

Identification and Purification of Antioxidant Peptides from Lentils (*Lens Culinaris*) Hydrolysates

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Abstract: Powder derived from Lentils was hydrolyzed sequentially using five different enzymes and their combinations for production of antioxidant peptide. For identifying antioxidant peptides, Lentils hydrolysate was fractionated using Ultra filtration and size exclusion chromatography (Superdex Peptide 10/300 GL) methods. The lentils peptide and its fractions demonstrated antioxidant activities as revealed from studies using a β -carotene-linoleate model system, hydroxyl radical-scavenging, the DPPH radical-scavenging activity assay, and a reducing power evaluation. Molecular characteristics of individual Lentils protein was investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results demonstrated that Lentils peptide fraction III exhibited the highest antioxidant activity compared to the other Lentils Fraction. The molecular weight and sequence were identified by LC-MS analysis as 587.31 Da and the amino acid sequence was as follows: Ala- Leu- Gly- Pro- Val- Met.

Keywords: Lentils, Lentils Peptide Hydrolysate, Antioxidant Properties, Sdspage

1. Introduction

Lentil (*Lens culinaris*) is an important pulse crop in America Australia, Afghanistan, Bangladesh, Canada, Egypt, Ethiopia, Egypt, India, Iraq, Iran, Morocco, Nepal, Pakistan, Sudan, Syria, Turkey and Tunisia. The annual production of Lentils accumulated to is 495 Ktons in the year of for 2013 (1). Lentil is a powerhouse of nutrition and a good source of potassium, calcium, zinc, niacin and vitamin K, but are also particularly rich in dietary fiber, lean protein, folate and iron. Lentils need short time for cooking compared with other pulses and its higher protein level with a lower level of anti-nutritional factors, make lentils very suitable for human consumption(2).

Reactive oxygen and nitrogen species (RONS) is scavenged by natural antioxidant from plant endogenous to food which formed energy supplier, detoxification, immune function and chemical signaling superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species (3), researchers have suggested that these may be of great

importance in preventing the onset of oxidative diseases (4)

The antioxidant activity responsible compounds could be isolated and used for preventing and treating of free radical related disorders (5). Lentils are a leguminous seed that have high levels of natural antioxidants (6,7,8). Lentils has the highest antioxidant capacity when measured as 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity in comparison with green pea, yellow pea and chickpea(9). 1, 1-Diphenyl-2-picryl-hydrazyl is a stable free radical which has an unpaired valence electron at one atom of Nitrogen Bridge (10). Repeatedly ingestion of legumes, as part of healthy diet, has been conversely conjoined with cardiovascular disease (CVD) (11), colon cancer, diabetes (12).

The in vitro digestion of the lentil proteins by pepsin/trypsin/ α -chymotrypsin, alcalase/flavourzyme and papain has been reported to significantly reduce the bile salt binding capacity compared to the undigested samples except in the case of sodium deoxycholate where no significant differences in bile salt binding were observed before and after hydrolysis (13). Recently, some study findings have shown that the lentils hydrolysate peptide has angiotensin I-

converting enzyme, Antioxidant and antihypertensive properties [11, 13, 14]

The aim of this current study was to determine the effect of using different enzymes in lentil protein hydrolysis on antioxidant activities and to further determine the bioactive peptides based on molecular weight distribution. In addition, purification of lentil peptides was also carried out.

2. Materials and Methods

2.1. Materials

Lentils used in this study were purchased from a super market in Bangladesh and later transported to People's Republic of China where further analysis was conducted. Trypsin, Alcalase and Flavorzyme were purchased from a shanghai canspec scientific instruments co. ltd. DPPH was obtained from TCI, Shanghai, China. Pepsin, papain, tert-Butylhydroquinone (TBHQ) and ascorbic acid were all purchased from Sigma Chemical Co. (China). All the other solvents and chemicals used were of analytical grade.

2.2. Protein Extraction

Extraction of water-soluble protein from Lentils was prepared using the extraction method as described by Suliman [15] with minor modifications. Lentils were ground into powder using a blender. The powder was then soaked in water in the ratio of 1:15(w:w). The solution was homogenized for 1 hour at room temperature after the pH was adjusted to 8.5 using 0.5 M NaOH. It was further

homogenized for 30 minutes and was then left for 5 hours. The solution was then centrifuged at $4500 \times g$ for 10 mins and the clear supernatant was collected. The pH of the collected supernatant was adjusted to 4.5 using 1M HCl and was left for 30 mins at room temperature. Later, the solution was centrifuged at $4500 \times g$ for 10 mins and the pellet was washed with water and centrifuged again at $4500 \times g$ for 10 mins. The collected protein was later freeze dried.

2.3. Protein Analysis

The total nitrogen content of the sample was estimated by the kjeldahl method [16] and the nitrogen conversion factor of 6.25.protein percentage was 92%. Lowery method [17] and Bradford Protein Assay [18] were also used for determining the protein content in liquid form.

