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ORIGINAL PAPER

Multifunctional hydrolysates from kenaf (*Hibiscus cannabinus* L.) seed protein with high antihypertensive activity in vitro and in vivo

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Abstract

Kenaf (*Hibiscus cannabinus* L.) seed is an underutilized protein-rich resource considered as a by-product of the kenaf fiber processing industry. Its high protein content (34%) makes it a promising candidate as a source of bioactive protein hydrolysates. In this study, the potential of enzymatically hydrolyzed kenaf seed protein to generate multifunctional bioactive peptides was evaluated. Kenaf seed protein concentrate was hydrolyzed using four different proteolytic enzymes (papain, alcalase, bromelain, and flavourzyme) at their respective optimum pH and temperature. The choice of enzyme affected the bioactivities to a certain degree as KSPH were shown to possess high ACE inhibitory activity and low-to-moderate DPP-IV and antioxidant activity. Papain KSPH showed the highest ACE inhibitory activity with 95% inhibition compared to other enzymatic hydrolysates, and therefore was chosen for further investigation of its antihypertensive activity. Papain KSPH was profiled for its hydrophobicity by RP-HPLC and revealed that the majority of late-eluting fractions exerted the highest ACE inhibitory activity. Spontaneously hypertensive rats showed a decrease of approximately 18–46 mmHg in their systolic blood pressure (BP) from 0 to 24 h after oral administration of papain KSPH at dosages of 100 mg/kg, 300 mg/kg, and 500 mg/kg. However, the effect was not dose-dependent. As a novel protein source, future research should aim to demonstrate the safety of kenaf seed protein and its hydrolysates, and validate its bioactivity through human intervention trials. Overall, kenaf seed protein has the potential to generate antihypertensive hydrolysates with multifunctional bioactivities as part of a functional food ingredient.

Keywords Kenaf seed · Protein hydrolysate · Multifunctional bioactivity · Antihypertensive · Spontaneously hypertensive rat

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Introduction

The growing world population has provoked an increased urgency in finding novel solutions to address food security issues. This is compounded by the threat of non-communicable diseases (NCD), including diabetes, cardiovascular diseases, and cancer, responsible for up to 71% of deaths worldwide [1]. While the problem lies partly with how food is manufactured and marketed, the solution may come in the form of food themselves [2, 3]. There's a growing body of evidence that certain bioactive components from food possess beneficial physiological effects that can help to ameliorate the effect of NCDs. Bioactive protein hydrolysates generated from enzymatic hydrolysis of protein substrates have shown potential as functional food ingredients [4]. Functional food ingredients produced from underutilized

agricultural by-products presents a promising avenue to address sustainability while contributing towards value addition [3]. Kenaf, a fibrous plant belonging to the Malvaceae family, is cultivated in more than 20 countries including China, India, and Thailand [5]. With approximately 229,700 tons produced in 2015/2016, kenaf has considerable potential as a versatile, multipurpose crop [6]. Kenaf is primarily grown for the production of paper, textile, rope, fiberboard, and other biocomposite materials [7]. While the stem is the primary resource harvested, kenaf seed has yet to be fully exploited for its nutritional and functional properties as food ingredients [8]. Kenaf seed is rich in dietary fiber, oil, and protein, of which the oil is recognized to possess substantially high antioxidant activity while the protein demonstrated satisfactory functional properties and prevented hydroperoxide decomposition in a food system [9, 10]. While the compositional and functional properties of kenaf seed protein have been elucidated [4], few studies have been conducted to evaluate its bioactive potential.

Hypertension is a chronic medical condition that is significantly associated with heart, brain and kidney diseases. It is estimated that 1.13 billion people worldwide are affected by hypertension, with two-thirds populated in low and middle income countries [11]. Angiotensin I-converting enzyme (ACE) plays a crucial physiological role in moderating BP via the renin-angiotensin and kinin–kallikrein systems. ACE causes the conversion of angiotensin I into a potent vasoconstrictor, angiotensin II, while inactivating the vasodilator, bradykinin. The net effect causes an elevated blood pressure, leading to the development of hypertension. Furthermore, angiotensin II is able to increase oxidative stress by stimulating a free radical chain reaction, causing the formation of intracellular reactive oxygen species and lipid peroxidation. The hydroxyl radical generated can easily react with biomolecules such as amino acids and proteins, leading to physiological disorders and causes the occurrence of diabetes, cardiovascular disease, neurodegenerative disorder, and cancer, among others [12]. Oxidative stress has been reported to play a vital role in the cause and development of type-2 diabetes and hypertension. Free radicals cause oxidative damage to pancreatic β-cells, involved in the production of insulin, leading to impaired insulin function. Diabetes and hypertension are increasingly prevalent especially among the elderly, which increases the risk of coronary and peripheral artery disease, stroke, and kidney disease [13]. Diabetes has been linked with hypertension due to its broad similarity in terms of cardiometabolic disorders including vascular dysfunction, sodium retention, and kidney damage among others [14].

Since these two comorbidities frequently exist in the presence of one another, a study encompassing antihypertensive, antioxidative, and antidiabetic properties is noteworthy. To date, the potential of kenaf seed proteins to serve as a source of bioactive peptides and functional food ingredient is yet to be fully explored. No work has been reported yet on the multifunctionalities (antihypertensive, antidiabetic, and antioxidative properties) of kenaf seed proteolysate produced from enzymatic hydrolysis, let alone its in vivo efficacy. Thus, this study aims to assess the multifunctionality of kenaf seed protein hydrolysates using various proteases, followed by an animal study to evaluate the BP lowering efficacy of the resulting proteolysate in spontaneously hypertensive rats.

Materials and methods

Materials

Kenaf seed (*Hibiscus cannabinus* L. var. V36) was obtained from National Kenaf and Tobacco Board, Malaysia from a farm located in Kelantan, Malaysia. The seed was packed in sealed bags and stored at 4 °C until further use. Enzymes papain and bromelain were purchased from Acros Organics (Geel, Belgium) while alcalase and flavourzyme were purchased from Novozyme (Bagsvaerd, Denmark). ACE from rabbit lung (2 U/mg), dipeptidyl-peptidase IV (DPP-IV), N-Hippuryl-His-Leu-hydrate (HHL), *o*-phthaldialdehyde (OPA), 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma-Aldrich (Missouri, MO, USA). Acetonitrile, trifluoroacetic acid, and methanol were of HPLC grade. All other reagents and chemicals used were of analytical grade.

