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Kinetics of angiotensin -1 converting enzyme inhibition and antioxidative properties of *Azadirachta indica* seed protein hydrolysates

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ABSTRACT

Neem (*Azadirachta indica*) seed protein hydrolysates were investigated for *in vitro* antioxidant and angiotensin 1-converting enzyme (ACE)-inhibitory activities. Neem seed proteins were hydrolysed using pepsin, trypsin and Alcalase. The degree of pepsin hydrolysis of neem seed protein was significantly higher ($p < 0.05$) than those of trypsin and Alcalase hydrolysis. Proteolytic hydrolysis of the isolate resulted in hydrolysates with improved Arg/Lys ratio, with pepsin hydrolysates still being able to maintain an acceptable level of essential amino acids comparable to that of the isolate. At 2.5 mg/mL, pepsin neem seed protein hydrolysate (NSPH) demonstrated the strongest antioxidant activity with 67.15 % and 50.07 % DPPH- and superoxide anion radical-scavenging activities, respectively, while trypsin NSPH had the highest ferric-reducing power. Using N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate, NSPHs strongly inhibited ACE (69.20–80.39 %) in a concentration-dependent manner. Pepsin NSPH had higher ACE-inhibitory activity than trypsin and Alcalase NSPHs. Kinetic studies showed the mechanism of ACE inhibition to be mixed-type with *Ki* values of 0.62, 0.84, 1.5 for pepsin, trypsin and alcalase NSPH, respectively. These results suggest that NSPH can be used as a potential nutraceutical with antioxidant capacity and inhibitory activity against ACE.

1. Introduction

Bioactive peptides are peptides which have biological activity and could influence human health. They are often inactive or less active within the sequence of their parent protein and need to be released by digestion or in vitro hydrolysis before becoming active [1]. They widely exist in natural sources such as milk, eggs, plant and animal parts. Usually, bioactive peptides are small and consist of 2–20 amino acids [2, 3]. Bioactive peptides have been reported to possess several activities among which are immunoregulatory, ACE-inhibitory, opioid, antimicrobial and antioxidant potentials [4].

Studies on protein hydrolysates have been undertaken extensively, and some of the identified active hydrolysates and peptides have been incorporated into functional foods [5]. However, many of these studies have only been able to achieve individual bio-functionality (rather than multifunctional) when incorporated into food formulations. Hence, peptides and hydrolysates with multifunctional abilities have been of particular relevance and importance [5, 6].

Hypertension, also referred to as high blood pressure, is a medical condition characterised by chronically elevated blood pressure [7]. As a progressive disorder that leads to several chronic diseases, about a quarter of the world's adult population suffers from hypertension, and the number is projected to escalate to 29 % by the year 2025 [8,9]. The renin–angiotensin system is the primary physiological pathway described for the control and management of blood pressure [10]. Angiotensin 1-converting enzyme (ACE) performs a critical role in the control of blood pressure. It catalyses the cleavage of the C-terminal dipeptide from inactive angiotensin I to the active angiotensin II (a potent vasoconstrictor), and also inhibits the activity of the vasodilator bradykinin. Therefore, inhibition of the catalytic action of ACE can suppress the elevation of blood pressure [11]. Synthetic ACE inhibitors like Captopril, Enalapril, Lisinopril and others, have several adverse

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effects which include cough, loss of taste, renal impairment etc. [12]. Hence, new alternatives, such as bioactive compounds from natural resources have been explored extensively as replacements of these drugs [13], and various studies have reported that bioactive peptides could be employed as potential antihypertensive agents [14, 15].

The strong connection between oxidative stress and lifestyle-related as well as cardiovascular diseases has become well recognised. Oxidative stress results when free radical generation and levels of active intermediates exceed the body's ability to neutralize and eliminate them [16]. To neutralise this oxidative stress, human cells possess a robust antioxidant defence system, which is made up of several antioxidant enzymes in conjunction with a number of low molecular-weight antioxidant molecules such as α-tocopherol and glutathione, ascorbic acid, cysteine, vitamins, etc. However, this defence system could be overwhelmed by several pathological or environmental factors, leading to ROS escaping destruction and forming the far more reactive radicals [17, 18]. In an attempt to expand treatment options, exploring approaches that seek to simultaneously target reduction of oxidative stress levels and antihypertensive potentials are of interest [19, 20, 21, 22].

Neem (*Azadirachta indica*) seed is indigenous to east India and Burma. It is found in much of South East Asia as well as West Africa, and in recent times the Caribbean, South and Central America. Neem leaves have been reported to possess antioxidant and antihypertensive potentials [23, 24]. The seed is often seen as the most essential piece of the plant given its oil content and many reported bioactive components. The protein and amino acid compositions of neem seeds have also been reported, and the amino acids are rich in the essential ones leucine, valine, glycine and threonine [25]. To date, there is no information on the potential enzymatic release of bioactive peptides from neem seed. Therefore, the objective of this work was to determine the *in vitro* antioxidant and ACE-inhibitory activities as well as the enzyme inhibition kinetics of neem seed protein hydrolysates.

2. Materials and methods

2.1. Materials

Oval-shaped and ripe neem fruits were harvested in the month of September, 2017 from different locations in Katsina, Katsina state, Nigeria. They were authenticated at the herbarium of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria where a Voucher No. UILH/001/860 was deposited for record purposes. Trizma-base, n-hexane, Bovine Serum Albumin, pepsin (from porcine gastric mucosa), trypsin (from bovine pancreas), Alcalase (protease from *Bacillus licheniformis*), angiotensin 1-converting enzyme (ACE; from rabbit lung), N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) and DPPH (1,1-diphenyl-2-picrylhydrazyl) were products of Sigma-Aldrich (USA). All other reagents used were of analytical grade.

2.2. Preparation of defatted neem seed powder

The exocarp and pulp of the ripe neem fruits were removed, and the light-colored endocarp was air-dried and cracked to obtain the brown seeds. The neem seeds were dried and pulverized. The powder was defatted using a method described previously [26] with some modifications. The meal was extracted four times with n-hexane (60–80 °C) at a meal/solvent ratio of 1:10 ($^{w}/_{v}$). The meal was then dried at 40 °C to remove excess solvent. The defatted flour was then sieved to obtained fine powder and refrigerated until later use.