2.4. Moisture Content

The moisture content was determined using the method developed by Nielsen [19]. Samples were taken out of the refrigerator and brought to room temperature before they were exposed to microwave heating. A sample of approximately 2.5 g was weighed in a paper. Then the samples were transferred into crucible. The crucibles were placed in a micro-oven. The temperature of the micro-oven was 113°C . After six hours the samples were placed into the micor-oven. The crucibles and sample were taken out, weighed for four different times. The sample is heated under specified conditions and the loss of weight is used to calculate the moisture content of the sample

$$\% \text{ of Moisture content} = \frac{\text{wt of water in the sample}}{\text{wt of wet sample}} \times 100$$

2.5. Preparation of Lentils Hydrolysates

Lentils hydrolysates were prepared according to the method described by Cabanillas [20] under controlled hydrolysis conditions of pH, temperature, enzyme concentration and stirring speed using five enzymes and their combinations. Each enzyme was added separately to the lentil protein concentrate slurry using a specific enzyme/substrate (E/S) ratio (w/w) (based on protein content of the concentrates) and digested for a specific time while maintaining the optimal temperature and pH of the enzyme. Briefly, Lentil protein was suspended in distilled water (0.5 g/20 mL) and added under optimal conditions (55°C , 1 h, and pH 8.0). In order to dissolve the protein at the beginning, the pH was set at 11 using 0.5 M NaOH. After one hour, the pH was adjusted using 1M HCL. The tryptic digestion (E/S: 1/25) was conducted for 3 to 24 h at 37°C and pH 6.5. The Alcalase digestion (E/S: 1/8) was conducted for 30 min to 120 min at 55°C and pH 8. The pepsin digestion (E/S: 1/100) was conducted at 1 h to 3 h at 37°C and pH 2. The papain digestion (E/S: 1/25) was conducted for 15 min to 60 min at 37°C and pH 6.5 and the flavorzyme digestion (E/S: 1/10) was conducted for 30 min to 120 min at 37°C and pH 7. LH was hydrolysed using sequential digestion with

Trypsin/pepsin (TP), Trypsin/Alcalase (TA), Alcalase/Flavourzyme (AF), Alcalase/Pepsin (AP), Pepsin/Papain (PP). The hydrolysate was subsequently transferred to a water bath (90°C) for 10 min to inactivate the enzyme. After cooling, the supernatant was obtained by centrifugation at $4500 \times g$ for 10 min.

2.6. Determination of Total Phenolic Compound of Lentils

The total phenolic content was determined by the Folin-Ciocalteu colorimetric method according to Sarac [21]. 200 mL of lentils extract solution was prepared at a concentration of 1 mg/mL and transferred into test tube. 5 mL of 0.2 N Folin-Ciocalteu reagents was added and the test tube was shaken. The mixture was allowed to stand at room temperature for 3 mins. then 10 ml of 7 % (w/v) sodium carbonate was added, mixed gently and made the volume to 25 mL with distilled water. The test tube was incubated for one and half hours in dark with intermittent shaking. . The absorbance of all samples was measured at 760 nm.

Content of phenolic compound was determined as mg gallic acid equivalents per gram of extract (mg/g GAE extract) using the following linear equation based on the calibration curve: $A = 0.294 C$, $R^2 = 0.996$ where A is the

absorbance and C gallic acid equivalents.

2.7. Amino Acid Composition

Amino acid compositions of three fractions of lentils were determined by High Performance Liquid Chromatography (HPLC) according to (22). The samples were hydrolyzed with 6 N HCL for 24 h at 110 °C in sealed vials maintained under inert atmosphere (N₂). Amino acid composition was determined using an HPLC system (Agilent 1100 series) and a reverse phase column ((250*4.6) mm 5 μm ODS HYPERSIL) at 40 °C. The solvent used was sodium acetate (25 mM), containing Triethylamine (0.02%) at pH 7.20±0.05 in acetonitrile at a flow rate of 1.0 mL/min. Tryptophan was determined at 338nm, 262nm (Pro,Hypro) after basic hydrolysis with 4 M NaOH for 4 h at 110 °C.

2.8. Determination of Antioxidant Activity

2.8.1. Dpph Radical-Scavenging Activity

DPPH radical-scavenging activity was determined using the method of Sharma [10] and Xiaohong (23) with minor modifications. Two milliliter of the Lentils protein hydrolysate solution in distilled water was mixed with two mL of 0.1mmol/L DPPH in methanol. The mixture was then vigorously mixed and allowed to stand at room temperature in the dark for 30 mins. The absorbance of the mixture was measured at 517 nm using a spectrophotometer. The DPPH control was conducted in the same manner, but ethanol was used instead of DPPH. The sample control group contained distilled water instead of the sample. The range of accuracy for spectrophotometric measurements falls within an absorbance of 0.221–0.698[10].

DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity \%} = \left(1 - \frac{A_t - A_b}{A_c}\right) \times 100,$$

Where A_t is the absorbance of sample; A_b is the absorbance of the sample control; and A_c is the absorbance of the DPPH control.

