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Antibacterial and Antioxidant Activities of an Enzymatic Hydrolysate Kidney Bean (*Phaseolus vulgaris* L.) Protein Isolates

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ABSTRACT

The aim of this study was to hydrolyze kidney bean (*Phaseolus vulgaris* L.) protein isolates by papain enzyme and evaluate the antibacterial effect against selected bacteria and antioxidant activities of these hydrolysates to test their possibility use as bio-preservatives in food. The range of protein hydrolysis by papain was evaluated by considering the degree of hydrolysis (DH) and electrophoresis estimation. The hydrolysate gained after 240 min regression had the highest DH (29%). While, hydrolysates gained after 60, 120 and 180 min had DH values of 8 %, 13%, and 18 % respectively. The antibacterial activity of kidney bean protein hydrolysates (100 mg/mL) with different degree of hydrolysis on bacterial growth for *B. licheniformis* and *E. coli* (O157:H7) was evaluated by disc-diffusion method. The hydrolysate with the highest antibacterial activity (DH= 29%) was selected for the antibacterial evaluation *in situ* and *in vitro*.

Keywords: kidney bean, Papain, Antioxidant activities, Antibacterial activity

INTRODUCTION

One of the major problems affecting the the length of time for which an item remains usable of food and which also may cause health hazards is the microbial contamination. Thus, many chemicals are used as a substance used to preserve food stuffs to increase the preservation time of food products .Owing to the rise of consumer concerns about the harmful effects of chemical agents and the increasing preference for natural components, Researches have concentrated on the production of natural additives producing which elucidate antimicrobial importance, in order to use them in the food industry (Osman *et al*., 2013).

Antibacterial peptides, mostly normal antibacterial peptides, play a critical role in food preservatives. Various studies elucidated that many natural AMPs have wide-spectrum antibacterial activities against Gram+ and Gram- bacteria (Sitohy *et al*., 2012 and Li *et al*., 2014).

Kidney bean (*Phaseolus vulgaris* L.) is vastly grown and used in Egypt. This bean usually contains20-30% protein, which has a perfect amino acid composition of aromatic amino acids

In this work papain obtained from the papaya plant (*Carica papaya* L.) is used. It has been presented to have a wide proteolytic action against numerous proteins, and has previously been used to hydrolyse kidney bean and pea proteins to improve the functional properties of the original material (Wani *et al*., 2015) and produce the antibacterial and the antioxidant peptides from milk proteins (Abdel-Hamid *et al*., 2016; Salami *et al*., 2010).

In the current study kidney bean (*Phaseolus vulgaris*) protein isolates was hydrolyzed by papain enzyme and evaluate the antibacterial activity against some pathogenic and spoilage bacteria and antioxidant activities of these hydrolysates to check their possibility utilize as bio-preservatives in food.

MATERIALS AND METHODS

1. Plant material

Kidney bean (*Phaseolus vulgaris* L.) seeds were obtained from market, Zagazig, Sharkia, Egypt.

2. Chemicals and reagents

The enzyme papain (from *Carica papaya,* E.C. 3.4.22.2) was obtained from Sigma Chemical Company (St. Louis, MO, USA).chemicals for electrophoresis were purchased from Bio Rad laboratoriese (Richmond, CA, USA). All other reagents used in tests were of analytical class.

3. Sample preparations

Kidney bean seeds were cleaned and ground by a mixer (Type 716, France) and the meal were ground to pass through a 1 mm^2 sieve. . The flour was defatted using chloroform: methanol (3:1, v/v) for 8 h. . Solvent was evaporated and dried-defatted flour was stored at 4 °C until used. Extraction of protein isolates

Protein isolates were isolated according to the method of Fan and Sosulski (1974), with slight modifications. Defatted flours were distributed in distilled water (5% w/v), and the pH of the dispersions was adjustedto 8 with 2 N sodium hydroxide. The obtained disperission were gentlystirred at 25 °C for 120 min, then centrifuged at 8 Kg at 20 °C for 60 min. The precipitate were ignored, and the supernatants were were regulated to pH4.5 with 2 N hydrochloric acid to isolate the proteins. Finally, the precipitatesobtained by centrifugation at 5 Kg for 25 min were re-dispersedin distilledwater. The dispersions were homogenized and adjustedto pH 7.0 with 2 N NaOH.

