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Screening of Hydrolysis Conditions for Green Pea Protein with Bromelain: A Time-Efficient Approach for CKD-Oriented Nutritional Therapy

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Abstract

Chronic kidney disease (CKD) is an emerging public health problem in Indonesia, in which dietary management, particularly protein source selection, plays an important role. Plant-based proteins such as green pea protein are considered more kidney-friendly and may provide additional functional benefits when enriched with bioactive peptides. This study aimed to optimize the enzymatic hydrolysis conditions of green pea protein using bromelain to increase the production of the antifibrotic peptide LERGDT, which may be beneficial for CKD-oriented nutrition. A factorial experimental design was used to evaluate the effects of hydrolysis time (8–72 h), enzyme concentration (5–20%), and water-to-protein ratio (5–7, v/w). Protein degradation was analyzed using SDS-PAGE, LERGDT was quantified by HPLC (retention time 11.5 min), and protein concentration was determined using the Bradford assay.

The results showed progressive protein hydrolysis and increased peptide formation with longer incubation times. The highest LERGDT content (2.66%) was obtained at 72 h; however, comparable levels were already observed at 12 h. Prolonging the hydrolysis time beyond 12 h did not result in a significant increase in peptide yield, indicating a plateau effect. Therefore, a 12-hour hydrolysis process was identified as an efficient condition for producing bioactive peptides from green pea protein. In conclusion, this study demonstrates the potential of producing time- and cost-efficient plant protein hydrolysates with possible application in dietary management for patients with CKD.

1. Introduction

Chronic kidney disease (CKD) is an escalating public health challenge both globally and in Indonesia. According to the 2023 Indonesian Health Survey [1], CKD affects approximately 0.22% of the population, impacting over 600,000 individuals nationwide. Due to its often-asymptomatic nature in the early stages, CKD frequently goes undetected until it reaches more advanced phases. This highlights the urgent need for effective preventive and therapeutic strategies, particularly those that can alleviate the metabolic burden on the kidneys. Among these, nutritional interventions—especially those focused on protein intake—are critical in slowing disease progression.

In the management of CKD, the source and quality of protein are key considerations. For patients with later-stage CKD, transitioning from animal-based to plant-based protein is often recommended. Plant proteins produce fewer nitrogenous waste products, exert a lower renal workload, and are naturally rich in beneficial phytochemicals and antioxidants [2, 3]. Among plant-based protein sources, *Pisum sativum* (green peas) is notable for its high protein content, excellent digestibility, and presence of bioactive peptides with therapeutic potential [4]. Previous studies have demonstrated the benefits of green pea protein hydrolysates in reducing blood pressure and supporting renal recovery [5, 6, 7, 8].

Since 2017, our research group has developed a functional protein hydrolysate derived from green peas using bromelain, an endopeptidase extracted from pineapple stems. This formulation—Protein Hydrolysate of Green Peas with Bromelain (PHGPB)—has exhibited both antifibrotic and antioxidant properties in CKD animal models and mesangial cell cultures [9, 10]. Further in silico analysis identified

LERGDT (Leu-Glu-Arg-Gly-Asp-Thr) as a bioactive peptide within PHGPB that may modulate fibrotic signalling through RGD-integrin interactions [11]. In vitro studies confirmed that both PHGPB and LERGDT suppress the expression of pro-fibrotic genes—SMAD2, SMAD3, and SMAD4—while downregulating TGF-β1 and upregulating the anti-fibrotic SMAD7 gene in glucose-induced MES13 SV40 cells. These findings underscore the therapeutic potential of PHGPB and LERGDT in mitigating renal fibrosis via targeted modulation of the TGF-β/SMAD signalling pathway [11]. In particular, the presence of the RGD motif in the LERGDT peptide sequence was associated with decreased levels of fibrosis markers such as fibronectin and TGF-β1 in diabetic glomerulosclerosis cell models. Beyond its favourable nutritional profile, PHGPB supports mesangial cell recovery and holds promise as a functional dietary component for kidney protection in CKD [10].

