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Original Article

IN-VITRO FREE-RADICAL SCAVENGING POTENTIAL OF OLIGOPEPTIDES DERIVED FROM WHEAT AND MUNG BEAN

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ABSTRACT

Objective: Therapeutic potential of peptides were well established, but only few information were available on the *in vitro* antioxidant activities of peptides isolated from wheat and mung bean. Therefore, the aim of this present work is to evaluate the *in vitro* antioxidant potential of low and high molecular weight oligopeptides separately isolated from one-week old wheat [*Triticum aestivum* L.] and mung bean [*Vigna radiata* Wilczek.] seedlings.

Methods: Peptides of different molecular weight range were isolated through cryo-crushing followed by solvent partitioning and ion-exchange column and were filtered through the Millipore ultrafiltration system. Purified peptides derived from both samples were assessed for their antioxidant activity.

Results: Both ranges of peptides were found to possess significant antioxidant activity. But peptides of low molecular weight (LMW) range (0.5 to 3 kDa) seems to be comparatively more efficient in scavenging free radicals such as DPPH, $ABTS^{+}$, nitric oxide as well as superoxide than peptides of high molecular weight (HMW), ranging between 3 to 10 kDa. Peptides of LMW from wheat highly responded to metal chelating in comparison to peptides of LMW isolated from mung bean.

Conclusion: These peptides may constitute an important part of the antioxidant defense system and could be used for the formulation of Functional foods and Nutraceuticals.

Keywords: Oligopeptide, Antioxidant, Ultrafiltration, Wheat, Mung bean.

INTRODUCTION

Reactive Oxygen Species (ROS) like hydrogen peroxide, hydroxyl radical, superoxide anion are constantly produced in the form of free radicals in the living cell through oxidative metabolic pathways [1]. These free radicals are responsible for causing diseases like cancer, multiple sclerosis, inflammation, coronary heart disease, cardiovascular disease, arthritis, etc. Antioxidant components are capable of minimizing the effects of these free radicals and thus, help in the prevention of these diseases [2] and also used for preservation in many food industries [3]. The inhibitory response of proteins towards oxidation reactions makes them a vital component of an antioxidant based defense system [4].

In recent years, numerous naturally occurring bioactive peptides have been isolated from different living organisms such as bacteria, fungi, plant, and animals, which are found to be involved in the regulation of physiological processes in plants like, induction of α-amylase synthesis, regulation of stomatal opening in the dark etc. [5]. Recently, the antioxidant potential of peptides derived from different plant samples has created a great attention among scientists. The peptides isolated from various plants like soybean [6], maize [7], chickpea [8], pea seed [9], buckwheat [10], alfalfa leaves [11] have been reported to possess potent antioxidant activity. The natural antioxidants based on peptides isolated from plant sample are effectively used for extending the storage period of food [12-14]. The antioxidant activities are affected by the molecular weight of peptides and also the composition and sequences of amino acid present in that particular peptides [15].

Several works have been done on high and low molecular weight peptides of various legumes and grains reporting their biochemical and physiological roles, but the variation in their antioxidant potential is yet to be revealed. In this present study, an attempt was made to determine the antioxidant potential of peptides isolated from wheat and mung bean seedlings. The peptides of different molecular weight, ranging between 0.5 to 3 kDa and 3 to 10 kDa were worked out and a clear line of difference was established on the antioxidant activity of these isolated peptides.

MATERIALS AND METHODS

Plant culture

Seeds of mung bean [*Vigna radiata* (L) Wilczek. cv. Sonali B1] and wheat [*Triticum aestivum* L. (cv. Sonalika RR-21), collected from the Central Pulses Research Institute (C. P. R. I.), Berhampur, West Bengal, India was weighed out (250 mg each) and allowed to culture in sterile Petri-plates with absorbent cotton supplied with the modified Hoagland solution with one-half strength major nutrients and full-strength micronutrients. The nutrient solution was aerated continuously. Experiments were conducted inside a controlled environment growth chamber with the following conditions: 14h light period with a light intensity of 350 μmol. m^{-2}. s^{-1}, 25°C/20 °C day/night temperature, and 80% relative humidity. The plant materials were grown for one week or specific germination hours, and used for definite experimental purposes.