2.3. Extraction of neem seed proteins

The protein component of the defatted meal was extracted using a method previously described [27] with slight modifications. Defatted neem seed meal was suspended in 0.1 M NaOH, pH 12.0 at a ratio of 1:10, and stirred for 1 hr to allow for alkaline solubilisation. This was then centrifuged at 18 °C, 3000 g for 10 min. The residue from the centrifugation step was further subjected to extraction twice using the same amount of 0.1 M NaOH and the supernatants were pooled. The pH of the supernatant was adjusted to 4.0 for acid-induced protein precipitation using 0.1 M HCl; the precipitate formed was recovered by centrifugation. Distilled water was used in washing the precipitate and the pH was adjusted to 7.0 using 0.1 M NaOH. The resulting precipitate was lyophilized and the neem seed protein isolate (NSPI) was stored at 4 °C in an air-tight container until required for further analysis.

2.4. Preparation of neem seed protein hydrolysates (NSPH)

The neem seed protein isolate was hydrolysed using a previously reported method [28] with some modifications. Digestion was performed at the specified conditions of each enzyme, including pepsin (pH 2.2, 37 °C), trypsin (pH 8.0, 37 °C) and Alcalase (pH 8.0, 60 °C) for 4 hrs. The NSPI was dissolved in the appropriate buffer (phosphate buffer, pH 8.0 for trypsin and Alcalase; and glycine buffer, pH 2.2 for pepsin) at 5 % ($^{w}/_{v}$). The enzyme was added to the slurry at an enzyme-protein ratio (E:P) of 1:100 ($^{w}/_{w}$). Digestion was performed at the specified conditions for 4 hrs with continuous stirring. The enzyme was inactivated by boiling in water bath (95–100 °C) for 15 min and undigested proteins was precipitated by adjusting the pH to 4.0 followed by centrifugation at 4000 g for 30 min. The supernatant containing target peptides was then collected. Peptide yield (%) was calculated as the ratio of peptide weight of lyophilized hydrolysate to the protein weight of protein isolate as previously reported [20]. Protein content of samples was determined using the biuret assay method [29] with bovine serum albumin (BSA) as standard.

2.5. Determination of degree of hydrolysis

Degree of hydrolysis (DH) was estimated by calculating the percentage of soluble protein in 10 % trichloroacetic acid (TCA) in relation to total protein content of the neem seed protein isolate according to the method previously reported [20]. The degree of hydrolysis was calculated based on the quantification of the soluble protein content following precipitation with TCA. Protein hydrolysate (1 mL) was added to 1 mL of 20 % TCA to produce 10 % TCA soluble material. The mixtures were left to stand for 30 min for precipitation, followed by centrifugation at 4000 g for 20 min. The supernatant was analysed for protein content using the biuret method [29] with bovine serum albumin (BSA) as standard. The degree of hydrolysis (DH) was computed as shown below:

$$\text{Degree Hydroloysis } (\%) = \frac{\text{Soluble peptide in 10\% TCA } (\text{mg/mL})}{\text{Total protein content of isolate } (\text{mg/mL})} \times 100\%$$

2.6. Amino acid analysis

Amino acid content of the neem seed protein hydrolysates (NSPH) and isolate was determined by employing Amino Acid Analyzer LC98I (BIOBASE, China). Pepsin NSPH (PNSPH), Alcalase NSPH (ANSPH) and trypsin NSPH (TNSPH) were lyophilized and then hydrolysed for 24 hrs at 110 °C with 6N HCl [30]. Hydrolysed samples were stored in sodium citrate buffer (pH 2.2) at 4 °C until the time of analysis when 50μL of the isolate/hydrolysate was injected for analysis. Tryptophan was determined differently by hydrolysis of the sample with NaOH. Cysteine and methionine were measured after performic acid oxidation prior to hydrolysis in 6 N HCl, and determined as cysteic acid and methionine sulphone, respectively [30].

2.7. DPPH radical-scavenging activity

DPPH radical-scavenging activity of NSPH was measured using the assay method previously described [31]. Briefly, 1 mL of NSPH

(dissolved in 0.1M sodium phosphate buffer, pH 7.0) at different concentrations (1.0–2.5 mg/mL) was added to 1 mL 0.1 mM DPPH dissolved in 95 % ethanol. The mixture was shaken vigorously and incubated in the dark and at room temperature for 30 min. The absorbance was read at 517 nm. Ethanol (95 %) was used as a blank. The control solution consisted of 1.0 mL of 95 % ethanol and 1.0 mL of DPPH solution. Percentage inhibition of DDPH radical was calculated thus:

$$\text{DPPH}^{\cdot}- \text{ scavenging Activity } (\%) = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100\%$$

The concentration of NSPH that scavenged 50 % DPPH radical (EC_{50}) was interpolated from a non-linear regression plot of DPPH-scavenging activity versus hydrolysate concentrations. All experiments were performed in triplicates.

2.8. Superoxide anion radical-scavenging activity

The superoxide-scavenging activity was determined according to the method previously developed [32] and some modifications were made. An aliquot (800 μL) of NSPH sample was mixed (0.5–2.0 mg/mL) with 800 μL of 50 mM Tris–HCl buffer (pH 8.3) containing 1 mM EDTA. An aliquot (400 mL) of 1.5 mM pyrogallol in 10 mM HCl was added directly into test tubes in the dark. The control reaction contained Tris–HCl buffer in place of NSPH. Absorbance was measured at 420 nm for 4 min at room temperature.

$$\dot{O}_{2^-} - \text{scavenging Capacity } (\%) = \frac{(\Delta\text{Abs/min})_{control} - (\Delta\text{Abs/min})_{sample}}{(\Delta\text{Abs/min})_{control}} \times 100\ \%$$

2.9. Ferric-reducing antioxidant power

The ferric-reducing antioxidant power (FRAP) of NSPH was determined according to a previously described method [20]. NSPH sample or ascorbate was prepared in 0.2 M phosphate buffer (pH 8.0). An aliquot of 1 mL of different concentrations (0.2–0.8 mg/mL) of NSPH were mixed with 1 mL of 1 % ($^{w}/_{v}$) potassium ferricyanide solution. The mixture was incubated at 50 °C for 30 min followed by the addition of 1 mL 10 % ($^{w}/_{v}$) TCA. A portion (1 mL) of the incubation mixture was added with 1 mL of distilled water and 0.2 mL of 0.1 % ($^{w}/_{v}$) ferric chloride in test tubes. After a 10 min reaction time, the absorbance of resulting solution was read at 700 nm. Higher absorbance suggested stronger reducing power. Ascorbic acid was used as the reference antioxidant. An aqueous solution of Fe (II) prepared at varying known concentrations ($FeSO_4{\cdot}7H_2O$ at 2.0, 1.0, 0.5, 0.25, and 0.125 mM) was used for calibration. Results were expressed as mM of Fe^{2+} released. All tests were performed in triplicates.