Proximate analysis of kenaf seed

Protein, moisture, ash, fat and fiber determination was performed according to the AOAC methods [15]. The protein content was calculated using 6.25 as the N (%) conversion factor.

Preparation of defatted kenaf seed flour

Kenaf seed was ground by a stainless steel grinder (GW-RT-02A, Good And Well Sdn. Bhd., Malaysia). The resulting flour was passed through a sieve (0.5 mm size). The kenaf seed flour was then defatted by mixing with petroleum ether at 1:3 (w/w) ratio and stirred continuously for 30 min at room temperature (28 °C). The solvent was then decanted and extraction was repeated 2 more times to achieve maximum defatting. The defatted kenaf seed flour was then dried overnight under a fume hood.

Preparation of kenaf seed protein concentrate

Defatted kenaf seed flour was mixed with distilled water at a 1:30 ratio (w/v) and adjusted to pH 9 using 4 M NaOH.

The mixture was agitated (80 rpm, 50 °C, 1 h) followed by centrifugation at 10,000 rpm (4 °C, 15 min). The supernatant was collected and the pH was adjusted to 4.5 using 0.1 M HCl. Protein precipitate was collected after centrifugation at 10,000 rpm (4 °C, 15 min), washed with water, and freeze dried to obtain kenaf protein concentrate (KPC).

Amino acid analysis of KSPH

The amino acid composition was determined according to Deng et al. [16] with slight modifications. Briefly, the sample was injected into a C18 column (Hypersil GOLD, Thermo Scientific, 250 mm × 4.6 mm, 5 µm), with the mobile phases (MP) composed of MP A (0.1 M ammonium acetate, pH 6.5) and MP B (0.1 M ammonium acetate/acetonitrile/methanol, 44:46:10 v/v/v, pH 6.5). Column temperature was set at 43 °C, and the elution followed a programmed gradient of MP A (100–0% in 50 min) and MP B (0–100% in 50 min) at 1 mL/min. Absorbance was recorded at 254 nm. Amino acids were quantified by comparing their peaks with an external standard.

Hydrolysis of KPC by enzymatic method

Hydrolysis was carried out following a previously established method with some modifications [17]. KPC was mixed with a suitable buffer for each enzyme at a ratio of 1:25 (w/v) for papain (50 mM phosphate buffer, pH 6.5, 65 °C), alcalase (50 mM phosphate buffer, pH 7.5, 55 °C), bromelain (50 mM acetate buffer, pH 5.0, 55 °C), and flavourzyme (50 mM Tris–HCl buffer, pH 8.0, 55 °C). Enzyme was added at an enzyme:substrate ratio of 1:50 (w/w) and hydrolysis was conducted for 6 h. Samples were taken initially at 30 min and every hour thereafter, and immersed in boiling water for 10 min to inactivate the enzyme. The reaction mixture was then cooled and centrifuged at 10,000 rpm for 25 min, and then filtered through 0.45 µm syringe filter to remove any insoluble, residual material. The supernatant was collected and stored at − 40 °C.

Degree of hydrolysis

An OPA-based spectrophotometric assay was performed to measure the degree of hydrolysis (DH) of KPC according to the method described by Church et al. [18] with some modifications. OPA solution was prepared freshly on the day of experiment by mixing three solutions: 7.62 g of sodium tetrahydroborate and 200 mg of sodium dodecyl sulfate dissolved in 150 mL of deionized water (Solution A); 160 mg of OPA dissolved in 4 mL of methanol (Solution B); 400 µL of β-mercaptoethanol adjusted to 50 mL with deionized water (Solution C). A volume of 36 µL of hydrolysate was added to 270 µL of OPA solution and incubated at room temperature for 2 min prior to measurement at 340 nm using a microplate reader (PowerWave X340, Biotek Instruments Inc., Winooski, VT, USA). DH was calculated using the following equation:

$$\mathrm{DH}\ (\%) = \left(\mathrm{A}_{340}\mathrm{Sample} - \mathrm{A}_{340}\mathrm{Protein}\right) / \mathrm{A}_{340}\mathrm{Total} \times 100$$

where, A_{340} Sample = absorbance after hydrolysis, A_{340} Protein = absorbance before hydrolysis (at 0 h) and A_{340} Total = absorbance after reacting unhydrolyzed KPC with 6 N of HCl and heated at 110 ºC for 24 h.

ACE-inhibitory activity

ACE inhibitory activity was determined using the method of Jimsheena and Gowda [19] with some modifications. Ten µL of ACE (100 mU/mL) was preincubated with 15 µL of KSPH or control solution for 10 min prior to addition of substrate solution. Then, 50 µL substrate solution composed of 5 mM HHL in 0.2 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, was added. The mixture was incubated for 60 min at 37 °C. The reaction was terminated by addition of 75 µL of 1 M HCl. Subsequently, 150 µL pyridine and 75 µL benzene sulphonyl chloride was added, immediately vortexed and then placed in an ice bath. From the mixture, 200 µL was transferred to a 96-well plate and absorbance was read at 410 nm. One unit of ACE inhibitory activity was defined as 1 µmol hippuric acid released per minute at 37 ºC. ACE inhibition was calculated using the equation below:

$$\mathrm{ACE\ inhibition}\ (\%) = \left[\left(\mathrm{A}_{410}\mathrm{Control} - \mathrm{A}_{410}\mathrm{Sample}\right) / \left(\mathrm{A}_{410}\mathrm{Control} - \mathrm{A}_{410}\mathrm{Blank}\right)\right] \times 100$$

where the control contained ACE and substrate, the blank contained only the substrate and the sample contained hydrolysate, ACE and substrate.

DPP-IV inhibitory activity

DPP-IV inhibition was determined as described by Lacroix and Li-Chan [20]. A total of 25 µL of sample was mixed with 100 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl and 1 mM EDTA, followed by the addition of substrate (25 µL of 0.2 mM Gly-Pro-ρ-nitroanilide) to the mixture. To initiate the assay, 50 µL of DPP-IV (0.0025 U/mL) was added and the solution was incubated for 60 min at 37 ºC. The reaction was terminated by the addition of 100 µL of 1 M sodium acetate buffer (pH 4.0) and the reaction was then read at 405 nm. Diprotin A (5 µM) was used as a control. DPP-IV inhibition was calculated using the following equation:

$$\text{DPP} - \text{IV inhibition } (\%) = \left[\left(A_{405}\text{Control} - A_{405}\text{Sample}\right) / \left(A_{405}\text{Control} - A_{405}\text{Blank}\right)\right] \times 100$$

where the control contained DPP-IV and substrate, the blank contained only the substrate and the sample contained hydrolysate, DPP-IV and substrate.

Antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activity was measured using the method described by Hwang et al. [21]. In a 96-well plate, 50 µL of sample solution was placed in each well mixed with an equal amount of distilled water and 100 µL of DPPH solution (0.2 mM, in 100% methanol). The mixture was incubated in the dark at room temperature for 45 min and the absorbance was measured at 517 nm. The blank consisted of distilled water and DPPH solution at 1:1 ratio. The scavenging activity was determined according to the following equation:

$$\text{DPPH radical scavenging activity } (\%) = \left[\left(A_{517}\text{Control} - A_{517}\text{Sample}\right) / A_{517}\text{Control}\right] \times 100$$

Metal ion-chelating activity

The chelating activity of antioxidants in the sample was determined according to the method described by Decker and Welch [22] with some modifications. Briefly, 100 µL of sample (or distilled water for blank) was mixed with 5 µL of ferrous chloride solution (2 mM) and 185 µL of distilled water. The mixture was then added with 10 µL of ferrozine solution (5 mM), vortexed and incubated at room temperature for 10 min. The absorbance was read at 562 nm to determine the chelating activity, using the following equation:

$$\text{Metal chelating activity } (\%) = \left[\left(A_{562}\text{Control} - A_{562}\text{Sample}\right) / A_{562}\text{Control}\right] \times 100$$

Ferric reducing power

The reducing power of the hydrolysates was measured according to the method described by Ismail et al. [23] with some modifications. Working FRAP solution was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM of TPTZ solution in 40 mM HCl and 20 mM ferric chloride solution at a ratio of 10:1:1 (v/v/v). Working FRAP reagent (3.0 mL) was added to 50 µL of sample, mixed thoroughly and incubated at 37 °C for 30 min. The absorbance was measured at 593 nm. The concentration of antioxidant activity was determined from the standard curve constructed using 5–100 µg/mL ferrous sulphate.

Reverse-phase high performance liquid chromatography (RP-HPLC)

The method used was as described previously by Zarei et al. [24]. Freeze-dried samples were diluted in MP A [0.1% trifluoroacetic acid (TFA) in deionized water] and filtered using a 0.2 µm nylon syringe filter. Then, 500 µL of 75 mg/mL filtered sample was loaded into a C-18 semi-preparative column (9.4 × 250 mm, Agilent Technologies, Santa Clara, CA, USA), after pre-conditioning of the column using the same mobile phase. Forty-five fractions were collected during a total run time of 67.5 min, eluted at a flow rate of 4.0 mL/min, and gradient elution of up to 70% of MP B (0.1% TFA in acetonitrile) between 10 and 55 min. The first 10 min involved only MP A whereas the last 12.5 min involved only MP B. Peak formation was monitored at 205 nm. All fractions were freeze-dried prior to further analysis.

Antihypertensive efficacy of KSPH on spontaneously hypertensive rats (SHR)

Thirty male SHR, aged 12–13 weeks old were obtained from the Animal Experimental Unit, Faculty of Medicine, University of Malaya, Kuala Lumpur. Each cage containing 2 rats were ventilated in room temperature (22–24 °C) with relative humidity of 50–60%, under automated 12-h light/dark cycle. Standard, fortified pellet diet (Altromin, Lippe, Germany) and tap water were available ad libitum. Rats were randomly assigned into 5 groups (n = 6), and treated with either distilled water, captopril at 50 mg/kg body weight (bw), papain KSPH at 100 mg/kg bw, 300 mg/kg bw, or 500 mg/kg bw dosage, using the oral gavage method. Samples were prepared by dissolving the different treatments in 1.0 mL distilled water, based on individual rat bw. The tail cuff method using the CODA non-invasive BP system (Kent Scientific, Torrington, CT, USA) were used to measure the systolic BP (SBP) of SHR via the pulses at the tail of the rats at 0, 2, 4, 6, 8, and 24 h after oral administration. Rats were placed on the warming plate for 10 min prior to measurement to ensure adequate blood flow to tail for accurate measurements. Two to three cycles of BP measurement of the same rat were conducted, where each cycle consist of 15 readings. All rats were handled gently, to avoid stress that might cause variation to the BP.

Statistical analysis

Data are presented as the mean of triplicate measurements ± standard deviations, and differences between means of data were analyzed by two-way ANOVA followed by Tukey's test ($p < 0.05$), using MINITAB 17 (Minitab Inc., State College, PA, USA).

Results and discussion

Proximate composition and isolation of protein

Table 1 shows the proximate composition of kenaf seed. The crude protein content was found to be higher than Mariod et al. [10] who also attempted protein extraction from kenaf seed variety V36 and obtained 29.8% crude protein. While seasonal, varietal, soil, and climate variations may contribute towards variations in protein content, differences between inter- and intra-plant seed protein content have been noted previously [25]. High protein content is advantageous in producing protein hydrolysates containing numerous bioactive peptides, as well as being a source of amino acids for nutrition. Defatted kenaf seed protein was extracted using alkaline conditions followed by isoelectric precipitation. The protein extraction yield was 11.6%, almost similar to Mariod et al. [10] who obtained 13.04% from kenaf seed variety QP3 and 10.56% yield from V36. Mir et al. [26] and Subagio [27] also obtained similar protein yields within the range of 7–13%, from their respective samples. The protein content of the concentrate was 55.6%, while other researchers obtained values of 63.1% [28] and 56.5% [29] from seeds of *Prosopis cineraria* and *Buchholzia coriacea*, respectively.

Protein extraction yields from kenaf seed utilizing the present method seems to be low when compared to other protein sources such as rapeseed and hempseed [30, 31]. Protein extraction could have been affected by the polysaccharide matrix surrounding the protein, hampering solubilization. Besides that, coextraction of polyphenols have been reported to affect protein extraction yields as they bind with proteins, creating polyphenol-protein complexes with altered solubility [32]. According to Mariod et al. [10], the water-soluble albumin fraction is the main protein type present in kenaf seed, followed by the salt-soluble globulin fraction. This suggests that the solvent used to solubilize kenaf seed protein could be influential and further optimization in terms of varying salt concentrations, pH, in combination with other factors may help to maximize extraction yields. Protein solubility for plant proteins are known to be highly soluble at higher pH values and could be used to increase extraction yield. However, extremely alkaline pH is also associated with formation of toxic lysinoalanine from crosslinking reactions between or within protein molecules [33]. Careful consideration is needed in balancing protein extraction parameters with process outcomes to avoid extremes that negatively affect the resulting protein concentrate/isolate from a health perspective.

