



CHEMICAL CHARACTERIZATION, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF 7S AND 11S GLOBULINS ISOLATED FROM PEA (*Pisum sativum*)

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ABSTRACT: The aim of this work was to investigate chemical characterization, antibacterial and antioxidant activities of 7S and 11S globulins isolated from pea (*Pisum sativum*). The minimum inhibitory concentration (MIC) of 7S and 11S globulins isolated from pea was determined against Gram positive (*Bacillus licheniformis* and *Bacillus thuringiensis*) and Gram negative [*E. coli* (O157:H7) and *E. coli* (E32511)] bacteria using agar well diffusion assay under the influence of different concentrations (0-800 µg/ml). The minimum inhibitory concentration (MIC) of 7S and 11S globulins against the four studied bacteria was 800 and 200 µg/ml, respectively. The concentration of the sample that scavenges 50% of the DPPH radicals (SC₅₀) was 100 and 110 µg/ml for 7S and 11S globulins, respectively. According to the results it could be concluded that 7S and 11S globulins were might be used in manufacturing of new functional products with increased nutritional value as the first step toward increasing its consumption and preserving food systems, as it is rich in nutrients and lower in cost.

Key words: Pea, pea protein isolate, 7S globulin, 11S globulin, antioxidant activity, antibacterial activity.

INTRODUCTION

Pea (*Pisum sativum*) is an abundant source of proteins that have been recognized for their high nutritional value (Boye *et al.*, 2010) and nutrient density (Azarpazhooh and Boye, 2012). Pea seeds are legumes, and possess high protein content approximately 23% (Costa *et al.*, 2006). The major protein component of pea seeds are globulins which constitute 65-80% of all proteins (Shcroeder, 1982). Legume globulins represent the majority of soybean seed proteins and can be subdivided into two main types according to their sedimentation coefficients: glycinin (11S) and β-conglycinin (7S). Glycinin has a molecular mass of 350 kDa and is composed of 5 constituent subunits, each of which consists of an acidic (37–42 kDa) and a basic polypeptide (20 kDa), linked together by a disulphide bond (Nielsen, 1985). Like other legumes, pea globulins consist of two major proteins, the legumine-like 11S globulin and the

vicilin-like 7S globulin (Rubio *et al.*, 2014). The antibacterial activity of these two main pea protein subunits (7S and 11S globulin) have never been investigated. But, the antibacterial activity of 7S and 11S globulin from soybean have been investigated (Yang *et al.*, 2008; Sitohy *et al.*, 2012; Osman *et al.*, 2013; Osman *et al.*, 2016a, b)

Microbial contamination is one of the main problems that may affect the shelf life of food and may also cause consumer illness. Therefore, many chemicals are used as food preservatives to increase shelf life of food products. Consequently, the need to explore novel antibacterial safe agents to replace current control strategies is urgent. Researchers have focused on the production of natural products that demonstrate antimicrobial significance to be used in the food industry (Osman *et al.*, 2013; Osman *et al.*, 2016a,b). The similarity between protein components of soybean and those of other legumes (Barker *et al.*, 1976; Derbyshire

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et al., 1976) suggests similar functions and applications. Therefore, the aim of this work was to investigate the antibacterial activity against selected bacteria [*Bacillus licheniformis*, *Bacillus thuringiensis*, *Escherichia coli* (O157: H7) and *Escherichia coli* (E32511)] and antioxidant activity (DPPH radical-scavenging activity) of pea protein isolates and their fractions (7S and 11S globulins).

MATERIALS AND METHODS

Materials and Chemicals

Pea (*Pisum sativum*) seeds were purchased from local market, Zagazig city, Sharkia Governorate, Egypt. *Bacillus licheniformis*, *Bacillus thuringiensis*, *Escherichia coli* (O157 : H7) and *Escherichia coli* (E32511) strains used in this study were kindly obtained from Microbiology Department, Faculty of Agriculture, Zagazig University, Egypt. All chemicals used in the experiments were of analytical grade.