2.10. Determination of ACE-inhibitory activity of NSPH

The ability of the NSPH to inhibit the activity of ACE *in vitro* was measured according to a modified spectrophotometric method [33] using FAPGG as substrate. Briefly, 500 μL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 mM NaCl, pH 7.5) was mixed with 20μL of ACE (0.1 U/mL; final activity of 2 mU) and 100 μL of NSPH (0.5–2.0 mg/mL) in 50 mM Tris–HCl buffer. The decreased absorbance at 345 nm, due to cleavage of the Phe-Gly peptide bond of FAPGG, was recorded at regular intervals for 5 min at room temperature. Tris–HCl buffer was used instead of hydrolysate solution in the blank experiment. ACE activity was expressed as rate of disappearance of FAPGG (ΔAbsorbance/min) and inhibitory activity was calculated using the equation below:

$$\textit{ACE Inhibition } (\%) = \frac{(\Delta\text{Absorbance/min})_{blank} - (\Delta\text{Absorbance/min})_{sample}}{(\Delta\text{Absorbance/min})_{blank}} \times 100$$

$(\Delta\text{Absorbance/min})_{\text{sample}}$ and $(\Delta\text{Absorbance/min})_{\text{blank}}$ are the reaction rates in the presence and absence of NSPH, respectively. The concentration of NSPH that inhibited ACE activity by 50 % (IC_{50}) was calculated using a non-linear regression plot of ACE inhibition versus sample concentrations.

2.11. Determination of kinetic parameters of ACE inhibition

Kinetic parameters of ACE inhibition by NSPH were determined according to a previous method [14] with slight modifications. The kinetics of ACE-catalysed conversion of FAPGG to N-(3-[2-furyl]acryloyl)-phenylalanine was studied in the absence and presence of three different concentrations of NSPH with 0.0625, 0.125, 0.25 and 0.5 mM FAPGG. The mode of inhibition of ACE and kinetic parameters (K_m, K'_m, V_{max} and V'_{max}) were estimated from Lineweaver–Burk plots. The catalytic efficiency (*CE*) of ACE in the absence and presence of the NSPHs was calculated using the equation below.

$$\text{CE} = \frac{\text{V}_{\text{max}}}{\text{K}_{\text{m}}} \text{ or } \frac{\text{V}'_{\text{max}}}{\text{K}'_{\text{m}}}$$

V_{max} (or V'_{max}) and K_m (or K'_m) are the maximum reaction velocity (or apparent value) and Michaelis constant (or apparent value), respectively. The enzyme-inhibitor dissociation constant (K_i) was determined as the intercept on the X-axis of a secondary plot of the slopes of the Lineweaver–Burk plots versus concentrations of inhibiting hydrolysate.

2.12. Statistical analysis

Data, expressed as mean of triplicates ± standard deviation (SD), were subjected to analysis of variance (ANOVA) and Tukey's multiple range tests using GraphPad Prism version 6.0 [34]. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Percentage protein yield, peptide yield and degree of hydrolysis

The percentage protein yield of isolation from neem seed was found to be 12.52 %. The percentage peptide yields of NSPI for pepsin, trypsin and Alcalase digestions are shown in Table 1. The peptide yields of pepsin, trypsin and Alcalase digestions were 35.61 %, 82.23 % and 48.25 %, respectively. The degree of hydrolysis for each enzyme employed is shown in Table 1. The degree of pepsin hydrolysis (27.9 ± 1.2%) was significantly higher ($p < 0.05$) than those of trypsin (23.3 ± 0.6%) and Alcalase (14.7 ± 0.6%); while the degree of trypsin hydrolysis was significantly higher than that of Alcalase.

3.2. Amino acid analysis

The amino acid composition of the hydrolysates and that of the isolate

Table 1
Percentage peptide yield and degrees of pepsin, trypsin and alcalase hydrolysis of neem seed protein isolate.

Hydrolytic Enzyme	Peptide Yield of Isolate (%)	Degree of Hydrolysis (%)
Pepsin	35.61	27.88 ± 1.24^{a}
Trypsin	82.23	23.34 ± 0.56^{b}
Alcalase	48.25	14.73 ± 0.55^{c}

Values of degree of hydrolysis represent mean of triplicate determinations ±SD. Values with different superscripts are significantly different at $p < 0.05$.

were analyzed and compared (Table 2). The percentage of polar amino acids in the NSPI was found to be 59.6 % with positively charged residues being 13.6 %, negatively charged residues being 18.6 % and uncharged polar residues being 27.4 %. Non-polar residues in NSPI make up 36.0 %. These levels are not very different from those of PNSPH, with marked difference only observable in the percentage of non-polar aliphatic amino acids having a 4.0 % decrease. The levels of all categories of amino acids are notably lower for TNSPH and even much lower for ANSPH with very lower values observed for polar uncharged amino acids, 22.3 % for TNSPH and 20.5 % for ANSPH as compared to 27.4 % for NSPI and 26.2 % for PNSPH. Particularly, levels of OH-containing residues (Ser and Thr) and Gly were noticeably reduced in TNSPH and ANSPH. Val and Trp contents are generally low in NSPI and hence in the hydrolysates. The essential amino acid content in PNSPH (30.7 %) is comparable to that of NSPI (36.8 %), but those of TNSPH (28.5 %) and ANSPH (26.9 %) are much lower. Arg/Lys ratio was improved for all the hydrolysates, 1.86 for PNSPH, 1.96 for TNSPH and 1.88 for ANSPH compared to the isolate (1.62).