Despite these promising results, the original PHGPB production protocol [12] requires 72 hours of enzymatic hydrolysis, presenting limitations in terms of scalability, cost-efficiency, and clinical feasibility. Recent evidence suggests that enzymatic hydrolysis conditions—particularly duration—can be optimized to reduce processing time without compromising peptide bioactivity [13, 14. 15, 16, 17]. Therefore, this study aims to systematically screen the hydrolysis parameters of PHGPB—including hydrolysis time, water-to-substrate ratio, and enzyme concentration—to develop a more efficient and reproducible method suitable for laboratory-scale production and future clinical application.

2. Materials and Methods

2.1 Materials

Split green peas (*Pisum sativum*) were sourced from Trinidad Benham Corp. (Denver, CO, USA; Split 200116). The peas were ground and sieved using a 120-mesh screen. Bromelain enzyme extract (purity ≥ 98%, food-grade) was derived from *Ananas comosus* (pineapple stem) and prepared as a crude aqueous extract. Analytical-grade reagents included Coomassie Brilliant Blue G-250, phosphate buffers, and HPLC-grade solvents (Merck, Darmstadt, Germany). The synthetic LERGDT peptide standard (purity 93%) was synthesized according to Hidayat's method [18] for quantification assays.

2.2 Methods

2.2.1 Experimental Design for Hydrolysis Screening

A factorial screening design was implemented to evaluate three key parameters: hydrolysis time (8 and 72 hours), water-to-substrate ratio (5× and 7× weight), and bromelain concentration (5% and 20% w/w) (Fig. 1**).** Eight combinations were generated using the R Open-Source statistical program. In each run, 40 g of pea powder was mixed with bromelain solution in the designated water volume and incubated under continuous stirring at room temperature (23–25°C).

2.2.2 SDS-PAGE Analysis

Protein breakdown was assessed via SDS-PAGE using 12% polyacrylamide resolving gels. Electrophoresis was conducted at 120V for 90 minutes using Tris-glycine-SDS buffer. Samples were prepared in reducing Laemmli buffer and heated at 95°C for 5 minutes. Protein bands were visualized using Coomassie blue staining and compared to a molecular weight marker ranging from 10 to 250 kDa. Both undiluted and 10× diluted hydrolysates were evaluated.

2.2.3 HPLC Quantification of LERGDT Peptide

LERGDT quantification was performed using reversed-phase HPLC (Merck LiChroCART 250 mm × 4.6 mm column, RP-18 guard column, 5 µm particle size). The mobile phase consisted of 20 mM KH_2PO_4 (Solvent A) and Milli-Q water (Solvent B), eluted under gradient conditions at a flow rate of 2.0 mL/min. Detection was performed at 210 nm using a UV-Vis detector. Retention times and peak areas were compared to the LERGDT standard to calculate relative peptide abundance.

2.2.4 Bradford Assay for Hydrolysis Progress

The Bradford protein assay was employed to estimate relative peptide size distribution over time. Sample absorbance at 595 nm was measured using a microplate reader. Because peptides ≤ 3 kDa do not induce a colorimetric response, lower absorbance was interpreted as indicative of smaller hydrolysates. Limitations of this method for accurate size determination were acknowledged [19, 20].

2.2.5 Univariate Optimization of Hydrolysis Time

To refine the most influential parameter (hydrolysis time), univariate experiments were conducted at fixed enzyme (20%) and water (5×) conditions. Samples were collected every 12 hours over 168 hours. LERGDT content and absorbance trends were monitored via HPLC and Bradford assay, respectively, and plotted to determine the point of hydrolysis saturation.

3. Results

3.1 SDS-PAGE Analysis of Protein Hydrolysis

SDS-PAGE analysis confirmed successful enzymatic hydrolysis of green pea protein in all eight factorial experiments. As shown in Fig. 2A, untreated green pea powder (lane 9) exhibited distinct high–molecular-weight protein bands. In contrast, lanes 1–8, representing the hydrolyzed samples, showed a progressive reduction in band intensity, indicating effective proteolysis by bromelain.

Figure 2B presents the same samples analyzed at a 1:10 dilution, revealing similar degradation patterns with improved band resolution. The disappearance of major protein bands and the appearance of lower–molecular-weight fragments demonstrate extensive protein breakdown following enzymatic treatment.

