

## Green Peas Protein Hydrolyzed by Bromelain in Simple Procedure to Improve Kidney Function in Cisplatin-induced Rats

### Abstract

**Context:** Chronic kidney disease (CKD) can lead to terminal kidney failure. Previous study has shown that protein hydrolysate in yellow peas (*Pisum sativum* L.) can be used as a natural remedy for CKD. **Aims:** To obtain hydrolysate protein that is most effective in improving kidney function of cisplatin (CP)-induced Wistar rats, based on urea, creatinine, atrial natriuretic peptide (ANP), cyclooxygenase-1 (COX-1), and renin levels of CP-induced nephrotoxicity Wistar rats. **Materials and Methods:** Methods of Kjeldahl, Bradford, Kunitz, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were used to determine the content of the eight types of protein hydrolysates. In *in vivo* experiment, the samples were administered to CP-induced nephrotoxicity Wistar rats, with urea, creatinine, ANP, COX-1, and renin as parameters. **Results:** Total neutrase activity was 40.65 U/mg, and bromelain was 35.77 U/mg. Total specific activities of both enzymes were almost identical. Protein hydrolyzed using bromelain had small fractions (<14.4 kDa). On the 30<sup>th</sup> day of treatment, urea and creatinine levels of all groups of treatment were significantly different from CP control ( $P < 0.01$ ). The lowest level was shown by the group which was treated with bromelain-hydrolyzed green pea protein. Among ANP, COX-1, and renin measurements, only the result of COX-1 showed the promising result. **Conclusions:** Green peas protein hydrolysate hydrolyzed by bromelain are suggested as the most effective in improving kidney function based on urea, creatinine, and COX-1 levels of CP-induced nephrotoxicity Wistar rats.

**Keywords:** Atrial natriuretic peptide, bromelain, cisplatin-induced nephrotoxicity, cyclooxygenase-1, green peas, protein hydrolysate, renin

### Introduction

Chronic kidney disease (CKD) is one of the diseases that can progress to terminal kidney failure, cardiovascular disease (CVD), and death. Moreover, there are 39% and 28% of CKD cases which are caused by uncontrolled Type I and Type II diabetes mellitus and hypertension, respectively.<sup>[1]</sup> In Indonesia, the Basic Health Research Data of Indonesia 2013 showed that hypertension has the highest prevalence. The potential of the prevalence of CKD increases with age, and the highest prevalence is found at the age of  $\geq 75$  years (0.6%).<sup>[2]</sup> In addition to hypertension and diabetes, overweight or obesity has been proven to increase the risk of CKD.<sup>[3]</sup>

It is generally recognized that grain legumes contribute positively to a balanced diet and can prevent noncommunicable disease deterioration, including Type II diabetes and CVD. Legumes such as

soybean (*Glycine max*), nuts (*Phaseolus* spp.), peas (*Pisum sativum*), lupins (*Lupinus* spp.), or lentils (*Lens culinaris*) had been extensively studied in attempts to find a cure for hypertension and diabetes mellitus.<sup>[4]</sup> Aukema *et al.* investigated the effects of typical hemp, peanut, and soy protein in kidney disease progression and associated cardiac hypertrophy in experimental polycystic kidney disease rats.<sup>[5]</sup> Maghsoudi *et al.* reported that amylase inhibitor-rich fraction from white common bean (*Phaseolus vulgaris*) extract was considered as a drug-design target for the treatment of diabetes.<sup>[6]</sup>

A study conducted by Li *et al.* showed that protein hydrolysate in yellow peas (*P. sativum* L.) can be used as a natural remedy for high blood pressure and CKD. The proposed mechanism of pea protein in lowering blood pressure is by stimulating the production of cyclooxygenase-1 (COX-1), a protein that

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can improve kidney function.<sup>[7]</sup> The protein works similarly to the angiotensin-converting enzyme (ACE) inhibitor antihypertensive drug. However, the active substances in these peas are still yet to be investigated. On the other hand, in the utilization stage, the results showed that yellow peas in a natural state do not provide the same health benefits as the yellow pea protein hydrolysate (PPH) extract. It is because the bioactive peptide in proteins must be released from the main protein by special treatments.<sup>[8]</sup>

CKD is closely linked to diabetes and high blood pressure or hypertension. Natriuretic peptide has been shown to have a beneficial effect on hypertension and plasma volume expansion. Research on the physiological and pathophysiological functions of natriuretic peptide and its implications was being studied extensively for the treatment of patients with CVD associated with CKD.<sup>[9]</sup> Atrial natriuretic peptide (ANP) plasma level is elevated in complicated CKD patients with deteriorating renal function, but the relationship between plasma level of ANP and CKD deterioration still needs to be investigated. Hypothesis of kidney protection effect from ANP by inhibiting mesangial cells proliferation and renal fibrosis.<sup>[10]</sup>

Renin-angiotensin system (RAS) hyperactivity is associated with the progression of renal damage. Approximately 80% of CKD patients develop hypertension and impaired renal function and blood pressure, and this is related to the physiological and pathological conditions of renin and ACE in the “vicious cycle.”<sup>[11]</sup>

In this study, two beans originating from Indonesia (gude beans and green peas) and two kinds of peas from Canada (yellow peas and pea protein isolate [PPI]) were hydrolyzed using two protease enzymes, either neutrase or bromelain. The aim of this study is to obtain hydrolysate protein that is most effective in improving kidney function based on urea, creatinine, ANP, COX-1, and renin levels of CP-induced nephrotoxicity Wistar rats.

