Original Article

Role of Pea Protein Hydrolysates as Antinephrotoxicity

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Abstract

Introduction: Renal damage can be caused by various causes. One of them is drugs that are toxic to the renal, such as cisplatin (CP). In an attempt to find a remedy for antinephrotoxicity, several hydrolyzed proteins were investigated. This study was conducted to find out the effects of 8 peas protein hydrolysates (PPH) hydrolyzed using simple procedure to renal organ indexes (OIs) and histopathological features of CP-induced nephrotoxicity Wistar rats. **Materials and Methods:** Protein hydrolysates of yellow peas, gude beans, green peas, and pea protein isolate (PPI) which hydrolyzed using neutrase or bromelain were administered to 50 female Wistar rats.The treatments were given for 30 days, and on day 7, all groups of rats, except negative control group, were injected CP intraperitoneal. Renal OIs were measured and kidneys were histopathological analyzed, which the results were converted to scoring system. Data were analyzed using ANOVA, LSD, Kruskal–Wallis, and Mann–Whitney test. **Results:** Data of renal OIs were homogenous and normally distributed but were not significantly different between groups ($P > 0.05$).The nephrotoxicity of CP were not changing the renal OI but worsen the histopathological features of renal tubules in CP-induced rats ($P < 0.01$). All protein hydrolysate treatment groups showed less histopathological score than CP group. Green PPH hydrolyzed by bromelain-treatment group showed the lowest scores. **Conclusion:** All PPH hydrolized with neutrase or bromelain improve the CP-induced nephrotoxicity rats. Green PPH with bromelain hydrolyzed had a promising potency as antinephrotoxicity.

Keywords: *Antinephrotoxicity, bromelain, cisplatin, green pea protein hydrolysate, kidney histopathological, organ index*

Introduction

Renal damage can be caused by various causes. One of them is drugs that are toxic to the renals, such as cisplatin (CP). The proximal tubules of the renal become the most affected organ since CP enters the cell through the transport of the copper transporter 1 and organic cation transporter 2 membranes that are widely present in the cell.[1] The primary targets are proximal straight and distal convoluted tubules where it accumulates and promotes cellular damage, by multiple mechanisms including oxidative stress, DNA damage, and apoptosis.[2-4] The role of reactive oxygen species (ROS) in the pathogenesis of nephrotoxicity has been proved by many studies.[5] CP induces free radical production causing oxidative renal damage, possibly due to depletion of nonenzymatic and enzymatic antioxidant systems. Abnormal production of ROS may damage some macromolecules to induce cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids, protein denaturation, and DNA damage.[6] Acute tubular necrosis is the most common form of hospital-acquired acute renal failure. Patient with abnormal renal function before nephrotoxic insult to the renals is at greater risk not only for acute or chronic renal disease but also for end-stage renal disease.[7] Chronic renal disease, irreversible destruction of renal, is characterized by gradual loss of renal function over time. In Indonesia, it is estimated that there are 100 people per million population or about 10,000 new chronic kidney disease (CKD) cases in a year. Once a person suffers from CKD, though at first stage, this condition cannot be reversible. The maximum effort that can be done is to slow the progression of the renal damage. Therefore, prevention of the disease becomes very important.[8] Until certain limit, good diet and nutrition gave benefits to prevent the worsened of the renal.

A previous study indicated that protein hydrolysate in yellow peas (*Pisum sativum* L.) can be used as a

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natural remedy for high blood pressure and CKD.[9] The antioxidant and antihypertensive properties of enzymatic green peas protein hydrolysate (PPH) have been shown in several studies.[10,11] 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. Compared with glutathione, peptide fraction has significantly higher antioxidant ability ($P < 0.05$) in inhibiting oxidation of linoleic and chelate metals.[11] However, the active substances in these peas still need to be investigated; however, in the utilization stage, the results showed that yellow peas in a natural state do not provide the same health benefits as yellow PPH extract because the effective proteins can only be activated with special enzymes.[12]

In our study, we have analyzed the effects of eight kinds of hydrolysates which obtained from four sorts of beans hydrolyzed using two sorts of enzymes, neutrase and bromelain (a protein breaking enzyme obtained from pineapple) to renal function of CP-induced Wistar rats. The results showed that all PPH showed an improved renal function in CP-induced Wistar rats, while green PPH which hydrolyzed by bromelain gave the least score of damage in kidney histopathological feature.