4. Protein content estimation

Total protein concentration of kidney bean isolate (KPI) was estimated by strike the totalnirogen by 6.25. The total nitrogen was estimated using micro Kjeldahel protocol according to AOAC (1996).

5. SDS-PAGE of proteins

An amended method of discontinuous SDS-Polyacrylamide slab gel electrophoresis based on the method of Laemmli (1970) was used in the fractionation of KPI.

6. Amino acids composition estimation

Total amino acids composition of kidney bean seeds hydrolysate was estimated by amino acid analyzer model Eppendorf LC3000.

7. Iso-electric point (PI) estimation

The PI were calculated from the solubility at different pH as the pH at which the protein is minimal soluble. Protein solubility was tested in the pH range of 2-10 according to the method summarized byChobert*et al.* (1991) with some modification. 125 mg of each sample was soluted in 25 ml distilled water and the pH was regulated to 2-10 using either 0.5 M HCl or 0.5 M

NaOH. The slurries were stirring for 60 min at 30 °C befor centrifugation at 1.2 Kg for 25 min at 4 °C. The supernatant was clarified. Protein content in the supernatant was assessed by Kjeldahl protocol (AOAC, 1996). Triplicate determination were done and the solubility was gained by plotting means of protein solubility against pH:

$$Solubility\ (\%) = Amount\ of\ protein\ in\ the\ supernatant / Amount\ of\ protein\ in\ the\ sample\ X\ 100$$

8. Enzymatic hydrolysis of Kidney bean protein isolate (KPI)

Lyophilized KPI was dissolved in 0.1M Na2HPO4-NaH2PO4 buffer pH 6.0 (10 % w/v) and hydrolysed by treating with papain (Enzyme/substrate ration of 1:200 W/W) at 37 °C and pH 6. The hydrolysis was pliable to complete for 4 h, at the finish of the reaction, the enzyme was stopped byheating in the boiling water for 10 min. Hydrolysate was purified by centrifugation at 4 Kg for 25 min at °C to separa insoluble fractions, and the supernatant was lyophilized and frozen at -20 °C until further use.

9. Extent of hydrolysis

The extent of hydrolysis was assayed after 60, 120, 180 and 240 min according to the procedure described by Hoyle and Merritt (1994)

$$Degree\ of\ hydrolysis\ (\%) = \frac{(10\%\ TCA - Soluble\ nitrogen\ in\ the\ sample)}{(Total\ nitrogen\ in\ the\ sample)} X\ 100$$

1. SDS-PAGE

SDS–PAGE of kidney bean protein hydrolysates (KPH) with papain at different times (60, 120, 180 and 240 min) was estimated according to Laemmli (1970) in 3% and 12 % acrylamide for the stacking and resolving gels, respectively.

10. Antibacterial activity evaluation

1. Bacterial strains

Two gram+ bacteria, i.e., *Bacillus licheniformis*, *Bacillus thuringiensis* and two Gram- bacteria, *Escherichia coli* (O157:H7) and *Escherichia coli* (E32511) were used to estimate the antibacterial activity of kidney bean protein hydrolysates after 60, 120, 180 and 240 min. using a diffusion method to select the high activity hydrolysate. The strains were obtained from Department of Microbiology, Faculty of Agriculture, Zagazig University, Egypt.

2. Agar well diffusion assay

3. Kidney bean protein hydrolysates (KPH) :after 60, 120, 180 and 240 min.using a diffusion method to select the high activity hydrolysate. Kidney bean protein hydrolysates after 240 min (the highest activity hydrolysates) were checked for antibacterial activity against *Bacillus licheniformis*, *Bacillus thuringiensis*, *Escherichia coli* (O157:H7) and *Escherichia coli* (E32511) by conventional well-diffusion assay (Nanda and Saravanan, 2009). The pure culture of bacterial strains wer sub-cultured on nutrient broth at 37 °C on a rotary shaker at 200 rpm. The exponential phase culture of these strains regulated to a concentration of 1 × 109 CFU mL-1. Each strain was spread uniformly onto the individual plates using sterile cotton swabs. Wells of 6-mm diameter were made on Müller Hinton Agar (MHA) plates using a gel puncturing tool. Thirty micoleter of the KPH solutions (0, 25, 50, 100, 200, 400 and 800 µg/mL) were transferred into each well. After incubation at 37 °C for 24 h, the diameter of inhibition zone was measured using a transparent ruler Minimum inhibitory concentration (MIC)