## Materials and Methods

### Materials

Four types of peas/beans sample: (1) Dry yellow Canadian pea (*P. sativum*) was obtained from a local market in Canada. (2) Gude beans (*Cajanus cajan*) were obtained from Cijengkol Village, Puncut, Bandung, West Java. (3) Green peas (*P. sativum* L) were from maica leaf, Magelang Plantation, East Java, Indonesia. (4) Pea protein (*P. sativum*) isolate was purchased from canadianprotein.com (Product#: EMW lot 161216100-PEAP). Two kinds of proteases are as follows: (1) Neutrase enzyme, given of kindness from Brenntag Connecting Chemistry (Asia Pacific). Neutrase® is a bacterial protease produced by a selected strain of *Bacillus amyloliquefaciens*. (2) Bromelain enzyme was obtained from fresh stem pineapple (*Ananas sativus*) juice that were grown in Subang, North Bandung, Indonesia, from local retail. Cisplatin (CP) for injection/i.p was

purchased from Dankos Farma (Jakarta, Indonesia). Reagent kit of ANP (QY-E11002), COX-1 (QY-E10736, and renin (QY-11096) was from Qayee-bio (Shanghai) for ELISA methods.

### Subject

Fifty female Wistar rats (5–6 weeks old), weighing 148–190 g, nullipara, and nonpregnant, were from the Department of Biology, Institut Teknologi Bandung (ITB), Indonesia.

### Methods

#### *Analysis protein characteristics of beans and protease enzymes*

The quantitative protein of the beans was assessed using micro Kjeldahl methods.<sup>[12]</sup> Neutrase and bromelain as protease enzymes were used to hydrolyze the beans, based on modified previous method.<sup>[13]</sup> Protein concentration of proteases was determined using Bradford methods<sup>[14]</sup> and tryptophan as a standard.<sup>[15]</sup> In addition, total specific activity of neutrase and bromelain was measured based on Kunitz method.<sup>[16]</sup>

#### *Protein hydrolysate preparation procedure*

Protein hydrolysates were prepared using method of Hidayat Copyright EC00201810615 based on previous method with modification.<sup>[17]</sup> Four types of peas powder have been sieved through the MESH sieve no. 120. Each was weighed for 50 g and dissolved in 200 mL water (400 mL for PPI). Neutrase or bromelain as much as 10% was added to each solution (based on the study conducted by Restiani, 2016, which stated that bromelain enzyme concentration of 10% yields the highest degree of hydrolysis in enzymatic hydrolysis of nyamplung [*Calophyllum inophyllum*] seed meal protein)<sup>[18]</sup> and then left for 72 h<sup>[19]</sup> (based on the study by Hale *et al.*, 2005: proteolytic activity of the bromelain solution remains relatively stable at least 1 week at room temperature), on a stirrer at room temperature (25°C–30°C) (based on the study by Poh and Abdul Majid, 2011: The enzymatic activity of bromelain decreases gradually from 25°C to 95C, stable at room temperature).<sup>[20]</sup> After stirred at room temperature and left for 72 h, then each solution was transferred to a tube and centrifuged at 6000 g for 10 min. The supernatant was filtered using a filter paper. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate and determine the molecular weight of protein hydrolysates.<sup>[21]</sup>

#### *In vivo experiment*

Fifty female Wistar rats were divided into 10 groups with different treatments. We used female animals because CP-induced nephrotoxicity show a gender difference in a rat model. CP-induced nephrotoxicity kidney damage is more severe in male than in female rats. It correlates with the expression of organic cation transporter 2 (OCT2) which male rats express more OCT2 in proximal renal tubules than female rats.<sup>[22]</sup>

These rats were induced with CP i.p 10 mg/kg BW before, based on Dugbartey *et al.*'s study that stated CP injection concentration >0.5 mg/mL may result in tissue cellulitis, fibrosis, and necrosis to produce CKD in rats.<sup>[23]</sup> Rats were provided hydrolysates proteins for 30 days, except negative control group. This animal experiment has been approved for the ethical clearance from Ethical Committee of Maranatha Christian University (No. 031/KEP FK UKM-RSI/II/2017). Each rat was treated with 100 mg/kg BW/day dose of protein hydrolysate as follows:

1. Protein hydrolysate of yellow Canadian pea, hydrolyzed by neutrase (PHYPN)
2. Protein hydrolysate of yellow Canadian pea, hydrolyzed by bromelain (PHYPB)
3. Protein hydrolysate of gude beans, hydrolyzed by neutrase (PHGBN)
4. Protein hydrolysate of gude beans, hydrolyzed by bromelain (PHGBB)
5. Protein hydrolysate of green peas, hydrolyzed by neutrase (PHGPN)
6. Protein hydrolysate of green peas, hydrolyzed by bromelain (PHGPB)
7. Protein hydrolysate of PPI, hydrolyzed by neutrase (PHPPIN)
8. Protein hydrolysate of PPI, hydrolyzed by bromelain (PHPPIB)
9. Negative control: the rats were not given any treatments
10. CP control: The rats were given only CP 10 mg/kg BW (only 1 time).

The treatment period was for 30 days. Groups 1–8 were administered with protein hydrolysates since day 0. On the 7<sup>th</sup> day, all rats were injected with CP i.p 10 mg/kg BW except rats of Group 9 as negative control. The rats were weighed every 7 days.

#### Sample collection

Urea and creatinine were measured 4 times on the 7<sup>th</sup>, 12<sup>th</sup>, 20<sup>th</sup>, and 30<sup>th</sup> day. The serum urea and creatinine levels were measured using COBAS ROCHE 311 with spectrophotometric methods. The level of normal urea for 8–16-week-old female rats is 13.2–27.1 mg/dL, while normal creatinine level rat females for that age is 0.2–0.6 mg/dL.<sup>[24]</sup>

Left kidneys of all rats were prepared to obtain kidney homogenates for the measurement of ANP, COX-1, and renin levels.

#### Kidney homogenate making procedure

The kidneys were cut into pieces and then put into a particular cuvette glass, under which were given an ice bath, and then added 2 mL PBS buffer for each gram of kidney. Kidney pieces are smoothed using blender waring until crushed. The slurry obtained was centrifuged at 7000 rpm at 4°C for 15 min. Supernatant was taken to be measured levels of ANP, COX-1, and renin using ELISA

method. Final measurement: Sate is read by ELISA reader at 450 nm wavelength.

