Journal of Animal and Veterinary Advances 7 (11): 1458-1463, 2008
ISSN: 1680-5593

Antimicrobial Effects of Lysozyme in Combination with *Zataria multiflora* Boiss. Essential Oil at Different pH and NaCl Concentrations on *E. coli* O157:H7 and *Staphylococcus aureus*

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Abstract: The antibacterial efficacy of unheated lysozyme (L) and Heat-Treated Lysozyme (HTL) at 62, 72 and 80°C, separately and in combination with *Zataria multiflora* Boiss. essential oil (Z.EO) on *E. coli* O157:H7 and *S. aureus* at different pH (5, 6 and 7) and NaCl concentrations (0.5, 1.5 and 3%) was studied. A micro-broth dilution assay was applied to define the Minimal Inhibitory Concentrations (MICs). The inhibitory action of L on *S. aureus* was increased along with pH deduction and NaCl enhancement, whereas *E. coli* O157:H7 wasn't inhibited up to 1024 μg mL^{-1} at similar circumstances. Both bacteria were inhibited by HTL (at 72 and 80°C) at NaCl 0.5%, but at ≥1.5% of NaCl concentration, the inhibition was stopped. However, the inhibitory activity of HTL was greater than L. Z.EO was effective against both bacteria and inhibition was stronger at lower pH and higher NaCl concentrations. MICs of L and HTL combined with Z.EO were decreased. Enhancement of Z.EO concentrations was led to more prominent inhibition, whereas, by increasing NaCl concentration, inhibition was reduced considerably. The lowest MICs of L and HTL were achieved by increase of Z.EO to 300 μg mL^{-1} along with decrease the pH and NaCl to 5 and 0.5%, respectively.

Key words: Lysozyme, *Zataria multiflora* Boiss, essential oil, combination, *E. coli* O157:H7, *S. aureus*

INTRODUCTION

Lysozyme as an enzyme has the potency of lysis of bacterial cells (Scaman *et al*., 2006). Lysozymes are detected as widespread enzymes among animals and plants and involved as a natural defiance against pathogenic bacteria (Nakimbugwe *et al*., 2006b). Most of lysozyme, is commercially produced from egg white. Enzymatic activity of lysozyme carried out through its lytic function on glycosidic bonds present between N-acetyl muramic acid and N-acetyl glucosamine of cell wall peptidoglycan (Chung and Hankock, 2005; Gill and Holley, 2003).

Lysozyme activity is variable relatively, but in general gram positive bacteria are more sensitive than gram negative bacteria and among gram positive bacteria thermophilic sporeformers are more susceptible. The main reason for resistance of gram negative bacteria resulted from lipoprotein-lipopo lysaccharide layer (Nattress and Baker, 2003; Vannini *et al*., 2004). Lysozyme is used for special food products, such as hard cheeses (to prohibit of late blowing) and wine products (to prevent of infection) as well as preservative for sea-foods, vegetables, fruits and as a gredient of pharmaceutical products (Nakimbugwe *et al*., 2006a). This natural enzyme also is used in products that have been sterilized by heat, to decrease thermal treatments (Hughey and Johnson, 1987).

Essential Oils (EOs) are plant originated materials that can be extracted by some methods such as fermentation, enfleurge and specially steam distillation (Burt, 2004). These secondary metabolites have a considerable spectrum of anti-inflamatory, antioxidant and anticarcinogenic activities. EOs also as biocidal substances have a broad portion of antibacterial, antifungal, antiviral and insecticide activities (Kalemba and Kunicka, 2003). The hydrophobicity of EOs enable them to penetrate in the lipids of membrane of bacterial cell wall, that can be led to ion linkage and other cell components and finally to cell death. The antibacterial activity of EOs is slightly more efficient on gram positive bacteria than gram negative bacteria because the outer membrane of gram negatives limits hydrophobic compounds diffusion via their lipopolysaccharide layer (Burt, 2004).

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Avishane Shirazi is the native name for *Zataria multiflora* Boiss in Iran and its essential oil has been applied usually to treatment of some illnesses like respiratory tract infections and irritable bowel syndrome (Sarififar *et al*., 2007) and it`s antimicrobial activity was reported in some studies (Basti *et al*., 2007; Misaghi and Basti, 2007; Sarififar *et al*., 2007).

A number of studies have been accomplished on antibacterial properties of lysozyme lonely or in combination with other factors. Different factors such as pH values, salt concentrations and temperatures were regarded as interesting subjects to study (Makki and Durance, 1996; Razavi-Rohani and Griffiths, 1996). The inhibitory action of NaCl on *E. coli* O157: H7, *S. typhimorium*, *S. grimessi* and *S. putrefaciens* has been proved (Gill and Holley, 2003).