Extraction and isolation of low molecular weight peptides

Peptides from wheat and mung bean seedling were isolated according to the methods of Jha *et al.* [16] with some modifications. Briefly 100g of 7d old whole wheat and mung bean seedlings for each set, were washed separately with tap water and cut into pieces, washed with water again and 0.2% sodium hypochlorite solution to avoid excessive contamination and finally washed with distilled water. The seedling pieces were frozen in liquid nitrogen and crushed and extracted with chilled distilled water with a measured amount by blender at 4 °C in a cold room. The material was centrifuged at 10,000 rpm for 30 min using protease inhibitor PMSF at 4 °C to remove the unwanted materials. The supernatants were subjected to ether wash at acidic pH to remove endogenous hormonal impurities, fats, lipids and oil as impurities and stored in deep freeze (-20 °C) for further study.

Ion exchange chromatography

The extracts were purified through cation and anion exchange resin (Dowex 50 and Dowex 1, Sigma Chemical Co., USA), filled inside two-glass column (60 cm × 2.9 cm, 1.6 meq/ml.) [17]. Freshly prepared 3(N) ammonia and 1(N) HCl were used for the elution of those peptides from the cation and anion exchange column respectively. The ammonia and hydrochloric acid were made free from extract solution through a liquid nitrogen trap fitted to a Lyophilizer (Lyolab BII). The whole extract was freeze dried to smaller volumes.

Ultrafiltration

The lyophilized material obtained from each set of respective germination hours were separately undergone ultra-filtration through a Millipore stirred cell fitted with 10 kDa (YM10), 3 kDa (YM3) and 0.5 kDa (YC05) cut-off ultrafilter membrane (Amicon made) and the filtrate between 3 to 10 kDa and 0.5 to 3 kDa were separately collected [18]. Precautions were made for removal of amino acids from plant extract by using 0.5 kDa cut-off ultrafiltration for three times in each case. The ultrafiltered samples were lyophilized and dissolved in 10 ml distilled water (for each set) and stored in deep freeze (-20 ˚C) for further use. The samples were repetitively filtered and finally purified, and dry extracts were obtained.

Purification and analysis of isolated peptides through HPLC

The semi-purified, concentrated peptide(s) extract from 7d old seedlings of wheat and mung bean were passed through C_{18} HPLC, Waters™486 reverse phase column in 10% spectral grade methanol as running solvent fitted with 515 HPLC pump, runtime 60 min, absorbance at 250 nm, column length 3.9×150 mm, injection volume 20 µl, and purified by using pump pressure 4000 psi with flow rate setting at 0.5-1.0 ml/min [16]. The peptide(s) appeared at different retention time were repeatedly tried and purified, concentrated and collected in deep freeze under-20 ˚C. Each peak was isolated with their retention time and re-injected into the column to check its repetitive occurrence.

DPPH-Scavenging activity

DPPH has been extensively used to determine the free radical-scavenging activity of samples due to its relative stability. The radical scavenging activity was measured by the DPPH method [19]. The reaction mixture contained 1.8 ml of 0.1 mM DPPH and 0.2 ml of aqueous extracts. The reaction mixture was kept in dark at room temperature for 30 min. The absorbance was measured at 517 nm. As a control, only reaction mixture without sample was taken. Percentage of inhibition was calculated according to this equation:

$$\text{Percentage inhibition of DPPH activity} = \{(A_0 - A_1)/A_0\} \times 100$$

Where,

A_0 = absorbance value of the blank sample.

A_1 = absorbance value of the tested sample.

A curve of inhibition percent or percent scavenging rate against sample concentrations was determined from where IC_{50} (concentration of the sample required to inhibit half of free radicals) of testing samples were calculated.