2.8.2. Antioxidant Activity as Determined by B-Carotene-Linoleate Model System

The antioxidant activity of the lentil crude extracts was determined in an emulsion system according to (8). Briefly, methanolic solutions (0.2 mL) containing 2 mg of the crude extract was pipetted into a series of test tubes to which 5 mL of a prepared emulsion of linoleic acid and β-carotene stabilized by Tween 40 were added. Immediately after addition of the emulsion, the zero-time absorbance reading at 470 nm was recorded for each tube. Samples were held in a water bath at 50 °C and their absorbance values were recorded over a 120-min period at 15-min intervals. The assay was replicated three times for each sample and means ± standard deviation were reported (8).

2.9. Reducing Power

The reducing power of the hydrolysates was determined using Wang's [24] method with minor modifications. The

sample solution (4 mL) in ionized water with a solid concentration in the range of 2 mg/mL was mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH=6.6) and 2.5 mL of 1 % potassium ferric cyanide solution. The mixture was then kept in a water bath at 50 °C for 20 min. The resulting solution was cooled rapidly, and then mixed with 2.5 mL of 10 % TCA (m/V), and centrifuged at 3000 rpm for 10 min. Finally, the supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride solution. The absorbance of the resulting mixture was measured at 700 nm after the reducing reaction for 10 min. High absorbance indicates strong reducing power.

2.10. Hydroxyl Radical-Scavenging Activity

The hydroxyl radical-scavenging assay was determined using the method as described by Yanhong [25] with minor modifications. Both 1, 10-phenanthroline (2.5 mM/L) and FeSO₄ (2.5 mM/L) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. 30% H₂O₂ (20mM/L) and LPH fractions (1.5 mg/ml) were added. The mixture was incubated at 37°C for 60 min, and the absorbance was measured at 536 nm. Results were determined using the following equation:

$$\text{Antioxidant Activity} = \frac{A_s - A_o}{A_c - A_o} \times 100$$

Where A_s , absorbance of the sample; A_o , absorbance of control solution containing 1, 10-phenanthroline, FeSO₄ and H₂O₂; A_c , absorbance of blank solution containing 1, 10-phenanthroline and FeSO₄.

2.11. Sds-Polyacrylamide Gel Electrophoresis

SDSPAGE was carried out using the technique described by Laemmli [26] with a 4% stacking gel and a 7.5 % resolution gel. Electrophoresis was carried out at 70V to 120 V for Tris-HCl SDS-PAGE. The running buffers were 0.1 M Tris-HCl, 0.1% (w/v) SDS, for the SDS-PAGE of the protein concentrates and 0.1 M Tris-HCl, 0.1 M tricine, with 0.1% (w/v) SDS, for the SDS-PAGE of the hydrolysates [27]. Sample solutions (30μL) were prepared from 10 mg freeze-dried protein extract or hydrolysate dissolved in 10 μL sample buffer (distilled water, 0.5 M Tri- HCl pH 6.8, glycerol, 10% SDS, 1% bromophenol blue and β-mercaptoethanol) heated at 98 °C for 5 min, then applied to the sample wells. The standard protein marker (Precision Plus Protein Standard, Bio-Rad, USA) which contained myosin (250,000 Da), β-galactosidase (150,000 Da), phosphorylase b (100,000 Da), serum albumin (75,000 Da), ovalbumin (50,000 Da), carbonic anhydrase (37,000 Da), trypsin inhibitor (25,000 Da), lysozyme (20,000 Da) and aprotinin (10,000 Da), was used to prepare a standard curve for molecular weight estimation [28].

2.12. Peptide Fractionation by Ultra Filtration

Lentils Hydrolysate was fractionated consecutively through UF membranes [29] having MWCO of 5 kDa, 3kDa and 1 kDa. The first UF separation was performed

using a regenerated cellulose 5 kDa-membrane (Master Flex, 88 cm² * 0.11 M², Millipore Corp., Cassette Holder) at a transmembrane pressure of 5-10 bar. The permeate collected in this first stage was further ultrafiltered using a regenerated cellulose 5 kDa-membrane. This process yielded four fractions for LH: a retentate enriched in peptides >5 kDa, a permeate mainly composed of peptides <5 kDa, a second retentate enriched in peptides 5 -3 kDa, and a second permeate containing peptides >3 kDa, the third retentate enriched in peptides 3 - 1 kDa, and permeate containing peptides <1 kDa and lyophilized into powder form.

2.13. Purification of Antioxidant Peptide

Ultrafiltration fraction was subjected to Superdex Peptide 10/300 GL, prepacked Tricorn high-performance column for high-resolution gel filtration of peptide by using Akta Pure. Adsorbed materials were eluted with 10 mM Tris-HCl Buffer (pH 7.6) [30] gradient at 0.5 ml/min flow rate, 1.05 psi in the 280 nm wavelength region. All samples were clarified by centrifugation at 10000g for 10 min followed by filtration through 0.22 µm in-line filters. The column flow-through and wash fraction was first collected into a 1 ml loop and then applied to a customized superdex peptide 10/300 GL equilibrated with storage buffer containing 10 mM Tris-HCl. Protein was eluted with storage buffer, peaks were collected in 2 ml fractions and analyzed by the SDS PAGE stained with coomassie Brilliant Blue R. purification of ultrafiltration fraction takes about 7 hrs. The column was washed and re-equilibrated prior to the next purification cycle. The column, tubing and loop were washed between chromatography steps to avoid cross-contamination. The final peak fractions and all solutions that could contain target protein were collected [31].

