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NUTRACEUTICAL FROM HEMP (*CANNABIS SATIVA* L.) AND FUNCTIONAL EVALUATION

Ranjana Das*[1]

[1]*Department of Chemical Engineering, Jadavpur University, Kolkata, India.

ABSTRACT

Hemp (Cannabis sativa L.) seed, an inevitable byproduct during commercial exploitation of valuable hemp fibre, is rich source of high quality oil (30%) and protein (25%). This protein has superior essential amino acid composition and easily digested. Hemp seed protein isolate have poor solubility relative to soy protein isolate owing to protein aggregation which greatly deteriorates functional properties of hemp protein isolate. The poor activity of hemp protein isolate could be effectively improved by enzymatic hydrolysis to add nutritional value and potential health effects. This investigation aims utilisation of nonconventional hemp proteins for human nutrition. Results present significant radical scavenging activity, reducing activity and Fe^{+2} chelating activity at 2h of hydrolysis which decreases on further hydrolysis. In comparison to synthetic antioxidant hemp protein hydrolysate requires about 1.8 times higher does for similar radical scavenging activity representing a good potential of hemp protein hydrolysate to be applied as safe antioxidant.

KEYWORDS

Hemp, Metallo-endopeptidase, Protein hydrolysate, Functional peptide and DPPH radical.

Author for Correspondence:

Ranjana Das,

Department of Chemical Engineering,

Jadavpur University, Kolkata, India

Email: ranjanads78@gmail.com

INTRODUCTION

Hemp, *Cannabis sativa* L. belongs to Cannabaceae family. Hemp is a widely cultivated plant of industrial importance, as an important source of food components, medicinal components and fiber. Historically hemp has been used primarily for the fiber cultigens and its fiber preparations. *C. sativa* was most valued as a fiber source, and only to a limited extent as an oilseed crop. In Canada, hemp is grown under license (under the Controlled Drugs and Substances Act) is presented as a crop of immense

nutritional prospect. Because of the lacking of extensive researches in the field of the hemp utilization practices, this study stresses on the potential of hemp based products; with special emphasis on hemp seed based value added components. Hemp seed has high levels of vitamins A, C and E and beta carotene, and it is also rich source of protein (25%)[1], carbohydrates, minerals and fiber[2]. Hemp seed is described as a rich source of protein and sulphur containing amino acids, and the concentration of methionine in protein isolates is higher in hemp than in soy bean[3]. A low THC form of industrial hemp is now legal to grow in several countries, and the global market for low THC containing hemp is increasing speedily[4]. Hemp seed has been confirmed as an excellent source of nutrition when fed to laying hens[5] and pigeons[6]. Thus, the proteins from hempseed have good potential to be applied as a valuable source of protein nutrition. The proteins (mainly edestin and albumin) in hempseed have well balanced amino acid compositions and easy digestibility. Osborne reported some properties of the globulin protein (edestin and edestan) from hempseed[7]. The functional properties of hemp protein isolate (HPI), as reported in several publications are poor as compared to soy protein isolate (SPI). In the hemp protein isolate (HPI), the edestin contributes about 80% of total hemp protein content. Hemp being good source of protein but its utilisation in food system is restricted due to poor protein solubility. The poor functional properties significantly limit the application of hemp protein in different food formulations. Among different approaches (physical, chemical and enzymatic) of protein structural modifications, enzymatic treatments have been widely applied to modify the functional properties of animal and plant and proteins, with modification of protein structure. Typically, the enzymatic modification is preferred because of milder process conditions required, stress-free control of the reaction and minimum possibilities of by-products formation[8,9]. In enzymatic modification processes, enzymatic hydrolysis has been widely used to improvise the functional properties of proteins, such as solubility, emulsification, water- and fat- holding capacities, gelation properties, foaming ability, and to tailor the functionality of certain proteins to achieve specific needs[10]. The aim of the present study is to synthesis hemp protein hydrolysate using a novel type enzyme membrane reactor with immobilized enzyme to add value to the hemp meal and to improve the functionality hemp protein isolate.

MATERIAL AND METHODS

MATERIAL

Low tetrahydrocannabiol (THC) containing hemp seeds were purchased from Granny Organics, Dehradun with specification as presented in Table No.1. α α′ -diphenyl-1-picryl-hydrazyl (DPPH) was purchased from Sigma Aldrich Chemical Co., USA. 'Protease A Amano 2G' enzymes was kind gift from Amano enzyme Inc. Nagoa Japan. The enzymatic membrane bioreactor used for synthesis of the protein hydrolysate is described in previous study[11] (Das *et al*, 2009). The module is a dead end module with volume 500mL. The enzyme used in the study is a metallo- endopeptidase was a kind gift from Nagoa Japan.