Synergism phenomenon between EOs and other agents or processes such as salts, fatty acids, low pH, nisin and mild heat treatment has been demonstrated (Burt, 2004; Kalemba and Kunicka, 2003).

The objective of the present study was to determine the best combination by lysozyme and *Zataria multiflora* essential oil (50, 150 and 300 μg mL^{-1}) at different pH (5, 6 and 7), salt concentrations (0.5, 1.5 and 3%) and lysozyme temperatures treatments to enhance the synergism effect against *E. coli* O157: H7, *S. aureus* and replacement of such combination to minimize food destructive heat processing or chemically-based food preservation methods.

MATERIALS AND METHODS

This study was carried out during winter of 2008 at Department of Food Hygiene and quality control, Urmia University.

Lysozyme preparation: Chicken egg white lysozyme chloride (60000 units per mg of protein: one unit will produce a A_{450} of 0.001 per min at pH 6.24 at substrate, in a 2.6 mL reaction mixture) was purchased from Sigma-Aldrich (Steinhem, Germany). Different amounts of lysozyme powder were dissolved in 66 mM potassium phosphate buffer (Sigma-Aldrich Steinhem, Germany) adjusted in pH 6.24 at 25°C, to obtain the lysozyme solutions by concentrations of 128, 256, 512 and 1024 μg mL^{-1} and then were sterilized by filtration via 0.22 μ microfilters.

Lysozyme thermal treatment: For thermal treatment, different temperature conditions (unheated lysozyme solutions at environment temperature 25°C and heat-treated) were applied. In essence, the screw-capped tubes containing lysozyme solutions were put in a water bath at 62, 72 and 80°C for 20 min and then were cooled immediately. Because of aggregation of insoluble materials arising from heating, solutions were centrifuged (3000 × g, 15 min). The supernatants were separated and sterilized via re-filtration for being used in assay.

Extraction of the essential oil: Plant materials (*Zataria multiflora* Boiss) were purchased from local grocery and authenticated at Institute of Medicinal Plants, Karaj, Iran. The dried aerial parts were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. Colorless oil, with characteristic odor, was obtained and dried over anhydrous sodium sulphate and stored at 4°C before use.

Extract yield was 1.71% (volume of extract/weight of dry matter ×100). Essential oils were prepared at three levels: 50, 150 and 300 μg mL^{-1}. The 5% (v/v) Dimethylsulfoxide (DMSO) (Merck, Schauchardt OHG, Hohenbrun, Germany) as dispersing solvent was added to broth media (Misaghi and Basti, 2007).

Inoculums and media preparation: The test microorganisms were *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* O157:H7 ATCC 25922 were provided graciously from Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. Stock bacteria were kept in granule containing cryo-tubes at -70°C. Each granule was transferred on tryptic soy agar (Merck, Darmstadt, Germany) and incubated at 37°C overnight. Fresh cultures were prepared by inoculating a single colony of TSA to 10 mL tryptic soy broth followed by 24 h incubation at 37°C. These suspensions were approximately included 10^9 cfu mL^{-1} and were finally prepared spectrophotomtrically at 600 nm to a concentration of 10^6 cfu mL^{-1} for being applied in procedures of assays. The pH values (5, 6) were adjusted by 1 N hydro chloric acid. The NaCl concentrations were prepared at 0.5, 1.5 and 3%.

Antimicrobial assays: A micro-broth dilution method was employed to determination of minimal inhibitory concentration (MIC) values of lysozyme in combination by other factors. The 96-well sterile tissue culture microtiter plates (Sarsted, inc. USA) were used. In each test the first well was filled by 280 μL TSB plus 40 μL of L, HTL or Z.EO solution and the next wells were only filled by 160 μL TSB and then two-fold dilutions were prepared for each concentration. Then 20 μL of inoculum (1.8×10^5 cfu per well) was added to each well to achieve a final volume equal to 180 μL $well^{-1}$. The lids of micro plates were put and micro plates shacked for 30 sec at 250 rpm in microplate thermo shaker (PST-60 HL PLUS, BOECO, Germany), then incubated at 37°C for 24 h.

The MICs and MBCs (minimal bacteriocidal concentration) of tests were determined by spreading 100 μL of each clear well on TSA following incubated at 37°C for 24h. MIC was defined as the lowest concentration at which produces ~ 90% kill of the test microorganisms following incubation and MBC as the lowest concentration of antimicrobial that produces ≥99.9 kill of the test microorganisms (Davidson *et al*., 2005).

