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Characterization of Antioxidant Peptides of Soybean Protein Hydrolysate

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***Abstract*—**In order to characterize the soy protein hydrolysate obtained in this study, gel chromatography on Sephadex G-25 was used to perform the separation of the peptide mixture and electrophoresis in SDS-polyacrylamide gel has been employed. Protein hydrolysate gave high antioxidant activities, but didn't give any antimicrobial activities. The antioxidant activities of protein hydrolysate was in the same trend of peptide content which gave high antioxidant activities and high peptide content between fractions 15 to 50. With increasing peptide concentrations, the scavenging effect on DPPH radical increased until about 70%, thereafter reaching a plateau. In compare to different concentrations of BHA, which exhibited higher activity (90%), soybean protein hydrolysate exhibited high antioxidant activities (70%) at a concentration of 1.45 mg/ml at fraction 25. Electrophoresis analysis indicated that, low-MW hydrolysate fractions (F1) appeared, on average, to have higher DPPH scavenging activities than high-MW fractions. These results revealed that soybean peptides probably contain substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules.

***Keywords*—**Antioxidant peptides, hydrolysis, protein hydrolysate, peptide fractions.

I. Introduction

FREE radicals generated by exogenous chemicals or endogenous metabolic processes in food systems may cause oxidative damage by oxidizing biomolecules resulting in cell death and tissue damage [1]. In the past few years, there has been increasing interest in research into antioxidants such as phenolic compounds and antioxidant peptides, since they can protect the human body from free radicals and retard the progress of many chronic diseases [2], [3]. The antioxidant activity of proteins, peptides and amino acids has been widely studied since this effect was firstly reported by [4]. Recent articles deal with the protection of protein fractions and peptides from porcine proteins [5], [6], eggs [7], fish [8]-[11] and from legumes and oilseeds [12] offer against oxidation. A number of studies have been devoted to assess the antioxidant potential of soy protein fractions as well as the isolation and structural characterization of the most active peptides, which contained 5–16 amino acid residues which affect the antioxidant power due to their sequence [13], [14]. The relationship structure activity has been established for soybean peptides, which present hydrophobic amino acids and one or more residues of His, Pro, Tyr and Trp. As it has been observed for other antioxidants, the activity of peptides depends on the assay considered [15], [16]. The antioxidant properties of soy protein hydrolyzates have been ascribed the cooperative effected by the a number of properties, including their ability to scavenge free radicals, to act as metal-ion chelator, oxygen quencher or hydrogen donor and to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets [17]. Several biological activities (angiotensin converting enzyme inhibition, anti-thrombotic, antihypertensive, antioxidative properties) have been reported for soy peptides [18]. The operational conditions employed in the processing of protein isolates, the type of protease and the degree of hydrolysis affect the antioxidant and biological activities [19].

The structural analysis of antioxidative peptides from soybean protein isolate hydrolysate (b-conglycinin) was studied by [20] and several studies investigated the antioxidant activities of proteins upon hydrolysis [21], [22].

This study aimed to produce of hydrolyzed peptides from soybean in order to analysis its structural and to investigate its antioxidant activities.

II..Materials and Methods

A.Materials

Plant materials: soybean (*Glycine ma*) was obtained from Agriculture Research Center, Giza, Egypt.

B.Methods of Analysis

1. Soy Protein Isolates (SPI)

One hundred grams of soybean were grounded and defatted using hexane using Sohxlet apparatus according to the method described by [23].

C.Enzymatic Hydrolysis

The enzymatic hydrolysis was carried out according to [20]. SPI (1.0g) was dissolved in 33ml of distilled water and 30mg protease was added to the protein solution after the pH was properly adjusted. The enzymatic hydrolysis was performed at pH 8.0, 38^{o}C for Papain. After digestion, hydrolysates were heated in boiling water for 3min to inactivate proteases, and then the contents were neutralized and centrifuged at 4000Xg for 20min. The supernatants were stored at –18^{o}C until use.