Isolation of pea Protein Isolates (PPI) and Subunits (7S and 11S Globulins)

Pea seeds were ground and the resulting powder was defatted using n-hexane by soxhlet apparatus for 8 hr. Pea protein isolate was separated from defatted seed meal using the procedure of Johnson and Brekke (1983). 7S and 11S globulins were isolated from defatted seed meal of pea as described by Kimura *et al.* (2008) with some modification. Ten grams from defatted seed meal of pea were dissolved in 150 ml buffer (0.03 mol/l trisHCl at pH 8.5, 0.4M NaCl, 10 mM β -mercaptoethanol, 1mM EDTA, 0.02% (W/V) NaN_3). The solution was stirring for 1 hr., at 45°C in water bath. 11S and 7S globulins were precipitated with ammonium sulphate (50-65% and 65-85%, respectively).

Chemical Characterization of PPI, 7S and 11S Globulins

SDS-PAGE

Twenty milligrams of PPI, 7S and 11S globulins from pea were dissolved in 1ml SDS 10% with 100 μ l β -mercaptoethanol for 15 min with vortexing every 2 min. The extract was then centrifuged at 10000xg for 5 min. Twenty μ l of extract were mixed with 20 μ l of SDS-loading

sample buffer (SDS 4%, β -mercaptoethanol 3%, glycerol 20%, TrisHCl 50mM pH 6.8 and bromophenol blue traces), heated at 96°C for 5 min and 10 μ l aliquot was electrophoresed (10 μ l of protein/lane) and analyzed by SDS-PAGE according to Laemmli (1970).

Urea-PAGE

Lyophilized pea protein isolate (PPI), 7S and 11S globulins from pea were dispersed (10 mg/ml) in a pH 6.8 buffer containing 0.25 M Tris-base, 50% glycerol and bromophenol blue traces. Samples were centrifuged at 15000 xg for 5 min at 20°C. Supernatants were analyzed by Urea-PAGE (10 μ l of protein/lane) in 3% and 12% stacking and resolving gels, respectively, according to Williams and Evans (1980).

pH-solubility curve

Protein pH-solubility curves was assayed for PPI, 7S and 11S globulins from pea in the pH range of 2–10 according to Chobert *et al.* (1991). The iso-electric points were estimated from the protein pH-solubility curves, being the pH corresponding to the least protein solubility.

Amino acid analysis

A sample of PPI or 7S or 11S globulins (0.3 g) from pea were hydrolyzed with 6 N HCl (10 ml) for 24 hr., at 110°C in a sealed tube. Amino acid composition was detected using amino acid analyzer apparatus model "Eppendorf LC3000", with hydrolysate column Eppendorf LC 3000 (250 \times 4.6). The determination was carried out at 47°C, detection wavelength 440 for proline and 570 nm for the other amino acids and flow rate 0.2 ml per minute. Amino acid composition was reported as g per 100 g protein.

Antibacterial activity estimation

The antibacterial activities of PPI, 7S and 11S globulin from pea were tested against Gram positive (*Bacillus licheniformis* and *Bacillus thuringiensis*) and Gram negative bacteria (*Escherichia coli* O157: H7 and *Escherichia coli* E32511) using agar well diffusion method and turbidity liquid media assay.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined using agar well diffusion method of Nanda and Saravanan (2009). The pure cultures of bacterial strains were sub-

cultured on nutrient broth at 37°C on a rotary shaker at 200 rpm. The exponential phase cultures of these strains were adjusted to a concentration of 1×10^9 CFU mL⁻¹. Each strain was spread uniformly onto the individual plates using sterile cotton swabs. Wells of 6-mm diameter were made on nutrient agar plates using a gel puncturing tool. Aliquots (30 µl) of PPI or 7S or 11S globulins (0, 50, 100, 200, 400 and 800 µg/ml) were transferred into each well. After incubation at 37°C for 24 hr., the diameter of the inhibition zones were measured using a transparent ruler (Ehinmidu, 2003). MIC of an antimicrobial is taken as the lowest concentration (µg/ml) that will inhibit the visible growth of a microorganism after overnight incubation.