Figure 2. SDS-PAGE electrophoregram of PHGPB samples: (A) undiluted samples, lanes 1–8 correspond to factorial experiments 1–8, lane 9: untreated pea powder, lane 10: molecular weight marker; (B) diluted (1:10) samples, lane 1: pea powder, lane 2: marker, lanes 3–10: samples 1–8.

3.2 HPLC Quantification of LERGDT Peptide

HPLC analysis consistently detected the antifibrotic peptide LERGDT at a retention time of approximately 11.5 minutes across all hydrolysate samples. The highest peak area percentage (2.66%) was observed in sample 7 (72 h hydrolysis, 5× water-to-protein ratio, and 5% enzyme concentration), indicating the most favourable condition for LERGDT production among the tested combinations.

The quantitative results for all eight factorial experiments are summarized in Fig. 3.

Figure 3. Overlay chromatograms of PHGPB samples and LERGDT standard showing the characteristic peak at a retention time of 11.5 minutes.

3.3 Bradford Assay Results

Bradford assay measurements demonstrated a marked decrease in protein concentration with increasing hydrolysis time. Absorbance values for samples 1–8 ranged from 0.8002 (sample 1) to 0.0418 (sample 7), indicating progressive protein degradation and the formation of low–molecular-weight peptides. The Bradford absorbance readings for the PHGPB screening experiments are summarized in **Table 1**.

Detailed absorbance responses of individual hydrolysate samples are presented in **Table 2**, while the overall absorbance responses of hydrolyzed samples measured with the Bradford reagent are shown in **Table 3**. These data further confirm the progressive reduction in protein content across the experimental conditions.

The influence of hydrolysis time, water-to-protein ratio, and enzyme concentration on Bradford absorbance is illustrated in **Fig. 4**. The decreasing absorbance trend observed under these conditions is consistent with the SDS-PAGE results, confirming extensive enzymatic hydrolysis of green pea protein.

3.4 Univariate Optimization of Hydrolysis Time

Time-course analysis revealed a continuous decline in protein absorbance from 8.87 at 0 h to 0.894 at 84 h, followed by a plateau phase extending to 168 h, indicating that most protein degradation occurred within the first 84 hours (Fig. 5). The corresponding absorbance values are presented in **Table 4**.

HPLC analysis showed that production of the antifibrotic peptide LERGDT became detectable by 12 h and increased progressively up to 84 h. Beyond this time point, no substantial increase in LERGDT peak area was observed (Fig. 6), suggesting that prolonged hydrolysis provides minimal additional benefit.

Together, these results indicate that hydrolysis beyond 84 h does not significantly enhance peptide production, and that shorter hydrolysis durations are sufficient to generate bioactive peptides from green pea protein.

Figure 5. Time-dependent degradation of PHGPB monitored by Bradford assay, showing a plateau after 84 h.

Table 4. Time-course Bradford absorbance values during univariate optimization.

Figure 6. HPLC chromatograms showing increasing LERGDT peak area from 0 to 84 h, with significant production observed by 12 h.

4. Discussion

This study provides a systematic evaluation of enzymatic hydrolysis parameters for protein hydrolyzed of green pea using bromelain (PHGPB) and demonstrates that strategic optimization can substantially improve process efficiency without compromising bioactive peptide production. The combined use of SDS-PAGE, HPLC, and the Bradford assay offered a comprehensive assessment of protein degradation, peptide formation, and hydrolysis kinetics [21].

SDS-PAGE analysis confirmed that bromelain effectively cleaved high-molecular-weight green pea proteins into smaller peptide fragments. This observation is consistent with previous reports describing bromelain's specificity toward legume proteins and its ability to generate functional peptides through fragmentation of larger protein chains [22, 23]. The gradual disappearance of intense protein bands and the concurrent appearance of lower-molecular-weight peptides with increasing hydrolysis time indicate robust proteolytic activity.