2.14. Determination of Molecular Weight Distribution

The lentils peptide fractions from AKTA pure filtered through a 0.45-µm cellulose acetate filter (Millipore) before HPLC analysis. The chromatographic system comprised a HPLC system consisting of LC-10AD Vp pumps, UV-VIS SPD-M10A Vp photo-diode array detector, SCL-10A Vp system controller. The samples were injected into a TSK Gel G2000SW_{XL} (5 µm, 7.86 x 300 mm). The analytical conditions for separation are described by Magdalena [32]. Briefly, the mobile phase was employed for elution: Acetonitrile / water / trifluoroacetic acid (45/55/0.1 (V/V)). The flow rate was 0.5 mL/min from the time 0 to 30 min. The column was re-equilibrated between sample injections with 10 mL of acetonitrile and 25 mL of mobile phase. Diode array detection was performed by scanning over a wavelength within the range from 220 nm to 280 nm at an acquisition speed of 1 s. Cytochrome C (MW12384), Bacitracin (MW1450), Gly-Gly-Tyr-Arg (MW451), Gly-Gly-Gly (MW189) were used as molecular weight standards. Samples were analyzed in triplicate.

2.15. LC Mass

To determine the molecular mass and amino acid sequence of the purified Lentils peptide, MS/MS experiments were performed [33]. Approximately 5ml of the purified peptide solution was injected into BEH 130 2.1X100mm 1.7µm connected to the waters acquity uplc. the mass spectrometer with a speed of 400mL/min, and the molecular mass was determined by a single charged (M+H)⁺¹ state in the mass spectrum via the electrospray interface. The ESI nebulizing gas and auxiliary gas used high purity nitrogen. Spectra were recorded over the mass/charge (m/z) range of 150-2000. The following parameters were used: electrospray voltage of 1700 V; heated capillary temperature of 400°C; the desolvation gas flow was 700 lit/hr and the cone gas flow was 50 lit/hr. collision energy of 6 volts; and activation time of 30 ms. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem mass spectroscopy (MS/MS) analysis. Data were processed using Masslynx (v4) software.

3. Results and Discussions

The total phenolic content of lentils extract measured by Folin-Ciocalteu colorimetric method was found 55 mg GAE/g extract. Other studies have previously reported a value of 58 mg/gm for the red lentils [8]. Amino acid compositions of Lentils and Lentils hydrolysates showed in table 1. The amino acid compositions of Lentils extract indicated that it possesses high proportions of Asp and Glu, which are usually storage proteins of legumes.

Table 1. Amino Acid composition of Lentils fractions (g/100g Protein).

Amino Acids	extrac protein	Hydrolysed ultrafiltration fractions	AKTA pure fractions
asp	10.31	9.98	1.33×10 ⁻¹
glu	17.50	17.63	1.28×10 ⁻¹
ser	3.61	3.50	6.63×10 ⁻²
his	2.29	2.18	5.47×10 ⁻²
gly	3.43	3.33	7.51×10 ⁻²
thr	2.69	2.51	4.86×10 ⁻²
arg	7.21	7.08	3.52×10 ⁻¹
ala	3.59	3.37	3.39×10 ⁻¹
tyr	2.45	2.26	7.19×10 ⁻³
cys-s	3.06×10 ⁻¹	2.72×10 ⁻¹	1.82×10 ⁻²
val	5.12	4.94	1.14×10 ⁻¹
met	8.32×10 ⁻¹	8.04×10 ⁻¹	2.44×10 ⁻²
phe	5.36	4.66	1.37×10 ⁻¹
ile	4.97	4.34	6.67×10 ⁻²
leu	8.04	7.15	1.74×10 ⁻¹
lys	6.44	6.33	1.01×10 ⁻¹
total	84.21	80.43	1.84

3.1. Sds-Page of the Lentils Protein of Different Concentration

Results of protein composition of lentils as determined by SDS-PAGE are shown in Fig 2. The protein profile of the lentils extract can be seen in lane 1,2,3 in different

percentages. SDS-PAGE pattern contained five major protein bands and seven weak bands. Lentils protein concentrates showed a multitude of bands ranging in size from approximately 25–100 kDa. Electrophoretic profile shows the extract was composed of at least seven polypeptides ranging from 25 to 100 kDa. All the concentrates showed bands in the range of 25–37 to 38–45 kDa corresponding to the basic and acidic subunits respectively. There are two bands that had molecular weights corresponding to 48 kDa and 65 kDa which probably represent the subunits of vicilin (48 kDa) and convicilin (63 kDa), respectively [34,35,36]. There are no significant differences among all the three lanes.