Protein Isolate Preparation from Hemp Seed and Determination of Protein Content

Hemp meal (HM) was prepared by cold extraction using hexane at 24±1°C. Hemp protein isolate (HPI) was prepared from defatted hemp meal using isoelectric point precipitation method[11]. HPI was used as the substrate for enzymatic hydrolysis process to get hemp protein hydrolysate. Defatted hemp meal was mixed with 20 times (w/v) deionized water at temperature of 30±1°C, and the solution was adjusted at pH 9.0 with 1(N) NaOH and agitated for 1 h. Samples were centrifuged at 10000×g for 20 min at 25°C. The supernatant obtained after centrifugation was adjusted to pH 4.9 with 1 (N) HCl, and the precipitate was collected again by centrifugation (10000×g, 10 min). The collected mass was freeze-dried to produce HPI. Soluble protein content of hemp protein isolate (HPI) was measured by standard method of Lowry et al. (1951)[12], using bovine serum albumin (BSA) as standard at 750 nm.

Protein Hydrolysate Preparation, Evaluation of Degree of Hydrolysis and Mass Distribution of Hydrolysate

Precipitated protein isolate was mixed in DI water (1: 20 w/v) and pH was adjusted according to the properties of the enzyme used with continuous stirring to achieve desired extent of hydrolysis. To the homogeneous solution of the protein isolate, enzymes were added with changing enzyme to substrate ratio of 0.1-2 (w/w), and hydrolysis was carried out at 35±2°C. Hydrolysate was immediately immersed in boiling water bath (95°C) for 1 min to inactivate the enzyme action. The solution then stored at 4°C for further analysis.

Degree of hydrolysis was determined following the method of Alder–Nissen, (1979) using 2, 4, 6-trinitrobenzene sulphonic acid[13]. For protein hydrolysate and different peptide fractions the molecular weight distributions were examined in RP-HPLC system (Cyber Lab, Millbury, USA) with C18 column (Phenomenex; dimention: 4.6×250 mm, 5µm) at 25°C. Elution profile was monitored at 220nm.

Radical scavenging activity (RSA) of protein hydrolysate

RSA of hemp peptide fractions were estimated in (DPPH•) system. DPPH•-radical scavenging activity of peptides was determined following the method as described by Yen and Wu, (1999)[14]. Radical scavenging activity of the hydrolysates /peptide fractions were expressed as IC_{50}, the concentration (µg/L) required to scavenging half of the initial free radical concentration in the reaction system (IC_{50}). Results are also expressed as percent inhibition of DPPH as shown in equation.

$$\text{Inhibition percent} = [\text{Abs(control)} - \text{Abs(sample)}] / \text{Abs(control)} \times 100$$

Evaluation of reducing activity and fe^{+2}chelation activity

The reducing activity of the peptide fractions are evaluated according to the method of Tang *et al*. (2009) with some modifications[15]. The reducing activity of HPH and HPI was observed as absorbance at 700nm. Higher absorbance of the reaction mixture indicated superior reducing power.

The Fe^{+2} ion chelating ability was determined by the method of Decker and Welch (1990)[16]. 5mL sample was mixed with 0.2mmole/L $FeCl^{+2}$ and 0.2mL 5mmole/L ferrozine solution. After 10 minutes of reaction absorbance was recorded at 562nm. Low absorbance signifies high Fe^{+2} ion chelation capacity. Results are presented as percent chelation capacity of the sample.

STATISTICAL ANALYSIS

All results are presented as mean± standard error of mean (SEM), of three determinations. Statistical analysis was done using Analysis of Variance (ANOVA) approach and value of $p < 0.05$ was considered statistically significant. MATLAB 7.0 software was used for statistical analysis.

RESULTS AND DISCUSSION

Characteristics of Hemp Protein Isolate and Respective Hydrolysate

Chemical composition analysis of HPI showed relatively less ash content and fiber content in protein isolate and is illustrated in Table No.1. Protein isolates of about 1g were used for controlled hydrolysis with varying enzyme concentration from 0.1-2 w/w %, to get respective protein hydrolysate, which is regarded as source of small peptides. Analysis of Variance shows that, enzyme type and enzyme dose have significant effect on degree of hydrolysis (p=0.008), but no synergistic/interactive effect of these two parameters (p=0.8771) was observed. With an aim to optimize the peptide population and their activity, enzyme dose was maintained upto 2%; further increase in enzyme dose was found to lower functional activity of protein hydrolysates, probably due to formation of more free amino acids and too small peptides with irrelevant activity. Further studies were carried out to estimate the activity profile of protein hydrolysate as extent of hydrolysis cannot simply be correlated with activity of protein hydrolysates.