Severity of the tissue toxicity appears to be related to the concentration of the CP solution. Infusion of solutions with a CP injection concentration >0.5 mg/mL may result in tissue cellulitis, fibrosis, and necrosis.[13] The weight of renal may change in CP-induced nephrotoxicity.[14] The study shows that animals treated with CP, the normalized renal weight increased, and it was correlated with intensity of tissue damage.[4,15-17] CP may also affect another organ weight (OW).

The aim of this study is to find out the effects of administration of protein hydrolysates of yellow peas, gude beans, green peas, and pea protein isolate (PPI), respectively, hydrolyzed by neutrase and bromelain for 30 days to renal organ index (OI) and kidney histopathological imaging of CP-induced Wistar rats.

Settings and design

This study used true experimental laboratory with randomized controlled trial and comparative design were used in this study.

Materials and Methods

Materials

Yellow Canadian pea (*P. sativum)* was obtained from local market. Gude beans (*Cajanus cajan*) from Cijengkol village, Punclut, Bandung, West Java. Green Peas (*Pisum sativum* L) from Maica leaf, Magelang Plantation, East Java. Pea protein (*P. sativum)* isolate was purchased from canadianprotein.com (Product#: EMW lot 161216100-PEAP). Neutrase enzyme containing proteases was purchased from Brenntag Connecting Chemistry (Asia Pacific). Bromelain enzyme was obtained from fresh pineapple (*Ananas sativus*) juice. CP for injection/ intravenous was purchased from Dankos Farma, Jakarta, Indonesia.

Subjects

Fifty female Wistar rats (5–6 weeks old), weighing 148–190 g, nullipara, and nonpregnant, were from Sekolah Ilmu dan Teknologi Hayati (School of Life Sciences and Technology), Institut Teknologi Bandung, Indonesia.

Methods

Protein hydrolysate preparation

Protein hydrolysates were prepared using the previous method with modification.[13] 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 of PPI). Neutrase or bromelain was added to each solution. It was then stirred at room temperature and left for about 72 h. Following the incubation, each solution was transferred to a tube and centrifuged at 6000 g for 10 min. The supernatant was filtered using filter paper. SDS-PAGE was employed to separate and determine the molecular weight of protein hydrolysates.[18]

In vivo test in laboratory rats

As many 50 female Wistar rats were divided into ten groups with different treatment. These rats were induced with CP before, and then the OI and histopathological renal feature were measured, with tubules degeneration, nuclear necrosis, and hyaline cast as parameters. The animal experiment has been approved for the ethical clearance from the Ethical Committee of Maranatha Christian University (084/ KEP FK UKM-RSI/III/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 (YPN)
2. Protein hydrolysate of yellow Canadian pea, hydrolyzed by bromelain (YPB)
3. Protein hydrolysate of gude beans, hydrolyzed by neutrase (GBN)
4. Protein hydrolysate of gude beans, hydrolyzed by bromelain (GBB)
6. Protein hydrolysate of green peas, hydrolyzed by bromelain (GPB)
7. Protein hydrolysate of PPI, hydrolyzed by neutrase (PPIN)
8. Protein hydrolysate of PPI, hydrolyzed by bromelain (PPIB)
9. Negative control: The rats were not given any treatments
10. CP control: The rats were given only CP 10 mg/kg BW (once).

The study was performed for 30 days; since the beginning until the end, the Groups 1–8 were given the protein

hydrolysates. On the 7th day, each group member was injected with CP intraperitoneal 10 mg/kg BW, except Group 9. Body weights were weighed every week (7 days). On day 30, all rats were killed. Left kidneys were weighed to measure the OW, and then the results were divided with their each body weight to obtain OI value. Right kidneys were made into histopathological slides and were seen under light microscope magnification of 40 times from five viewing fields with parameters and scores: Tubules degeneration (cloudy swelling): 0 none; 1 focal; 2 diffused. Nuclear necrosis: 0 none; 1 focal; 2 diffused. Hyaline cast: 0 none; 1 focal; 2 diffused.

Tubules degeneration is an energy metabolism disorder in the cell, especially in transport active mechanisms in Na/K/ATPase; therefore, the cell cannot pump out Na ion from the cell. This condition occurs caused by CP, which has light molecular weight, therefore easily filtrated by glomerulus, carried by renal tubules cell, and reached the highest gradient in proximal contortus tubules of renal.[13] Nuclear necrosis is a further form of degeneration, nuclear cell death begins with a picnotic feature which is indicated by thickening of the nucleus chromatin followed by carioeccyst then cariolysis. The hyaline cast was produced because of inflammation of the tubules cell; then, leaky protein occurred and sedimented as cast.[3]

