). Simultaneously, consumption of nutritional supplements has become an increasing trend due to growing health awareness. Both of these trends have driven increased demand on plant-based protein supplements which makes it a growing market (Fortune Business Insights, 2024).

Protein powder is a type of concentrated protein supplement containing both essential (EAAs) and non-essential amino acids (NEAAs) (Lopez and Mohiuddin, 2020). It is widely used for dietary and bodybuilding purpose, especially among athletes (Kårlund et al., 2019). Protein powders can be classified into animal-based and plant-based categories. Animal-based protein powders including whey, casein and egg whites contain all nine EAAs in sufficient quantities, making them as complete proteins. In contrast, plant-based protein powders such as soy, pea,

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hemp and brown rice have incomplete amino acid profile, often lacking one or more EAAs, particularly in leucine, lysine and methionine content (Ahnen et al., 2019; Gorissen et al., 2018; Qin et al., 2022). Plant-based protein powders also possess lower bioavailability due to the presence of antinutritional factors (ANFs) such as phytic acid and tannins that will affect digestibility (Berrazaga et al., 2019). The protein digestibility-corrected amino acid score (PDCAAS) for animal proteins is typically 1.0 (100%), while most plant proteins score lower, with the exception of soy (Herreman et al., 2020).

Despite the flaws, benefits offered by plant-based proteins outweighs their drawbacks. They serve as alternatives for certain groups of people including vegans and consumers with other dietary restrictions. They also contain less saturated fat and no cholesterol, making them a healthier option (Lamberg-Allardt et al., 2023).

Incomplete amino profile of plant-based protein can be resolved via protein complementation, an approach of compensating for missing amino acids by combining different plant-based proteins (Marcus, 2013). One of such combinations is legumes and cereals (Preedy and Watson, 2010) such as pea and brown rice protein powders.

This research studies the nutritional value and bioavailability of commercialized pea and brown rice protein powders in terms of digestibility by performing a series of *in vitro* digestive system simulation. This is to ensure sufficient amount of proteins are absorbed by the digestive system. Protein complementation is done by mixing pea and brown rice protein powders using different ratios and the best ratio is determined based on its digestibility profile to ensure the blend contains sufficient amount of amino acids that can be digested efficiently. Pea protein powder is chosen for its high nutritional value (Taylor et al., 2021), rather complete amino acid profile (Babault et al., 2015), sustainability (Tulbek et al., 2017) and less common allergies compared with other legumes such as soy and nut (Taylor et al., 2021). Brown rice protein powder is chosen as its raw material is highly available, rich in methionine content and possesses hypoallergenic properties (Jayaprakash et al., 2022; Zhao et al., 2023)

The objectives of this research are to evaluate the digestibility of pure and mixed pea and brown rice protein isolate and to determine the best mixing ratio of pea and brown rice protein based on digestibility and structural analysis. Furthermore, this research also aims to compare the release kinetics of digested protein of different protein blend mixtures. Understanding digestion kinetics provides critical insights into the rate and extent of protein hydrolysis, which influences amino acid availability and the functional performance of protein-based food products.

MATERIALS AND METHOD

Materials

Plant-based protein samples of Soluxe Nutrition Original Pea Protein Isolate Unflavoured and Soluxe Nutrition Brown Rice Protein Isolate Original Unflavoured were purchased online from Soluxe Nutrition, Selangor, Malaysia. For *in vitro* digestibility test, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared using Minekus (2014) method with analytical grade chemicals purchased from QRec and Sigma-Aldrich. Pepsin, pancreatin and bile solution were purchased from Solarbio. Pierce™ Bovine Serum Albumin Standard Ampules used in BSA standard curve was purchased from Thermo Fisher.

Sample Preparation

Samples were prepared by mixing pea and brown rice protein isolates at different ratios of 1:0, 4:1, 3:2, 2:3, 1:4, and 0:1 in terms of w/w percentage. The samples were homogenized using a lab mixer (ZX3 Vortex Mixer, VELP Scientifica).