ABTS⁺ scavenging activity

The spectrophotometric analysis of $ABTS^+$ radical cation (s) scavenging activity was determined according to Re *et al.* [20] method. This method is based on the ability of antioxidants to quench the $ABTS^+$ radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of BHT. The $ABTS^+$ was obtained by reacting 7 mM $ABTS^+$ radical cation (s) in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 12h. Before usage, the $ABTS^+$ solution was diluted to get an absorbance of 0.750±0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, 2 ml of $ABTS^+$ solution was added to 1 ml of the aqueous extract. After 30 min, absorbance value was recorded at 734 nm, relative to a blank absorbance. The percentage inhibition of the samples was calculated as:

$$\text{Percentage inhibition} = \{1 - (A_1/A_0)\} \times 100$$

Where,

A_0 = absorbance at 734 nm of the blank

A_1 = absorbance at 734 nm of the sample mixture.

Reducing power

The reducing power assay was performed to follow the method of Oyaizu [21] with some modifications. Sample extracts were diluted at different concentrations with 2.5 ml of the 0.2 M phosphate buffer (pH: 7.0) and 2.5 ml of 1% potassium ferricyanide solution were then added. The mixtures were incubated at 50˚C for 20 min in a water bath. After cooling at room temperature, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. 2.5 ml upper layer of the supernatant was mixed with 2.5 ml of distilled water and 250 µl of 0.1% aqueous ferric chloride. The fluorescent green colour appeared and the absorbance of the final solution was recorded at 700 nm.

Metal chelating activity

Determination of chelation of iron (II) ions by extracts was carried out as described by Dinis *et al.*, [22]. To a mixture of 400 µl of extracts and 1.6 ml of methanol, 40 µl of 2 mM $FeCl_2$ solution and 80 µl of 5 mM ferrozine were added. After 10 min of incubation, the absorbance of the solution was recorded at 562 nm.

Nitric oxide scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction [23]. 216 µl Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride), 360 µl (5 mM) sodium nitroprusside-PBS solutions and 320 µl extract was mixed. The reaction mixture was incubated at 25 °C for one hour. Lastly, 2 ml water was added, and finally absorbance was taken at 546 nm.

Radical scavenging activity was expressed as percentage inhibition from the given formula:

$$\text{Percentage inhibition of NO radical} = \{(A_0 - A_1)/A_0\} \times 100$$

Where,

A_0 = absorbance of control

A_1 = absorbance of the sample.

Superoxide scavenging activity

According to Nishikimi *et al.* [24] methods, the superoxide radical scavenging activity was measured with slight modification. The reaction mixture contained 1 ml of methanolic extract, 1 ml of NBT solution and 1 ml of NADH solution. 100 µl of PMS was added to the reaction mixture after 5 min incubation. The reactant was incubated at 25˚C for 30 min and absorbance was measured at 560 nm against methanol as a control.

Data analysis

Principal component analysis (PCA) of antioxidant activity of different source of peptides was analyzed by using XLSTAT 2015 software. Pearson (n) type PCA was used for data analysis.

RESULTS

DPPH & $ABTS^{\cdot+}$scavenging activity

The results of DPPH and $ABTS^+$ free radical-scavenging activity of purified peptides are shown in table 1. Both free radical-scavenging activity of peptide samples isolated from wheat and mung bean increased in a concentration-dependent manner. The result indicated that the DPPH and $ABTS^+$ radical scavenging activities of the peptides are molecular weight dependent. The LMW peptides exhibited better DPPH radical scavenging activities than the HMW. Higher antioxidant activity, *i.e.* lower IC_{50} values was exhibited by LMW wheat peptides in comparison to HMW peptides which showed higher IC_{50} values. Mung bean peptides also showed a similar trend like wheat where LMW peptides have better scavenging potential than HMW peptides.

Reducing power assay

In reducing power assay, the reducing capacity of a biological compound plays a significant indicator of its potential antioxidant activity [25]. As shown in table 1, the low molecular peptides exhibited effective reducing ability which increased with an increase in sample concentration. The reducing ability of the peptides was determined by ascorbic acid equivalent. The lower ascorbic acid equivalent value indicates lower reducing the ability of samples. LMW peptides of wheat exhibited higher ascorbic acid equivalent value when compared with that of mung bean. The LMW peptides had significantly higher reducing power when compared to those of HMW peptide(s).