HPLC analysis further demonstrated the formation of the antifibrotic peptide LERGDT, which reached its highest abundance (2.66%) under conditions of 72 h hydrolysis, 5× water-to-substrate ratio, and 5% enzyme concentration. This finding aligns with the reported role of LERGDT in RGD–integrin–mediated antifibrotic and Renal protective pathways [10]. Importantly, time-course analysis revealed that substantial levels of LERGDT were already produced at 12 h, indicating that prolonged hydrolysis does not result in a proportional increase in peptide yield and may be inefficient for practical applications.

The Bradford assay provided kinetic support for these observations. Total protein absorbance decreased progressively with hydrolysis time, reflecting the accumulation of peptides below the assay's detection threshold (< 3 kDa) [19, 20, 24]. Although the Bradford method has limitations in detecting small peptides, the consistent decline in absorbance corroborates the extensive protein fragmentation observed in SDS-PAGE analysis.

These findings are in agreement with recent studies demonstrating that hydrolysis time can be significantly shortened without compromising functional peptide yield when process conditions are properly optimized. For instance, hemp seed oil cake hydrolysates achieved optimal antioxidant activity within 60 min using moderate bromelain concentrations (~ 3%) and elevated temperature (~ 40°C) (Appl. Sci. 2024; García Arteaga et al., 2022; Matthew et al., 2024). Similarly, optimization of pea protein hydrolysis through control of moisture content, temperature (~ 70°C), and enzyme dosage resulted in

favourable degrees of hydrolysis (up to ~ 38%) within much shorter durations than traditional protocols [25].

Beyond peptide yield, enzymatic pretreatment also induces structural modifications that influence protein functionality. Reductions in β-sheet content and improvements in protein digestibility, as reported for soy protein systems, have been associated with accelerated release of low-molecular-weight peptides [26]. These structural and functional changes support the present Bradford and SDS-PAGE findings and suggest broader implications for improving protein bioavailability and nutritional quality.

From an industrial perspective, the observed plateau in hydrolysis beyond approximately 72–84 h, combined with early saturation of LERGDT production at 12 h, indicates considerable potential for reducing processing time, energy consumption, and operational costs. This pattern mirrors results reported in both plant- and animal-based protein systems, in which optimized enzymatic conditions reduced hydrolysis duration by 80–90% while maintaining bioactivity [11, 17].

Nevertheless, several limitations should be acknowledged. Although the optimized protocol shows promise at the laboratory scale, further studies are required to confirm reproducibility at the industrial scale and to validate the in vivo efficacy of LERGDT. Future work should also explore assisted hydrolysis approaches, such as ultrasound, high-pressure processing, or microwave pretreatment, to further enhance peptide yield and functional properties [23]. In addition, characterization of other bioactive peptides presents in PHGPB and evaluation of their biological activities may broaden its therapeutic and nutritional applications.

5. Conclusion

This study demonstrates that screening of enzymatic hydrolysis parameters, particularly hydrolysis time, can markedly improve the efficiency of green pea protein hydrolysate (PHGPB) production using bromelain without reducing bioactive peptide yield. Although the antifibrotic peptide LERGDT reached its highest abundance at 72 h, substantial levels were already obtained after only 12 h, indicating that hydrolysis duration can be significantly shortened. These findings support the development of a more efficient, cost-effective, and scalable production process for PHGPB, with potential application as a functional dietary component for chronic kidney disease management.

Declarations

During the preparation of this work, the author(s) used ChatGPT to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Tables

Tables are available in the Supplementary Files section.