#### Statistical analysis

Values are presented as mean  $\pm$  standard deviation. Data obtained were analyzed by ANOVA followed by *post hoc* least significant difference test for multiple comparison. Differences were considered to be statistically significant when  $P \leq 0.05$  and highly significant if  $P \leq 0.01$ . Result data of average ANP, COX-1, and renin levels were interpreted descriptively.

## Results

### Protein content

The protein content of the dried weight is 26.66%, 23.87%, 25.71%, and 70.21% for yellow Canadian peas, gude beans, green peas, and PPI, respectively.

### Total specific activity of the enzyme

Neutrase activity and protein amount in neutrase were 0.499 U/mL and 1.23 mg, respectively. It resulted in 40.650 U/mg for the total specific activity of neutrase. On the other hand, bromelain activity and the amount of protein in bromelain were 0.215 U/mL and 0.601 mg, respectively. As a result, the total specific activity of bromelain was 35.773 U/mg. These results showed that the total specific activities between the two enzymes are almost the same.

### Protein concentration and pH of the hydrolysates

After 72 h, the total amount of the solution products, pH, and the amount of protease were measured based on BSA equation and the absorbance at 280 nm. The calculated amount of protein from each sample is seen in Table 1.

### Molecular weight of protein hydrolysates

Figure 1 shows the results of protein hydrolysates using neutrase and bromelain. Protein hydrolysates using neutrase generally had a wide molecular weight, ranging from <14.4 to 25.0 kDa. Both PHYPN and PHGBN had several protein bands between 18.4 and 14.4 kDa. On the other hand, PHGPN had protein bands below 14.4 kDa, while PHPPIN had the most wide range protein bands below 14.4 kDa and more than 25.0 kDa. On the other hand, protein hydrolysates using bromelain had smaller molecular weight than neutrase. PHYPB, PHGBB, PHGPB, and PHPPIB samples have protein bands below 14.4 kDa.

### In vivo test in laboratory rats

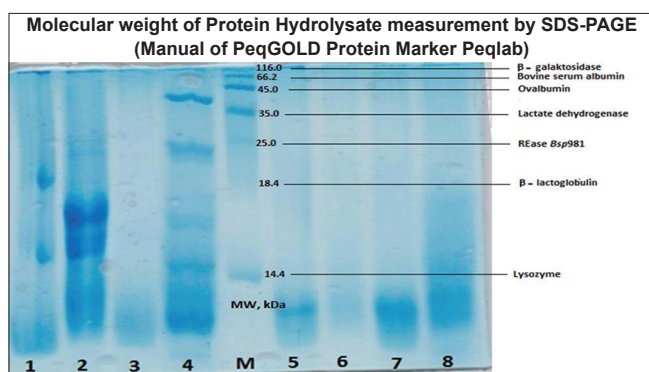
The mean results of urea and creatinine levels on day 7 (D7), D12, D20, and D30 are shown in Figures 2-5. Those figures show the comparison of each sample from hydrolyzed protein using neutrase or bromelain with negative control and CP control.

Based on the results of urea level measurement, there were no significant differences in all groups ( $P = 0.379$ )

**Table 1: Results of protein hydrolysates hydrolyzed by neutrase or bromelain**

Protein hydrolysates	Volume (ml)	pH	Concentration of protein (mg/mL)*
Yellow peas neutrase (PHYPN)	142.5	3.9	0.8169/0.7387=40.84 mg/mL
Yellow peas bromelain (PHYPB)	150	3.8	0.408/0.7387=27.61 mg/mL
Gude bean neutrase (PHGBN)	145	4.2	0.4865/0.7387=32.93 mg/mL
Gude bean bromelain (PHGBB)	135	4.2	0.3295/0.7387=22.30 mg/mL
Green peas neutrase (PHGPN)	145	3.6	0.8375/0.7387=56.69 mg/mL
Green peas bromelain (PHGPB)	115	3.7	0.699/0.7387=47.31 mg/mL
Pea protein isolate neutrase (PHPPIN)	350	7.3	0.91/0.7387=61.59 mg/mL
Pea protein isolate bromelain (PHPPIB)	300	7.2	0.555/0.7387=37.56 mg/mL

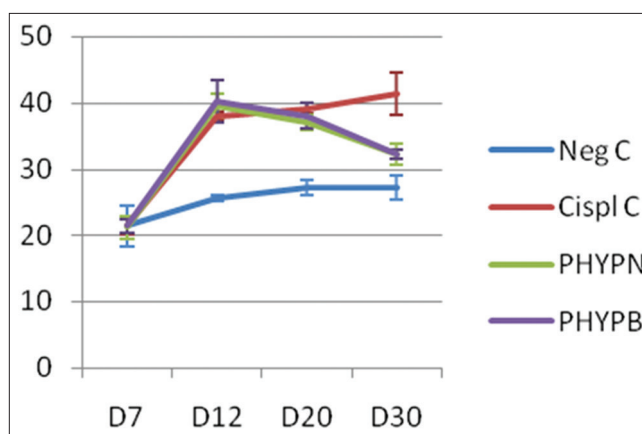
\*Concentration of protein was calculated by dividing the absorbance of the hydrolysate sample in diluted 50x (0.02 samples + 0.98 aquadest) at the A280 wavelength with the BSA equation. BSA: Body surface area, PHYPN: Protein hydrolysate of yellow peas Neutrase, PHYPB: Protein hydrolysate of yellow peas bromelain, PHGBN: Protein hydrolysate of gude beans Neutrase, PHGBB: Protein hydrolysate of gude beans bromelain, PHGPN: Protein hydrolysate of green peas Neutrase, PHGPB: Protein hydrolysate of green peas bromelain, PHPPIN: Protein hydrolysate of pea protein Isolate Neutrase, PHPPIB: Protein hydrolysate of pea protein isolate bromelain



