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Potential use as a bio-preservative from lupin protein hydrolysate generated by alcalase in food system

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ABSTRACT

In this study, we evaluated the antibacterial activity of hydrolysates from lupin protein *in vitro* against gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria and in food system (in minced beef under refrigerated condition).Hydrolysis of lupin protein with Alcalase was monitored for 4 h. Hydrolysates obtained after 1, 2, 3 and 4h had degree of hydrolysis values of 9%, 16%, 23% and 25%, respectively. The minimal inhibitory concentration (MIC) of LPH was determined against all bacterial strains. MIC value of LPH against all bacterial strains was 100 µg ml^{-1}. The results showed that gram positive bacteria were more sensitive than gram negative bacteria. Adding LPH at different concentrations (100, 150 and 200 µg/g) to minced beef showed antibacterial activity during storage at 4°C compared to nisin (200 µg/g) as a positive control. The results of this study showed that enzymatic hydrolysis by Alcalase at pH 7.8 and 55 °C for 240 min is an easy tool to increase the antibacterial activity of lupin protein against both gram negative and gram positive bacteria and can be used as a bio-preservative in food system. Extending the technological validity of minced beef will help avoid big losses of minced beef and enhance its chances to be incorporated into many meat products while avoiding hygienic, chemical and technological deterioration.

1. INTRODUCTION

The problems of spoilage and food poisoning, mainly by oxidation processes or by microorganism activity, during production and storage are still concerns for both the food industry and consumers, despite the use of synthetic chemical additives and various preservation methods [1-3]. Ground beef is a perishable product with noted food safety concerns because it provides a favorable medium for the growth of both spoilage and food-borne microorganisms. It is frequently contaminated by microorganisms due to excessive handling, (i.e. slaughtering, processing and transporting). In fact, food poisoning is still a threat for both consumers and the food industry despite the use of preserving processes. Meanwhile, consumers are concerned about the safety of foods containing preservatives. Therefore, there has been a growing interest in new and effective techniques to reduce cases of food-borne illnesses. Preservatives in foods are regarded as 'chemical' additives and 'unnatural' by many consumers and are rejected for this reason. Natural alternatives, in contrast, have a much better image. For several years, basic proteins, spices, herbs and herbal extracts with antimicrobial activities have been discussed in this context [4-15]. The biodiversity of plants provides an important source of chemical compounds, which have many therapeutic applications such as antiviral, antibacterial, antifungal and anticancer activities [16]. Legumes play an important role in the traditional diets of many regions throughout the world [17-18]. In particular, lupin seeds are characterized by a virtually non-existent starch and high protein content (total protein content of approximately 34%) in comparison to other legumes such as beans and peas [19].

A number of strategies have been suggested to improve the antimicrobial activities of proteins, including chemical modification such as esterification [20] and enzymatic hydrolysis using digestive proteases , microbial and plant Proteolytic enzymes [21, 22]. Alcalase is a bacterial extract from *Bacillus licheniformis* [23] containing several proteinases with different specificities.

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Alcalase has been used extensively to prepare soluble hydrolysates of soy protein [24] and fish protein [25] as well as to produce bioactive peptides [26, 27]. The antibacterial activity of lupin seeds protein hydrolyzed with alaclase has been not studied. Therefore, the aim of this work was to hydrolyze lupin protein by alcalase and evaluate the antibacterial activity of these hydrolysates (LPH) against selected pathogenic and spoilage bacteria to test their potential use as bio-preservatives in food.

2. MATERIAL AND METHODS

2.1 Plant Material

Lupinseeds (*Lupinus angustifolius* L.) seeds were purchased from local market, Zagazig, Sharkia governorate, Egypt.

2.2 Meat Samples

The fresh raw beef purchased from local market in Zagazig city, Sharkia government, Egypt was finely minced in sanitized meat mincers. The samples of the minced meat were transferred to sterilized polyethylene sachets.