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1.Determination of Degree of Hydrolysis

The determination of free amino groups was determined by the method described by [24]. The protein hydrolysate was dissolved/ dispersed in hot 1% sodium dodecyl sulfate at a concentration of 0.25-2.5 X 10-3 amino equivalents/l. A sample solution (0.250ml) was mixed with 2 ml of 0.2125M sodium phosphate buffer (pH 8.2) and 2ml of 0.1% trinitrobenzenesulfonic acid (TNBS) and the mixture was incubated in dark for 60min. at 50°C. The reaction was quenched by adding 4ml of 0.1 N HCl and the absorbance was measured at 340nm using Unicam UV 300 Thermo N.J. L-leucine solution 1.5mM was used as the standard. Transformation of the measured leucine amino equivalents to degree of hydrolysis was carried out by means of a standard curve for each particular protein substrate. The degree of hydrolysis (DH) was calculated according to the following equation:

$$DH = \frac{\text{Free amino group}}{\text{Total peptide bonds}}$$

2.Estimation of the Average Peptide Chain Length

The average peptide length (PCL) in the hydrolysate was estimated according to [25] by the determination of the free amino group using TNBS reaction as described above and the PCL was calculated according to the following equation:

$$PCL = \frac{100}{\%\, DH}$$

D.Protein Characteristics

1.Size Exclusion Chromatography

The soy protein hydrolysate and Natto were dissolved in 20 ml of 0.1N acetic acid (30g/100ml) and applied on a column (5.0X90cm) filled with sephadex G-25. The elution was realized at a flow rate of 10ml/min at room temperature using 0.1N acetic acid as a mobile phase. Eluted compounds were detected at 254nm. Twenty ml of the fractions were collected and storage at -18ºC until use [26].

2.Determination of Peptide Content

Peptide was determined according to the o-phthaldialdehyde method of [22]. A volume of 50µl of the sample was added to 2ml o-phthaldialdehyde (OPA) mixture 50ml of a mixture containing 25ml of 100mM sodium tetraborate, 2.5ml of 20% (w/w) sodium dodecyl sulfate (SDS), 40mg of OPA dissolved in 1ml methanol, 100µl of β-mercaptoethanol, and 21.4ml distilled water. After the incubation for 2min at room temperature, the absorbance was read at 340nm. The peptide content was calculated on the basis of the standard curve constructed by using L-glutathione (reduced form) as standard.

3.Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This analysis was performed using the procedure described by [27]. Runs were carried out with 40g/l and 120g/l acrylamide gels (stacking and separating, respectively). The following continuous buffer system was used: 0.375mol/l Tris-HCl, pH 8.8, 1g/l SDS for the separating gel; 0.025mol/l Tris-HCl, 0.192mol/l glycine and 1g/l SDS, pH 8.3 for the running buffer, and 0.125mol/l Tris-HCl, pH 6.8, 200ml/l glycerol, 10g/l SDS, and 0.5g/l bromophenol blue as sample buffer. For runs under reducing conditions the sample buffer contained 50ml/l 2-mercaptoethanol (2-ME) and samples were heated for 60s in a boiling water bath. The following protein molecular mass standards were used: phosphorylase b (94 kDa); bovine serum albumin (67kDa); ovalbumin (45kDa); carbonic anhydrase (30kDa); trypsin inhibitor (20.1kDa); a-lactalbumin (14.4kDa). Gels were fixed and stained with Coomassie Brilliant Blue Stain. Gels images were acquired with the Gel Doc 1000 Image Analysis System (Bio-Rad, Richmond, CA, U.S.A.) and analyzed with the Molecular Analyst Software (Bio-Rad) in order to determine the molecular masses of the polypeptides and the relative intensity of the bands.

4.Amino Acids Analysis

Samples were hydrolysis with 6N HCl at 110°C for 24 hs and free amino acids (FAA) were determined without acid hydrolysis according to the method described by [20]. Total amino acids (TAA) and free amino acids (FAA) were estimated by Automatic Amino Acid Analyzer LC3000 Eppendorf (Germany), with the following conditions: flow rate 0.2ml/min pressure of buffer 0 - 50 bar pressure of reagent 0 –150 and reaction temperature 123°C.