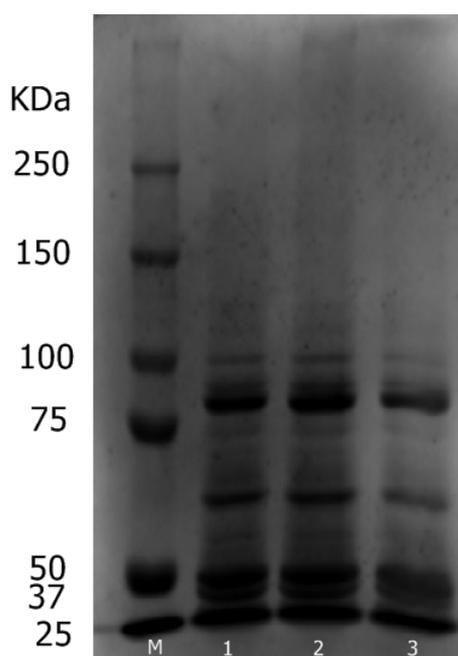


Fig. 2. SDS-PAGE separation of lentil protein in different concentrations; 1: 5% with loading buffer; 2: 3% with loading buffer; 3: 1% with loading buffer.

The enzyme Alcalase prefers to cleave peptides at the interior of the polypeptide chain and it can hydrolyze into peptide which has better and nutritional characteristics than the original proteins. Alcalase also can generate bioactive peptides [36]. Flavorzyme is a protease complex from

Aspergillus oryzae with endopeptidase and exopeptidase activities [23]. Pepsin was found to cleave peptides that fit well into the active site. Therefore it preferentially cleaves some peptides better than others [37]. Papain, a widely commercial protease in bioprocesses, used for the obvious morphology change and the enhanced catalytic efficiency indicated the practical value of the new p-CLEAs on catalysis of macromolecules [38].

3.2. Enzymatic Hydrolysis of Lentils Protein

Lentils proteins were hydrolyzed by using Alcalase, Flavourzyme, Pepsin, Trypsin and Papain with their identified optimum conditions for producing the antioxidant peptides. The antioxidant activity of the Lentils hydrolysates was determined using DPPD, Free radical scavenging activity, reducing power and β -carotene-linoleate model system as describe previously. In free radical scavenging activities against DPPH, pepsin and combination of pepsin-trypsin hydrolysate revealed the most potent free radical scavenging ability. SY Kim [39] previously reported that peptic hydrolysate had the highest antioxidant activity, further antioxidant lentils peptide has produced by hydrolyzing of combination of two different enzymes. Results for the analysis using DPPD are presented in Figure 1(a,b). It is evidently clear that the combined use of trypsin and pepsin had the highest antioxidant activity and the Lentils hydrolysates prepared by pepsin alone also showed high antioxidant activity. The lentils hydrolysate by pepsin and pepsin-trypsin further separated eight molecular weight groups using ultrafiltration membranes (MWCO=5,3 and 1 kDa) into > 5 kDa, 5-3 kDa, 3-1 kDa, <1 kDa. The antioxidant activity of the four enzymes as determined by DPPD assay showed differences as reflected in picture (c). The result indicated that the < 1kDa group of pepsin-trypsin has the highest antioxidant activity than the other groups. After ultrafiltrating the pepsin fractions, it did not show the obvious ability to scavenge the DPPH free radical. So for further investigation, the pepsin-trypsin <1 kDa fraction was used. The IC_{50} value is 1.395 mg/ml as shown in the figure (d).