2.3 Microorganisms

Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), were kindly obtained from Laboratory of Microbiology, Department of microbiology, Faculty of Science, Zagazig University, Egypt.

2.4 Animals

Healthy male white albino rats (*Rattus norvegicus*), Wistar strain (160 ± 10 g, body wt) were obtained from Organization of Biological Products & Vaccine (Helwanfarm, Cairo, Egypt) and housed in plastic cages in groups of 5 animals/cage. The experimental animals were allowed to acclimatize under the laboratory conditions (temperature of 25 ± 5 °C; relative humidity 50–70% and normal light/ dark cycle) for 2 weeks at least prior the experiment. They were provided with balanced pelleted diet (23% protein) and tap water *ad libitum* throughout the adaptation and experimental period.

2.5 Lupin Protein Isolate (LPI) Isolation

Lupin seeds were manually cleaned and ground using a Moulinex mixer (Type 716, France) at a maximum speed and the meal was ground to pass through a 1 mm^2 sieve. The powder was then defatted using chloroform: methanol (3:1v/v) for 8 h. Solvent was evaporated by rotary-evaporator and dried-defatted meal was used to separate LPI from Lupin seeds at pH 4.5 according to Johnson and Brekke [28].

2.6 Preparation of Lupin Protein Hydrolysate (LPH)

Lyophilized LPI was dissolved in distilled water (100g/L) and hydrolyzed batch-wise by treatment with Alcalase (E/S ratio of 1:200 (w/w)) at 55 °C and pH 7.8. The hydrolysis was allowed to proceed for 240 min, the pH was kept at 7.8 during hydrolysis by addition of 1M NaOH and the degree of hydrolysis (DH) was determined every 60 min during hydrolysis according to Adler-Nissen [29]. At the end of hydrolysis, the enzyme was inactivated by heating at 80 °C for 20 min. Hydrolysate was clarified by centrifugation at 4000 g for 30 min at 16 °C to remove insoluble substrate fragments, and the supernatant was lyophilized and freeze at -20°C until further use.

2.7 Acute Toxicity

According to OECD guideline for testing of chemicals [30]. Twenty male Wistar Albino rats (160 ± 10 g, body wt.) were divided into four groups, 5 rats each. All treatments were delivered by gavage as dissolved in 2mLdistilled water. The first group received 2 mL distilled water free from any external treatment. The groups 2, 3 and 4 received one single dose of LPH of 2000, 2500 and 5000 mg/kg body wt.), respectively. All rats were kept under observation for 24 h for recording any symptoms of toxicity or mortality and maintained for further 14 days to observe behavioral and body weight changes.

2.8 Antibacterial Activity of LPH Against Gram Positive and Gram Negative Bacteria

LPH was tested for antimicrobial activity against gram positive bacteria (*S. aureus* and *B. subtilis*) and gram negative bacteria (*P. aeruginosa* and *E. coli*) by conventional well-diffusion assay [31]. The pure cultures of bacterial strains were sub-cultured on Mueller Hinton broth (MHB) and incubated on a rotary shaker at 200 rpm at 37 °C (*S. aureus, P. aeruginosa* and *E. coli*) or 28 °C (*B. subtilis*) for 24h. An aliquot (0.1 ml) of the last culture was transferred into 10 ml MHB and incubated at 37 °C (*S. aureus, P. aeruginosa* and *E. coli*) or 28 °C (*B. subtilis*) for 24 h to reach a count of 1.05×10^9CFU/ml. Each strain was spread uniformly onto individual plates using sterile cotton swabs. Wells of6-mm diameters were made on Mueller Hinton Agar (MHA) plates using gel puncture. Aliquots (40 µl) of LPH concentrations (50, 100, 150, 200 and 250µg/ml) were transferred onto each well of all plates. Negative control (sterilized distilled water) was carried out. After incubation at at37 °C (*S. aureus, P. aeruginosa* and *E. coli*) or 28 °C (*B. subtilis*) for24 h, the different levels of zones of inhibition were measured using a transparent ruler and the diameter was recorded in mm to conclude the minimum inhibitory concentration (MIC).