**Figure 1: Polyacrylamide gel electrophoresis (SDS-PAGE) of protein hydrolysates (peqGOLD Protein Marker peqlab). 1: Protein hydrolysate of yellow Canadian pea, hydrolyzed by neutrase, 2: Protein hydrolysate of gude beans, hydrolyzed by neutrase. 3: Protein hydrolysate of green peas, hydrolyzed by neutrase. 4: Protein hydrolysate of pea protein isolate, hydrolyzed by neutrase. M. Protein Marker peqGOLD peqlab. 5: Protein hydrolysate of yellow Canadian pea, hydrolyzed by bromelain. 6: Protein hydrolysate of gude beans, hydrolyzed by bromelain. 7: Protein hydrolysate of green peas, hydrolyzed by bromelain. 8: Protein hydrolysate of pea protein isolate, hydrolyzed by bromelain**

at D7. Subsequently, there were ANOVA highly significant differences ( $P \leq 0.01$ ) on D12 ( $P \leq 0.01$ ), D20 ( $P \leq 0.01$ ), and D30 ( $P \leq 0.01$ ), respectively. Furthermore, on D30, all groups of treatments showed a highly significant improvement of urea level compared to CP control group ( $P \leq 0.01$ ). The lowest urea levels at D12, D20, and D30 were shown by Group 6 (PHGPN).

The mean creatinine level for each group is shown in Figures 6-9. The graphs showed the comparison of each sample of protein hydrolysate using neutrase or bromelain, with negative control and CP control. There were significant ANOVA differences, i.e.  $P = 0.003$ ,  $P \leq 0.01$ ,  $P = 0.030$ ,  $P \leq 0.01$ , for D12, D20, and D30, respectively. Furthermore, all groups of treatments were observed to have significant difference at creatinine level from CP control group ( $P \leq 0.01$ ) at D30. The lowest creatinine levels at D12, D20, and D30 were shown in Group 6 (PHGPB) [Figure 8].



**Figure 2: Urea mean level after treatments of protein hydrolysate of yellow Canadian pea, hydrolyzed by neutrase and protein hydrolysate of yellow Canadian pea, hydrolyzed by bromelain. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection**

The lowest results for both urea and creatinine levels in relation to kidney function were shown in Group 6, PHGPB. The results of urea and creatinine measurement in that group at the 4<sup>th</sup> week were 30.6 mg/dL and 0.754 mg/dL, respectively, while in negative control were 27.2 mg/dL and 0.738 mg/dL.

### Result of atrial natriuretic peptide, cyclooxygenase-1, and renin measurements

ANP, COX-1, and renin measurements were performed on left kidneys homogenate of CP-induced nephrotoxicity rats, using the ELISA method with Qayee-Bio Production kit, 3 repetitions (Triple), and were interpreted descriptively.

Results of average ANP level in negative controls showed mean levels of 144.1 pg/mL while CP control showed highly different results, i.e. 225.1 pg/mL. The treatment groups showed poor results, no results below or near the negative control results. Five groups showed lower results than the CP control group, while three groups showed

higher results than CP control group, i.e. PHGBB, PHGPB, and PHPPIB groups [Figure 10].

In COX-1 level measurement, negative control showed mean levels of 211.4 pg/mL while CP control showed highly different result, i.e. 151.9 pg/mL. The treatment groups showed fairly good results although no results were higher than negative controls. All the groups showed higher results than the CP control and the highest COX-1 levels showed by PHGPB group (207.4 pg/mL) [Figure 11].

In renin measurement, negative controls showed mean levels of 153.8 pg/mL while CP control showed highly different results, i.e., 227.4 pg/mL. The treatment groups

showed poor results. All the groups showed higher results than the CP control group. The group that showed lower results than the CP control was only the PHGPN group with levels of 224.1 pg/mL [Figure 12].

## Discussion

In this study, we have compared four types of pea/bean samples. The protein content of the dried weight was 26.66%, 23.87%, 25.71%, and 70.21% for yellow Canadian peas, gude beans, green peas, and PPI, respectively. It is clear that PPI has the highest protein content, but its hydrolysate protein did not give certainty to give the best results.

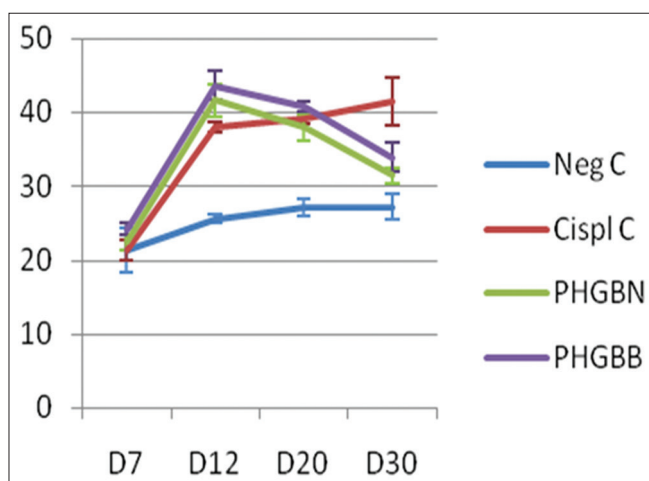


Figure 3: Urea mean level after treatments of protein hydrolysate of gude beans, hydrolyzed by neutrase and protein hydrolysate of gude beans, hydrolyzed by bromelain. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection

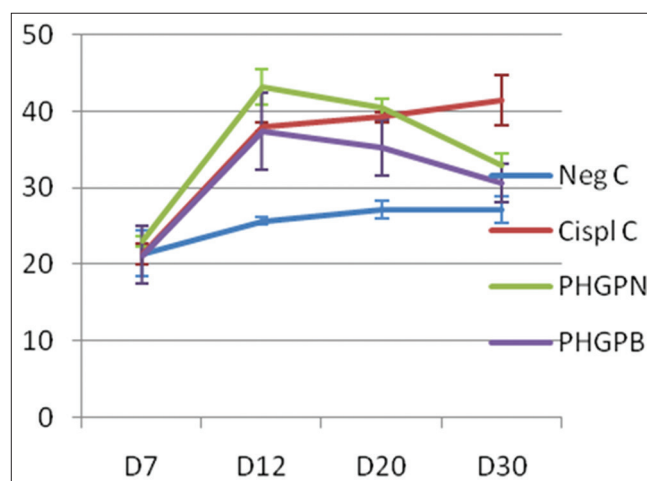


Figure 4: Urea mean level after treatments of protein hydrolysate of green peas, hydrolyzed by neutrase and protein hydrolysate of green peas, hydrolyzed by bromelain. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection

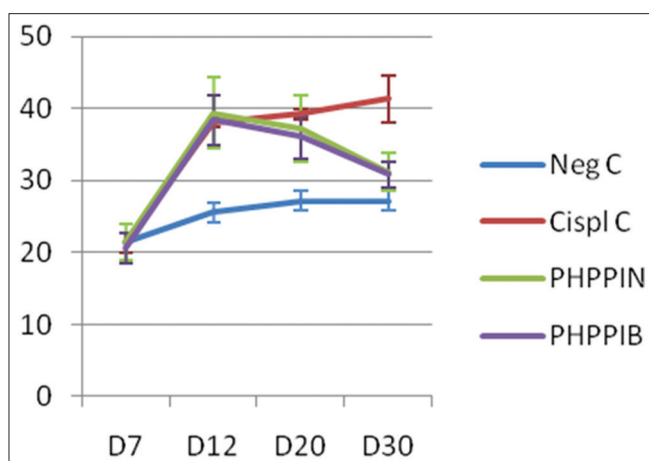


Figure 5: Urea mean level after treatments of protein hydrolysate of pea protein isolate, hydrolyzed by neutrase and protein hydrolysate of pea protein isolate, hydrolyzed by bromelain. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection

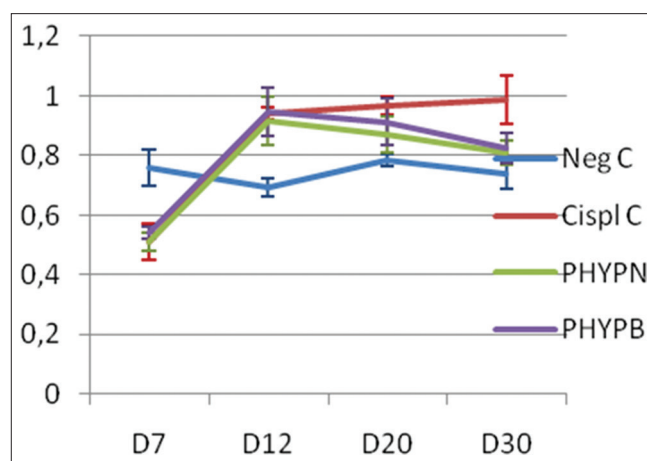


Figure 6: Creatinine mean level after treatments of protein hydrolysate of yellow Canadian pea, hydrolyzed by neutrase and protein hydrolysate of yellow Canadian pea, hydrolyzed by bromelain. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection

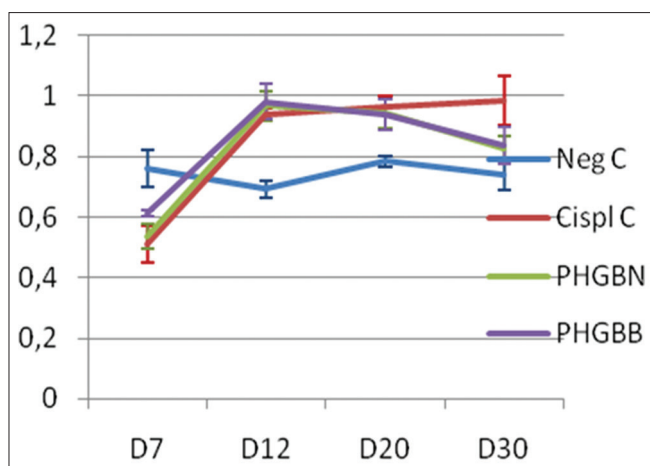


Figure 7: Creatinine mean level after treatments of protein hydrolysate of gude beans, hydrolyzed by neutrase and protein hydrolysate of gude beans, hydrolyzed by bromelain protein hydrolysates. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection

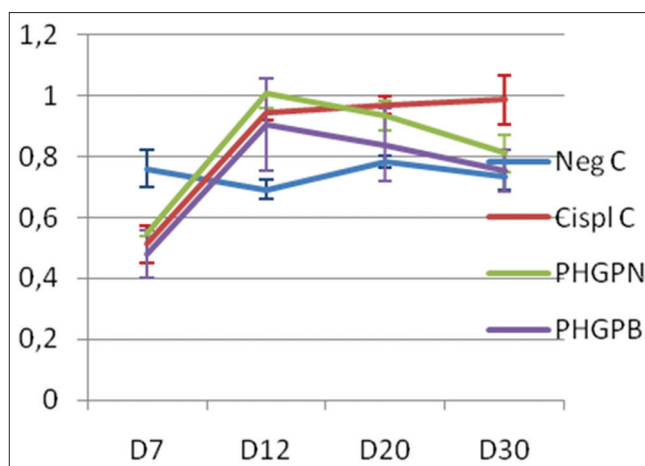


Figure 8: Creatinine mean level after treatments of protein hydrolysate of green peas, hydrolyzed by neutrase and protein hydrolysate of green peas, hydrolyzed by bromelain. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection

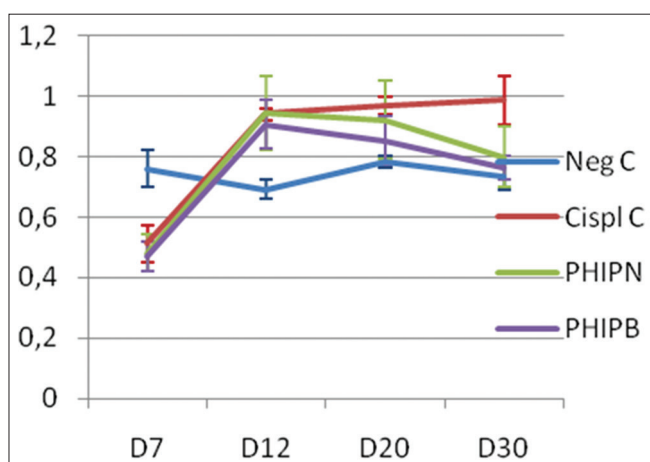


Figure 9: Creatinine mean level after treatments of protein hydrolysate of pea protein isolate, hydrolyzed by neutrase and protein hydrolysate of pea protein isolate, hydrolyzed by bromelain. D7: First urea level measurement. Before cisplatin injection, D12: Second urea level measurement, after 5 days cisplatin injection, D20: Third urea level measurement, after 12 days cisplatin injection, D30: Fourth urea level measurement, after 23 days cisplatin injection

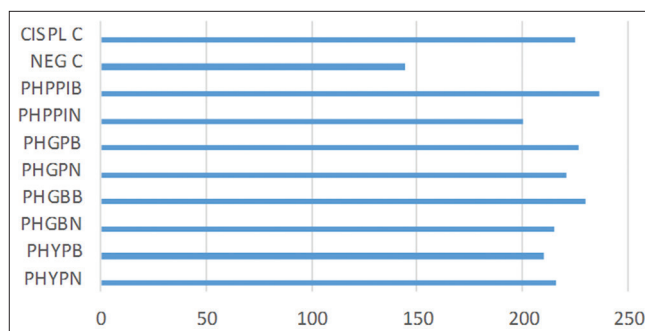


Figure 10: Atrial natriuretic peptide mean level

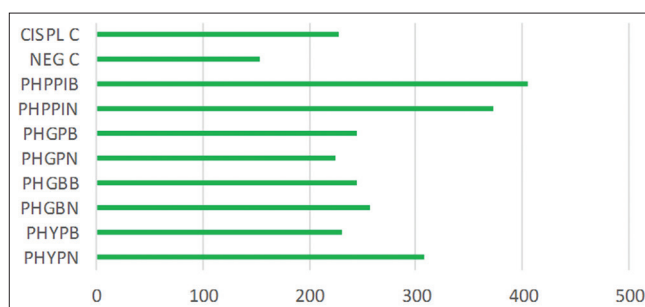


Figure 12: Renin mean level

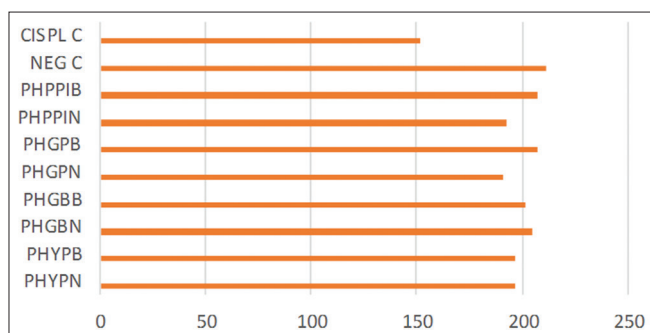


Figure 11: Cyclooxygenase-1 mean level

Protein hydrolyzed using bromelain had small fractions (<14.4 kDa). The smaller the molecular weight of a

protein or peptide, the easier it will be to be absorbed. PPH, which the primary effect is lowering blood pressure, is a peptide containing <3 kDa.<sup>[7]</sup> Stanisavljević's *et al.*'s study, which measured the antioxidant activity of the pea hydrolysate fraction, showed that the fraction formed from a hydrolysate of <10 kDa molecules weight obtained from fermented pea protein purified by *Lactobacillus rhamnosus* BGT10, contained the basic peptide with the highest antioxidant activity.<sup>[25]</sup> It was suggested that protein hydrolyzed using bromelain in this study may have high antioxidant activity.

**Table 2: Mean result of Urea Level of Cisplatin-induced Wistar rats after Treatments**

Number	Group	D7	D12	D20	D30
1	PHYPN	21.2±1.72	39.6±1.85	37.0±1.10	32.2±1.60
2	PHYYPB	21.4±1.02	40.2±3.12	38.0±2.00	32.2±0.75
3	PHGBN	22.4±1.02	41.6±2.15	37.8±1.72	31.4±1.02
4	PHGBB	24.2±0.75	43.6±1.96	40.8±0.75	34.0±2.00
5	PHGPN	23.0±0.63	43.2±2.32	40.4±1.20	33.0±1.41
6	PHGPB	21.2±3.76	37.4±5.00	35.2±3.71	30.6±2.58
7	PHPPIN	21.4±2.50	39.4±4.96	37.2±4.71	31.2±2.56
8	PHPPIB	20.6±2.06	38.4±3.44	36.0±2.97	30.8±1.72
9	Neg C	21.4±3.01	25.6±0.49	27.2±1.17	27.2±1.72
10	Cispl C	21.4±1.36	38.0±0.63	39.2±0.75	41.4±3.26

D12: Second measured urea level after 5 days cisplatin injection, D20: Third measured urea level after 12 days cisplatin injection, D30: Fourth measured urea level after 23 days cisplatin injection. PHYPN: Protein hydrolysate of yellow peas Neutrase, PHYYPB: Protein hydrolysate of yellow peas bromelain, PHGBN: Protein hydrolysate of gude beans Neutrase, PHGBB: Protein hydrolysate of gude beans bromelain, PHGPN: Protein hydrolysate of green peas Neutrase, PHGPB: Protein hydrolysate of green peas bromelain, PHPPIN: Protein hydrolysate of pea protein Isolate Neutrase, PHPPIB: Protein hydrolysate of pea protein isolate bromelain, Neg C: Negative control, Cisp C: Cisplatin control