Metal chelating activity

The chelation of transition metal ions $Fe^{2+}$and $Cu^{2+}$by antioxidative peptides could check the oxidation reaction [22]. From table 1, it can be stated that the metal chelating activity is higher in mung bean in comparison to wheat.

Nitric oxide scavenging activity

The nitric oxide generated from sodium nitroprusside reacts with oxygen and form nitrite. The extract inhibits nitrite formation by contending with oxygen to react with nitric oxide directly and also inhibits its synthesis [26]. In these experiment peptides from wheat showed higher nitric oxide scavenging activity than peptides from mung bean. In HMW IC_{50} value of wheat and mung bean was much higher than their LMW counterparts. Like previous observations, nitric oxide scavenging activities of these peptides are molecular weight dependent.

Superoxide scavenging activity

Superoxide is considered as an initial free radical which is formed from mitochondrial electron transport systems, to create other cell-damaging free radicals, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen [27]. Peptides can protect the cell against toxic effects of some superoxide free radical [28]. The results shown in table 1 clearly indicates that peptides isolated from wheat have higher superoxide scavenging potential than the mung bean peptides because wheat perceived low IC_{50} values, *i.e.*, highly effective antioxidant activity. It was also observed that in both the samples low molecular weight peptides exhibited stronger superoxide activity than large size peptides.

Table 1: Antioxidant activities [IC_{50} values (µg/ml)] of peptide(s) isolated from 7d old seedling of wheat and mung bean at two different molecular weight ranges (0.5-3 kDa & 3-10 kDa)

Free radical scavenging attributes	Wheat	Mung bean	Wheat	Mung bean
	0.5-3 kDa		3-10 kDa	
DPPH	21.66±0.49[a]	98.89±0.673[b]	529.85±0.514[c]	701.753±0.867[d]
$ABTS^{+}$	174.64±0.81[a]	248.97±0.92[c]	243.56±0.84[b]	702.70±1.03[d]
Superoxide	389.14±0.94[a]	594.89±0.83[c]	515.97±0.76[b]	691.67±0.97[d]
Nitric oxide	949.33±1.05[a]	1490.33±0.94[c]	1205.86±0.89[b]	1788.00±1.04[d]
Metal chelating	325.29±0.61[a]	452.50±0.57[b]	616.15±0.72[c]	665.56±0.49[d]
Reducing power	190±0.43[a]	94.36±0.66[c]	149.9±0.57[b]	81.66±0.81[d]

Results are represented as mean±SEM of three replicates (n = 3). Values with different letters (a, b, c and d) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT).