Bacterial growth curve

Turbidity (A_{600}) was used to estimate CFU/ml in nutrient broth media suspensions as an indicator of the bacterial growth over time. 7S and 11S globulins (at their MIC values) were added to the medium (10 ml) containing 100 µl G⁺ or G⁻ bacteria (10^9 CFU/ml) and examined for their growth as compared to control (without adding any substance). All treatments were incubated at 37 °C for different time periods (0, 6, 12, 18 and 24 hr.) before measuring the turbidity.

Antioxidant activity evaluation

The antioxidant activity of 7S and 11S globulins at different concentrations (0, 25, 50, 100, 200, 400 and 800 µg/ml) isolated from pea was evaluated by their ability to scavenge DPPH radicals according to the method described by Hanato *et al.* (1988). Three hundred µl of each concentration added to 3 ml of 0.1 mM DPPH dissolved in ethanol. After incubation period of 30, min at room temperature, the absorbance was determined against a control at 517 nm. The SC₅₀ (the concentration of the sample that scavenges 50% of the DPPH radicals) was calculated by linear regression of curves showing percentage scavenging versus sample concentration.

RESULTS AND DISCUSSION

Chemical Characterization of PPI, 7S and 11S Globulins

The SDS-PAGE patterns of pea protein isolate (PPI) and subunits (7S and 11S

globulins) of pea proteins are shown in Fig. 1. Pea protein isolate consisted of two main fractions 11S globulin (with molecular weight of 11S globulin subunits which had 20 and 40 KD corresponding the basic and acidic subunits) and 7S globulin (with molecular weight of 7S globulin subunits which had 75, 60 and 52 KD corresponding the α, α and β subunits).

Globulin 11S is a hexamer with a molecular weight between 330 and 410 kD and each monomer of 60 kD can split into acidic (approximately 40 kDa) and basic (approximately 20 kDa) polypeptides *via* disulphide bond reduction (O'Kane *et al.*, 2004). The migration in Urea-PAGE into cathode direction (Fig. 2) indicated that 7S and 11S globulins were much faster than their respective pea protein isolate referring to bigger positive charges. 7S globulin indicated further lower migration rate than 11S globulin in accordance with the fact that 11S globulin is originally more basic than 7S globulin (Osman *et al.*, 2016a).

The pH solubility curve (Fig. 3) of PPI, 7S and 11S globulins isolated from pea showed the least soluble point at pH 4, 5 and 7 for PPI, 7S and 11S globulins, respectively. The isoelectric points of the 7S and 11S fractions (5 and 7) reflect the more basic nature of the 11S fraction. Osman *et al.* (2016a) recorded that the isoelectric point of 7S and 11S globulin isolated from soybean is 5 and 6.5, respectively. The similarity between protein components of soybean and those of other legumes (Barker *et al.*, 1976; Derbyshire *et al.*, 1976) who suggested similar functions.

Amino acid composition

The amino acid contents of defatted pea flour were recorded in Table 1. The contents of the hydrophobic amino acid residues (Pro, Gly, Ala, Val, Ile, Leu and Phe) were around 33.93% (5.85 + 4.71 + 4.33 + 5.11 + 3.63 + 6.41 + 4.17, respectively) of the total amino acid. The content of the acidic amino acid residues (asp + glu; 11 + 15.8, respectively) was lower than that of the basic amino acids (arg + lys + his; 13.98 + 9.2 + 3.96, respectively), *i.e.*, 26.8 and 27.14%, respectively.