The urea measurement results showed that the urea level in protein hydrolysate groups hydrolyzed by bromelain was lower than which were hydrolyzed by neutrase. It was shown by less value of the urea level between PHGPB and PHGPN, PHPPIB, and PHPPIN. The urea level of PHGPB and PHPPIB was lower than PHGPN and PHPPIN [Figures 4 and 5]. Moreover, the creatinine level seems to show similar results. However, the creatinine level in PHYPN and PHGBN were lower than PHYYPB and PHGBB. The lowest results for urea and creatinine levels were found in Group 6, PHGPB (as seen in Tables 2 and 3), although the value was still higher than the normal value for female rats of that age. A prolonged treatment may be needed to reach the normal value. Based on the results of this study, the protein content of the beans was not correlated with the *in vivo* experiment. PPI has very high protein content (70.21%), but its effect to kidney function was not as great as that of green peas (25.71%) hydrolyzed by bromelain. The potential of protein hydrolysates depends on its bioactive peptide, and it still needs to be investigated.

Enzymatic hydrolysis breaks down proteins and produces peptides of smaller size that have many beneficial biological functions; it significantly increases the solubility of hydrolysate proteins up to 62% over a wide pH range of 2–12.<sup>[26]</sup> According to Udenigwe *et al.*'s study, hydrolysis using neutrase will produce the highest hydrolysate protein (23.4%). Neutrase hydrolysate showed the highest radical scavenging activity of 2,2-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (inhibitory concentration 50 = 3.6 mg/mL). Neutrase is summed up as the optimal

**Table 3: Mean result of Creatinine Level of Cisplatin-induced Wistar rats after treatments**

Number	Group	D7	D12	D20	D30
1	PHYPN	0.510±0.03	0.916±0.08	0.872±0.06	0.808±0.04
2	PHYYPB	0.542±0.02	0.948±0.08	0.914±0.08	0.826±0.05
3	PHGBN	0.536±0.04	0.968±0.05	0.942±0.05	0.828±0.04
4	PHGBB	0.612±0.01	0.982±0.06	0.940±0.05	0.838±0.06
5	PHGPN	0.546±0.01	1.008±0.05	0.934±0.05	0.810±0.06
6	PHGPB	0.480±0.08	0.904±0.15	0.838±0.12	0.754±0.07
7	PHPPIN	0.482±0.06	0.944±0.12	0.920±0.13	0.798±0.10
8	PHPPIB	0.470±0.05	0.906±0.08	0.854±0.08	0.762±0.04
9	Neg C	0.760±0.06	0.692±0.03	0.784±0.02	0.738±0.05
10	Cispl C	0.512±0.06	0.940±0.02	0.968±0.03	0.986±0.08

D7: First measured ureum level before cisplatin injection, D12: Second measured ureum level after 5 days cisplatin injection, D20: Third measured ureum level after 12 days cisplatin injection, D30: Fourth measured ureum level after 23 days cisplatin injection. PHYPN: Protein hydrolysate of yellow peas Neutrase, PHYYPB: Protein hydrolysate of yellow peas bromelain, PHGBN: Protein hydrolysate of gude beans Neutrase, PHGBB: Protein hydrolysate of gude beans bromelain, PHGPN: Protein hydrolysate of green peas Neutrase, PHGPB: Protein hydrolysate of green peas bromelain, PHPPIN: Protein hydrolysate of pea protein Isolate Neutrase, PHPPIB: Protein hydrolysate of pea protein isolate bromelain, Neg C: Negative control, Cisp C: Cisplatin control

enzyme to hydrolyze white egg proteins to produce high antioxidant peptides.<sup>[27]</sup>

The value of total enzyme activities between neutrase and bromelain was nearly identical. Even though bromelain has a slightly less activity compare with neutrase, it showed a better result. As a nephroprotective agent, the effectiveness of an enzyme does not depend on the high value of total enzyme activity but also on its ability to effectively cleave protein to smaller peptide fragments. As a proteolytic enzyme, bromelain breaks down proteins into their amino acid building blocks through a hydrolysis reaction in amino acid residue: lysin, alanine, or tyrosine.<sup>[27,28]</sup> According to Glider and Hargrove study, bromelain activity depends on some intrinsic and extrinsic factors; among those, temperature and pH significantly affect bromelain activity. The optimum bromelain activity against casein is at a temperature range from 10°C to 20°C at acid pH, from 30°C to 40°C at a base pH value, and from 40°C to 60°C at neutral pH (pH 6.8–7.1).<sup>[29]</sup> In this study, the hydrolyzed procedure of PHGPB was optimized in pH 3.7 at 25°C.

Bioactive peptides, obtained by enzymatic hydrolysis of food proteins, have been shown to exhibit promising effects on human health and illness, such as antihypertensive activities, antioxidant activities, anti-inflammatory properties, and lipid-lowering properties. The production of potential peptides should be based on the parameters of important organ functions, and in this case, the kidneys. The enzymatic hydrolysis of food proteins had great benefits in producing bioactive peptide sequences from other inactive complexes of inactive molecules.<sup>[30]</sup>