Fourier Transform Infrared (FTIR) Analysis

FTIR analysis (Nicolet 6700 FT-IR spectrometer) was conducted on the protein blend samples (powder form) before *in vitro* digestion using mid infrared wavelength. Parameter was set at 4000−650 cm^{-1} wavelength. 1 g of each sample was used and the data obtained was interpreted as transmittance values.

In Vitro Digestibility Analysis

The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to Minekus (2014), as tabulated in **Table 1**.

Table 1 Composition of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

Reagent	Stock conc. ($g.L^{-1}$)	SGF pH3		SIF pH7	
		Vol. of stock (mL)	Conc. ($mmol.L^{-1}$)	Vol. of stock (mL)	Conc. ($mmol.L^{-1}$)
KCl	37.3	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.9	0.9	0.8	0.8
$NaHCO_3$	84	12.5	25	42.5	85
NaCl	117	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.5	-	-
For pH adjustment					
HCl (6 $mol.L^{-1}$)	-	1.3	15.6	0.7	8.4

For the digestibility test, 1 g of protein sample was mixed with 20 mL of distilled water, 15 mL of SGF, 0.01 mL of 0.3 $mol.L^{-1}$ $CaCl_2(H_2O)_2$, 3.2 mL pepsin and 0.4 mL of 1 mol HCl. The samples were left at a 37 °C water bath for 2 h. After 2 h, 20 mL of each sample were mixed with 11 mL SIF, 0.04 mL of 0.3 $mol.L^{-1}$ $CaCl_2(H_2O)_2$, 5 mL pancreatin, 0.15 mL of 1 mol NaOH and 2.5 mL bile solution. The mixtures were once again incubated at the 37 °C water bath for another 2 h. Sampling was done every 20 min starting at 0 min (initial) where 0.5 mL of each sample was centrifuged at 500 rpm, and 0.1 mL supernatant was mixed with 3 mL Bradford reagent. The mixtures were measured using UV-Vis spectrophotometer at 595 nm wavelength. Optical densities measured were interpreted into total soluble protein concentrations by referring to the BSA standard curve. The digestibility percentage was calculated based on the total soluble protein content after *in vitro* digestion, indicated by Equation (1) (Haliza et al., 2021), where P_t is the total protein content before *in vitro* digestion and P_r is the total protein content after *in vitro* digestion.

$$\text{Digestibility}(\%) = 100\% - \left(\frac{Pt}{Pr} \times 100\%\right) \qquad (1)$$

Kinetic Modelling

Optical density data obtained via 20-min time intervals for *in vitro* digestibility analysis was interpreted into protein

concentrations using BSA standard curve. Release data of total soluble protein was then evaluated kinetically using zero order model, first order model, Higuchi model and Hixson-Crowell model, by plotting graphs to determine the best fit model (Tee et al., 2022). The models are represented by Equation (2) until (5).

$$\text{Zero order} : C_0 - C_t = K_0 t \quad (2)$$

$$\text{First order} : \log C = \log C_0 - K_1 t/2.303 \quad (3)$$

$$\text{Hixson-Crowell} : C_0^{1/3} - C_t^{1/3} = K_{HC} t \quad (4)$$

$$\text{Higuchi} : \mathrm{Q} = K_H t^{1/2} \quad (5)$$

C_t is the amount of protein released at time interval t, C_0 is the initial concentration of protein at time t=0, C is the remaining protein at time t, Q is the amount of protein released in time t per unit area, K_0 is the zero-order rate constant, K_1 is the first-order rate constant, K_{HC} is the Hixson-Crowell constant and K_H is the Higuchi dissolution constant.