Histopathological analysis of right renal organ female Wistar rats

The kidneys were weighed, soaked in a solution of 1% formalin, with stained with H and E for histopathology preparations. Then, they were analyzed under a light microscope with ×100 and ×400 and conducted semi-quantitative interpretation by scoring. The parameters used to assess are the modification from the study by Suhita *et al.* in Indonesia.[19] Proximal and distal tubules of contortus observed whether cloudy swelling degeneration, nuclear necrosis, and the hyaline cast of the tubules were present. Comparison of damage of each group, the result of interpretation was converted into median scores and summed up. From each group, three renal histopathological rats were measured and three parameters (cloudy swelling degeneration of rats renal tubules, nuclear necrosis, and hyaline cast) were seen on five fields of view and the result was recorded in form of a score from 0 to 2.

Statistical analysis

Data values are presented as mean ± standard deviation. Data of OI were analyzed by ANOVA followed by *post hoc* LSD test for multiple comparisons. Differences were considered to be statistically significant when $P < 0.05$ and highly significant if $P < 0.01$. Histopathological analysis statistical tests were performed using Kruskal–Wallis continues with Mann–Whitney test ($\alpha = 0.05$), significant when $P < 0.05$ and very significant if $P < 0.01$.

Results

Effects of protein hydrolysates to organ index

Effects of protein hydrolysates of yellow peas, gude beans, green peas, and (PPI) hydrolyzed with neutrase or bromelain to kidneys' OW and OI of CP-induced rats are shown in Table 1. Data of renal OI were homogenous and normally distributed but were not significantly different between groups ($P > 0.05$).

Effects of protein hydrolysates to renal tubules histopathological

Effects of protein hydrolysates of yellow peas, gude beans, green peas, and PPI hydrolyzed with neutrase or bromelain to renal tubules' histopathological features of CP-induced rats are shown in Figures 1-3.

Figure 1 shows a decrease in score averages of cloudy swelling tubules degeneration which was gradual from CP control group with maximal score (2), then GBN, GBB, GPN, PPIN, and PPIB (1), YPN and YPB (0.67), until GPB and NC (0). Average of cloudy swelling tubules degeneration score of negative control and CP control group had a highly significant difference ($P < 0.01$). It indicated that CP caused cloudy swelling tubules degeneration in renal rats. Cloudy swelling scores of YPN and YPB group when compared with CP control group

Table 1: Comparison of the renal organ weight and organ index between various pea protein hydrolysate treatment groups and control groups

Group	Renal OW	Renal OI
YPN	1.24±0.14	0.007±0.000
YPB	1.27±0.08	0.007±0.001
GBN	1.15±0.12	0.007±0.001
GBB	1.33±0.31	0.008±0.000
GPN	1.28±0.16	0.007±0.001
GPB	1.30±0.17	0.007±0.000
PPIN	1.45±0.12	0.008±0.000
PPIB	1.36±0.09	0.007±0.000
NC	1.55±0.34	0.009±0.002
CispC	1.47±0.36	0.008±0.000
Kolmogorov-Smirnov	**Normal distribution**	**Normal distribution**
Levenne	3.625	3.235
ANOVA	0.184	0.374

YPN: Protein hydrolysate of yellow Canadian pea, hydrolyzed by neutrase-treatment group, YPB: Protein hydrolysate of yellow Canadian pea, hydrolyzed by bromelain-treatment group, GBN: Protein hydrolysate of gude beans, hydrolyzed by neutrase-treatment group, GBB: Protein hydrolysate of gude beans, hydrolyzed by bromelain-treatment group, GPN: Protein hydrolysate of green peas, hydrolyzed by neutrase-treatment group, GPB: Protein hydrolysate of green peas, hydrolyzed by bromelain-treatment group, PPIN: Protein hydrolysate of pea protein isolate, hydrolyzed by neutrase-treatment group, PPIB: Protein hydrolysate of pea protein isolate, hydrolyzed by bromelain-treatment group, NC: Negative control group, CispC: Cisplatin control group, OW: Organ weight, OI: Organ index

showed a significant difference ($P = 0.05$). The GPB group compared to CP control group showed a highly significant difference ($P = 0.01$).

Scores of nuclear necrosis of all treatment groups are lower than CP control group with the lowest score in GPB group. Scores of hyaline cast of all treatment groups are lower than CP control group with the lowest score in YPN and GPB group. The comparison of nuclear necrosis and hyaline cast between all treatment groups and negative control group with CP control group showed highly significant differences ($P = 0.01$).

Renal histopathology imaging (cloudy swelling degeneration, nuclear necrosis, and hyaline cast) of rats' renal tubules is shown in Figure 4.