3.3. DPPH radical-scavenging capacity of NSPHs

The ability of varying concentrations of NSPHs to scavenge DPPH radical was determined (Fig. 1A). There was an increase in the scavenging activity of ascorbic acid, pepsin, trypsin and Alcalase NSPHs with increase in concentration. At all concentrations studied, the DPPH-scavenging activity of ascorbic acid was higher than those of the hydrolysates. Pepsin NSPH demonstrated higher radical-scavenging activity than other NSPHs. However, trypsin NSPH radical-scavenging activity was higher than that of Alcalase NSPH which had the lowest scavenging activity.

The EC_{50} values for DPPH radical-scavenging activity of ascorbic acid and NSPHs are shown in Table 3. Ascorbic acid had the lowest EC_{50} (0.51 $\pm$ 0.06 mg/mL), followed by pepsin NSPH (0.91 $\pm$ 0.04 mg/mL), trypsin NSPH (2.68 $\pm$ 0.09 mg/mL) and then Alcalase NSPH (2.90 $\pm$ 0.04 mg/mL).

Table 2
Amino acid composition of neem seed protein isolate and hydrolysates (g/100g sample).

Amino acid	NSPI	PNSPH	TNSPH	ANSPH
Asp	6.8	6.6	6.3	6.2
Glu	11.8	10.7	8.6	8.0
His	3.4	3.1	2.9	2.8
Lys	3.9	2.8	2.5	2.4
Arg	6.3	5.2	4.9	4.5
Ser	7.6	7.3	4.7	4.7
Thr	5.3	4.9	4.3	4.2
Gln	1.6	1.4	1.5	1.0
Asn	3.9	3.8	3.6	3.4
Pro	5.9	5.8	5.3	5.0
Cys	3.1	3.0	2.9	2.2
Gly	9.4	8.2	7.0	7.1
Ala	5.3	4.8	4.2	4.0
Val	0.5	0.4	0.3	0.3
Ile	3.0	2.8	2.5	2.4
Leu	5.0	4.3	4.2	4.0
Met	4.0	3.2	3.1	3.0
Phe	4.7	3.5	3.4	3.1
Tyr	3.4	3.2	2.8	2.6
Trp	0.7	0.5	0.4	0.2
PAA	59.6	54.6	47.5	44.4
PCR	13.6	11.1	10.3	9.7
NCR	18.6	17.3	14.9	14.2
PUR	27.4	26.2	22.3	20.5
PUOHR	12.9	12.2	9.0	8.9
NAA	36.0	30.9	27.9	26.7
AR	27.2	23.7	21.3	20.8
ArR	8.8	7.2	6.6	5.9
EAA	36.8	30.7	28.5	26.9
Arg/Lys	1.62	1.86	1.96	1.88

NSPI – Neem Seed Protein Isolate; PNSPH – Pepsin Neem Seed Protein Hydrolysate; TNSPH – Trypsin Neem Seed Protein Hydrolysate; ANSPH – Alcalse Neem Seed Protein Hydrolysate.
PAA - Polar Amino Acids: Asp, Glu, His, Lys, Arg, Ser, Thr, Gln, Asn, Pro, Cys.
PCR - Positively Charged Residues: His, Lys, Arg.
NCR - Negatively Charged Residues: Glu, Asp.
PUR - Polar Uncharged Residues: Gln, Asn, Pro, Cys, Ser, Thr.
PUROH - Polar Uncharged Residues (OH-containing): Ser, Thr.
NAA – Nonpolar Amino Acids: Gly, Ala, Val, Ile, Leu, Met, Phe, Tyr, Trp.
AR – Aliphatic Residues: Gly, Ala, Val, Ile, Leu, Met.
ArR – Aromatic Residues: Phe, Tyr, Trp.
EAA – Essential Amino Acids: Phe, Met, Val, His, Thr, Arg, Trp, Lys, Ile, Leu.
Arg/Lys – Arginine to Lysine Ratio.

3.4. Superoxide radical-scavenging activity of NSPHs

The percentage superoxide radical-scavenging activity of the ascorbic acid, pepsin, trypsin and Alcalase neem seed protein hydrolysates is presented in Fig. 1B. Ascorbic acid and the NSPHs exhibited increase in superoxide-scavenging activity with increasing concentration. The superoxide-scavenging capacity of the hydrolysates was lower than that of ascorbic acid at all concentrations. However, among the hydrolysates, pepsin NSPH demonstrated a higher scavenging power than trypsin and Alcalase NSPHs at all concentrations. Trypsin NSPH had a higher scavenging activity than Alcalase NSPH at higher hydrolysate concentrations of 1.0, 1.5 and 2.0 mg/mL.

3.5. Ferric-reducing antioxidant power of NSPHs

The ferric-reducing power of NSPHs at varying concentrations in comparison with ascorbic acid is shown in Fig. 2. The ferric-reducing power of NSPHs as well as ascorbic acid was concentration-dependent, although there was no significant difference ($p > 0.05$) between the reducing power of pepsin NSPH at 0.60 and 0.80 mg/mL concentrations. At 0.2 mg/mL concentration, the reducing ability of the hydrolysates was not significantly different ($p > 0.05$) from that of ascorbic acid, except Alcalase NSPH which was significantly lower ($p < 0.05$). At 0.40, 0.60 and 0.80 mg/mL concentrations, trypsin NSPH exhibited higher ($p < 0.05$) ferric-reducing ability than pepsin and Alcalase NSPHs as well as ascorbic acid. However, pepsin and Alcalase NSPHs had significantly lower ($p < 0.05$) reducing capacity at all the concentrations studied. Pepsin NSPH demonstrated a rather weak response to increasing hydrolysate concentration and the results showed that at higher concentrations of 0.60 and 0.80 mg/mL, there was significant increase in its reducing ability.

3.6. ACE-inhibitory activity of NSPHs

The ACE-inhibitory activities of neem seed protein hydrolysates showed different trends as shown in Fig. 3. At all concentrations except 1.0 mg/mL, pepsin NSPH had significantly higher ($p < 0.05$) ACE inhibition than trypsin and Alcalase NSPHs, while the inhibitory activity of trypsin NSPH was significantly higher ($p < 0.05$) than that of Alcalase NSPH. The highest inhibitory activity of 80.39 $\pm$ 0.60% was exhibited by pepsin NSPH at 2.0 mg/mL. At 1.0 mg/mL NSPH concentration, there was no significant difference ($p > 0.05$) between the ACE-inhibitory activities of pepsin and trypsin NSPHs but they were significantly higher ($p > 0.05$) than that of Alcalase NSPH.