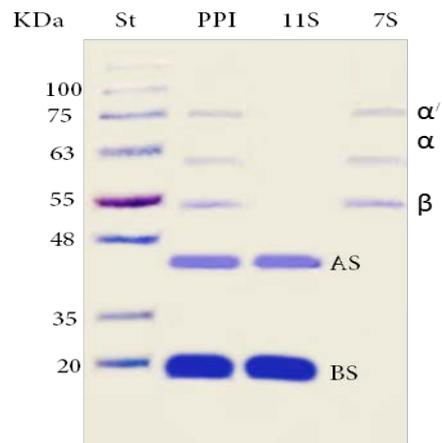


Fig. 1. SDS-PAGE of pea protein isolate (PPI), 7S and 11S globulins isolated from pea seeds. (AS; acidic subunit, BS; basic subunit)

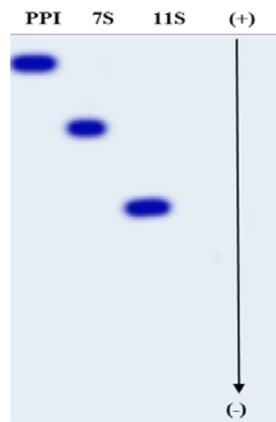


Fig. 2. Urea-PAGE of pea protein isolate (PPI), 7S and 11S globulins isolated from pea seeds

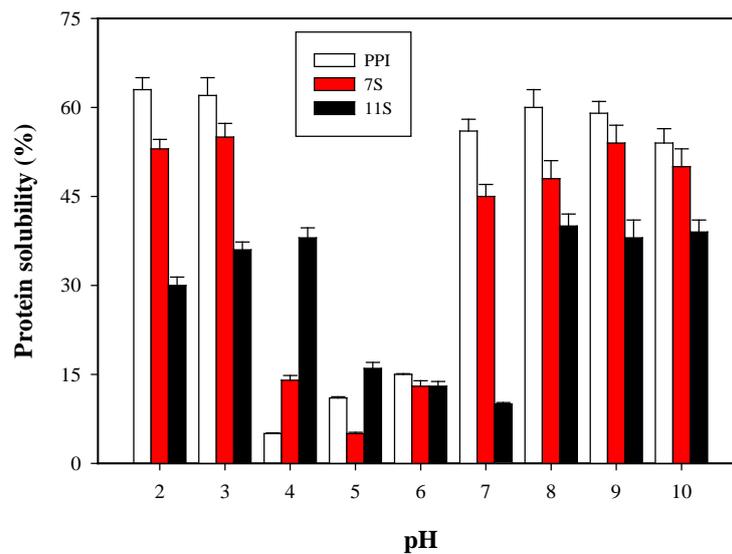


Fig. 3. pH-solubility histogram of pea protein isolate (PPI), 7S and 11S globulins isolated from pea seeds

Table 1. Amino acid composition (g amino acid / 100 g protein) of defatted pea flour

Amino acids	Concentration (g / 100 g defatted flour)
Aspartic	11
Threonine	3.7
Serine	4.9
Glutamic	15.8
Proline	5.85
Glycine	4.71
Alanine	4.33
Cysteine	0.52
Valine	5.11
Methionine	0.97
Isoleucine	3.36
Leucine	6.41
Tyrosine	3.18
Phenylalanine	4.17
Histidine	3.96
Lysine	9.2
Arginine	13.98

Antibacterial activity estimation

The minimum inhibitory concentration (MIC) of 7S and 11S globulins isolated from pea seeds were determined against Gram positive (*Bacillus licheniformis* and *Bacillus thuringiensis*) and Gram negative [*E. coli* (O157: H7) and *E. coli* (E32511)] bacteria using agar well diffusion assay (Table 2 and Fig. 4) under the influence of different concentrations (0-800 µg/ml). The results indicated that inhibition zone diameters are dependent on concentration of tested materials. The minimum inhibitory concentration (MIC) of 7S and 11S globulins against the four studied bacteria was 800 and 200 µg/ml, respectively.

Four tested G+ and G- bacteria were grown in their appropriate liquid media in the presence or absence of 1 MIC of 7S globulin (800 µg/ml) or 11S globulin (200 µg/ml) for 24 hr., at 37°C (Fig. 5).