RESULTS AND DISCUSSION

FTIR Analysis

Figure 1(a) illustrates the FTIR analysis result of all samples. According to the graph, all of the samples had portrayed few observable troughs, where two of the most obvious are recorded at the wavenumber range of 1700-1600 cm^{-1} and 1580-1400 cm^{-1}. The wavenumber region of 1700-1600 cm^{-1} can be interpreted as the amide I band whereas the region of 1580-1400 cm^{-1} represented the amide II band (Ji et al., 2020). The particular band region for amide I and amide II was enlarged and shown in **Figure 1(b)**. For the amide I band, pea to brown rice protein ratio of 4:1 showed the least through (negative peak) depth, i.e., the highest transmittance, followed by 3:2, 2:3, 1:4, 0:1, and 1:0. All of the samples' trough are recorded within the wavenumber range of 1627 to 1632 cm^{-1}, which indicates that the protein secondary structure of the samples mainly consists of β-sheets (Yang et al., 2015). For the amide II band, ratio 4:1 recorded the highest transmittance value, followed by 2:3, 3:2, 1:4, 1:0 and 0:1. This slightly different order may be caused by some of the samples having strongly ordered structures with strong C=O stretching in the amide I band but having weaker hydrogen bonding (N-H bending) at the amide II band.

Considering that the environmental conditions remained constant, studies suggested that the peak (or trough) intensities of the amide bands are influenced by the amount of secondary structures or hydrogen bonding (Andrade et al., 2019; Saxton & McDougal, 2021). This statement also abided to Beer-Lambert law which stated that the absorbance value being directly proportional to the concentration of the absorbing species, meaning that higher concentrations of functioning groups can lead to higher corresponding peak intensities, and since transmittance is inversely proportional to absorbance, a lower transmittance value indicates a stronger bond and vice versa. As such, it could be said that the sample with 4:1 mixing ratio has the weakest bond, making it easier to be broken down and digested.

By observing the overall changes, it was discovered that by mixing pea and brown rice protein isolates, all of the mixtures had somewhat improved digestibility as they all had higher transmittance values at the amide band troughs compared with pure pea (1:0) and brown rice (0:1) protein isolates. This would be further explained in digestibility analysis. The 4:1 ratio was observed to bear the highest transmittance value at each of the amide band, suggesting the highest digestibility amongst all samples.

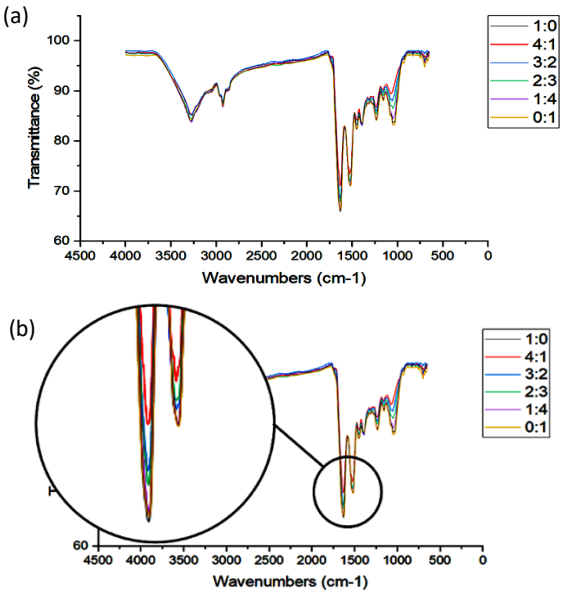

Figure 1 (a) Compilation of FTIR spectra (transmittance values) of protein mixtures made from different pea to brown rice protein isolate mixing ratios **(b)** Enlarged FTIR spectra showing the amide I and amide II band regions.