MIC of elected samples was estimated as described previously (Yamamoto *et al*., 2003). Aliquots (30 µL) of the KPH solutions (0, 25, 50, 100, 200, 400 and 800 µg/mL) were transferred into each well. The lowest concentration of the tested materials that presented visible clear area on MHA plates was observed as the MIC.

4. Antioxidant Activity Evaluation

Kidney bean protein hydrolysates (KPH) after 60, 120, 180 and 240 min.at 200 µg/mLwere evaluated as an antioxidative agents to select the highest activity hydrolysates.KPH with the highest activity (after 240 min) was estimated for antioxidant activity by using the following methods

5. Antioxidant activity estimation

The DPPH radical scavenging activity was estimated by the procedure of Gocer and Gulcin (2011) . The radical scavenging ability of the sample was recorded as a decrease in the absorbance of DPPH·(OD517) radical and it was studied using the following equation:.

$$Radical\ scavenging\ activity\ (\%) = \frac{Abs.control - Abs.sample}{Abs.control} x100$$

The SC50 value was recorded as an active concentration of KPH that is wanted to scavenge 50 % of radical activity (Bursal & Gulcin, 2011) Results and Discussion

11. Chemical Characterization of kidney bean protein isolate (KPI)

Total protein concentration in the KPI was 92% (data not shown). The data in Figure 1, presentation the type of the SDS-PAGE electrophoregram of kidney bean protein isolate. Ten bands with molecular weights ranging between 30 to 200 kDa, are showedin the protein isolate. The 46 and 55 kDa bands, conformable to phaseolin subunits (40 and 55 kDa according to Montoya *et al*. (2006) Montoya *et al*. (2006). This electrophoresis type is comparable to those reported by Monttoya *et al*. (2006) and Carrasco-Castilla *et al*. (2012) Carrasco-Castilla *et al*. (2012and mention that oure phaseolin preparation was enriched in the major reserve protein of *p. vulgaris* and contains almost no other pollute proteins. .

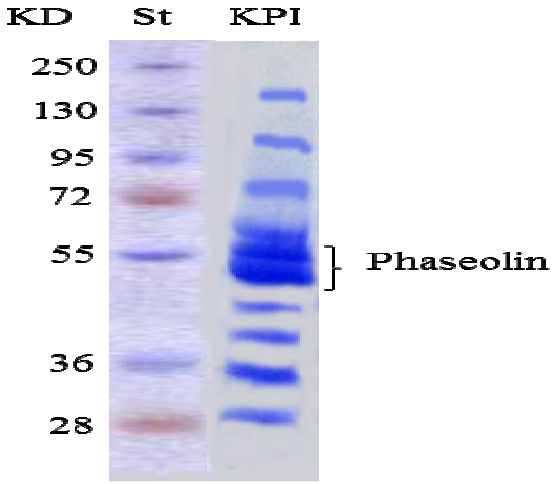

Fig. 1. SDS-PAGE of kidney bean protein isolate (KPI) Lane St =MW standards (kD).

The amino acids composition of samples were recorded in Table. 1 The amino acids patterns of protein isolate and flour were simillar. The contents of the hydrophobic amino acid for defatted flour and protein isolate (Pro, Gly, Ala, Val,Ile, Leu, Phe) are around [37.2% and 36.6%, respectively]. The content of the negatively charged amino acid residues for defatted flour and protein isolate (asp + glu) are around [28.1% and 27.5%, respectively] is higher than that of the positively charged amino acids (arg + lys + his) are around [18% and 17.5%, respectively]. These results are scomparable to those previously reported by Carrasco-Castilla *et al*. (2012).