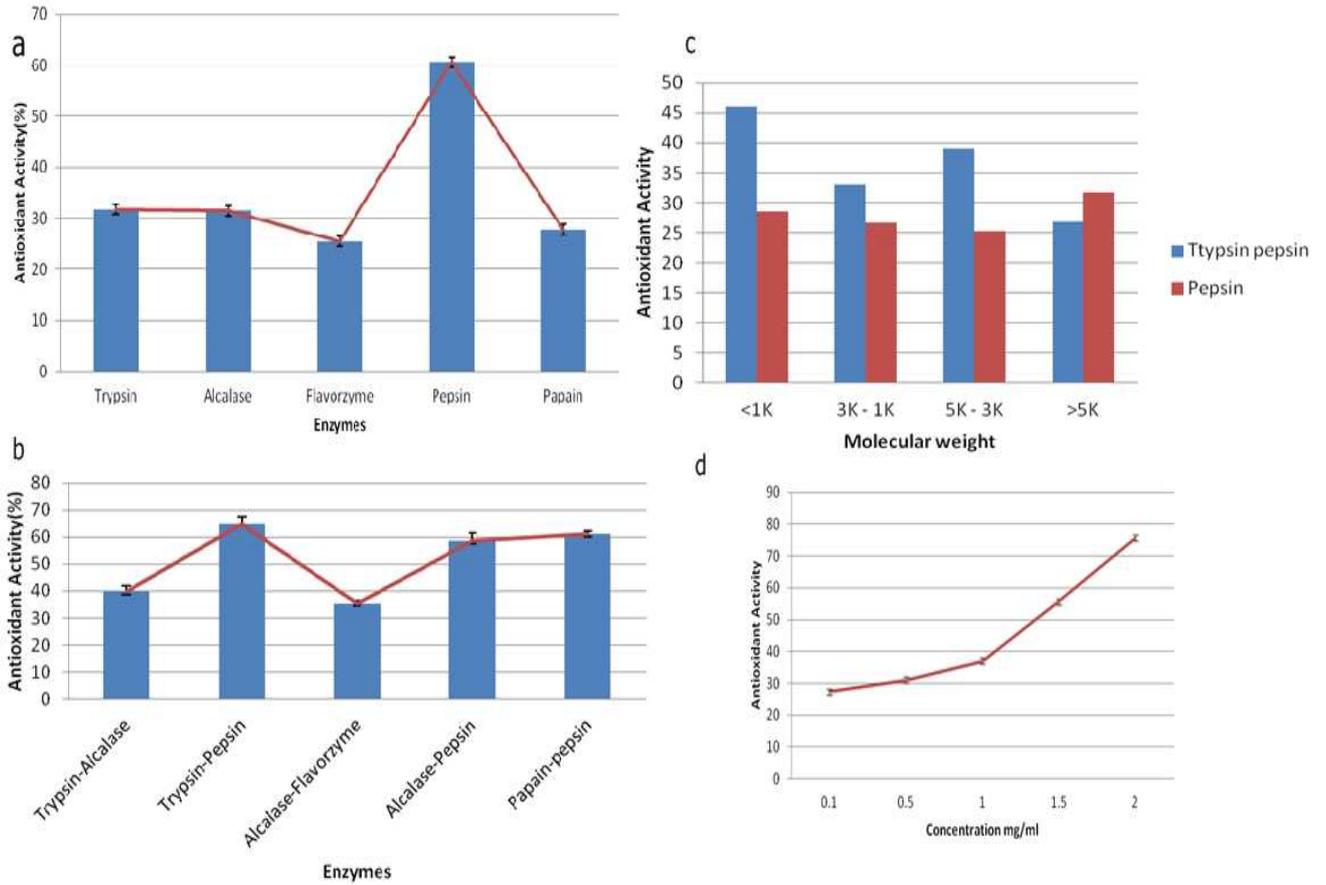


Fig. 1. The DPPH antioxidant activity of lentils hydrolysates by five different enzymes (a) , five different enzymes combinations (b), ultrafiltration fractions of Trypsin-pepsin and pepsin (c) and Trypsin-Pepsin hydrolysate in different concentration (d) with controlled conditions.