Table 1 Proximate composition, protein concentrate yield and protein content of kenaf seed flour

Parameter	Value (g/100 g dry basis)
Crude protein	34.13 ± 1.46
Ash	6.1 ± 0.12
Moisture	7.0 ± 0.20
Fat	19.13 ± 0.42
Fiber	19.74 ± 1.51
Carbohydrate	13.9 ± 3.54
Protein content in concentrate	55.6 ± 2.75

Values represent the mean from three replicates ± S.D

Amino acid analysis of kenaf seed flour and papain KSPH

The amino acid composition was analyzed to assess the protein profile of kenaf seed and possible effects of amino acid profile on KSPH bioactivity. Table 2 shows that Glu and Cys, are the most abundant amino acids in kenaf seed, while Met and Phe are the least, in agreement with the study by Mariod et al. [10] with slight differences. Meanwhile, Kim et al. [34] showed that several other kenaf cultivars produced seeds with Glu and Asp as the predominant amino acids, which indicated environmental and cultivar/varietal differences would affect the amino acid composition to a certain degree. From a nutritional standpoint, the value of dietary protein sources depends on their amino acid profile and digestibility, among other factors [35, 36]. Essential amino acids (EAA) such as Val, Leu, and Ile are associated with enhancing muscle protein synthesis, and consuming protein sources rich in those amino acids could be considered an effective strategy to counteract skeletal muscle loss among sedentary and elderly individuals [37, 38]. A study of various agro-food industrial waste streams showed that only a few protein sources had > 40% EAA from the total amino acid content, with a respectable amount being 35–40% [39]. Kenaf seed contains appreciable amounts of EAA (34.70% EAA) with a high protein content, thus kenaf seed protein derivatives could be an interesting source of protein for human nutrition. Kenaf seed also contained considerable amounts of sulphur amino acids (Cys and Met), higher than some of the legume, cereal, and nut-based sources, including soybean, faba bean, rice, and peanut [40].

Bioactive protein hydrolysates and peptides' effectiveness largely hinge upon peptide length/size, amino acid

Table 2 Amino acid composition of kenaf seed and papain KSPH

Amino acid	Concentration (mg/g)	
	Kenaf seed meal	Papain KSPH
Aspartic acid	25.99 ± 10.86	18.36 ± 0.67
Glutamic acid	68.02 ± 20.49	45.38 ± 2.46
Serine	15.30 ± 4.32	8.56 ± 0.51
Glycine	14.25 ± 0.45	6.44 ± 0.39
Histidine	12.25 ± 2.45	2.80 ± 0.08
Arginine	29.44 ± 4.54	20.38 ± 1.16
Threonine	16.43 ± 6.26	5.78 ± 0.14
Alanine	18.17 ± 1.02	11.86 ± 0.19
Proline	10.56 ± 2.04	7.96 ± 1.12
Tyrosine	12.33 ± 1.08	2.36 ± 2.62
Valine	12.18 ± 0.01	9.07 ± 0.76
Methionine	9.96 ± 0.6	2.82 ± 0.36
Cysteine	53.43 ± 2.38	12.99 ± 0.26
Isoleucine	21.77 ± 3.90	10.66 ± 0.79
Leucine	24.69 ± 3.6	22.74 ± 0.45
Phenylalanine	9.89 ± 5.38	6.59 ± 0.36
Lysine	24.34 ± 1.4	1.05 ± 0.30
% Total essential amino acid (EAA)	34.70	31.41
% Total nonessential amino acid (NEAA)	65.30	68.59

Individual amino acid values represent the mean from three replicates ± S.D

EAA: His, Ile, Leu, Lys, Met, Phe, Thr, Val

NEAA: Asp, Glu, Ser, Gly, Arg, Ala, Pro, Tyr, Cys

sequence and identity. ACE inhibitory activity has been reported to be influenced by short peptides with 2–12 amino acids containing acidic, basic, or hydrophobic amino acids [41]. Short peptides are preferable due to the cleft-shaped conformation of the ACE active site preventing access for large peptides [42]. Similarly, for DPP-IV inhibition, hydrophobic amino acids are thought to contribute towards inhibitory potency via enhanced hydrophobic interactions with the DPP-IV active site [43]. Antioxidative capacity of protein hydrolysates are related to the presence of hydrophobic and acidic amino acids [44]. The amino acid Cys, containing a sulfhydryl (SH) group could ameliorate hypertension by the free radical scavenging activity of its thiol group, relieving the oxidative stress by modulating nitric oxide levels, therefore contributing towards antioxidative activity [41]. Therefore, since appreciable amounts of amino acids relevant to the bioactive properties of protein hydrolysates or peptides were present in kenaf seed, enzymatic hydrolysis is expected to release those peptides composed of the necessary amino acids to exert bioactivity.

Generation of multifunctional kenaf seed protein hydrolysates by proteolytic enzymes

Figure 1 shows the degree of hydrolysis (DH) and bioactivities (ACE inhibition, DPP-IV inhibition, and antioxidation) of papain, alcalase, bromelain, and flavourzyme-generated hydrolysates. Enzymatic proteolysis of kenaf seed proteins generally improved the bioactivities investigated in this study. The DH of all KSPH showed initial rapid increase followed by a gradual reduction in rate of hydrolysis, a pattern similar with other plant-based protein hydrolysis such as horse gram [45] and sweet sorghum [46]. Depending on the enzyme used, different levels of DH were attained among the KSPH. This suggests that different enzymes affect substrates differently, attacking different sites on kenaf seed proteins, producing peptides in a range of molecular weights and amino acid composition [47]. Moreover, the rapid increase in DH during the initial hydrolysis phase also reflects the abundance of cleavage sites available to the enzymes. As hydrolysis progresses, the rate of increase in DH begins to plateau, which could be due to depletion of enzymatic cleavage sites or enzyme denaturation. The former case was illustrated by Rui et al. [48] when they performed sequential enzymatic digestion of several types of beans. After the initial hydrolysis by alcalase had slowed and approached a DH plateau, DH increased when they used flavourzyme as compared to papain, which only increased DH by a negligible amount. On the other hand, evidence suggests that enzyme denaturation may not be responsible for DH plateauing. Zarei et al. [17] showed that defatted palm kernel cake protein hydrolyzed with bromelain, flavourzyme, and papain produced hydrolysates with DH that remained relatively unchanged after 5 h of hydrolysis up to 24 h, even after additional enzymes was added every 6 h. Therefore, the availability of enzymatic cleavage sites may be the limiting factor affecting DH in this study. As mentioned earlier, enzymatic hydrolysis of proteins entails the production of peptides with varying lengths, structure, and amino acid composition, that exhibit different bioactivities at different potencies. This is reflected in Fig. 1, with the overall trend indicating that the enzymatic proteolysis generated hydrolysates with relatively high ACE inhibitory and metal ion chelating activity, as well as low-to-moderate levels of DPP-IV inhibitory, DPPH, and FRAP activities.