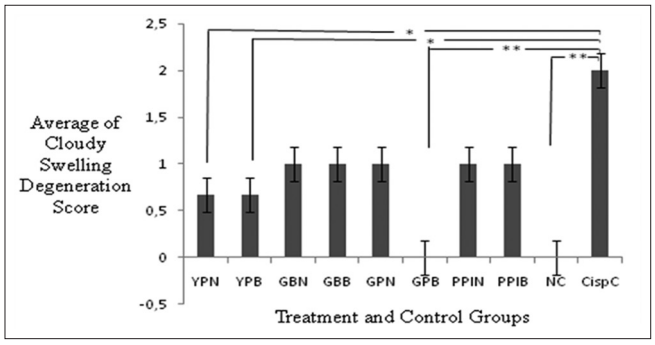

Figure 1: Calculation result of average and Mann–Whitney test for median score of cloudy swelling degeneration of rats renal tubules. YPN: Group of hydrolysate protein of yellow pea by neutrase, YPB: Group of hydrolysate protein of yellow pea by bromelain, GBN: Group of hydrolysate protein of gude beans by neutrase, GBN: Group of hydrolysate protein of gude beans by bromelain, GPN: Group of hydrolysate protein of green peas by neutrase, GPB: Group of hydrolysate protein of green peas by bromelain, PPIN: group of Hydrolysate protein of pea protein isolate by neutrase, PPIN: Group of hydrolysate protein of pea protein isolate by bromelain, NC: group of negative control, CispC: group of cisplatin control

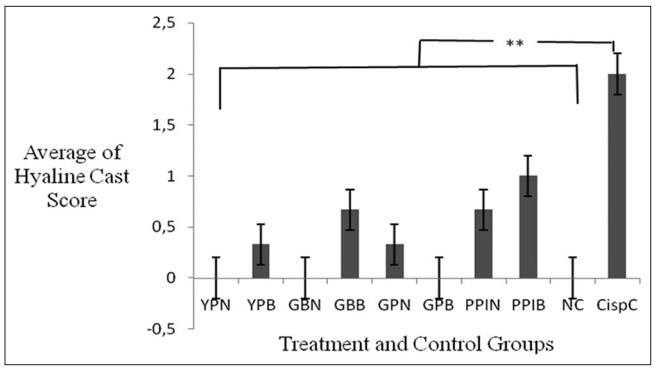

Figure 3: Calculation result of average and Mann–Whitney test for median score of hyaline cast of rat renal tubules. YPN: Group of hydrolysate protein of yellow pea by neutrase, YPB: Group of hydrolysate protein of yellow PEA by bromelain, GBN: Group of hydrolysate protein of gude beans by neutrase, GBN: Group of hydrolysate protein of gude beans by bromelain, GPN: Group of hydrolysate protein of green peas by neutrase, GPB: Group of hydrolysate protein of green peas by bromelain, PPIN: Group of hydrolysate protein of pea protein isolate by neutrase, PPIN: Group of hydrolysate protein of pea protein isolate by bromelain, NC: Group of negative control, CispC: Group of cisplatin control

Discussion

The CP-induced nephrotoxicity effect is reported to be gender related. Some substances that contained high antioxidants, such as pomegranate and Vitamin E, had a protective effect against CP-induced nephrotoxicity in rats but did not prevent in female rats. This may related to the female hormone, estrogen. Estrogen is nonnephron protector against CP-induced nephrotoxicity; moreover, estrogen can induce oxidative stress in the renal that leads to nephrotoxicity.[19-22]

The result of this research is not in line with the above theory. Renal OI of the CP control group was not significantly different with the negative control group. Provision of CP 10 mg/kg BW only one time has not caused changes in the weight of kidney organs. This study cannot be assessed the difference with the trial group. To cause severe changes in kidney, OW may be required provision of CP in a longer time.