The growth curves of the tested control bacteria reached a maximum turbidity after around 18 hr., at 37°C. At this time point, 7S reduced the growth of G+ bacteria by about 32.4-41.2%, while 11S inhibitory action was in the range of 82–86%. Similarly, 7S could inhibit the bacterial growth of the two gram negative bacteria by about 44-46% against 80-85% reduction by 11 Sglobulin. The antibacterial properties of proteins are dependent on their interaction with the bacterial cell wall and membranes (Hancock, 2004). Positively charged protein electrostatically bind to lipopolysaccharides on the outer membrane of gram negative bacteria or lipoteichoic acids on the surfaces of gram positive bacteria (Glinel *et al.*, 2012). The chemical structure of 11S globulin, indicating its separation into basic and acidic subunits may allow either domain to electrostatically interact with the bacterial cellular components affecting the cells integrity (Kuipers and Gruppen, 2008).

Table 2. The Inhibition zone diameter (mm) induced in Gram positive (*Bacillus licheniformis* and *Bacillus thuringiensis*) and Gram negative [*E. coli* (O157:H7) and *E. coli* (E32511)] bacteria using agar well diffusion assay under the influence of different concentrations (0-800 µg / ml) of 7S and 11S globulins isolated from pea seeds

Microorganism	Concentration (µg / ml)									
	7S globulin					11S globulin				
	50	100	200	400	800	50	100	200	400	800
Gram+										
<i>B. licheniformis</i>	-	-	-	-	40±0.9	-	-	50±1	52±1.1	55±1.2
<i>B. thuringiensis</i>	-	-	-	-	30±0.8	-	-	40±0.8	45±0.9	50±1.1
Gram-										
<i>E. coli</i> (O157:H7)	-	-	-	-	25±0.7	-	-	20±0.4	25±0.7	27±0.6
<i>E. coli</i> (E32511)	-	-	-	-	22±0.5	-	-	20±0.5	24±0.8	25±0.5

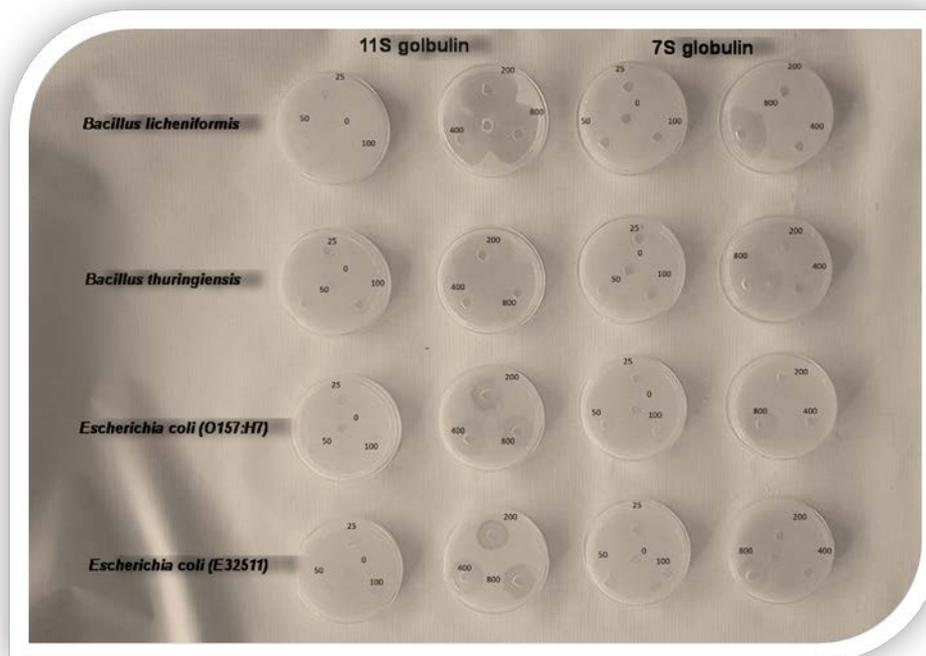


Fig. 4. Inhibition zone in two Gram positive (*Bacillus licheniformis* and *Bacillus thuringiensis*) and Gram negative [*E. coli* (O157:H7) and *E. coli* (E32511)] bacteria under the influence of different concentrations (0-800 µg / ml) of 7S and 11S globulins isolated from pea seeds