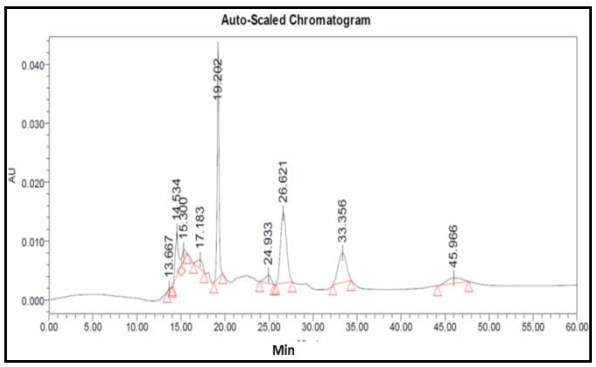

Fig. 1: HPLC Chromatogram of Wheat LMW peptide fraction

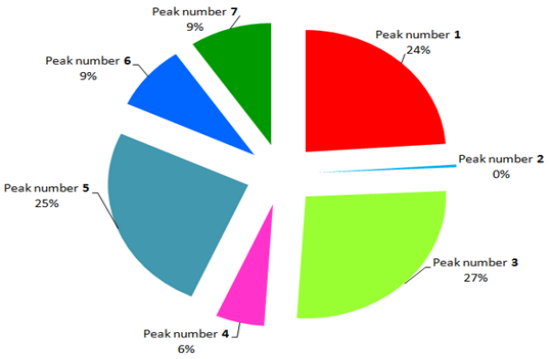

Fig. 3a: HPLC peak area of wheat LMW peptide fraction

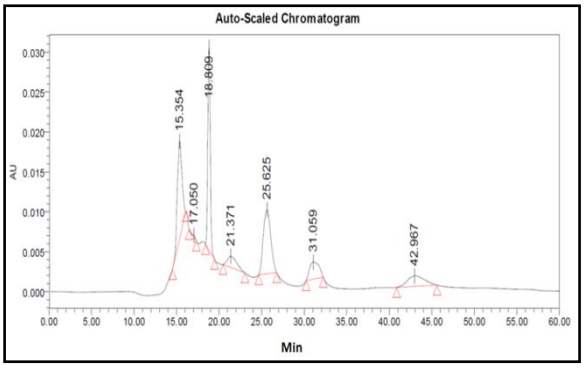

Fig. 2: HPLC Chromatogram of *Vigna* LMW peptide fraction

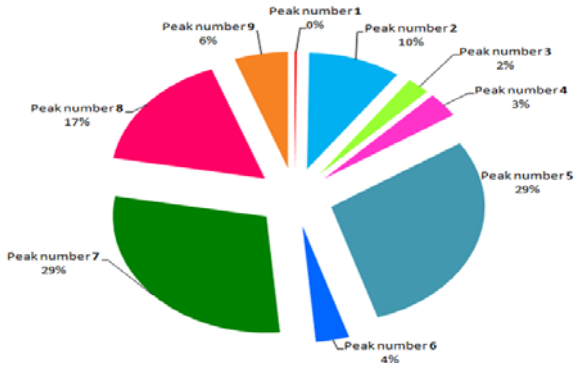

Fig. 3b: HPLC peak area of *Vigna* LMW peptide fraction

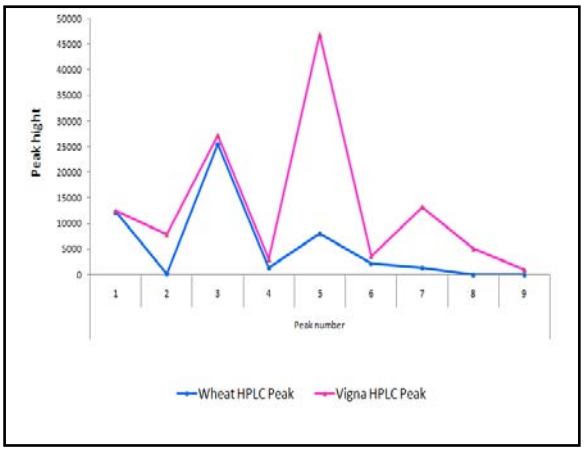

Fig. 4: HPLC peak height of wheat and *Vigna* peptides

HPLC analysis of isolated peptides

HPLC analysis was performed with LMW peptides because strong bioactivity was obtained only with LMW peptide fractions. The HPLC chromatogram profile of isolated LMW peptide(s) extract from 7d old seedlings of wheat and mung bean clearly revealed a specific pattern according to their stationary and mobile phase interaction (fig. 1, fig. 2). This separation pattern appears on the retention time of peptide and helped us to purify the exact peptide(s) fractions and concentrated it for repeated cycles. In the case of wheat, six peaks were clearly identified, whereas nine peaks were detected with mung bean peptides. The measurement of the amount (concentration) of a peptide in the sample is conducted by two ways, first by determination of the peak height from the baseline, and secondly, by determination of the peak area. In many cases, a linear relationship was found in between height or area and the concentration of peptides. The area and height of the wheat and the mung bean peptide peak appeared in HPLC chromatogram detector is represented in fig. 3 (a, b) and fig. 4 respectively.