Figures

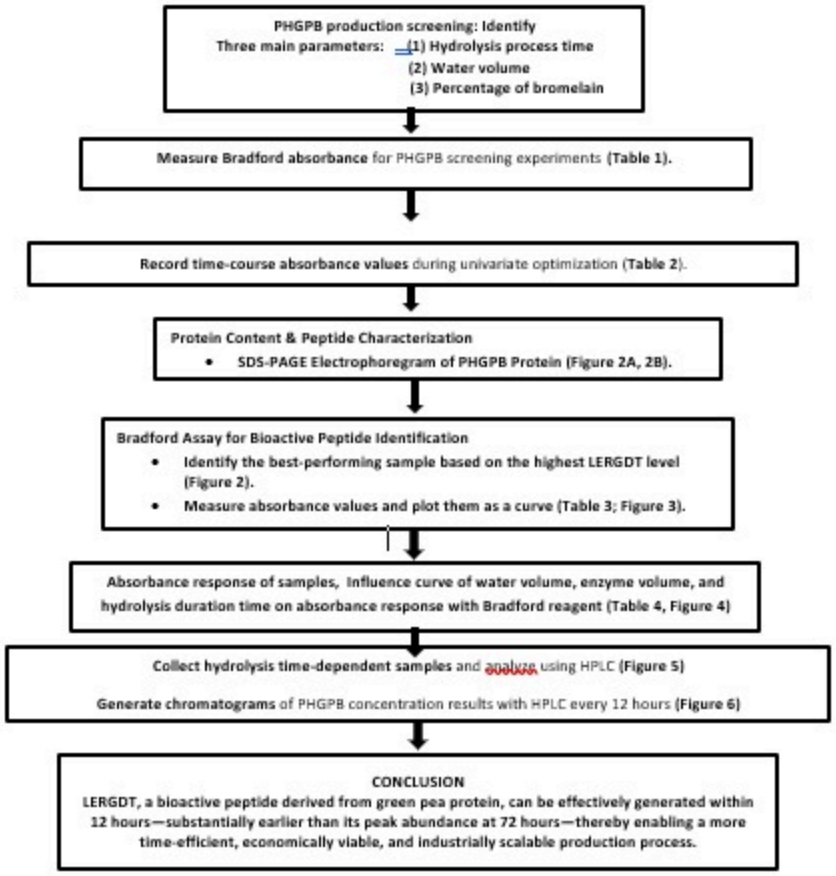


Fig 1. Research Methodology Steps

Figure 1

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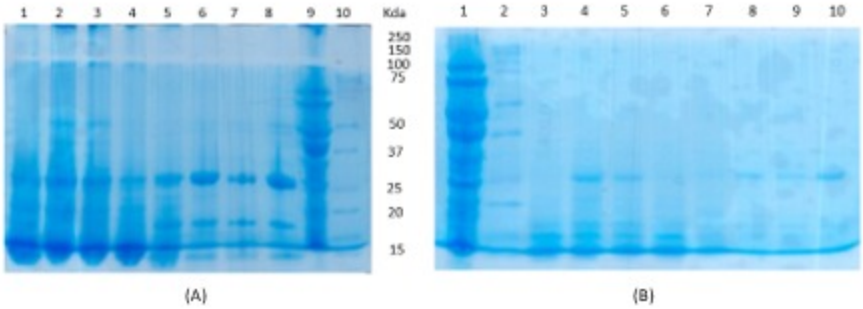


Fig. 2. SDS-PAGE Electrophoregram of PHGPB Protein. **A**. (1-8) Results of experiments 1-8; (9) Pea Powder; (10) Protein Marker. **B**. PHGPB diluted 10 times (1) Pea Powder; (2) Protein Marker; (3-10) Results of experiments 1-8; (10).

Figure 2

Fig. 2. SDS-PAGE Electrophoregram of PHGPB Protein. **A**. (1-8) Results of experiments 1-8; (9) Pea Powder; (10) Protein Marker. **B**. PHGPB diluted 10 times (1) Pea Powder; (2) Protein Marker; (3-10) Results of experiments 1-8; (10).