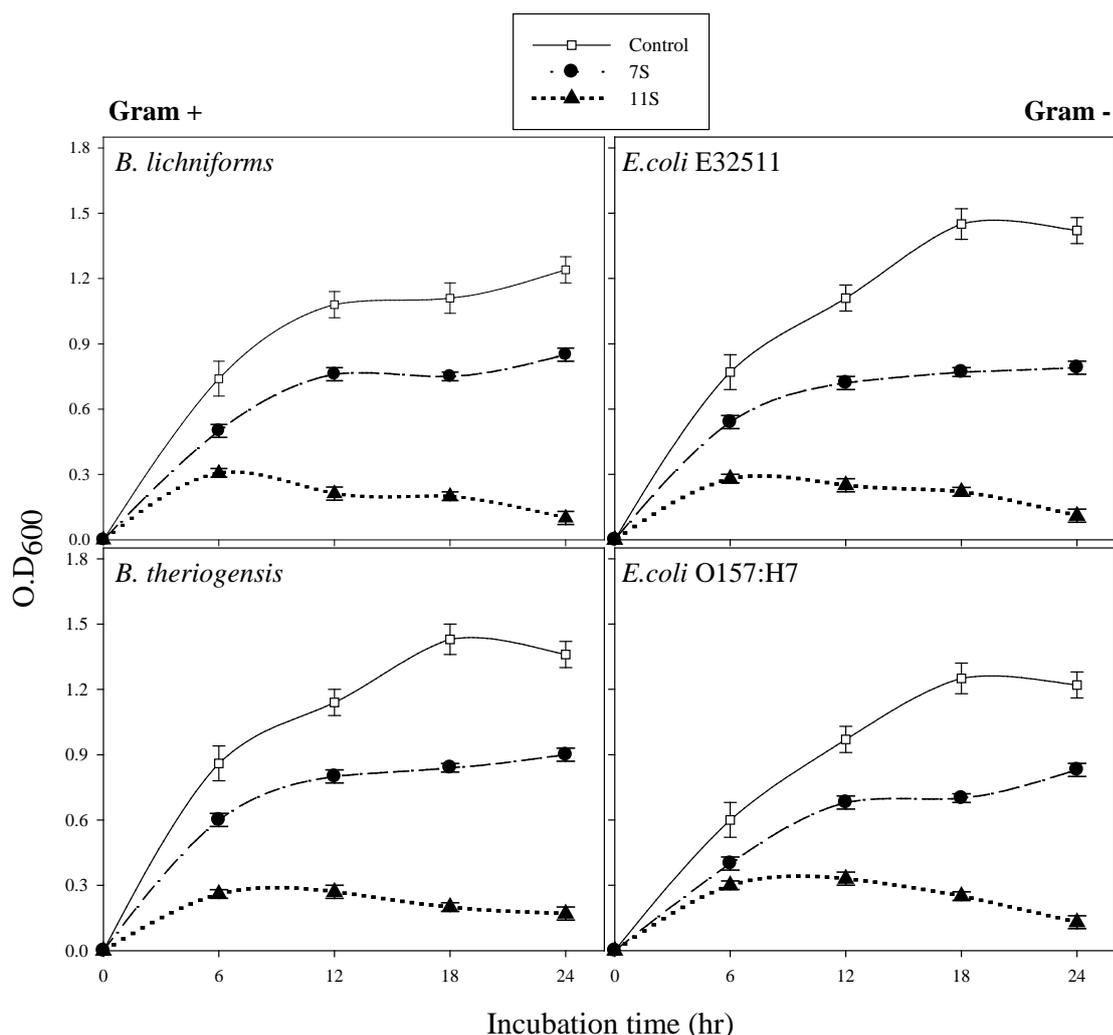


Fig. 5. Growth curve of G⁺ (*Bacillus licheniformis* and *Bacillus theriogenes*) and G⁻ [*E. coli* (O157:H7) and *E. coli* (E32511)] bacteria during 24 hr., at 37°C in the presence of 1 MIC of 7S and 11S globulins isolated from pea seeds

Based on the results of pH-solubility and Urea-PAGE reflect the more fundamental nature of the 11S part and high activity as an antibacterial agent compared to 7S globulin.