Table 1. Amino acids (g / 100 g) of defatted kidney bean flour and kidney bean protein isolate

Amino acid	Concentration (g / 100 g defatted flour)	
	Flour	Isolate
Aspartic	11.5	11.3
Threonine	4.3	4.6
Serine	7	7.3
Glutamic	16.6	16.2
Proline	2.4	2.6
Glycine	4.4	4
Alanine	4.2	4.3
Cystine	0.4	0.6
Valine	5.1	4.5
Methionine	0.5	0.4
Isoleucine	5.7	6.2
Leucine	9.2	9
Tyrosine	2.8	3.1
Phenylalanine	6.2	6
Histidine	3.2	3.8
Lysine	7.1	6.9
Arginine	7.2	7.3

The pH-solubility curve KPI is showed in Figure 2. The solubility pattern of KPI mention that solubilityreduced as th pH increased from two to five, which conformable to its PI, after which next increase in pH increased protein solubility progressively.. The minimum solubility for KPI (10 %) was at pH 5 which corresponds to its isoelectric point (pI). The highest protein solubility (67%) was obtained at pH 10.

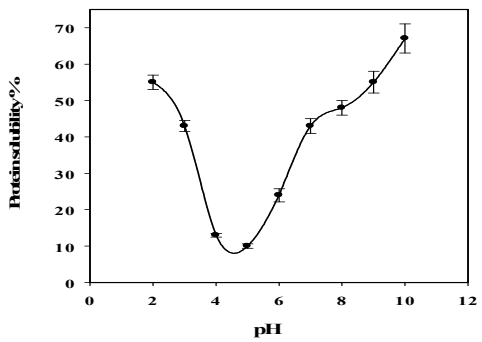

Fig. 2. PH-solubility curve of KPI

Solubility is one of the most vital advantage of proteins. Good solubility of proteins is wanted in many functional usages, because more soluble proteins supply a homogenous dispersibility in the colloidal systems (Zayas, 1979).

Kidney bean protein hydrolysate production and fractionation

The extent of protein hydrolysis by papain was determined by estimating the DH and electrophoretic analysis. The hydrolysate gained after 240 min degeneration had the highest DH (29%). While, hydrolysates obtained after 60, 120 and 180 min had DH values of 8%, 13%, and 18% respectively as shown in Figure 3.

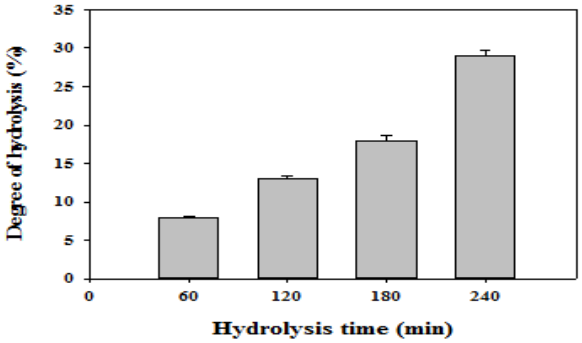

Fig. 3. Progression of hydrolysis with time for kidney bean protein isolate when acted by papain (Enzyme/substrate ration 1:200, W/W) at 37 °C and pH 6.

Extent of hydrolysis was showed in the range of 3.13-4.70% when KPIs were processed with 0.1% papain for 30 min (Wani *et al*., 2015). Extent of hydrolysis in the range of 6.71–8.63% was showed in KPIs hydrolysed for 60 min and considrable difference were showed in DH. Barac *et al*. (2011) presented DH from 3.9 to 4.7% for pea protein isolates hydrolyzed with chymosin up to 60 min of hydrolysis. Govindaraju and Srinivas (2006) recorded DH in the range of 3.4-14.7% for arachin protein processed with papain. Comparing with the percent DH of the above result the percent DH in our study reflects the papain wide specificity (hydrophobic-Argor Lys)X except Val) and the value obtained in the current work were in the range of previously presented values.

Ten protein bands with molecular mass ranging from 30 to 200 kDa, are showed in the protein isolate (lane,1) The electrophoretic profile of kidney bean protein hydrolysates after 240 min proteolysis by papain reported that all kidney bean protein fractions were completely hydrolyzed (Figure 4, lane 5), in which is in convention with the highest DH.