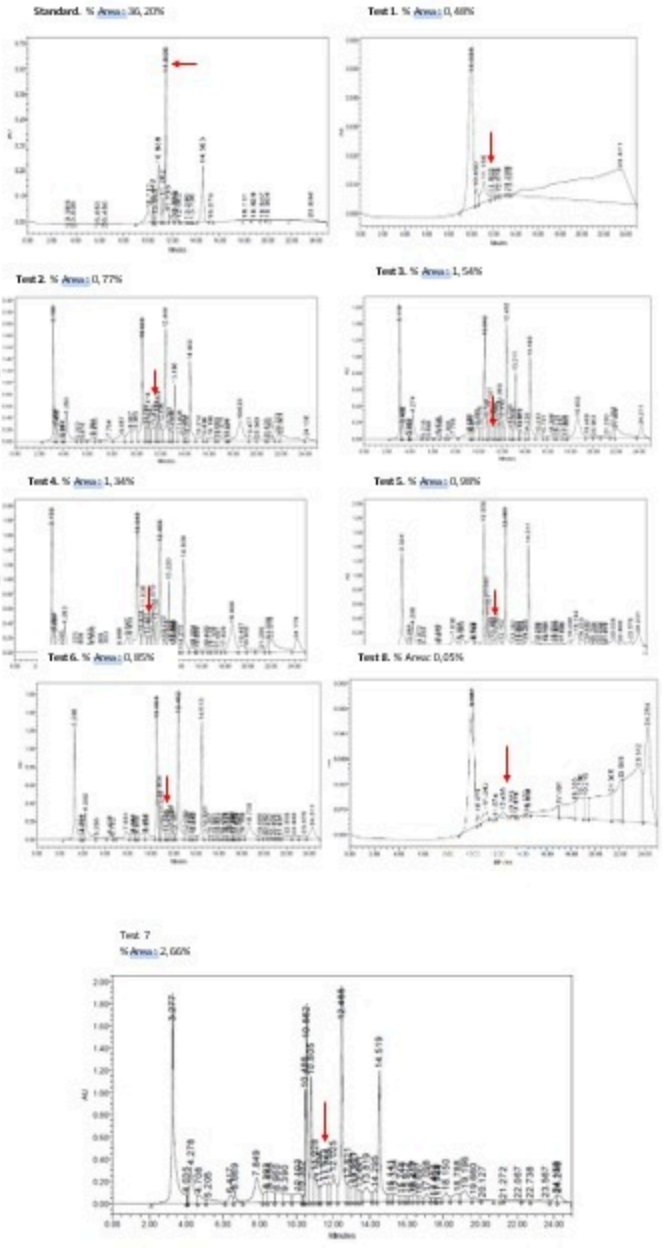


Fig. 3. Chromatogram of 8 PHGPB characterization results using the HPLC method.

Figure 3

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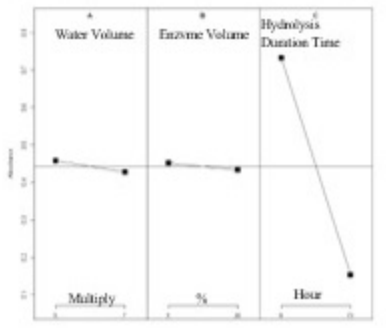


Fig. 4. Influence curve of water volume, enzyme volume, and hydrolysis duration time on absorbance response (sample test results using the Bradford method)

Figure 4

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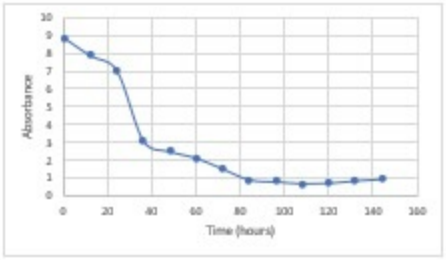


Fig. 5. Monitoring curve of protein hydrolysis time every 12 hours

Figure 5

Fig. 5. Monitoring curve of protein hydrolysis time every 12 hours

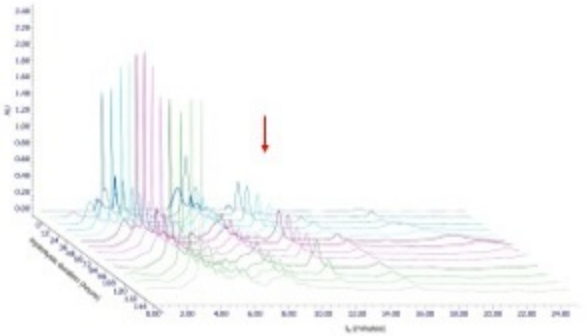


Fig. 6. Chromatogram of PHGPB characterization results with HPLC every 12 hours.

Figure 6

Fig. 6. Chromatogram of PHGPB characterization results with HPLC every 12 hours.

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