Results from the experiments were analyzed by using the SPSS 11.5 statistical package (SPSS Ltd., Woking, UK). All experiments were accomplished in triplicate and the results reported are averages.

RESULTS AND DISCUSSION

The enzymatic function of lysozyme has been observed at a range of pH from 3.5-7 (Wang and Shelef, 1992). Along with decreasing pH, some gram positive bacteria were inhibited by lysozyme (Razavi-Rohani and Griffiths, 1996). For instance, in case of a gram positive organism like *L.monocytogenes*, by decreasing the pH to 5.5, growth inhibition was encouraged (Johansen *et al*., 1994). At pH values of 5, 6 and 7 L had no inhibitory effect in concentration up to 1024 μg mL^{-1} on *E. coli* O157:H7, whereas at pH 5 *S. aureus* was inhibited at 1024 μg mL^{-1}, but no inhibition was seen at pH values 6 and 7 (Table1).

Salt concentrations have critical effect on L activity. According to Razavi-Rohani and Griffiths (1996), inhibitory action of lysozyme only was effective at ≥5% of NaCl concentration. Low concentration may resulted in non specific activation of lysozyme, whereas its activity abolished at high salt concentration (Davidson *et al*., 2005). In current study, L concentration by 128-1024 μg mL^{-1} had no inhibitory effect at NaCl concentrations of 0.5-3% against *E. coli* O157:H7 and *S. aureus* (Table 1).

Heat-denaturation may result in strengthening the antibacterial effect of lysozyme. The results of our study were in agreement with Ibrahim *et al.* (1996a). They reported that thermal denaturation at 80°C (pH 7.2) led to dimerization of enzyme and dimeric form can be effective on both gram positive and gram negative bacteria. Heat-denaturation of lysozyme was abolished enzymatic activity but antimicrobial activity was maintained and membrane- disturbing activity of denatured lysozyme was affected bacterial and fungal cells. It was appeared that amphiphatic C-terminal domain involved in bactericidal and fungicidal effects (Düring *et al*., 1999). In relation of

Table 1: MICs and MBCs (μg mL^{-1}) of lysozyme at different pH values, NaCl concentrations and preliminary heating temperatures

		E. coli O157:H7		*S. aureus*	
Samples and treatment conditions		MIC	MBC	MIC	MBC
Unheated Lysozyme (L)					
pH	5	>1024	nd[A]	1024	>1024
	6	>1024	nd	>1024	nd
	7	>1024	nd	>1024	nd
NaCl %	0.5	>1024	nd	>1024	nd
	1.5	>1024	nd	>1024	nd
	3	>1024	nd	>1024	nd
Heat Treated Lysozyme (HTL)					
Temp°C	62	>1024	nd	>1024	nd
	72	1024	>1024	>1024	nd
	80	512	1024	1024	>1024

[A]Not detected

Table 2: MICs and MBCs (μg mL^{-1}) of HTL at 72 and 80°C with combination by pH and NaCl factors

				E. coli O157:H7		*S. aureus*	
Samples and treatment conditions	Temp°C	pH	NaCl %	MIC	MBC	MIC	MBC
Heat Treated Lysozyme (HTL)	72	5	0.5	512	1024	1024	nd
			1.5	>1024	nd[A]	>1024	nd
			3	>1024	nd	>1024	nd
		6	0.5	>1024	nd	>1024	nd
			1.5	>1024	nd	>1024	nd
			3	>1024	nd	>1024	nd
	80	5	0.5	256	512	512	1024
			1.5	>1024	nd	>1024	nd
			3	>1024	nd	>1024	nd
		6	0.5	1024	>1024	>1024	nd
			1.5	>1024	nd	>1024	nd
			3	>1024	nd	>1024	nd

[A]Not detected

Table 3: MICs and MBCs (μg mL^{-1}) of Z.EO at different pH values and NaCl concentrations

		E. coli O157:H7		*S. aureus*	
Samples and treatment conditions		MIC	MBC	MIC	MBC
pH	5	150	300	75	150
	6	>300	nd[A]	300	>300
	7	>300	nd	>300	nd
NaCl %	0.5	>300	nd	>300	nd
	1.5	300	>300	150	300
	3	150	300	75	150

[A]Not detected

Table 4: MICs and MBCs (μg mL^{-1}) of unheated lysozyme (L) in combination with *Z.multiflora* essential oil (50, 150 and 300 μg mL^{-1}) at pH 5 and different NaCl concentrations

	L+Z.EO						
Organism	NaCl (%)	MIC (50)	MBC (50)	MIC (150)	MBC (150)	MIC (300)	MBC (300)
E. coli O157:H7	0.5	512	1024	256	512	128	256
	1.5	1024	>1024	512	1024	256	512
	3	>1024	nd[A]	>1024	nd	1024	>1024
S. aureus	0.5	256	512	128	256	64	128
	1.5	512	1024	256	512	128	256
	3	1024	>1024	1024	>1024	1024	>1024