5.Measurement of Antioxidative Activity of Peptides (DPPH scavenging activities)

The antioxidant activity of the samples was measured using the modified method of [22]. An aliquot 0.1ml of the DPPH radical solution (0.25mM, in ethanol) and 0.1ml of the sample were added to the microplate and the mixture was shaken vigorously for 20min in the dark. The decrease in absorbance was read at 520nm against a blank (without sample). The DPPH radical scavenging activity of the sample was compared with that of a reference standard, BHA. Therefore, BHA equation value expressed the antioxidant activity of sample in terms of the mg of BHA. A standard curve was prepared by measuring the reduction in absorbance of the DPPH radical solution at different concentrations of BHA over a period of 20min. The DPPH radical scavenging activity was determined as follows:

$$\text{DPPH scavenging effect \%} = A_{blank}-A_{sample} / A_{blank} \times 100$$

where A = absorbance blank = all reagents except sample.

E.Statistical Analysis

Data were subjected to statistical analysis using the General Linear Models Procedure of the Statistical Analysis System [28]. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio [29]. All statements of significance were based on probability of $P < 0.05$. The correlation calculation was carried out using

ToolPack to determine whether two ranges of data move together.

III. Results and Discussion

As a result of enzyme activity, protein was hydrolysed into large, small peptides and amino acids. This was depending on the type of enzyme and the protein.

A. Degree of Hydrolysis and Peptide Chain Length (PCL)

For the evaluation of the degree of hydrolysis achieved by enzymes hydrolysates, trinitrobenzosulfonic acid (TNBS) was used by measuring TNBS reactant substances (NH2 groups, α–amino groups released as a result of enzyme activity) which was 22% allowing it to give a peptide chain length of 5-7 amino acid residues.

B. Isolation and Purification of Peptides

Hydrolysate was fractionated into peptide groups, these was made by Sephadex G-25 column. A 350ml of elution (18 fractions) was collected before hading the peptides. Fig. 1 shows peptides fractions of soybean protein hydrolysates. Every fraction was tested for its peptide content and antioxidant activities.

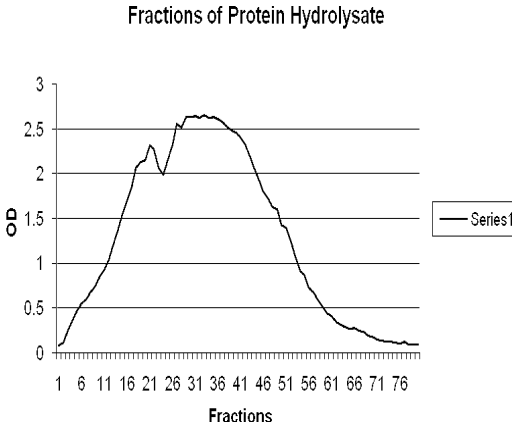

Fig. 1 Fractions of soybean protein hydrolysate

C. Electrophoresis

Electrophoresis in SDS-polyacrylamide gel has been employed. However, difficulties were occurred when these methods are applied to hydrolysates that contain low molecular weight peptides. Ionic and hydrophobic interactions between the solute and the gel used in exclusion chromatography are more pronounced in peptide separation and the logarithmic ratio between the molecular weight and the elution position is reduced. These ionic and hydrophobic effects resulted in differences in amino acid composition especially the aromatic ones. Predominate over the normal behavior of gel permeation in Sephadex G-25, which is dependent on the size of the peptides. These problems were similar to those reported by [25].

The data presented in Fig. 2 shows the SDS–PAGE of soy flour (F), protein Hydrolysate (H), protein Hydrolysate fractions (Hf), fermented soybean natto (N), and natto fractions (Nf): (lanes 3-13). The major protein subunits can be clearly identified in the initial soybean flour: 7S (β-conglycinin) is consisted of α, β subunits 11S (glycinin) is consisted of acidic subunits (A) and basic subunits (B) (lane2). After hydrolysis or fermentation (lane 4, 5), the 7S subunits of protein degraded more than 11S subunits to some extent (lane 6-13). Most of the 7S and 11S subunits in soybean were hydrolyzed to low-molecular weight peptides and recorded 45, 31, 20 kDa or amino acids after hydrolysis of soybean protein with protease (lane 4) whereas after fermentation with *Bacillus subtilis* the virtually all proteins have disappeared (lane 5) from the gel except 20kDa band. In comparison, the degree of hydrolysis of soybean protein hydrolysate was lower than that of the natto (lanes 4, 5). Furthermore, protein hydrolysate fractions showed varied range of molecular weight between 44kDa and 6kDa and some free amino acids (lane 6-9), However soybean fermented natto fractions mostly contain free amino acids and small amount of peptides with molecular weight 2-14kDa (lane10-13) which suggested that the bacteria used in this study degraded protein with exo-peptidase activities resulting in free amino acids. Similar to these observations were reported by [22] in okara hydrolysate.