DPPH-Radical Scavenging Behaviour of Hemp Protein Hydrolysate

The stable DPPH radical in ethanol is comprehensively used to test the radical scavenging ability of various compounds. DPPH in methanol

(absorption maxima at 517 nm) is very stable free radical system used to examine radical scavenging activity and antioxidant potential. In induced oxidation system, free radicals propagate the oxidative chain reactions and accelerate oxidation process. Components having the properties of scavenging free radicals serve the purpose of antioxidant. In publication by Shimada *et al*. (1994)[17] it was illustrated that, DPPH radical on encounter with a proton-donating substance (an antioxidant), get scavenged, thus terminating the radical chain reactions and the absorbance of control solution get reduced. In this study, IC_{50} value (sample concentration that effective in reducing initial DPPH concentration by 50%) is used to elucidate the scavenging activity. Lower the IC_{50} value signifies higher free radical scavenging ability. The IC_{50} value of the hydrolysates gradually decreased with increasing hydrolysis time from 60 to 240 min (Table No.2).

In our observation, protein hydrolysates exhibit radical scavenging activity when compared with synthetic antioxidant BHT (Table No.2). Radical scavenging activity of this enzyme modified protein hydrolysates found to vary significantly ($p<0.05$) with enzyme type and extent of hydrolysis. During hydrolysis a mixture of small peptides and free amino acids are produced depending on enzyme type, antioxidative potential of those peptide mixtures and amino acids depend on the nature of the amino acid residue at the side chain of generated peptides as well as sizes of the amino acids. June *et al*. (2004)[18] has also highlighted the dependence of antioxidant activity on protease nature and hydrolysis condition employed. 'Protease A' modified protein hydrolysate (P_A), exhibits maximum radical scavenging activity of 5.2% at degree of hydrolysis 30%. Variation of radical scavenging activity with dosage of enzyme is presented in Figure No.1. An optimum of radical scavenging ability for different protein hydrolysates was observed near 30% DH; activity further reduces on increasing degree of hydrolysis for the enzyme types used in this study. Inverse co-relation between radical scavenging activity and degree of hydrolysis may results due to the formation of single amino acid residues which may exhibits comparatively lower radical scavenging properties or overturn activity of short peptides.

The reducing capacity of a compound depicts inherent antioxidant potential. Reducing activity was assayed by measuring absorbance at 700 nm to monitor the amount of Fe^{+3} reduced to Fe^{+2}. The hydrolysates exhibited comparatively higher reducing power that compared to protein isolate and comparable activity to synthetic antioxidant BHT (Table No.2). This suggests that the hydrolysate obtained by 'Protease A' has the potency to exhibit excellent reducing power.

The antioxidant activity of protein hydrolysate is also related to the metal ion chelation capability as metal ions acts as pro-oxidants and propagate oxidative chain reactions. In this work, the Fe^{2+}chelating capability was assayed by the reduction of absorbance for 'Fe^{2+}-ferrozine' complex and results are presented as IC_{50} value (concentration of sample required to decrease initial absorbance value by 50%). The hydrolysates obtained in 60-180 minute exhibited Fe^{2+}chelating ability in range of 1.6–1.4mg/mL, while the Fe^{2+}chelating ability for synthetic antioxidant BHT was 0.98 mg/mL. The activity of hydrolysate significantly decreased when the hydrolysis time was increased up to 240 minute (Figure No.1). The Fe^{2+}chelating ability of these protein hydrolysate obtained by Protease A is highly significant in comparison to synthetic antioxidants.

Table No.1: Proximate analysis of hemp seed

S.No	Parameters	Percent content
1	Moisture	2.83±0.20
2	Carbohydrate	29.20±0.10
3	Protein	24.00±0.40
4	Oil	32.60±0.10
5	Ash	4.65±0.30
6	Fiber	17.80±0.20

Table No.2: Antioxidant properties of hemp protein hydrolysate

S.No	Sample	Antioxidant activity		
		DPPH scavenging activity (IC_{50}) (mg/L)	Reducing power Abs at 700nm	Fe^{+2} chelating activity (mg/L)
1	BHT[a]	1.2±0.1	0.58+0.03	0.98±0.02
2	HPH60[b]	3.2±0.1	0.39±0.02	1.6±0.10
3	HPH120 [b]	2.7±0.2	0.32±0.04	1.3±0.20
4	HPH180 [b]	2.5±0.1	0.29±0.02	1.4±0.10
5	HPH240 [b]	2.2±0.1	0.28±0.02	1.9±0.30