ACE inhibitory activity of KSPH

Minimal ACE inhibition prior to enzymatic hydrolysis was shown in Fig. 1b. Maximal ACE inhibitory activity was achieved by papain KSPH with 95.47% inhibition after 30 min of hydrolysis and decreased slightly afterwards showing > 88% inhibition up to 6 h hydrolysis. Flavourzyme-generated KSPH also exhibited high ACE

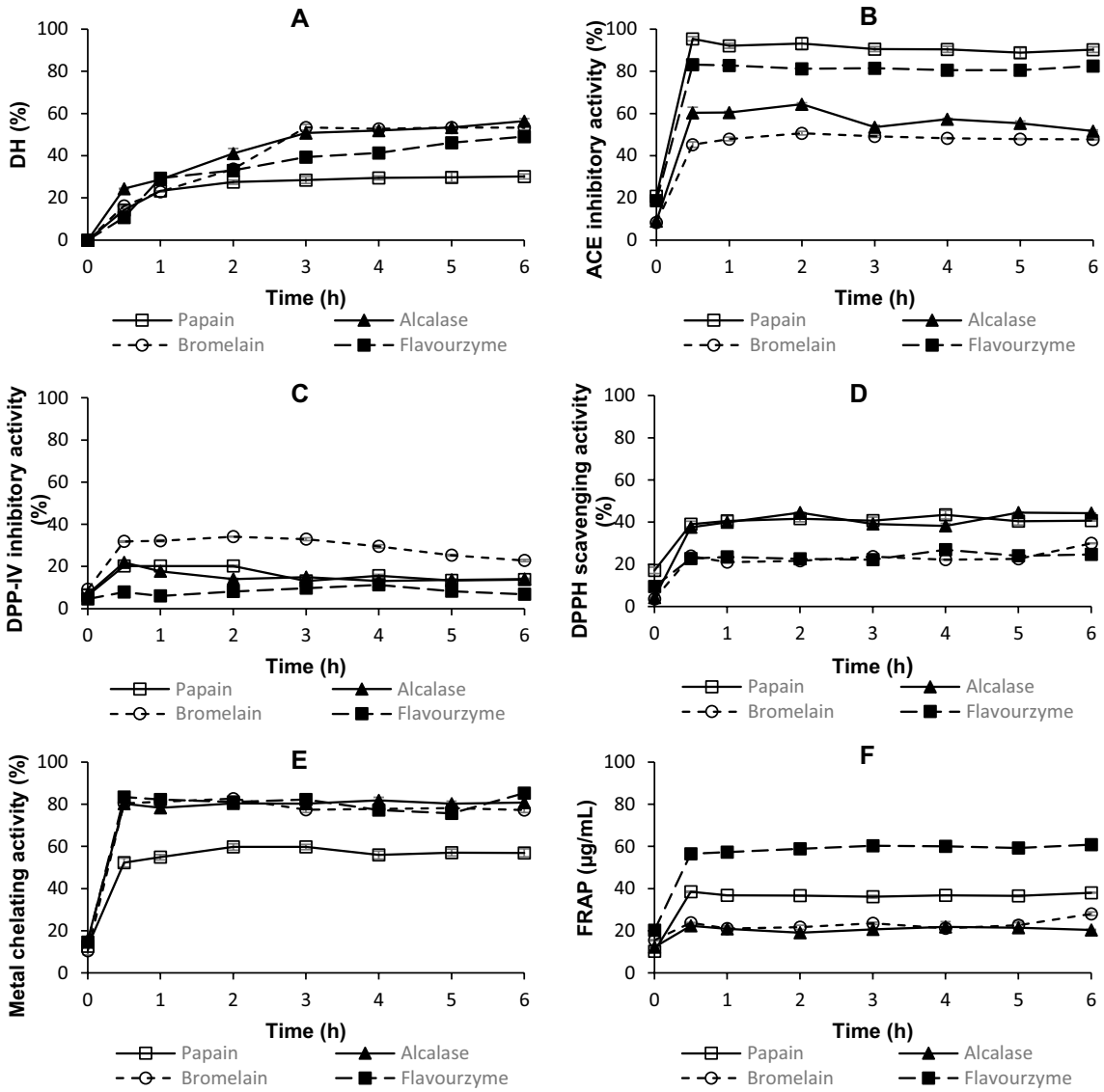

Fig. 1 Changes over time for kenaf seed hydrolysates produced using 4 different enzymes, in terms of DH (**a**), ACE inhibitory activity (**b**), DPP-IV inhibitory activity (**c**), DPPH scavenging activity (**d**), metal ion chelating activity (**e**), and ferric reducing power (**f**). Each data point represents the mean ± SD

inhibitory activity but lower than papain KSPH ($p < 0.05$), with > 80% inhibition after 30 min up to 6 h hydrolysis. Despite having the highest DH, both alcalase and bromelain KSPH had lower ACE inhibitory activity compared to papain and flavourzyme KSPH ($p < 0.05$). In contrast, flavourzyme and papain KSPH both recorded lower DH yet exhibited high ACE inhibitory activity. This lends credence to the opinion that different proteolytic enzymes are more effective against certain protein substrates, which may be derived from different sources. Extensive hydrolysis could degrade potent peptides into less potent fragments, suggesting that hydrolysis beyond a certain DH results in the reduction of ACE inhibitory activity, as reported by Mune, Minka, and Henle [49]. This was partially observed during this study. When DH increased throughout the hydrolysis, ACE inhibitory activity remained above 88% inhibition with no major decrease in inhibitory activity for papain KSPH, while alcalase KSPH showed a noticeable drop in ACE inhibitory activity ($p < 0.05$). The difference could be due to the nature of the enzyme, with alcalase being an endoprotease with broad nonspecific activity capable of cleaving more peptide bonds than papain, also an endoprotease, but preferred cleavage at specific amino acids [50]. ACE inhibitory activity of protein hydrolysates are influenced by several factors including the type of enzyme, hydrolysis conditions, peptide molecular weight, and the hydrophobic properties of the amino acids at the C-terminal region

of peptides [51]. A combination of those factors would produce peptides where high ACE inhibitory activity has been linked to the presence of Pro, Val, Leu, Ile, Tyr, Phe, Trp, Arg, and Lys within the peptide sequences [52].