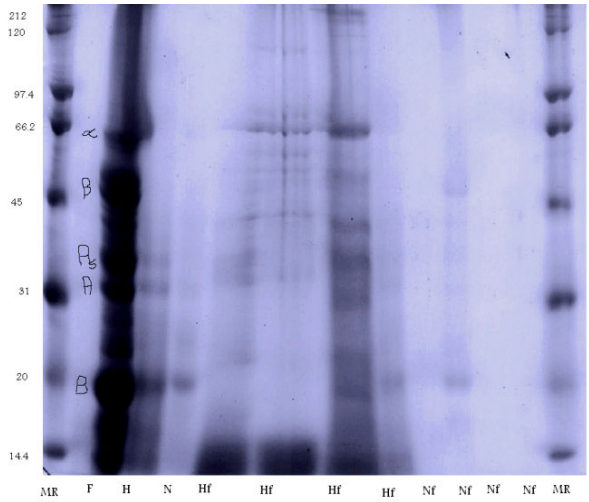

Fig. 2 SDS PAGE profile of soybean flour (F), soybean protein hydrolysate (H), fermented soybean natto (N), protein hydrolysate fractions (Hf), natto fractions (Nf) and protein marker (MR): Myosin (212kDa), β-Galactosidase (120kDa), Phosphorylase B (97.4kDa), Bovine Serum Albumin (66.2kDa), Ovalbumin (45kDa), Carbonic Anhydrase (31kDa), Soybean Trypsin Inhibitor (20kDa) and Lysozyme (14.4kDa)

D. Peptides Content of Fractions

The data of peptide concentration of each fraction was analyzed are presented in Fig. 3 These data shows the difference between the concentrations of fractions, which had a high content between fraction 10 and 60 and it was ranging from 0.1 to 1.45mg/ml of elution.

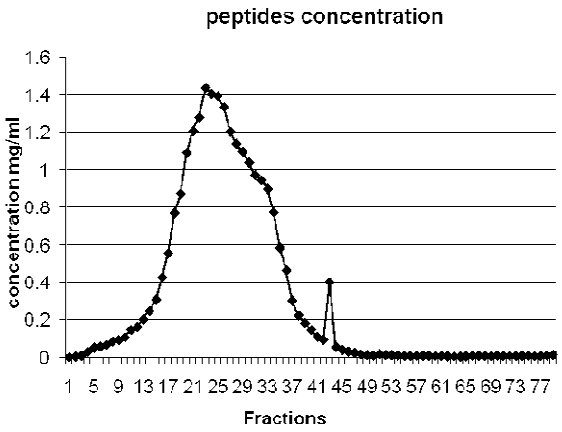

Fig. 3 Peptide concentration in mg/ml of protein hydrolysate fractions

E.Amino Acid Analysis

Table I shows the amino acid composition of each of soybean flour, soybean protein isolate (SPI), soybean protein isolate hydrolysate (SPIH) and free amino acids (FAA). The sample of SPIH was rich in glutamic acid (17.9%) compared to SPI (1.0%) although there was a reduction in the concentration of ASP, THR, PRO, LEU, ILE, PHE, TYR, HIS, LYS and ARG when compared to SPI. The free amino acids showed different trend; being rich in HIS (18.7%), ILEU (18.6%), TYR (13.1%), PHE (9.8%), LYS (7.9%), LEU (7.1%), and VAL (7.0%) and the remainder amino acids have lower levels [25]. Came previously to similar results and explained the large amount of the essential amino acids released as free form to the specificity of enzymes used.