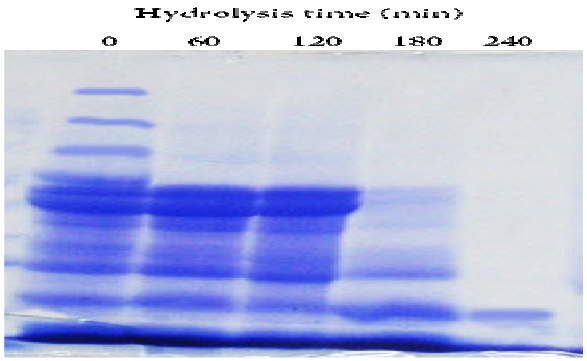

Fig. 4. SDS-PAGE of kidney bean protein hydrolysates produced using papain (Enzyme/substrate ration 1:200, W/W) at 37 °C and pH 6. at different times (0, 60, 120, 180 and 240 min).

Antibacterial activity of kidney bean protein hydrolysates

The antibacterial activity of kidney bean protein hydrolysates (100 mg/mL) with different degree of hydrolysis on bacterial growth for *B. licheniformis* and *E. coli* (O157:H7) was evaluated by disc-diffusion method.

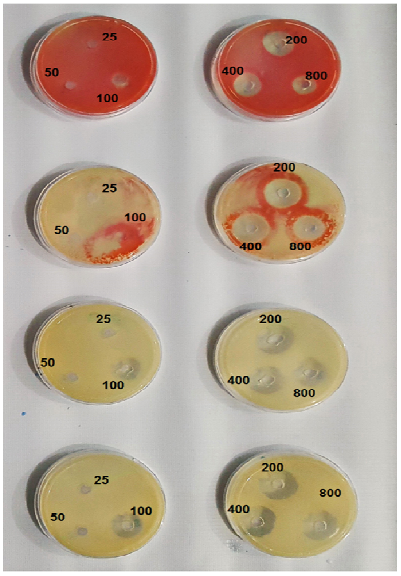

Fig. 5. Inhibition zones in two Gram+ (*Bacillus licheniformis* and *Bacillus theriogensis*) and Gram- [*E. coli* (O157:H7) and *E. coli* (E32511)] bacteria under the influence of different concentrations (0-800 µg / ml) of kidney bean protein hydrolysates (KPH) produced using papain (Enzyme/substrate ration 1:200, W/W) at 37 °C and pH 6.for 240 min..

The hydrolysate with the highest antibacterial activity (DH= 29%) as shown in Table 2 was selected for the antibacterial evaluation.

Table 2. Antibacterial activity of kidney bean protein hydrolysates (100 mg/ml) with different degree of hydrolysis on bacterial growth for *B. licheniformis* and *E. coli* (O157:H7) using well-diffusion assay.

Hydrolysis time (min)	DH (%)	Antibacterial activity	
		B. licheniformis	*E. coli* (O157:H7)
60	8 ±0.2	-	-
120	13 ±0.3	-	-
180	18 ±0.6	-	-
240	29 ±0.7	+	+

(-) means no activity
(+) means the high activity

The kidney bean protein hydrolysates with the highest degree of hydrolysis (DH= 29%) was applied at different concentrations (0-800 µg/mL) to Petri dishes containing MHA infected with two gram+ bacteria (*Bacillus licheniformis* and *Bacillus theriogensis*) and two gram- bacteria [*collie.coli* (O157:H7) and *E. coli* (E32511)], incubated at 37 °C for 24 h. the inhibition zone diameter are recorded in Table 3. Native KPI was applied similarly at the same applied concentrations. Control was exactlyprepared as the treatments except that protein was replacedby distilled water. It was spotted that either control or native proteins samples did not showed any inhibition.

Table 3. The Inhibition zones diameter (mm) induced in Gram+ (*Bacillus licheniformis* and *Bacillus theriogensis*) and Gram- [*E. coli* (O157:H7) and *E. coli* (E32511)] bacteria using agar well diffusion assay under the influence of different concentrations (0-800 µg / ml) ofkidney bean protein hydrolysates (KPH) produced using papain (Enzyme/substrate ration 1:200, W/W) at 37 °C and pH 6. for 240 min.