DPP-IV inhibitory activity of KSPH

In Fig. 1c, DPP-IV inhibitory activity was observed to be relatively low for all KSPH. Enzymatic hydrolysis improved DPP-IV inhibitory activity, to a certain extent. Bromelain-generated hydrolysates had the highest DPP-IV inhibitory activity at 34.15% after 2 h of hydrolysis. The level of DPP-IV inhibitory activity for all KSPH either remained low or decreased as hydrolysis progressed as in the case for bromelain hydrolysates. This could be an indication of DPP-IV inhibitory peptides being degraded by bromelain after 2 h of hydrolysis thus lowering its inhibitory activity. Another possibility is the enzymes selected herein was not able to produce peptides with the ability to inhibit DPP-IV to a high level. Some studies have shown that some proteins are hydrolyzable but lack DPP-IV inhibitory activity, while some proteins are resistant towards enzymatic hydrolysis preventing the release of active peptides [53]. This shows the importance of enzyme selection to produce hydrolysates with high DPP-IV inhibitory activity. Nongonierma and FitzGerald [54] stated that for peptides possessing half-maximal inhibitory activity (IC_{50}) of less than 200 µM, the consensus was that peptides containing Trp at the N-terminal, as well as Pro at the second position from the N-terminal was preferred for high DPP-IV inhibition. They also stated that peptides with IC_{50} more than 2000 µM seemed to have Ala at the N-terminal and Trp at the C-terminal positions. Based on amino acid composition, kenaf seed has relatively low Pro content and higher Ala content. Relatively low Pro content might be a contributing factor for the low DPP-IV inhibitory activity, while relatively moderate Ala content may not be enough to offset the lower DPP-IV potency in KSPH. DPP-IV inhibitory hydrolysates have been generated from various different food protein sources, including plants, marine, and milk sources, with varying potencies [43]. Connolly, Piggott, and FitzGerald [55] examined various proteolytic enzymes' effectiveness to generate DPP-IV inhibitory hydrolysates from brewers' spent grain over 4 h of hydrolysis. A clear dose–response relationship was observed whereby increasing hydrolysate concentration demonstrated significant increases in DPP-IV inhibition. An increase from 1.5 to 3.5 mg/mL resulted in approximately twofold increase in DPP-IV inhibition for some of the hydrolysates. Increasing KSPH concentration by utilizing methods such as ultrafiltration could yield greater DPP-IV inhibition.

Antioxidative activity of KSPH

Antioxidative potential of KSPH was evaluated using three different in vitro assays, to reflect the different possible antioxidant mechanisms. DPPH scavenging activity from the four enzyme hydrolysates ranged from 21.01 to 44.56%, as shown in Fig. 1d. Alcalase KSPH showed the highest radical scavenging activity at 44.56% after 6 h hydrolysis, followed by papain KSPH at 43.52% after 4 h hydrolysis. Scavenging activity of KSPH generally remained steady after hydrolysis, across the 6 h hydrolysis period, indicating the release of peptides with radical scavenging activities unaffected by proteolysis. Alcalase and papain KSPH showed almost similar radical scavenging activities, with bromelain and flavourzyme KSPH also showing similar radical scavenging values to each other but lower than alcalase and papain KSPH ($p < 0.05$). Moderate levels of DPPH radical scavenging shown can be related to the moderate amount of those amino acids in kenaf seed, as can be observed from the amino acid composition in Table 2. From the study done by Ghanbari et al. [56], hydrophobic amino acids such as Met, Val, Leu, and Ala play a crucial role in DPPH radical scavenging activity by hydrolysates/peptides. Kenaf seed has relatively high Leu and Ala content in contrast to its low Met and Val contents, which probably lowered the overall DPPH radical scavenging activity.

In Fig. 1e, Metal ion-chelating activity showed relatively high values from the generated KSPH (52.33–85.25%), with flavourzyme KSPH exhibiting the highest value at 85.25% after 6 h hydrolysis ($p < 0.05$). High metal ion-chelating activity shown by KSPH was likely influenced by the presence of high amounts of Glu, Asp, Arg, and Lys in kenaf seed. Hydrolysis may have enabled exposed carboxylic groups as in Glu and Asp to form ionic or electrostatic interactions, facilitating iron chelation [57, 58]. Additionally, the imidazole ring in His has been suggested to play a role in metal ion chelation [59]. Metal ion-chelation activity did not fluctuate much as hydrolysis progresses, indicating that peptide size may not be as influential as other factors, such as the peptide structure and availability of amino acids to interact with metal ions [60]. Phongthai, D'Amico, Schoenlechner, Homthawornchoo, and Rawdkuen [60], showed that ultrafiltration of in vitro gastrointestinal-digested rice bran protein hydrolysates produced a < 3 kDa fraction that had lower metal chelating activity than the 3–5 kDa fraction. Another study reported that unfractionated and 5–10 kDa had higher chelating activities than lower molecular weight fractions [61].

Figure 1f illustrates that enzymatic hydrolysis improved KSPH FRAP to a certain extent. FRAP capacity was relatively low except for the flavourzyme-generated hydrolysates with FRAP values remained relatively unchanged at > 56 µg/mL from 0.5 h of hydrolysis and onwards. Alcalase and

bromelain produced KSPH with the lowest FRAP. As generally observed from the DPPH and metal ion-chelating results, the hydrolysates' reducing power did not change significantly after hydrolysis, suggesting that an increase in DH did not influence FRAP capacity. This is in line with the study by Vieira, da Silva, Carmo, and Ferreira [62]. However, a study [63] showed that high molecular weight pigeon pea peptide fractions (> 10 kDa) displayed higher FRAP activity than lower molecular weight fractions, revealing that ultrafiltration decreased FRAP. Thamnarathip et al. [64] found that the FRAP of enzymatic hydrolysates of rice bran was lower than unhydrolyzed rice bran. This highlights the difference between different samples, and its composition as the determinant of antioxidant activity. Amino acids containing sulfur, such as Cys and Met, greatly influences ferric ion reduction due to the ability of the sulfhydryl group to take part in redox reactions through its various oxidation states [65, 66]. Overall, the antioxidative capacity of KSPH seems to be influenced by the type of enzyme used, in line with the research by Karami et al. [67]