The IC_{50} values of pepsin, trypsin and Alcalase NSPHs in ACE inhibition were estimated (Table 3). The IC_{50} values of trypsin (0.57 $\pm$ 0.14 mg/mL) and Alcalase (0.86 $\pm$ 0.11 mg/mL) NSPHs were not significantly different ($p > 0.05$) but were significantly higher ($p < 0.05$) than that of pepsin NSPH (0.21 $\pm$ 0.12 mg/mL).

3.7. Kinetic parameters of ACE inhibition by NSPHs

Lineweaver-Burk plots were used to estimate the kinetic parameters

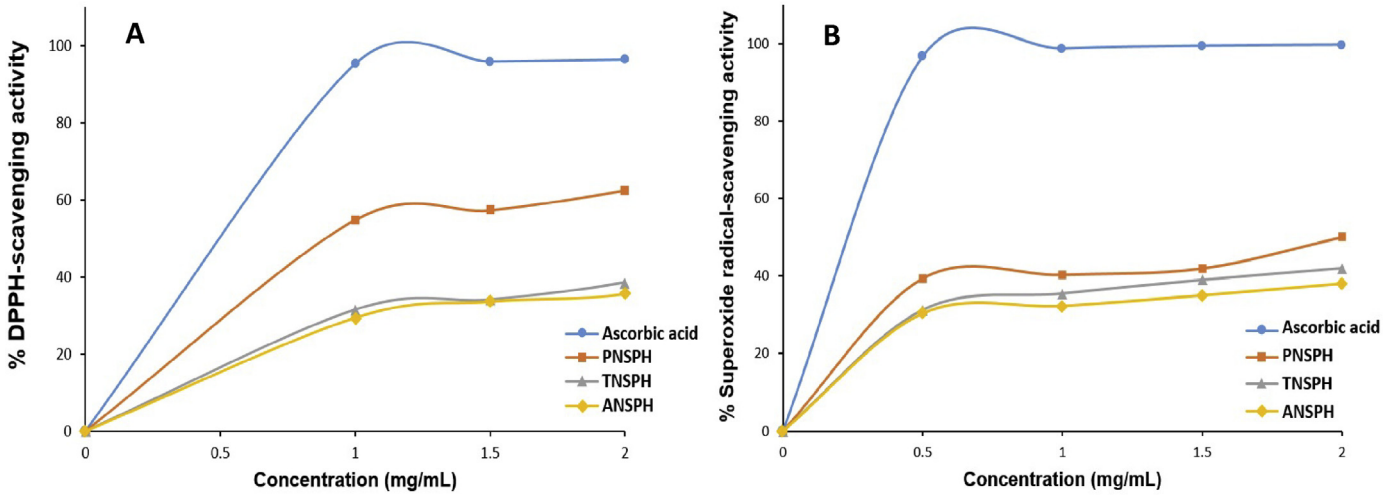

Fig. 1. DPPH-scavenging (A) and superoxide radical-scavenging (B) activities of pepsin, trypsin and Alcalase NSPHS. PNSPH – Pepsin Neem Seed Protein Hydrolysate; TNSPH –Trypsin Neem Seed Protein Hydrolysate; ANSPH – Alcalase Neem Seed Protein Hydrolysate.

Table 3
Fifty per cent (50%) effective/inhibitory concentration (EC_{50}/IC_{50}) of pepsin, trypsin and Alcalase NSPHS in radical-scavenging and ACE inhibition

Sample	DPPH radical-scavenging EC_{50} (mg/mL)	Superoxide radical-scavenging EC_{50} (mg/mL)	ACE inhibition IC_{50} (mg/mL)
Ascorbic Acid	0.51 ± 0.06^{a}	0.24 ± 0.00^{a}	-
PNSPH	0.92 ± 0.05^{b}	2.21 ± 0.04^{b}	0.21 ± 0.12^{a}
TNSPH	2.68 ± 0.09^{c}	2.85 ± 0.05^{c}	0.57 ± 0.14^{b}
ANSPH	2.91 ± 0.05^{d}	4.27 ± 0.03^{d}	0.86 ± 0.10^{b}

Values represent mean of triplicate determinations ±SD. Values with different superscripts are significantly different at $p < 0.05$.
PNSPH – Pepsin Neem Seed Protein Hydrolysate; TNSPH –Trypsin Neem Seed Protein Hydrolysate; ANSPH – Alcalase Neem Seed Protein Hydrolysate.

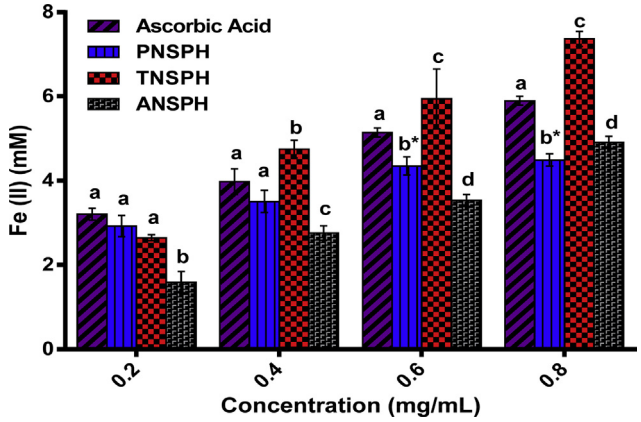

Fig. 2. Ferric-reducing power of pepsin, trypsin and Alcalase NSPHS. Values represent mean of triplicate determinations ±SD. Different letters (a–d) denote significant difference ($p < 0.05$) among different samples at the same concentration. Bars with asterisk (*) denote insignificant difference ($p > 0.05$) of the same sample at different concentrations. PNSPH – Pepsin Neem Seed Protein Hydrolysate; TNSPH –Trypsin Neem Seed Protein Hydrolysate; ANSPH – Alcalase Neem Seed Protein Hydrolysate.