Principal component analysis

Principal component analysis (PCA) was performed to find out the existing relationship (if any) among different antioxidant activities of isolated two peptide sources. Biplot of PCA analysis showed 98.27% cumulative variability (fig. 5) with PC1 and PC2. HMW Peptides formed a cluster, whereas it had a negative loading from LMW peptides. ABTS, DPPH and Metal chelating (MC) had negative loading from reducing power (RP), superoxide (SO) and nitric oxide (NO).

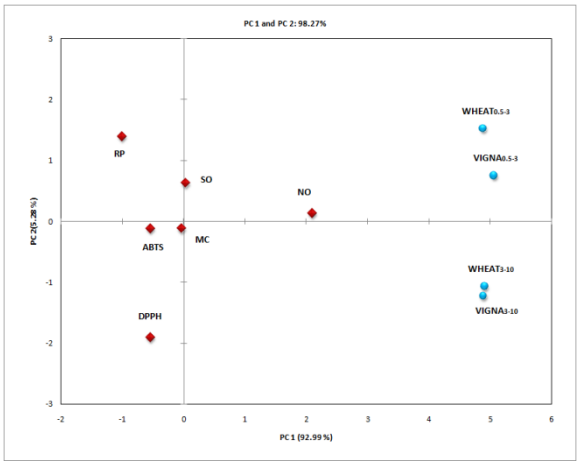

Fig. 5: Biplot of PCA analysis of different antioxidant activities of two peptides source

DISCUSSION

The antioxidant activity of isolated peptides was molecular weight dependent. Our result is in agreement with the results reported previously in quinoa [29] and hemp seed [30], which showed that LMW peptide fractions had higher DPPH scavenging activities than HMW peptides. In between 0.5-3 kDa, the higher antioxidant potential was demonstrated by peptides as observed in case of peptides isolated from mulberry leaves also [16]. It was also observed that wheat peptides have comparatively higher scavenging activity than mung bean peptides in both molecular ranges. The results signify that smaller size peptides exhibited better-reducing power than high molecular weight fractions. In contrast, Girgih *et al.* [30] reported that the reducing power activity was enhanced to increase in molecular weight of peptides in hemp seed protein. Through PCA analysis, it might be stated that superoxide, nitric oxide and reducing power characterized the antioxidant activities of wheat and *Vigna* peptides at 0.5-3 kDa ranges. On the other hand, ABTS and DPPH influenced antioxidant activities of HMW peptides.

Carboxyl and an amino group in the side chains of the acidic (Glx and Asx) and basic (Lys, His and Arg) amino acids play an important role in chelating metal ions [31]. Histidine residues have been reported to contribute to the metal chelating effect of protein hydrolysates, which is commonly related to its imidazole ring [32]. Similarly, Li *et al.* [8] reported that low molecular weight peptides from chickpea protein hydrolysates exhibited strong superoxide radical scavenging activity and was found to contain higher concentrations of Phenylalanine, Isoleucine, Leucine and Valine in comparison to other fractions. Therefore, it can be suggested that the superoxide scavenging activity may be related to the concentration of these hydrophobic amino acids. From HPLC analysis, it might be concluded that our isolated peptide fraction contains more than one peptide chains which appeared as separate peaks at different retention time.

CONCLUSION

The results of this study showed that peptides derived from seedlings of wheat and mung bean possesses antioxidant properties. Small sized peptides (0.5 to 3 kDa) seem to be more effective scavengers of free radical such as ABTS, DPPH, nitric oxide and superoxide. The peptides isolated from wheat seedling had better antioxidant properties than peptides of mung bean. Hence, these peptides might be used for the formulation of Functional foods and Nutraceuticals. Also, these seedlings might be explored as a natural source of antioxidants and preservatives in the food industry for storage of food products and to maintain freshness during production.

ABBREVIATION

DPPH**:** 1,1-diphenyl-2-picrylhydrazyl, $ABTS^{+}$**:**2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid) Diammonium salt, LMW**:** Low molecular weight, HMW**:** High molecular weight

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CONFLICTS OF INTERESTS

None of the authors have any competing interests to declare

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