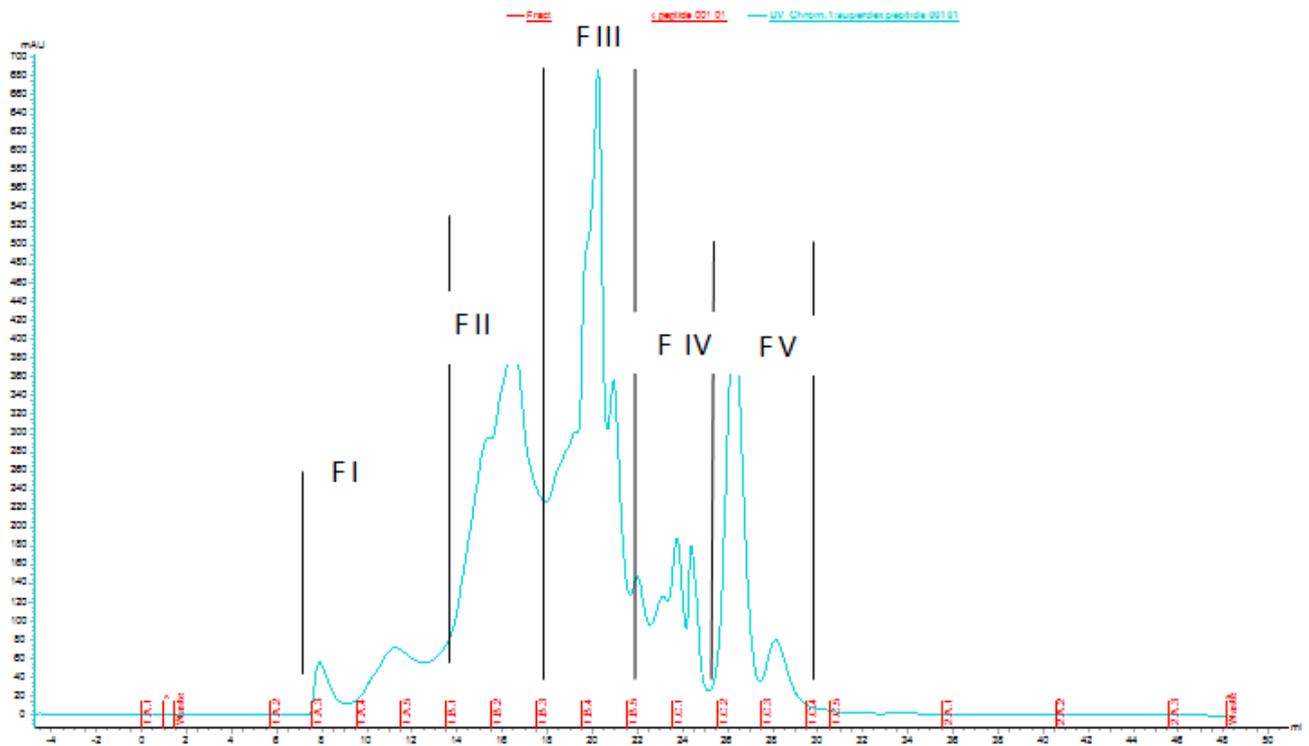


Fig. 3. Separation of antioxidant peptides from Lentils hydrolysate ultrafiltration fraction by Superdex Peptide 10/300 GL, prepacked Tricorn high-performance column for high-resolution gel filtration of peptide by using Akta Pure .Sample was eluted with Tris-HCl Buffer,Ph 7.6 at aflow rate of 0.5 mL/min in the 280 nm wavelength region. Five peptide fractions (Fra.I, Fra.II, Fra.III,Fra IV and Fra V) were obtained according to molecular size.

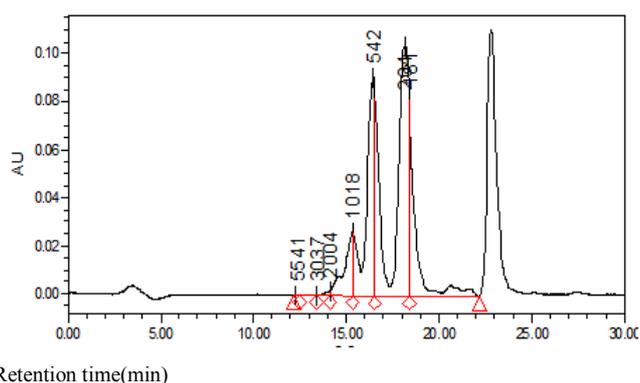


Fig. 4. HPLC pattern on a TSK Gel G2000SW_{XL} (5 μ m, 7.86 x 300 mm) column of the Fra.III fraction obtained from Superdex Peptide 10/300 GL at 220 nm. The resulting chromatograms contained two major peaks (retaining time 17.6 and 18.4 min) corresponding to the target peptide and a number of peaks corresponds to unidentified substances.

3.3. Antioxidant Activity of Fraction III

Results for the antioxidant Activity of the lentils fraction III are shown in Table 2. The absorbance was recorded at 2 mg concentration. The antioxidant activities of Lentils Fraction III were measured in β -carotene-linoleate model system, reducing power, hydroxyl radical-scavenging and DPPH radical-scavenging activity. As shown in Table. 2, the autoxidation of linoleic acid was 73%. During oxidation, a hydrogen atom was abstracted from the active bis-allylic methylene group of linoleic acid located on carbon-11 between two double bonds. The pentadienyl free radical so formed then attacked highly unsaturated β -carotene molecules in an effort to reacquire a hydrogen atom. As the β -carotene molecules lost their conjugation, the carotenoids lost their characteristic orange colour; this process was monitored spectrophotometrically. Unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal-ion chelation and enzyme inhibition, brought about by various additives [4]. Based on loss of absorbance at the 517 λ_{\max} for DPPH the Frac III has shown 63% scavenging activity. The results of the reducing power were weaker than the reference used. The reducing power of bioactive compounds had been reported to be associated with their antioxidant activity [40]. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound [25]. The reducing power assay is often used to evaluate the ability of antioxidant proteins and peptides to donate electrons. In this assay, the ability of lentils fraction III to reduce Fe^{3+} to Fe^{2+} was determined. The presence of antioxidants in tested samples results in the reduction of Fe^{3+} /ferricyanide complex to ferrous form. The reducing power of lentils fraction III has been shown in Table 2. Free radicals with the major species of reactive oxygen species (ROS) are unstable, and react readily with other groups or substances in the body, resulting in cell damage and hence human disease, especially; the chemical activity of

hydroxyl radical is the strongest among the ROS. It is easily react with biomolecules such as amino acids, proteins [41]. in this study Lentils fraction III has shown 40% of hydroxyl radical.

Table 2. Antioxidant activity of Lentils Fraction III

Reducing Power	0.288
Antioxidant activity as determined by β -carotene-linoleate model system(%)	73
DPPH radical-scavenging activity (%)	63
Hydroxyl radical-scavenging activity (%)	40

3.4. AKTA Pure Analysis

For further Purification of the pepsin-trypsin fraction AKTA purifier system was employed. After Hiload Superdex Peptide 10/300 GL column separation, chromatograms were recorded automatically as shown in figure 3. The operations of data collection were driven by UNICORN software which guarantees quick, simple communications between systems and users, and meets the stringent control and data handling procedures of modern laboratories. Five fractions were collected. The fractions were then concentrated and freeze dried. DPPH antioxidant activity was determined for each of the fraction. The activities of the fractions were 23%, 30%, 63%, 26% and 41% respectively. Fraction III was found to show the highest antioxidant activity among all the fractions.