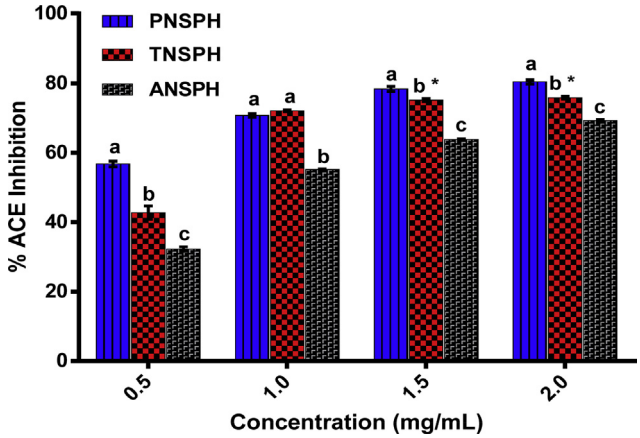

Fig. 3. ACE-inhibitory activity of pepsin, trypsin and Alcalase NSPHS. Values represent mean of triplicate determinations ±SD. Different letters (abc) denote significant difference ($p < 0.05$) among different samples at the same concentration. Bars with asterisk (*) denote insignificant difference ($p > 0.05$) of the same sample at different concentrations. PNSPH – Pepsin Neem Seed Protein Hydrolysate; TNSPH –Trypsin Neem Seed Protein Hydrolysate; ANSPH – Alcalase Neem Seed Protein Hydrolysate.

and modes of ACE inhibition by pepsin, trypsin and Alcalase NSPHs as shown in Fig. 4A-4C, respectively. The modes of ACE inhibition by pepsin and trypsin NSPHs were mixed-type inhibition patterns (Fig. 4A &4B). Alcalase NSPH also exhibited a mixed-type inhibition but tilting more towards non-competitive inhibition mechanism (Fig.4C). A summary of the kinetic parameters obtained from Lineweaver-Burk plots in the absence and presence of three different concentrations of each of pepsin, trypsin and Alcalase NSPHs is presented in Table 4. The presence of each of the three hydrolysates resulted in elevation of the value of Michaelis constant K_m (mM). Values of the apparent Michaelis constant K'_m (mM) in the presence of pepsin and trypsin NSPHs were higher than that of Alcalase NSPH at all hydrolysate concentrations. However, K'_m in the presence of trypsin NSPH was higher than that in the presence of pepsin NSPH at concentrations of 1.0 and 1.5 mg/mL. The presence of trypsin NSPH showed a clear concentration-dependent increase in K'_m.

In a concentration-dependent manner, pepsin, trypsin and Alcalase NSPHs caused a reduction in the reaction maximum velocity V_{max} (A/min) and catalytic efficiency *CE* (A/min/mM) of ACE (Table 4). Whereas V'_{max} values in the presence of pepsin and Alcalase NSPHs were lower than in the presence of trypsin NSPH, *CE* values were lower in the presence of pepsin and trypsin NSPHs than in the presence of Alcalase NSPH. At 1.5 mg/mL, trypsin NSPH (0.016 A/min/mM) caused the highest reduction in *CE* followed by pepsin NSPH (0.018 A/min/mM). K_i

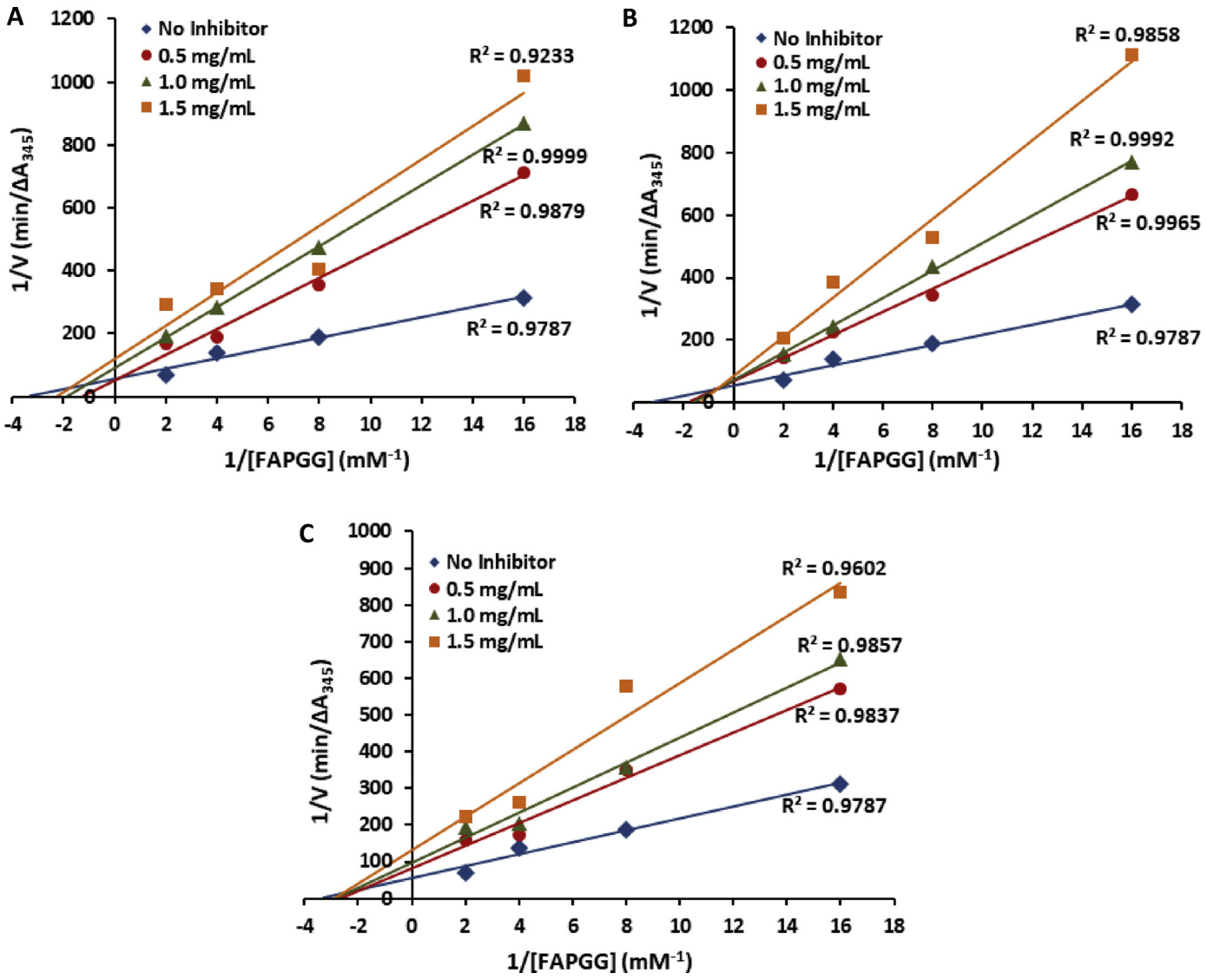

Fig. 4. Lineweaver–burk plot of ACE inhibition by different concentrations of pepsin (A), trypsin (B) and Alcalase (C) NSPHs at varying FAPGG concentrations.