Digestibility Analysis

Figure 2 (a) illustrates the digestibility of protein mixtures made from different pea to brown rice protein isolate mixing ratios. It can be seen that the blend with 4:1 ratio exhibited the highest digestibility amongst the samples, recorded at 75.14%, whereas 0:1 ratio (pure brown rice protein isolate) recorded the lowest digestibility percentage at 61.10%. Compared to the pure powders, the 4:1 blend exhibited a 5.60% increase in digestibility relative to pure pea powder (1:0) and a 22.98% increase compared to pure brown rice powder (0:1). This result justified the data obtained by FTIR analysis showing that the sample with 4:1 ratio being more susceptible to be broken down and digested as it has the weakest bond strength amongst other samples.

Next, using the same data, a best fit curve using polynomial fit was plotted to determine the optimal mixing ratio with the best digestibility (**Figure 2(b)**). From the curve, the highest peak was observed at 28.24% (~30%). Therefore, it can be inferred that the blend for an optimized digestibility can be produced by mixing 70% w/w of pea protein with 30% w/w brown rice protein, i.e., 7:3 pea to brown rice ratio.

The improvement in terms of digestibility when pea protein was mixed with brown rice protein can be related with their complementary amino acid profiles. Pea protein in general is rich in lysine but low in methionine content (Shanthakumar et al., 2022). Meanwhile, brown rice protein is known for its high methionine content yet lacks lysine (Lee at al., 2021). Blending these complementary protein sources resulted in a mixture that compensated the limitation of each individual protein, hence improving amino acid profile and its overall digestibility. This was justified by Hertzler et al. (2020), whose study found that mixing 40% to 90% of pea protein with rice protein would provide a PDCAAS score of 1.0, meaning it is fully digestible and possesses a complete amino acid profile. Additionally, mixing plant proteins was

found to mitigate anti-nutrients or anti-nutritional factors (ANFs) that could interfere with protein digestion and absorption. This is due to the overall dilution of ANFs when a protein source rich in ANFs is being mixed with another protein with lower concentrations of ANFs. Major ANFs in pea protein include tannin and trypsin inhibitors, whereas for brown rice protein they consist of phytates or phytic acid (Gilani et al., 2005). Thus, by mixing both plant proteins the ANF concentration can be significantly reduced, hence the improved digestibility.

Bradford assay was used to measure the total soluble protein concentration after digestion. The results shown in both **Figure 2** indicates an increment in total soluble protein concentration after *in vitro* digestion, which was expected since intact proteins were broken down into smaller peptides and free amino acids via enzymatic hydrolysis that remained soluble in water. Contents of total soluble protein include dipeptides, tripeptides, free amino acids such as lysine, methionine, leucine and glutamine as they possess high solubility characteristics (Nomoto et al., 2021). These molecules contribute to the measured protein concentration based on their interactions with the Coomassie Brilliant Blue dye which bonds with aromatic and basic amino acid residues. Therefore, the increase in total soluble protein portrayed the extent of protein breakdown which in turn could be interpreted as protein digestion efficiency.

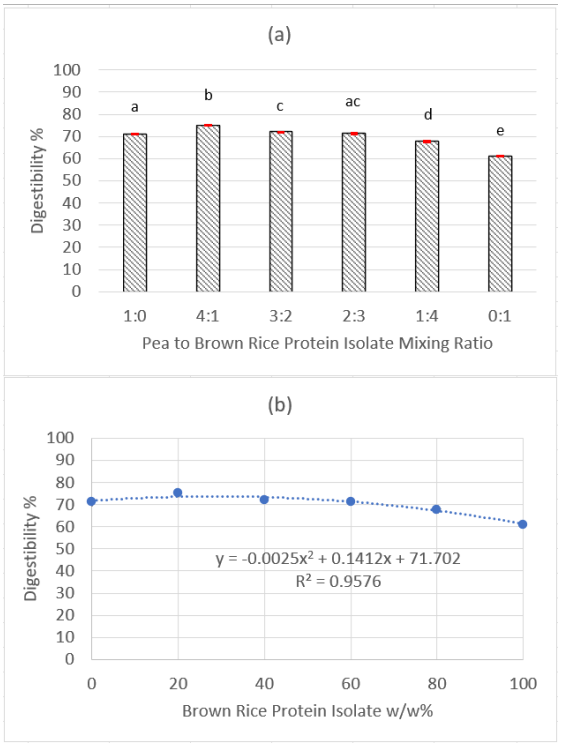

Figure 2 (a) Digestibility of protein mixtures made from different pea to brown rice protein isolate mixing ratios, where different letters above the bars indicate significant differences (p<0.05), **(b)** Best fit curve of protein digestibility against brown rice protein percentage.