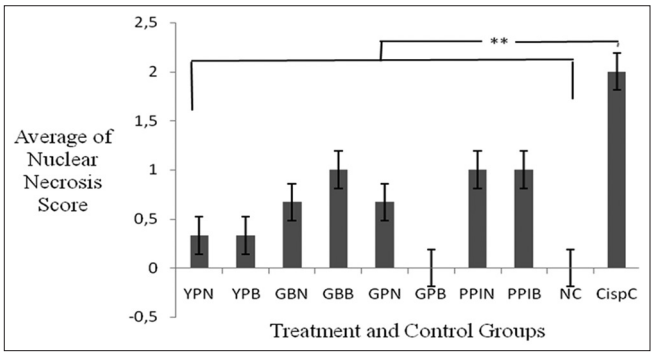

Figure 2: Calculation result of average and Mann–Whitney test for median score of nuclear necrosis of rat renal tubules. YPN: Group of hydrolysate protein of yellow pea by neutrase, YPB: Group of hydrolysate protein of yellow pea by bromelain, GBN: Group of hydrolysate protein of gude beans by neutrase, GBN: Group of hydrolysate protein of gude beans by Bromelain, GPN: Group of hydrolysate protein of green peas by neutrase, GPB: Group of hydrolysate protein of green peas by bromelain, PPIN: Group of hydrolysate protein of pea protein isolate by neutrase, PPIN: Group of hydrolysate protein of pea protein isolate by bromelain, NC: Group of negative control, CispC: Group of cisplatin control

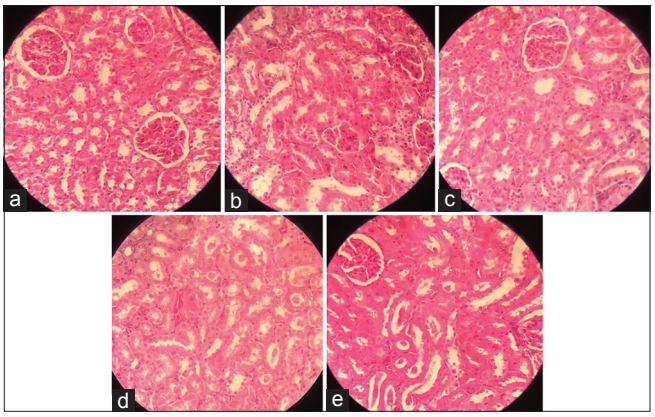

Figure 4: Histopathology imaging of kidney cisplatin-induced Wistar rats after treatments. (a) Score of cloudy swelling, nuclear necrosis and hyaline cast = 0, (b) cloudy swelling = 1, (c) nuclear necrosis = 1, (d) hyaline cast = 1, (e) cloudy swelling, nuclear necrosis and hyaline cast = 2

Bioactive peptides, obtained by enzymatic hydrolysis of food proteins, have been shown to exhibit good 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, in this case, the renals. The enzymatic hydrolysis of food proteins will produce bioactive peptide sequences from other inactive complexes of inactive molecules.[23] Legumes were claimed as a sustainable and inexpensive source of protein that high in antioxidants, high in energy, a source of micronutrients, antidiabetic and anticancer properties, no cholesterol, high protein, high dietary fiber, low fat, low glycemic index, and gluten-free.[24]

Green PPH improves the histopathological features of renal of CP-induced Wistar rats. Albumin degeneration (cloudy swelling) is the mildest degeneration that is reversible because the injury happened still not severe. Albumin pile exists in cytoplasm looks cloudy and swollen, usually found in renal tubules cells, and may be caused by fever, anoxia, poor nutrition, and circulatory disorders poisoning, or nephrotoxic drug, such as CP. In the cell, due to the low chloride concentration, the chloride bonds of CP are disrupted, and the empty part will be filled by water molecules from cell cytoplasm while the bonding on the CP will become highly reactive. Reactive CP can easily bind to guanine from the ruptured DNA of renal cells. This condition will stimulate the p53 gene to detect DNA damage, but cells that cannot be repaired, by the p53 gene, will be directed to apoptosis and lead to necrosis.[13,25]

The molecular weight of hydrolysate proteins which hydrolyzed using bromelain enzyme generally had smaller than which hydrolyzed using neutrase. Protein hydrolysate of GPB showed molecular weight protein smaller than 14.4 kDa.

It may be related to antioxidant and antinephrotoxicity activities. However, this assumption still needs to be investigated.[26] The antioxidant properties in green PPH may play a role in improving the renal tubules' histopathological feature. Antioxidant can relieve the inflammation of the renal which will ultimately improve renal damage. A study by Stanisavljević *et al.*, 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 having the highest antioxidant activity.[27]

The previous experiment results showed that green PPH hydrolyzed with bromelain showed a good effect in improving renal function in CP-induced Wistar rats compared with other protein hydrolysates, shown by the lowest urea serum level.[26] In this current study, green PPH which hydrolized with bromelain suggested that have good effect in improving the renal tubules' histopathological imaging.

Conclusion

All PPHs hydrolyzed with neutrase or bromelain improve the histopathological features of kidney CP-induced rats. Green PPH which hydrolyzed with bromelain had a promising potency as antinephrotoxicity.

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Conflicts of interest

There are no conflicts of interest.

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