2.9 Storage of Minced beef with LPH under Refrigeration Conditions

Minced beef samples (100 g) were placed in stomacher bags and homogenized in a stomacher for 2 min at room temperature. Following homogenization LPH was added to the samples. Treatment of the samples included no addition (negative control), addition of LPH at 100 μgg^{-1}, 150 $\mu gg^{-1}$and 200 μgg^{1}. Tests using nisin (200 μgg^{-1}) as positive control was carried out in parallel. All stomacher bags with samples from all treatments were wrapped and stored under aerobic conditions at 4 °C for 15 days.

Antioxidant analysis and microbiological analysis of samples were carried out at different intervals of preservation (0-15 days) at 4 °C.

2.1.1 Lipid peroxidation assay

Lipid peroxidation in the minced beef supplemented with LPH at different concentration (100 µgml^{-1}, 150 µgml^{-1} and 200 µgml^{-1}) was measured using the method of Niehius and Samuels on [32] after different intervals of preservation (0-15 days) at 4 °C. Five gram of each meat sample was homogenized. A volume of 10%w/v homogenate was prepared in 0.05 M phosphate buffer (pH 7) and centrifuged at 12,000 ×g for 60 min at 4 °C. The supernatant obtained was used for lipid peroxidation assessment.100µl from supernatant was treated with 2000µl of (1:1:1 ratio) TBA–TCA–HCl reagent (thiobarbituric acid 0.37%, 15% trichloroacetic acid and 0.25 N HCl). All the tubes were placed in a boiling water bath for 30 min and allowed to cool. The amount formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm using JENWAY 6405 UV/visible spectrophotometer (UK) against a reagent blank. Percentage inhibition was calculated using the equation:

$$\text{Lipid oxidation inhibition (\%)} = [1 - (\text{absorbance of sample}/\text{absorbance of control})]\ x100$$

2.1.2 Microbial analysis

Microbial analysis of minced beef supplemented with LPH at different concentration (100 µgml^{-1}, 150 µgml^{-1} and 200 µgml^{-1}) compared to nisin (200 µg/g) as a positive control was assessed after different intervals of preservation (0-15 days) at 4 °C followed the procedures outlined in APHA [33]. The samples (10 g) were transferred aseptically to a stomacher bag containing 90 ml of peptone saline diluent (1.0 g peptone and 8.5 g sodium chloride in 1 liter of distilled water) at room temperature and homogenized for 60 s. A serial 10-fold dilution series was prepared. Determinations were carried out for different bacterial counts using different specific selective media [34] as follows total viable count (TVC) was enumerated on Plate Count Agar (PCA, Merck, Darmstadt, Germany) at 25 °C after 72 h, psychrotrophs were counted on PCA (Merck, Darmstadt, Germany) at7 °C after 10 days and coliformbacteria was determined by MacConkey agar (Mast Group, Merseyside, UK) with a double layer of the same medium incubated at 37 °C for 24 h. Microbiological data were transformed into logarithms of the number of colony forming units (CFU/g).

2.10 Technological and Sensorial Qualities

2.10.1 Preparation of beef burger

Minced beef samples were used for beef burger manufacture with these ingredients: minced beef; 1000 g, fat; 21 g, fresh onion; 3 g, sodium chloride; 20 g, black pepper; 50 g, coriander; 30 g, clove; 5 g and cinnamon; 5 g) which were minced twice. Treatment of the samples included no addition (negative control), addition of LPH at 100 µgg^{-1}, 150 µgg^{-1} and 200 µgg^{-1}. Addition of nisin (200 µgg^{-1}) as positive control. Meat mixture was shaped manually using patty maker to obtain round disks 9.5 cm diameter and 0.5 cm thickness. Burgers were packed in polyethylene bags in foam dish.