[A]Not detected

Table 5: MICs and MBCs (μg mL^{-1}) of Heat-Treated Lysozyme (HTL) in combination with *Z.multiflora* essential oil (50, 150 and 300 μg mL^{-1}) at pH 5 and different NaCl concentrations

	HTL+Z.EO						
Organism	NaCl (%)	MIC (50)	MBC (50)	MIC(150)	MBC(150)	MIC(300)	MBC (300)
E. coli O157:H7	0.5	256	512	64	128	32	64
	1.5	512	1024	256	512	128	256
	3	1024	>1024	1024	>1024	1024	>1024
S. aureus	0.5	512	1024	256	512	64	128
	1.5	1024	>1024	512	1024	256	512
	3	>1024	nd[A]	1024	>1024	1024	>1024

[A]Not detected

heat-treatment, neither preliminary heating of lysozyme at 62°C nor unheated treatment were effective against *E. coli* O157:H7 and *S. aureus* at all concentrations, although when temperatures were exceeded to 72 and 80°C, the inhibitory properties against *E. coli* O157:H7 was increased and inhibition was more prominent than *S. aureus* (Table 1).

According to Ibrahim *et al.* (1996b), along with increasing NaCl concentration, antibacterial action of heat-denatured lysozyme on *E. coli* K12 and *S. aureus* was reduced. In the next step the interactions among all factors (In case of pH, two more efficient level of the factor were selected) were evaluated. To do these pH values of 5 and 6, salt concentrations of 0.5, 1.5 and 3% and HTL (at 72 and 80°C) were used. Among all combinations, at pH 5, salt concentration 0.5% and heating temperature 80°V, the MICs were at the lowest quantity: 512 and 256 μg mL^{-1} for *S. aureus* and *E. coli* O157:H7, respectively. The MICs were increased along with heating temperature deduction. At NaCl concentrations ≥1.5%, absence of inhibition was determined. At higher NaCl concentration there was an anti-synergism or antagonism effect in such situation. The best inhibition was at pH 5, NaCl 0.5% and heating temperature 80°C (Table 2).

Sharififar *et al.* (2007) were reported that Z.EO had a great activity on *S. aureus* and *E. coli* and MICs were 21 and 42 mg mL^{-1}, respectively. Combination of sodium chloride and mint essential oil resulted in synergism effect against *S. enteritidis* and *L. monocytogenes* (Burt, 2004). According to Basti *et al.* (2007), at pH 6 storage temperature ≤25°C up to 43 days and 0.06% Z.EO concentration, inhibition of *S. aureus* and *S. typhimurium* was complete, whereas at pH 7.3, storage temperature ≤35°C and ≤0.0015% Z.EO concentration no inhibition was seen. Increasing of an essential oil hydrophobicity at low pH was led to its more effective dissolving in bacterial cell membrane lipids and finally to increase of bacterial susceptibility (Burt, 2004). Along with pH deduction and NaCl concentration enhancement, the MICs of Z.EO were decreased, especially at pH 5 and 3% NaCl concentration (Table 3).

In final step, probable synergism or antagonism between L and HTL (at 80°C as most active lysozyme) with Z.EO at optimum pH (ie 5) and NaCl concentrations

from 0.5-3% was studied. The results were indicated that by increasing Z.EO concentrations and decreasing NaCl concentrations, MICs were decreased substantially, as *E.coli* O157:H7 and *S. aureus* were inhibited at 128, 64 and 32, 64 μg mL^{-1} by L+Z.EO and HTL+Z.EO combinations, respectively at 0.5% NaCl and 300 μg mL^{-1} Z.EO concentrations (Table 4 and 5).

CONCLUSION

On the basis of results, besides hurdle technology, changing or modification of involved intrinsic and extrinsic factors could be exhibited more effective inhibition against *E.coli* O157:H7 and *S. aureus*. MICs of lysozyme were decreased at low pH values, NaCl concentrations and high levels of Z.EO percentages. Heating was improved inhibitory activity gradually. The greatest inhibition was achieved by combination of HTL (80°C) and Z.EO (300 μg mL^{-1}) at pH 5 and NaCl 0.5%. Therefore it could be concluded that HTL and Z.EO could be used to prevent of food borne pathogens and naturally food preservation processes instead of chemically-based preservation methods.

ACKNOWLEDGEMENT

This project was supported by financial funding of Faculty of Veterinary Medicine of Urmia University, Iran.

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