Antioxidant activity estimation

The concentration of the sample that scavenges 50% of the DPPH radicals (SC₅₀) was 100 and 110 µg/ml for 7S and 11S globulins, respectively. In Table 3 the DPPH assay, low SC₅₀ values indicate strong radical scavenging activity as reported by Zhu *et al.* (2011).

Several studies have recorded the ability of the protein to inhibit lipid oxidation in food. Proteins originating from milk and soybeans

have been shown to exhibit antioxidant activity due to their abilities to inactivate reactive oxygen species, scavenge free radicals, chelate pro-oxidative transition metals, reduce hydro peroxides, enzymatically eliminate specific oxidants, and alter the physical properties of foods to separate reactive species (Elias *et al.*, 2008).

Some amino acids, such as histidine, tyrosine, methionine, and cysteine, have been reported to show antioxidant activity. In particular, histidine exhibited strong radical scavenging activity due to the decomposition of its imidazole (Xie *et al.*, 2008; Sarmadi and Ismail, 2010) described the mechanisms of action of amino acids: aromatic amino acids convert radicals to stable molecules

Table 3. Antioxidant activity (DPPH radical scavenging; SC₅₀) of 7S and 11S globulins isolated from pea

Sample	(SC ₅₀) DPPH (µg / ml)
7S	100
11S	110

by donating electrons while keeping their own stability; hydrophobic amino acids enhance the solubility of peptides in lipids, which facilitates accessibility to hydrophobic radical species; and acidic and basic amino acids contain carboxyl and amino groups in their side chains, which act as chelators of metal ions and as hydrogen donors. Dietary use of antioxidants has been shown to promote health by increasing antioxidant capacity (Samaranayaka and Li-Chan, 2011).

Conclusion

According to the present results it could be concluded that 7S and 11S globulins were might be used in manufacturing of new functional products with increased nutritional value as the first step toward increasing its consumption and preserving food systems, as it is rich in nutrients and lower in cost.

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التوصيف الكيميائي للجلوبوليينات 7S و 11S المعزولة من البسلة وخواصها المضادة للأكسدة والبكتريا

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الهدف من هذا البحث هو دراسة التوصيف الكيميائي، النشاط المضاد للبكتيريا ومضادات الأكسدة للجلوبوليينات المفصولة من البسلة حيث تم تحديد الحد الأدنى من التركيز المثبط من 7S و 11S جلوبيولين والمعزولة من البسلة ضد الميكروبات الموجبة والسالبة لجرام باستخدام طريقة Agar well diffusion تحت تركيزات مختلفة من صفر إلى ٨٠٠ ميكروجرام/ملل، والحد الأدنى من التركيز المثبط من 7S و 11S جلوبيولين ضد البكتيريا المختبرة ٨٠٠ ميكروجرام/ملل في حاله 7S، ٢٠٠ ميكروجرام/ملل في حاله 11S على التوالي، تم دراسة النشاط المضاد للأكسدة باستخدام DPPH وأظهرت النتائج أن التركيز الذى يعمل على تثبيط ٥٠% من الشقوق الحرة (SC50%) هو ١٠٠ ميكروجرام/ملل في حاله 7S و ١١٠ ميكروجرام/ملل في حاله 11S، نستنتج أن هذه الجلوبيولينات المعزولة من البسلة من الممكن أن تدخل في تصنيع الأغذية الوظيفية ذات القيمة الغذائية العالية كخطوة مبدئية تمهيدا لاستهلاكها حيث إن هذه الجلوبيولينات غنية بالمغذيات ومنخفضة التكاليف وتؤدي لإطالة مدة حفظ الغذاء.

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