All burger beef treatments were grilled on hot plate with little sunflower oil at 110 °C for 4 min. Then the cooking loss percentage was calculated from following equation according to A.O.A.C. [35].

$$\text{Cooking loss (\%)} = \frac{\text{Fresh burger weight} - \text{Cooked burger weight}}{(\text{Fresh burger weight})X100}$$

Shrinkage percentage was calculated from this equation according to A.O.A.C. [35].

$$\text{Shrinkage (\%)} = (a - b) + (c - d)X100$$

The thickness of uncooked burger (a), the thickness of grilled burger (b), the diameter of uncooked burger (c) and the diameter of grilled burger (d).

Sensory evaluation was conducted according to the method described by Mansour and Khalid [36]. Cooked burger samples were served warm to 10 panelists (staff of food science Department, Faculty of Agriculture, Zagazig University, Egypt) without care of age or sex. The panelists were subjected to sensory evaluation using an 8 point hedonic scale for appearance color, juiciness, tenderness flavor and overall acceptability. A numerical basis as assort of evaluation from 1-8 was used where (1=dislike extremely, 2= dislike very much, 3= dislike moderately,4= dislike slightly, 5= like slightly,6= like moderately, 7=like very much, 8= like extremely).

2.11 Statistical Analysis

All biological trials and measurements were conducted in triplicate and expressed as the mean plus the standard error. ANOVA variance analysis was used for the statistical analysis of data using the general linear models (GLM) procedure of the SAS software (version 9.1, SAS Institute, Inc., 2003). Least significant differences were used to compare means at $p< 0.05$.

3. RESULTS AND DISCUSSION

3.1 Production of LPH

Alcalase was used to produce bioactive peptides from proteins [21, 26]. In the present study, hydrolysis of LPI with Alcalase was monitored for 4h. Hydrolysates obtained after 1, 2, 3 and 4h had DH values of 9%, 16%, 23% and 25%, respectively. The high DH values are consistent with the broad specificity of Alcalase. Similar DH values were reported by Osman *et al*. [21] from Alcalase hydrolysis (4h) of goat milk proteins. Also from hydrolysis of barbel muscle protein by Alcalase nearly similar DH values were obtained after 1 and 2 h but the final DH was lower [27].

3.2 Acute Toxicity

No death or hazardous signs were recorded in rats during 14 days of observation after acute treatment by oral route with

LPH in doses of 2,000, 2,500 and 5,000 mg/kg body weight. The absence of mortality after single administration of very high doses (up to 5,000 mg/kg body weight/day) of LPH during acute toxicology test indicates the safety of this protein and suggesting a lethal dose 50% (LD_{50}) above 5,000 mg/kg body weight. No signs of overt toxicity were observed in any group 24 h after dosing and no abnormal breathing, impaired movements, etc. were noticed in any rat group. Observation of treated animals over the next 14 days showed no adverse effects of these treatments.

3.3 Antibacterial Activity

Antibacterial activity of LPH against gram negative and gram positive bacteria was evaluated by agar well diffusion method. LPH exhibited antibacterial activity against all tested bacteria (Table 1). The minimal inhibitory concentration (MIC) of LPH was determined against all bacterial strains. MIC value of LPH against all bacterial strains was 100 µg ml^{-1}. The results showed that G^+ bacteria were more sensitive than G^- bacteria due to a unique outer membrane in G^+ bacteria that determines permeability and susceptibility of the cells to the antibacterial agents [37].

3.4 Storage of Minced Beef with LPH under Refrigeration Conditions

3.4.1 Microbial analysis

The results presented in table 2 show the changes in the levels of total viable count (TVC) in minced beef preserved at 4 °C for 15 days as supplemented with LPH at different concentrations (100, 150 and 200 µg/g) compared to nisin (200 µg/g) as a positive control. It can be observed that TVC of all treated minced beef samples were significantly different from the negative control group. TVC increased gradually with time and reached the 7 log CFU/g after 7, 10, 10, 10 and 15 days for control, nisin (200 µg/g), LPH (100 µg/g), LPH (150 µg/g) and LPH (200 µg/g) respectively.