Protein Release Kinetics

Figure 3 illustrates the cumulative release of digested protein from protein blend samples. All samples achieved 100% release rates after 4 h. Notably, from 0 to 40 min all samples showed steep increment signifying the period of high enzymatic activity for pepsin during the SGF phase. Eventually enzymatic activity slowed down, possibly due to the lack of undigested substrate, in this case, protein. At the 120-min time frame, introduction of pancreatin in SIF had caused another increasing slope in all of the samples indicating further digestion and release of peptides and amino acids into the digestive solution.

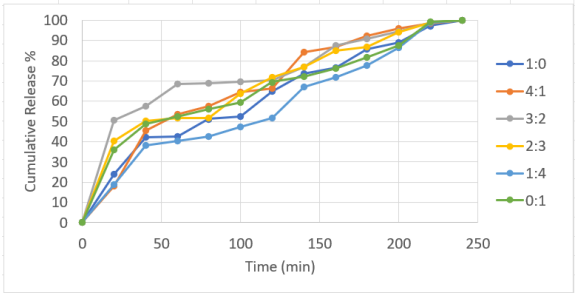

Figure 3 Cumulative release of digested proteins from protein blend samples.

Table 2 records the R^2 values based on the kinetic models. According to the table, the release kinetics for all of the samples were found to be best described using Higuchi model except for 1:4, which was best described by the zero-order model (R^2 value of 0.9663). Samples that have the best affinity with Higuchi model indicate that their release model follows the Fick's diffusion law. Fick's diffusion law states that the rate of diffusion doubles if the surface area or concentration gradient is doubled (Enderle, 2012). Higuchi model also suggests that diffusion is the primary mechanism which controls the release rate, which is initially fast but slows down over time (Paul, 2011). In the case of the zero-order kinetic model, it can be described as having a constant release rate of protein where the enzymatic activity is uniform, suggesting that the digestion might occur at the surface of the protein instead of needing to diffuse into the matrix (Sezer et al., 2011).

Table 2 R^2 values of different kinetic models for the plant protein mix digestibility.

Mixing Ratio (Pea : Brown Rice)	R^2			
	Zero Order	First Order	Higuchi	Hixson-Crowell
1:0	0.9515	0.8260	**0.9820**	0.8735
4:1	0.9057	0.9181	**0.9798**	0.9475
3:2	0.7816	0.8505	**0.9336**	0.8824
2:3	0.8960	0.8384	**0.9772**	0.9005
1:4	**0.9663**	0.7271	0.9484	0.8205
0:1	0.8992	0.7332	**0.9781**	0.8312

CONCLUSIONS

Combining pea and brown rice protein at a 4:1 ratio significantly boosts protein digestibility as proven by *in vitro* digestibility test results. Optimal ratio obtained from the best fit polynomial curve is 7:3 pea to brown rice ratio. FTIR analysis had provided insights on molecular interactions that may contribute to the enhanced bioavailability. All these findings support the concept of combining complementary plant proteins that improves amino acid profile and digestibility. The release kinetic study showed that majority of the protein mixtures followed Higuchi model's release kinetic, suggesting that the digestion of protein depends on its diffusion. As such, it can be said that this study

contributes to the development of plant-based protein alternatives with better quality and sustainability.

Acknowledgement

The authors acknowledge the Ministry of Higher Education Malaysia and Universiti Teknologi Malaysia (grant no. R.J130000.7851.4L945) for the support of this research.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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