TABLE I
AMINOACID COMPOSITION (MOL %) OF DIFFERENT ORGANIC SOYBEAN PRODUCTS

Amino acid	Total amino acid			Free amino acid of SPIH
	Flour	SPI	SPIH	
ASP	13.3	12.2	11.6	1.0
SER	3.7	3.3	3.9	0.8
THR	4.7	4.2	2.2	1.5
GLU	21.1	1.0	17.9	1.7
PRO	4.7	4.6	4.2	1.2
GLY	4.6	4.3	4.6	3.5
ALA	4.5	4.5	5.4	4.5
VAL	4.7	5.4	6.2	7.0
MET	0.3	0.5	0.7	0.9
LEU	5.0	5.5	4.8	7.1
ILE	8.0	8.3	7.7	18.6
PHE	0.1	1.0	0.5	9.8
TYR	0.2	1.9	0.5	13.1
HIS	1.7	3.3	1.9	18.7
LYS	5.8	6.5	5.2	7.9
ARG	6.0	10.1	6.0	1.0
NH4+	11.5	10.4	12.8	1.9

SPI: Soy protein isolate, SPIH: Soy proteins isolate hydrolysate

F. Antioxidative Activity of Protein Hydrolysate

Many proteins, peptides and amino acids have been investigated to have antioxidant activity Fig. 4 illustrates the scavenging activities of the fractions. It could be observed that the antioxidant activities give the same trend of peptide content which had high antioxidative activities and high peptide content between fractions 15 to 50. With increasing peptide concentrations, the scavenging effect on DPPH radical was increased until about 70%, thereafter reaching a plateau. In compare to different concentrations of BHA, which exhibited higher activity (90%), soybean protein hydrolysate exhibited also high antioxidant activities (70%) at a concentration of 1.45mg/ml at fraction 25 (Fig. 4). Electrophoresis analysis indicated that low-MW Hydrolysate fractions (F1) appeared, on average, to have higher DPPH scavenging activities than high-MW fractions. These results revealed that soybean peptides probably contained substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules. Previous studies also demonstrated that a number of food-derived peptides or protein hydrolysates were capable of interacting and quenching DPPH radicals. However, it is difficult to make comparison between different studies due to the lack of antioxidant standard and considerable influence by radical concentration and experimental conditions. In this concern, [30] studied DPPH scavenging assay to assess the antioxidant activity of the soy protein hydrolysate fractions and reported the same concept of antioxidant activities of the protein hydrolysates which depending on the molecular weight of fractions. Moreover, [31] demonstrated that hydrolyzed of rapeseed protein concentrates showed 2.3–3.0 times higher DPPH radical scavenging activity than non-hydrolyzed samples.

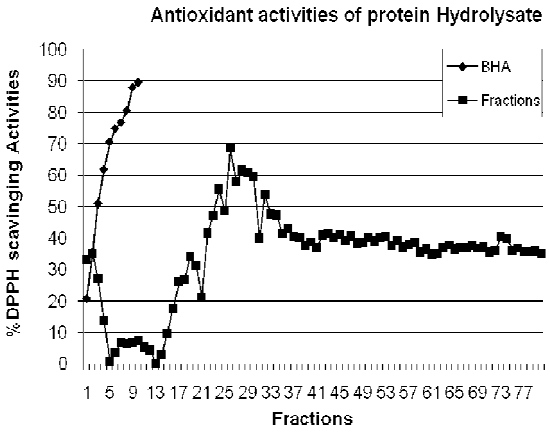

Fig. 4 Antioxidant activity of protein hydrolysate fractions

Saito et al. [32] compared the antioxidant capacity of 28 structurally related peptides to Leu-Leu-Pro-His-His, isolated from soybean protein digests, Pro-His-His was identified as an active center and demonstrated that His-containing peptides can act as metal-ion chelators, active-oxygen quenchers, and hydroxyradical scavengers and can contribute to the antioxidant activity of peptides. Also, after enzyme digestion of β-conglycinin and glycinin, the radical-scavenging activities were increased 3–5 times. Heating did not change the activity of the proteins, indicating that forming peptides was more critical than maintaining protein structure. These results indicated that increasing of Leu and His value after hydrolysis resulting in high antioxidant activities of soybean

protein hydrolysate. Chen et al. [20] mentioned that it is probable that the amino acid residues play a role in increasing the interaction between peptides and fatty resulting in antioxidative activity in food systems.

IV. Conclusion

In order to characterize the soy protein hydrolysate obtained in this study, Protein hydrolysate gave high antioxidant activities, The antioxidant activities of protein hydrolysate was in the same trend of peptide content which gave high antioxidant activities and high peptide content. Electrophoresis analysis indicated that, low-MW hydrolysate fractions (F1) appeared, on average, to have higher DPPH scavenging activities than high-MW fractions. These results revealed that soybean peptides probably contain substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules.

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