3.4.2 Lipid peroxidation

The inhibition against lipid oxidation in minced beef supplemented with LPH at different concentration (100, 150 and 200 µg/g) and storage at 4 °C for 15 days compared to control was shown in Figure 1.

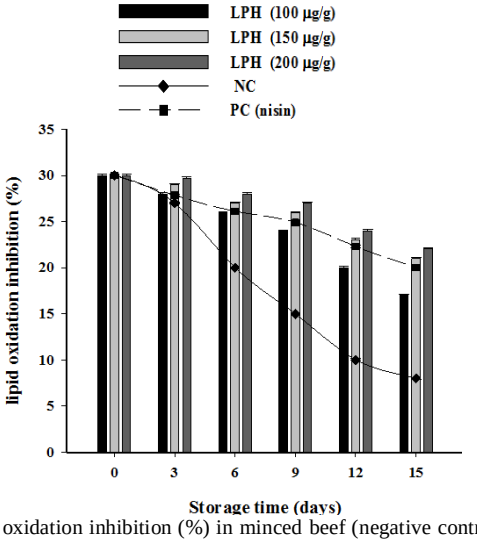

Fig. 1: Lipid oxidation inhibition (%) in minced beef (negative control; NC)as supplemented with LPH at different concentrations (100, 150 and 200 µg/g) and stored at 4°C for different periods (0-15 day) compared to nisin (200 µg/g) as a positive control (PC).

Untreated minced beef showed much decreasing in inhibition against lipid oxidation (8.67 ±0.053) than minced beef supplemented with LPH(100, 150 and 200 µg/g) and nisin (200 µg/g) (18 ±0.021, 22.34±0.043, 25 ±0.06 and 23.13±0.023) respectively, after 15 days storage at 4 °C compared to zero time (30 ±0.013).

Table 1: Antibacterial activity of LPH at different concentrations (50-250µg ml^{-1}) against $Gram^+$(*Staph. aureus* and *B. subtilis*) and $Gram^-$(*P. aeruginosa* and *E. coli*) bacteria using agar well diffusion assays.

Microorganisms	Inhibition zone diameter (mm)				
	50 µg ml^{-1}	100 µg ml^{-1}	150 µg ml^{-1}	200 µg ml^{-1}	250 µg ml^{-1}
Gram positive bacteria					
Staph. aureus	-	24^{a}±0.3	27^{a}±0.3	33^{a}±0.5	39^{a}±0.7
B. subtilis	-	15^{b}	21^{b}±0.21	27^{a}±0.4	33^{b}±0.5
Gram negative bacteria					
P. aeruginosa	-	11^{b}	13^{c}±0.2	17^{b}±0.3	25^{c}±0.5
E. coli	-	9^{bc}±0.11	12^{c}±0.11	16^{b}±0.4	21^{c}±0.22

Table 2: Total viable count in minced beef (negative control; NC)as supplemented with LPH at different concentrations (100, 150 and 200 µg/g) and stored at 4°C for different periods (0-15 day) compared to nisin (200 µg/g) as a positive control (PC).

Storage time (days)	NC	PC (Nisin; 200 µg/g)	LPH		
			100 µg/g	150 µg/g	200 µg/g
	Log cfu/g				
0	2^{c}±0.09	2^{c}±0.09	2^{c}±0.09	2^{c}±0.09	2^{c}±0.09
3	3.7^{c}±0.11	2^{c}±0.07	2.4^{c}±0.06	2.2^{c}±0.08	2^{c}±0.15
6	5.4^{b}±0.15	3.6^{c}±0.09	3.7^{b}±0.05	3.4^{c}±0.09	3^{c}±0.12
7	7.3^{b}±0.18	4.9^{c}±0.11	5.1^{b}±0,11	4.5^{b}±0.11	4.3^{b}±0.18
10	8^{a}±0.21	6.9^{b}±0.08	7.2^{a}±0.13	6.8^{a}±0.14	5.3^{b}±0.2
15	9.3^{a}±0.19	8.3^{a}±0.16	8.6^{a}±0.18	7.5^{a}±0.21	6.7^{a}±0.22