**Table 4: Mean result of ANP, COX-1, Renin Level of Kidney Homogenate Tissue of Cisplatin-Induced Wistar Rats**

Number/ Group	Result		
	ANP	COX-1	Renin
1.1	217.0	184.9	382.5
1.2	213.9	212.8	351.4
1.3	217.8	191.7	188.6
PHYPN	216.2	196.5	307.5
2.1	207.8	204.4	228.7
2.2	213.1	202.6	237.0
2.3	209.5	183.0	225.3
PHYPB	210.1	196.7	230.4
3.1	228.6	217.2	260.1
3.2	216.8	201.7	277.3
3.3	200.5	196.2	235.3
PHGBN	215.3	205.0	257.6
4.1	221.8	208.0	281.9
4.2	235.0	194.2	219.7
4.3	233.4	203.6	234.6
PHGBB	230.1	201.9	245.4
5.1	222.0	182.6	224.2
5.2	204.5	190.9	230.3
5.2	235.4	199.9	217.7
PHGPN	220.6	191.2	224.1
6.1	232.0	199.7	226.8
6.2	225.9	211.3	252.7
6.3	221.4	211.2	254.8
PHGPB	226.4	207.4	244.8
7.1	209.2	201.1	345.3
7.2	205.7	181.4	379.2
7.3	186.2	194.6	395.7
PHPPIN	200.4	192.4	373.4
8.1	243.6	207.1	423.8
8.2	232.0	218.5	400.6
8.3	234.5	195.3	389.9
PHPPIB	236.7	207.0	404.8
9.1	134.2	192.0	140.2
9.2	178.1	230.9	175.5
9.3	120.0	211.2	145.6
Neg C	144.1	211.4	153.8
10.1	237.6	144.6	241.4
10.2	233.6	110.2	225.2
10.3	204.0	200.9	215.7
Cisp C	225.1	151.9	227.4

COX-1: Cyclooxygenase-1, ANP: Atrial natriuretic peptide, PHYPN: Protein hydrolysate of yellow Canadian pea, hydrolyzed by neutrase, PHYPN: Protein hydrolysate of yellow peas Neutrase, PHYPB: Protein hydrolysate of yellow peas bromelain, PHGBN: Protein hydrolysate of gude beans Neutrase, PHGBB: Protein hydrolysate of gude beans bromelain, PHGPN: Protein hydrolysate of green peas Neutrase, PHGPB: Protein hydrolysate of green peas bromelain, PHPPIN: Protein hydrolysate of pea protein Isolate Neutrase, PHPPIB: Protein hydrolysate of pea protein isolate bromelain, Neg C: Negative control, Cisp C: Cisplatin control

The antioxidant properties in PHGPB may play a role in promoting the kidney function. The antioxidant can relieve

the inflammation of the kidney which will ultimately improve damage of the kidney, in this case demonstrated by decreased of urea and creatinine level. Antioxidant and antihypertensive properties of enzymatic green pea protein hydrolysate have been reported in several studies.<sup>[15,31]</sup> The eluted fraction contains higher levels of hydrophobic and aromatic acids when compared to the original PPH, exhibiting the most powerful radical and metal chelating activity; however, the hydrophobic character does not seem to contribute in reducing peptide strength. In comparison to glutathione, peptide fraction has significantly higher antioxidant ability ( $P < 0.05$ ) in inhibiting oxidation of linoleic and chelate metals.<sup>[31]</sup> PHGPB showed protein band in SDS-PAGE molecular weight smaller than 14.4 kDa, which may be related to high antioxidant and antinephrotoxicity activities. However, this assumption still needs to be investigated. According to the Girgih *et al.*'s study, protein PPH permeates (PPH-5) which mainly consists of low molecular weight peptide (<10 kDa), with 2–6 amino acids, containing rennin and angiotensin-converting inhibitor. This antihypertensive potential is due to the presence of easily absorbed hydrophilic peptides.<sup>[32]</sup>

The purpose administration of protein hydrolysates to the rats since the beginning was to investigate the preventive effects to CP-induced nephrotoxicity kidney rats. The urea and creatinine levels of Group 6 increased at the 12<sup>th</sup>, 20<sup>th</sup>, and 30<sup>th</sup> day of the study. However, this result was considerably lower than other treatment groups [Figures 2-9]. It was suggested that this protein hydrolysate potentially has a protective effect on kidney function.

The main targets of antihypertensive peptides are renin and ACE.<sup>[33]</sup> Renin mean level of negative control was supposed to be low. CP group showed high renin mean level [Figure 12]. Nevertheless, the results of this study showed that administration of protein hydrolysate did not show average renin levels as low as negative control [Table 4]. This may be due to the limited treatment time, or indeed, renal nephrons damage caused by CP was highly severe and irreversible.

Similarly, with ANP examination results, the results showed that the administration of protein hydrolysate did not produce low levels of ANP [Table 2]. ANP mean level of negative control supposed to be high, but the result showed low level [Figure 10]. However, the CP group showed high ANP mean level. These results were difficult to explain. Results of all treatment groups were almost similar with CP group. ANP is elevated in complicated CKD patients with deteriorating renal function;<sup>[10]</sup> this condition may correlate to the compensation mechanism of the body.

COX-1 mean level of negative control showed much higher level than the result of CP group [Table 2]. All of treatments group showed higher COX-1 mean level than CP group [Figure 11]. Among the three results of



measurement (ANP, COX-1, and renin), only COX-1 assessment showed promising results. This is in accordance with the results of Li *et al.*'s study, in which PPH can increase the production of COX-1 which can improve kidney function.<sup>[7]</sup> COX-1 is responsible for the production of prostaglandins that are important for homeostatic functions, such as maintaining the integrity of the gastric mucosa and regulating renal blood flow.<sup>[34]</sup> We propose that PHGPB improved the kidney function through this mechanism, instead of by RAS system or ANP.

Considering these results, it can be concluded that protein hydrolysate of green peas hydrolyzed using bromelain protease is promising enough to be investigated further as a candidate strain for the large-scale production of bioactive peptides from legume proteins.

The main caveats of this study are limited facilities, i.e., the tool for making homogenate due to the tissue breaker (mincer) can only work on a minimum volume. This constrains caused us to prepare the kidney tissue in groups; thus, the results data can only be analyzed per group from three repetitions and descriptively interpreted.

## Conclusions

Green peas protein hydrolysate hydrolyzed by bromelain is suggested as most effective in improving kidney function, based on urea, creatinine, and COX-1 levels of CP-induced nephrotoxicity Wistar rats.

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## Conflicts of interest

There are no conflicts of interest.

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