Table 4
Kinetic parameters of ACE-catalysed reaction in the absence and presence of pepsin, trypsin and Alcalase NSPHS.

Catalytic Parameter	Control (No Inhibitor)	PNSPH (mg/mL)			TNSPH (mg/mL)			ANSPH (mg/mL)		
		0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
K_m or K'_m (mM)	0.296	0.782	0.516	0.492	0.535	0.608	0.746	0.380	0.351	0.342
V_{max} or V'_{max} [a]	0.018	0.019	0.010	0.008	0.014	0.014	0.011	0.012	0.010	0.008
CE [b]	0.061	0.025	0.021	0.018	0.027	0.023	0.016	0.032	0.029	0.022
K_i (mg/mL)	—	0.621			0.841			1.532		

K_m (K'_m) – Michaelis constant in the absence (and presence) of hydrolysate; V_{max} (V'_{max}) – Maximum velocity in the absence (and presence) of hydrolysate; CE – Catalytic efficiency; K_i – Enzyme-inhibitor dissociation constant; a – (μmol/mg/min); b – (ΔAbsorbance/min/mM).
PNSPH – Pepsin Neem Seed Protein Hydrolysate; TNSPH –Trypsin Neem Seed Protein Hydrolysate; ANSPH – Alcalase Neem Seed Protein Hydrolysate; ACE – angiotensin -1 converting enzyme.

(enzyme-inhibitor dissociation constant; mg/mL) of ACE inhibition by pepsin NSPH (0.621 mg/mL) was lower than those of trypsin (0.841 mg/mL) and Alcalase (1.532 mg/mL) NSPHs. K_i of ACE in the presence of Alcalase NSPH was more than twice as much as in the presence of pepsin NSPH (Table 4).

4. Discussion

4.1. Percentage protein yield, peptide yield and degree of hydrolysis

The percentage protein yield obtained after isolation of neem seed protein was similar to the value previously reported by Djibril et al. [25] and slightly higher than the percentage protein yield of neem seed isolate (6.5–11.6 %) in another report [35]. This result was also higher than the

protein yield (1.76 %) reported for neem seed protein extraction using salt precipitation method [36]. This corroborates the finding from a previous study [37] that acid precipitation gives a higher yield in protein extraction than salt precipitation. The protein content of neem seed in comparison with reported values for other common Nigerian oil seeds, such as egusi melon, soybean, groundnut and palm kernel was slightly lower [38, 39]. This implies that the yield obtained is a function of the protein content of neem seed and may also depend on the method of isolation [40]. The protein content of neem seed protein isolate obtained in this study was higher than the previously reported percentage extractable protein [36] although salt precipitation method was used in that study. The value obtained in the present study was similar to the reported 76.02 % protein content of Australian canola seed [27] and 79 % in bambara groundnut [37].

Degree of hydrolysis gives an idea of how frequent a protease encounters its cleavage sites [41]. The trend of result obtained in this study for degree of hydrolysis (DH) was similar to that of common carp roe (egg) protein hydrolysed with pepsin, trypsin and Alcalase as reported by Chalamaiah et al. [42]. The degrees of pepsin, trypsin and Alcalase digestion of common carp roe protein were 30.0, 19.5, and 12.7 %, respectively [42]. The higher DH exhibited by pepsin (27.9 $\pm$ 1.2%) could be due to the reported presence of abundant quantity of aromatic amino acid residues in neem seed proteins [20, 25] for which pepsin has preferential specificity [43]. This was also similar to the result obtained for rainbow trout muscle in which pepsin had the highest degree of hydrolysis followed by trypsin and Alcalase, although with higher percentages [12].

4.2. Amino acid composition

In addition to characterizing the amino acids composition, data on the amino acid levels could also give information regarding the understanding of observed hydrolysate bioactivities. The high essential amino acid content of NSPI was in agreement with a previous report on neem seed [25]. The essential amino acid content of the isolate met both the FAO 35.0 % recommendation [44] and Joint FAO/WHO ~31.4 % recommended [45] for human nutrition. Thus, in addition to its potential therapeutic bioactivities, NSPI could support adequate human growth if provided as a source of dietary protein. Hydrolysis of the isolate affected the amino acid composition in varying ways. The improvement in Arg/Lys ratio was similar to what was reported by Malomo et al. [46]. An increased Arg/Lys ratio in the diet has been reported to facilitate hypocholesterolemic effects and hence could improve cardiovascular health [47]. Thus, the results show that the hydrolysates could offer better positive health effects on the cardiovascular system when compared to the isolate.

The high contents of Glu, Asp, Ser, Gly, Arg, Pro, Leu and Ala are similar to the observations in previous studies [10, 48]. Arg has been reported to play an essential vasodilatory role by being a precursor of Pro and Glu, promoting nitric oxide synthesis and reducing the risk of heart diseases [49]. Negatively charged residues (Asp and Glu) are known to be strong electron donors that are capable of enhancing interactions with cations present at enzyme active sites [15, 50]. Hydrophobic residues like Leu can facilitate the presence of peptides at the water-lipid interface and hence enhance access to scavenge free radicals generated at the lipid phase [51].

4.3. Radical-scavenging activity of NSPHs

The radical-scavenging activity of NSPHs was studied in terms of their DPPH-scavenging and superoxide radical-scavenging capacities. The higher radical-scavenging activity of pepsin NSPH compared to trypsin and Alcalase NSPHs was also represented by the EC_{50} values obtained for each of the NSPHs. EC_{50} is a parameter widely used to measure the antiradical efficiency [52], and a low EC_{50} is indicative of high scavenging activity. The highest scavenging activity of pepsin NSPH compared to other proteases was also reported for rapeseed [15], pink perch [53], yellowfin sole [54] and porcine haemoglobin [55]. Higher content of hydrophobic aromatic amino acids, such as His, Met, Cys, Phe and Tyr have been reported to contribute to higher radical-scavenging activity [42]. The difference in radical-scavenging activity of the hydrolysates might be due to the difference in amino acid constituents and sequence resulting from different degrees of hydrolysis [42]. The scavenging activity of NSPHs reveals that the hydrolysates were able to scavenge the DPPH and superoxide radicals by donating a proton, making it a stable diamagnetic molecule and stop the radical chain reaction [56]. The scavenging activity was also found to be concentration-dependent which is in agreement with studies on canola, rapeseed and soybeans hydrolysates [57, 58].