Enzymatic hydrolysis conditions used in this study seems to be favorable for production of ACE inhibitory hydrolysates. Attaining multifunctional bioactive hydrolysate presents some difficulty due to each bioactivity presenting different requirements pertaining to peptide amino acid composition, sequence, and size. Mune, Minka, and Henle [49] analyzed antioxidant, ACE inhibitory, and DPP-IV inhibitory activities of bambara bean protein hydrolysate generated by alcalase, thermolysin, and trypsin. They reported ACE inhibitory and DPP-IV inhibitory activities ranged from 28.98 to 58.30%, and 7.98–44.25%, respectively. However, the results were obtained after 24 h hydrolysis. Optimization through response surface methodology may be a feasible method to achieve improved multifunctional bioactive peptides. Some peptides with high in vitro ACE inhibitory potency was revealed to be unable to exert antihypertensive activity in animal studies [68]. This illustrates that other factors might also influence antihypertensive activity in vivo and ACE inhibitory activity on its own may not guarantee its efficacy. With that in mind, papain-generated KSPH was chosen for further analysis as papain-generated hydrolysate had the highest ACE inhibitory activity.

Profiling of papain KSPH according to its hydrophobicity

Figure 2 shows the ACE inhibitory activity of papain KSPH according to its hydrophobicity along the elution profile of 0–70% ACN. Fractions that eluted earlier contained highly polar or hydrophilic molecules, while fractions that eluted later contained more non-polar or hydrophobic molecules. Peak formation was monitored at 205 nm, where the absorbance intensity can be attributed to peptide bonds [69]. The chromatogram in Fig. 2a shows high absorbance intensities near the middle, indicating the majority of papain KSPH peptide mixture possessed moderate hydrophobicity. As can be seen in Fig. 2b, the ACE inhibitory activity was observed to be skewed, with high ACE inhibition from the late-eluting fractions. Early-eluting fractions up to fraction 25, contained peptides with more hydrophilic characteristics that are low-to-moderate in ACE inhibitory activity. This could be caused by the peptides not having the necessary amino acid composition or sequence to interact with ACE. Fraction 26 onwards had relatively high ACE inhibitory activity and this could be explained by the hydrophobic characteristics of the peptides that eluted later. This suggests that high ACE inhibition shown by papain KSPH could be influenced by highly hydrophobic peptide fractions. The chromatogram shows low absorbance intensities in this region suggesting the presence of highly potent peptides. Peptide sequences that contain amino acids with high ACE inhibitory properties might contribute to the increase in activity. Nevertheless, mildly hydrophobic fractions have been purified to obtain peptides with antihypertensive effect on SHR [70, 71]. Depending on the application, highly hydrophobic hydrolysates or peptides may present difficulties in terms of processing into functional food products [4].

Antihypertensive efficacy of papain KSPH in SHR

Figure 3 depicts the antihypertensive effect of several treatments on systolic blood pressure (SBP) in SHR. As expected, distilled water did not affect any change towards the SBP of SHR throughout 24 h ($p > 0.05$). Papain KSPH treatments was able to decrease SBP between the range of 18–46 mmHg from 0 to 4 h. Notable decreases in SBP from baseline were observed beginning after 2 h of oral administration for the three papain KSPH doses ($p < 0.05$). Captopril exhibited the highest BP reduction with a total decrease of 56 mmHg at 8 h ($p < 0.05$) from the baseline, reflecting its status as an antihypertensive drug. Among the three papain KSPH dosages, 500 mg/kg recorded the highest decrease in SBP, and reduced SBP by 46 mmHg after 8 h. Nevertheless, there was no significant difference on SBP observed between dosages from 0 to 8 h ($p > 0.05$), therefore 100 mg/kg can be used as the optimum dosage for the rats to consume papain KSPH. Overall, SBP was lowered significantly compared to the baseline across all tested dosages ($p < 0.05$), which indicates that papain KSPH can be considered as an effective means of lowering SBP in vivo. The ability of papain KSPH to lower SBP in this study does not seem to be dose dependent, suggesting the presence of potent peptide constituents working synergistically rather than a concentration-dependent effect to cause SBP reduction. Other plant-based protein hydrolysates have also been shown to reduce SBP in SHR. Previously, alcalase mung bean hydrolysate at 600 mg/