3.5 Technological and Sensorial Qualities

Lupins (*Lupinus angustifolius*) have been suggested as a potential substitute for soybeans in Asian Food. Much research has been done using lupin as a substitute for soybean in soy milk and tofu. Furthermore lupin is a high protein seed and their protein has many properties [38]. Therefore, the potential of using lupins protein as a nutraceutical food supplement and additive in Asian Foods is tremendous. Our own investigation in table 3 has shown that lupin protein can improve the color, water absorption, protein level and eating qualities of fortified burger, that through the reduction of cooking loss % and Shrinkage % gradually by increasing of concentration from (33.4%, and 23.1%) in control sample to 26.8%, and 10.5% in positive sample (nicin 200µg/g) respectively, which was nearest value compare to the highest concentration of lupin protein additives that probably due to the high total digestible nutrients (TDN) in the lupin [39]. The obtained results are in harmony with Mahmoud *et al.* [40] who observed decreased in cooking loss as the amount of protein was increased in meat products.

Table 3: Cooking loss and shrinkage percentages in beef burger (negative control) as supplemented with LPH at different concentrations (100, 150 and 200 µg/g) compared to nisin (200 µg/g) as a positive control.

Samples treatments	Cooking loss%	Shrinkage %
Negative control	33.4^{a}	23.1^{a}
Positive control (Nisin; 200 µg/g)	26.8^{a}	10.5^{c}
LPH (100 µg/g)	29.2^{a}	17.8^{b}
LPH (150 µg/g)	26.9^{a}	17.8^{b}
LPH (200 µg/g)	27.4^{a}	15.5^{b}

Organoleptic properties of burger were evaluated for appearance, color, juiciness, tenderness, flavor and overall acceptability. Table 3 illustrated sensory evaluation of beef burger containing different concentrate of LPH (100, 150 and 200 µg/g) compared to nisin (200 µg/g) as a positive control. The obtained results proved the high acceptability of all samples and the acceptability score for burgers produced were not significantly different ($p > 0.05$) from the control and the fortified samples [41].

Table 4: Sensory evaluation of beef burger (negative control; NC) as supplemented with LPH at different concentrations (100, 150 and 200 µg/g) compared to nisin (200 µg/g) as a positive control (PC).

Treatments	Appearance	Flavor	Tenderness	Juiciness	Color	Over-all acceptability
NC (without treatment)	7.8^{a}	5.0^{c}	6.3^{b}	6.6^{ab}	7.0^{a}	90^{a}
PC (Nisin; 200 µg/g)	7.0^{bc}	6.0^{a}	7.0^{a}	6.2^{c}	7.0^{a}	90^{a}
LPH (100 µg/g)	7.8^{a}	5.2^{c}	6.2^{b}	6.0^{bc}	6.8^{a}	89^{a}
LPH (150 µg/g)	7.0^{bc}	5.8^{ab}	6.8^{a}	7.0^{a}	8.0^{a}	90^{a}
LPH (200 µg/g)	7.6^{ab}	5.4^{bc}	6.8^{a}	7.4^{a}	7.6^{a}	90^{a}

4. CONCLUSION

The results of this study showed that enzymatic hydrolysis by alcalase at pH 7.8 and 55 °C for 240 min is an easy tool to increase the antibacterial activity of lupin protein against both gram negative and gram positive bacteria and can be used as a bio-preservative in food system. Extending the technological validity of minced beef will help avoid big losses of minced beef and enhance its chances to be incorporated into many meat products while avoiding hygienic, chemical and technological deterioration.

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