3.5. HPLC Analysis

In order to identify the peptides, Fraction III from AKTA pure was subjected to HPLC analysis. Results as presented in Fig 4 showed the chromatographic profile corresponding to the analysis of lentils AKTA pure fraction III. The resulting chromatogram contains two major peaks. The retaining time for the peaks was 17.6 and 18.4 min which corresponded to the target peptide and the unidentified substances.

3.6. LC Mass Analysis

The Fraction III was also subjected to LC/MS for the calculated m/z values of the different multiply charged fraction III forms. Molecular mass determination has been done for lentils AKTA pure fraction III. The MS spectrum is shown in Fig:5, and the MS/MS spectrum of a single charged ion with m/z 540 Da and this is in agreement with findings previously reported on chymotrypsin lentils hydrolysate which had a molecular weight of <556 Da [32].

Peptides were identified automatically by Masslynx software, Complete LC-MS/MS runs were saved as *.raw files. The peptide mass range was set with the range of 200-1800 amu. The precursor ions tolerance and fragment ions tolerance was set to 2 amu and 1.00 amu, respectively. The match result gave a putative amino acid sequence: Ala- Leu- Gly- Pro- Val- Met (587.32Da)

Some authors have previously reported that the antioxidant activity of peptide is the inverse of the molecular weight. This meant that low molecular weight peptide of less than 20

amino acids has highest antioxidant activity and those less molecular weight peptide can cross the intestinal barrier and exert biological effects(23).

20 biologically amino acids are potentially oxidizable, They contain reactive amino acids containing nucleophilic sulfur-containing side chain (cysteine and methionine) or

aromatic side chains (tryptophan, tyrosine, and phenylalanine) and they can also abstracted hydrogen easily. Some chains which contain Histidine are also oxidatively labile. On the other hand, cysteine, methionine, tryptophan, tyrosine, phenylalanine, and histidine, are formed by common oxidation(42).

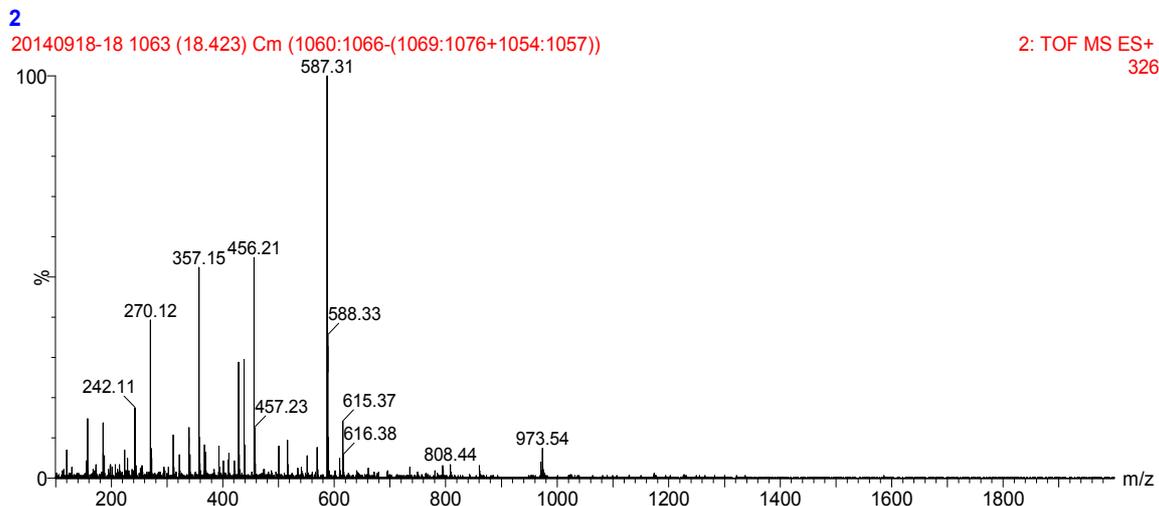


Fig. 5. The MS spectrum of Fraction-III (587.32Da).

4. Conclusions

The findings in this study has shown that lentils proteins were effectively hydrolyzed by five enzymes (Alcalase, Flavorzyme, Trypsin, pepsin, papin) to obtain peptides with the best antioxidant activities. The purification of the lentils depended on the DPPH radical-scavenging activity .The results of the present work indicated that Fra.III possessed high DPPH radical- scavenging activity and β -carotene-linoleate model system. By gel permeation chromatography on Superdex Peptide 10/300 GL, the Lentils fraction III antioxidant peptide was purified from Lentils hydrolysates, and the molecular weight and sequence were identified by LC-MS analysis as 587.31 Da and the amino acid sequence was as follows: Ala- Leu- Gly- Pro- Val- Met.

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