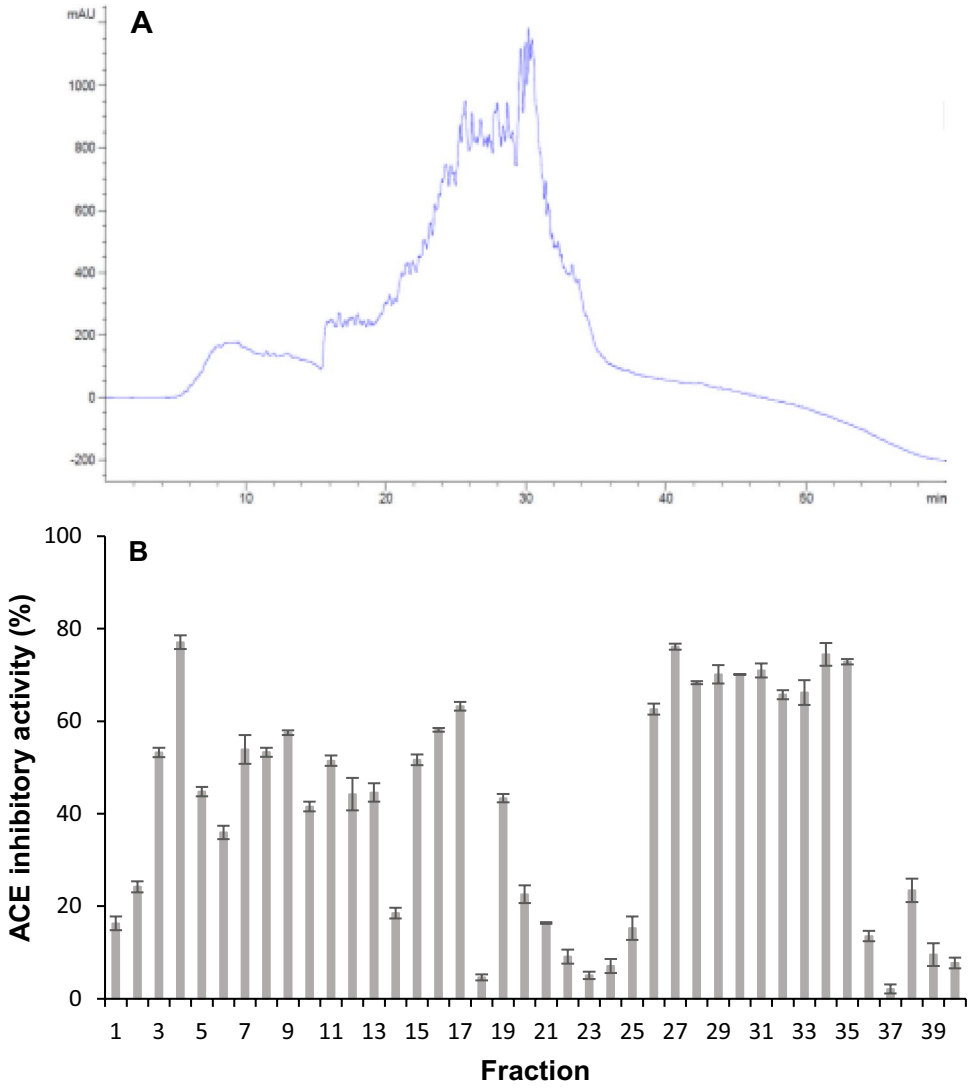

Fig. 2 RP-HPLC elution profile (**a**) and ACE inhibitory activity of fractionated papain KSPH (**b**). ACE inhibitory activity (%) is presented in mean ± SD

kg dose have shown antihypertensive activity in SHR with a maximum BP reduction of 30 mmHg in after 6 h, while thermolysin pea protein hydrolysate showed maximum SBP reduction at 19 mmHg after 4 h using 100 mg/kg dose [72, 73]. Cermeño et al. [74] showed alcalase-flavourzyme brewer's spent grain hydrolysate was able to reduce SBP by 37.8 mmHg in SHR, illustrating the potential of food protein hydrolysates to alleviate hypertension.

As pointed out by Giwa Ibrahim et al. [8], kenaf seed has yet to be utilized extensively as a source of food ingredients. Kenaf seed protein and its protein hydrolysates would be considered as a novel source of protein, peptides, and amino acids, therefore safety considerations need to ensure the processing methods do not lead to undesirable changes in nutritional aspects and potential allergenicity before being introduced widely [75]. Throughout the animal study, SHR appetite and their apparent behavior suggested no debilitating effects as a result of hydrolysate treatment, consistent with other protein hydrolysates tested on animals [76]. Although protein hydrolysates and bioactive peptides are known to be safer compared to synthetic drugs, an in-depth toxicity study should be performed to ascertain the potential acute or chronic toxic effects [77]. With that said, bioactive protein hydrolysates/peptides ultimately require human intervention trials to be sure that it will exhibit the claimed health effects [78]. This study serves to provide supporting evidence that protein hydrolysates, including KSPH, are promising candidates to be utilized as a weapon to combat NCDs.

Conclusion

Kenaf seed was evaluated for its potential to generate multifunctional bioactive peptides using proteolytic enzymes. Results showed that selecting the enzyme used is crucial to

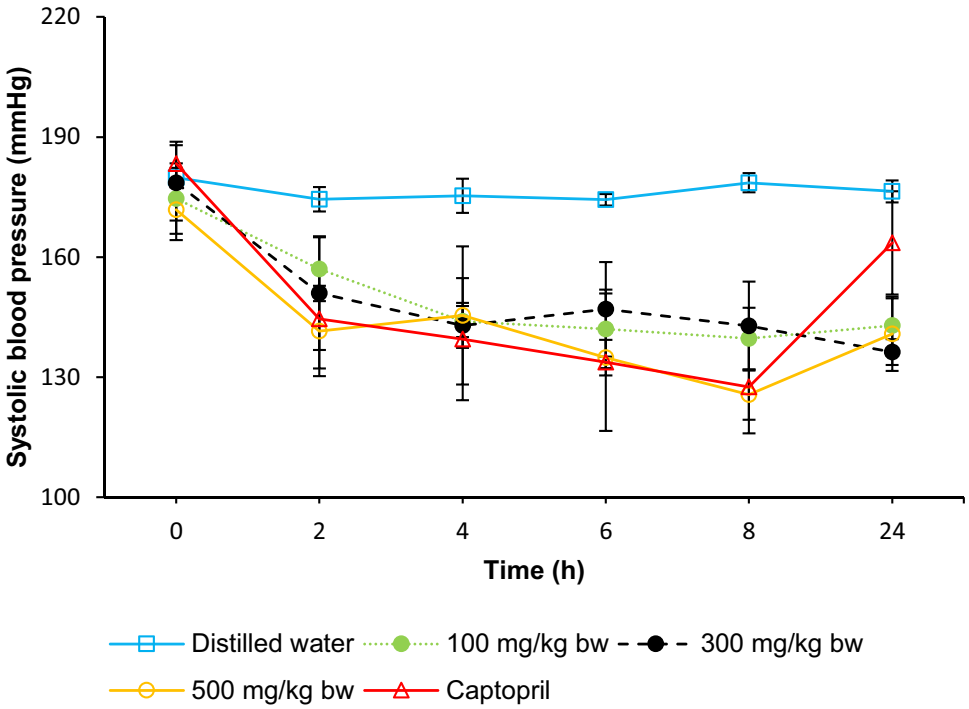

Fig. 3 Systolic blood pressure of SHR administered with captopril, distilled water, papain KSPH at 100 mg/kg bw, 300 mg/kg bw, and 500 mg/kg bw. Each data point represents the mean ± SD

produce kenaf seed hydrolysates with good multifunctional bioactivities. In general, KSPH had high ACE inhibitory activity and low-to-moderate levels of DPP-IV inhibitory and antioxidative activity. RP-HPLC profiling showed the high ACE inhibitory activity by papain KSPH was mostly influenced by the highly hydrophobic peptide constituents. Subsequently, papain KSPH exerted significant SBP reduction in SHR at all dosages tested, but SBP reduction was not dose-dependent. Overall, papain-generated KSPH showed potential as a functional food ingredient to combat hypertension.

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Author contributions NDZ conducted the experiments, analyzed and interpreted results, and drafted the manuscript. MAH and CSY interpreted results and revised the manuscript critically for important intellectual content. FSH, SMA, and MZ designed experiments, assisted in experiments, literature search, and data interpretation. SRS, WZWI, RK, and NS conceptualized, supervised the research, provided feedback, and are responsible for final approval of the version to be published.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The in vivo study was approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (Reference no.: UPM/IACUC/AUP-R071/2018).

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