Microorganisms	Inhibition zone diameter (mm/KPH: DH= 29%)						
	0	25	50	100	200	400	800
Gram+				Concentration (µg / ml)			
B. licheniformis	-	-	-	9±0.4	20 ±0.1	25 ±0.2	33 ±0.1
B. theriogensis	-	-	-	15 ±0.1	26 ±0.2	31 ±0.3	39 ±0.2
Gram-							
E.coli (O157:H7)	-	-	-	13 ±0.5	22 ±0.3	28 ±0.1	36 ±0.2
E. coli (E32511)	-	-	-	16 ±0.3	24 ±0.4	30 ±0.1	38 ±0.3

It is noticed in Table 3 and Figure 5that KPH gave high to concentration-subjected inhibition zones Mostly, there were no considrable differences between the gram + and – bacteria in their sensitivity to the tested agents at difrent concentrations indicating towide specificity antibacterial action of the hydrolyzed proteins. This result might be referred to the division ofantibacterial peptides by the action of papain. The same results were recorded byAbdel-Hamid *et al.* (2016)and Salami *et al*. (2010).

4. Antioxidant activity of kidney bean protein hydrolysates

1. Relationshipe between DH and the antioxidant activity

The hydrolysates were subjected for antioxidant evaluation Using DPPH assay. Our results propose that there is a direct relation between the hydrolysis period, DH and the antioxidant activity. After 4 h hydrolysis showed the highest activity actiona (89%) at a concentration of 200 µg/mLfor DPPH assay.

2. DPPH· assay

The DPPH· examination is one of the *in vitro* protocoles for the assesment of the ability of an antioxidant to reduce free radicals. The extent of color changes is correlated with the sample antioxidant activity (Xie *et al*., 2008).

The respective SC_{50} values were evaluated and the SC_{50} value of the KPH with the highest DH was 100µg/ml (Figure 6).

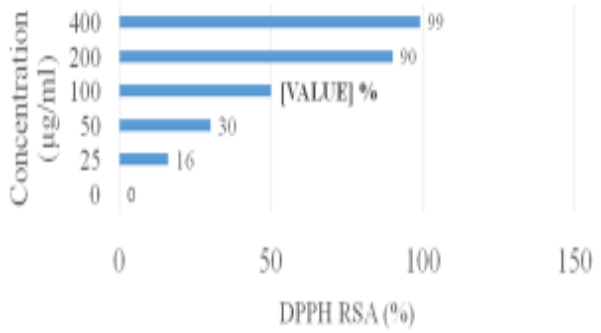

Fig. 6. DPPH radical scavenging activity (RSA) of kidney bean protein hydrolysates(KPH) at different concentration (0-400 µg/mL) produced using papain (Enzyme/substrate ration 1:200, W/W) at 37 °C and pH 6 for 240 min.

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الأنشطه المضادة للبكتيريا والمضادة للأكسدة للمعزول البروتينى للفاصوليا والمحلل بالانزيمات

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الهدف من هذا البحث هو اجراء التحلل الانزيمى للمعزول البروتينى للفاصوليا بواسطه انزيم البابين واختبار النشاط المضاد للبكتيريا الممرضة والمسببه للتلف وكذلك النشاط المضاد للأكسدة بغرض استخدامها كمادة حافظة طبيعية.تم دراسة مدى تحلل البروتين بواسطه قياس درجة التحلل والتفريد الكهربى للبروتين ولوحظت اعلى درجة تحلل بعد ٢٤٠ دقيقة (٢٩%) فى حين ان درجات التحلل التى حصلنا عليها بعد ٦٠ و ١٢٠و ١٨٠ دقيقة كانت ٨%و ١٣% و ١٨% على التوالى. تم تقدير النشاط المضاد للبكتيريا للبروتينات ذات درجات التحلل المختلفة عند تركيز (١٠٠ ملليجرام / ملل) ولوحظ أن البروتين ذو أعلى درجة تحلل هو الذى له نشاط مضاد للبكتيريا. وبقياس النشاط المضاد للأكسدة لوحظ زيادتة بزيادة درجة التحلل. وبالتالى يمكن استخدام هذه البروتينات كمواد حافظه طبيعية لما لها من نشاط مضاد للبكتيريا والأكسدة وكذلك كونها أمنه من الناحية الصحية.