[a]Butylated hydroxyltoluene;[b] Hemp protein hydrolysate (HPH) at respective times of 60, 120, 180 and 240 minutes

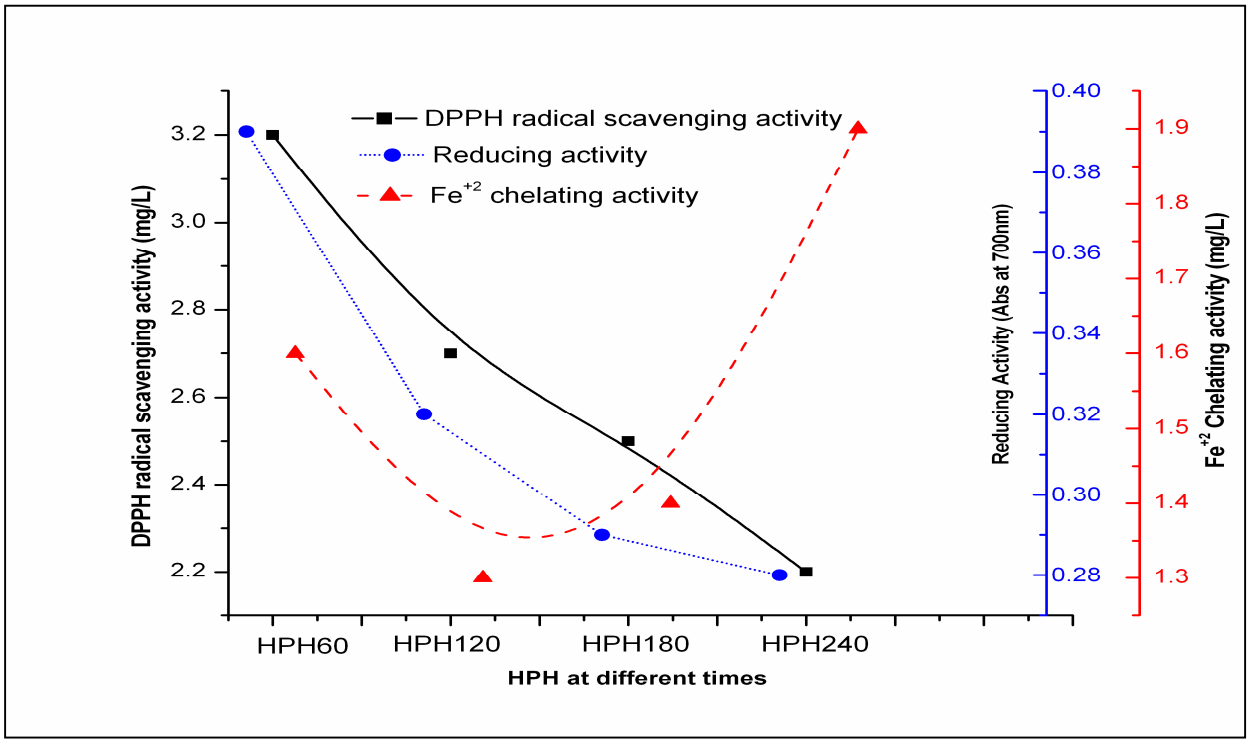

Figure No.1: Antioxidant potential of hemp protein hydrolysate at different time of hydrolysis

CONCLUSION

The principal goal of the study was to examine the activity of some commercially used endoproteinase for synthesis of protein hydrolysate from hemp seed meal as a source of bioactive peptides. Radical scavenging activity of protein hydrolysates were analyzed in simulated DPPH radical system. 'Protease A' modified protein hydrolysate, exhibited superior radical scavenging activity at 30% degree of hydrolysis; and shows better radical scavenging activity, reducing properties and metal ion chelation ability. The findings indicates about the relevance of metallo-endopeptidase 'Protease A' as promising proteinase for preparation of bioactive peptides from seed protein sources. In comparison to commercially used synthetic antioxidant butylated hydroxyl toluene (BHT), HPH requires about 1.8 times, 1.5 times and 1.5 times higher doses for similar radical scavenging activity, reducing activity and chelating activity respectively which presents promising antioxidant nature for hemp protein hydrolysate. Results point that, these protein hydrolysates can serve as food grade preservatives in improving the storage life of food emulsions as a radical scavenging agent. Determination of the amino acid profile and characterization of the protein hydrolysates hope to be reported in our upcoming publications.

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CONFLICT OF INTEREST

I declare that I have no conflict of Interest.

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