4.4. Ferric-reducing antioxidant power of NSPHs

The FRAP assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen, and some studies have shown that there is a direct relationship between antioxidant activities and reducing power of peptides [59]. The result shows that neem seed protein hydrolysates possess higher ferric-reducing power than ascorbic acid at the studied concentrations. In a concentration-dependent pattern, they were able to reduce the ferric ions to the ferrous form. This agrees with earlier studies of [42, 53]. Similarly, Naqash and Nazeer [53] also reported for protein hydrolysates from pink perch that the reducing power of hydrolysates varied with the protease used in hydrolysis. Compounds with higher reducing power are more capable of donating electrons or hydrogen, and thus have potential for use as antioxidants [60]. The strong reducing power of NSPHs may be attributed to the increased availability of protons as a result of the production of peptides by enzymatic hydrolysis [56].

4.5. ACE-inhibitory activity of NSPHs

The protease treatments of neem seed proteins liberated bioactive peptides from the native protein structure, and the hydrolysates led to a concentration-dependent loss of ACE activity. Pepsin NSPH displayed the most potent ACE-inhibitory activity which could be due to the presence of hydrophobic amino acid residues in pepsin hydrolysates of neem seed proteins [25]. The presence of hydrophobic amino acids has been reported to influence ACE inhibition positively [28, 61]. ACE-inhibitory activity of the hydrolysates obtained in this study was slightly lower than the values (69–87 %) previously reported for cod protein hydrolysate [62] at 1 mg/mL hydrolysate concentration. This could be attributed to the difference in the type of proteolytic enzyme and enzyme-substrate ratio used.

The Alcalase digested NSPHs exhibited the least ACE inhibition amongst the NSPHs. This is similar to the finding of an earlier study [12] in which pepsin hydrolysate of *Luffa cylindrical* seed proteins exhibited the highest ACE inhibition among the various enzymatic hydrolysates, and Alcalase hydrolysate exhibited the least activity. The IC_{50} value of pepsin NSPH was similar to that of Alaska pollack protein hydrolysates previously reported [60]. There may be a correlation between the degrees of hydrolysis by proteolytic enzymes and biological activities (including ACE inhibition) of hydrolysates [63]. Segura-Campos et al. [5] reported that inhibition of ACE activity by Chia protein hydrolysates depended significantly on the degree of hydrolysis. This is also in agreement with previous findings that antihypertensive peptides often contain only two to nine residues and are thus resistant to endopeptidase degradation in the digestive tract [64].

4.6. Kinetic parameters of ACE inhibition by NSPHs

K_m value of ACE activity in the absence of the inhibiting NSPHs was determined to be 0.296 mM FAPGG, which was similar to previously reported values of 0.30 mM [33], 0.292 mM FAPGG [65] and 0.306 mM [28]. Pepsin, trypsin and Alcalase NSPHs showed a mixed-type inhibition

mechanism which was characterized by their effects on the catalytic parameters at different hydrolysate concentrations. This was apparent in the concentration-dependent decrease in V'_{max} and *CE* as well as increase in K'_m of ACE (for trypsin NSPH) which indicated a decline in the maximal reaction rate, catalytic efficiency and affinity of ACE for FAPGG with increasing hydrolysate concentrations [66]. This result was similar to the mode of inhibition reported for Alcalase cationic flaxseed peptides [28] and chicken skin protein hydrolysates [67]. This shows that the NSPHs exhibited their inhibition probably by binding to both free ACE and substrate (FAPGG)-bound states to form enzyme-substrate or enzyme-substrate-inhibitor complexes thereby leading to reduced ACE-substrate interactions or loss of enzyme activity. This also suggests that the hydrolysed peptides could have affinities for sites other than the substrate-binding site resulting in change of enzyme active site conformation, hence inhibiting activity [14, 28, 68].

The lower K_i value for pepsin NSPH (0.621 mg/mL) than those of trypsin and Alcalase NSPH indicates that the binding affinity of pepsin NSPH for ACE is stronger than those of trypsin and Alcalase NSPHs. It was observed that the slopes of the double reciprocal plots demonstrated a concentration-dependent increase. Higher slope values indicated reduced rates of reaction which correspond to higher ACE inhibition. This agreed with the report from an earlier study on chicken skin protein hydrolysate [50].

5. Conclusion

The results from this study clearly indicate that neem seed protein hydrolysates are rich in bioactive peptides with ACE-inhibitory and antioxidant activities. The results also confirmed the influence of protease specificity on the antioxidant and ACE-inhibitory activities of the hydrolysates obtained. Proteolytic hydrolysis of the isolate resulted in hydrolysates with improved Arg/Lys ratio, with pepsin hydrolysates still being able to maintain an acceptable level of essential amino acids comparable to that of the isolate. Pepsin hydrolysate of neem seed proteins generally had more antioxidant and ACE-inhibitory abilities than the trypsin and Alcalase hydrolysates. The inhibition of ACE observed in this study could be said to depend on the ability of the proteases (pepsin, trypsin and Alcalase) to release peptides of varying degree of bioactivity resulting from their individual cleavage specificities. The mode of ACE inhibition by the hydrolysates was the mixed-type inhibition. An *in vivo* investigation of the observed bioactivities is necessary, and consequently, NSPH could be exploited as a potential alternative to synthetic antioxidants and antihypertensives.

Declarations

Author contribution statement

Rotimi O. Arise: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Marvellous A. Acho: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Abeeb A. Yekeen: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ibrahim A. Omokanye: Performed the experiments; Analyzed and interpreted the data.

Elizabeth O. Sunday-Nwaso: Performed the experiments.

Olatunbosun S. Akiode: Analyzed and interpreted the data.

Sylvia O. Malomo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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