

Extent and Mode of Action of Cationic Legume Proteins against *Listeria monocytogenes* and *Salmonella* Enteritidis

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Published online: 10 April 2013
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Abstract The methylated soybean protein and methylated chickpea protein (MSP and MCP) with isoelectric points around pI 8 were prepared by esterifying 83 % of their free carboxyl groups and tested for their interactions with *Listeria monocytogenes* and *Salmonella* Enteritidis. The two substances exhibited a concentration-dependent inhibitory action against the two studied bacteria with a minimum inhibitory concentration of about 100 µg/mL. The IC₅₀ % of the two proteins against *L. monocytogenes* (17 µg/mL) was comparable to penicillin but comparatively much lower (15 µg/mL) than that of penicillin (85 µg/mL) against *S. Enteritidis*. The two proteins could inhibit the growth of *L. monocytogenes* and *S. Enteritidis* by about 97 and 91 %, respectively, after 6–12 h of incubation at 37 °C. The constituting subunits of MSP (methylated 11S and methylated 7S) were both responsible for its antimicrobial action. Transmission electron microscopy of the protein-treated bacteria showed various signs of cellular deformation. The cationic proteins can electrostatically and hydrophobically interact with cell wall and cell membrane, producing large pores, pore channels and cell wall and cell membrane disintegration, engendering higher cell permeability leading finally to cell emptiness, lysis and death.

Keywords Antimicrobial · Cationic proteins · *Listeria* · *Salmonella* · Cell lysis

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Introduction

Listeria monocytogenes is an important food-borne pathogen causing listeriosis, a severe disease of high fatality rate in USA [1] and EU [2] where the incidence was 3 cases per million population in 2005. Capable of multiplication under high salt concentrations (10 % NaCl), broad pH range (pH 4.5–9) and different temperatures (0–45 °C), it can pose a potential risk to human health [3]. *Salmonella* spp. emerge in many foods by post-process recontamination, causing outbreaks of salmonellosis, another fatal food-borne disease. It can multiply to harmful levels during inappropriate storage conditions and can considerably grow at 8 °C [4]. An EC regulation on microbiological criteria for foodstuffs [5] was legislated and applied in force from January 2006, imposing the whole absence of *Salmonella* and a maximum permissible level of *L. monocytogenes* in foods of 100 CFU g⁻¹ [5].

Resistance to antimicrobial agents by pathogenic bacteria has emerged in recent years, representing a major health problem [6], so the identification of new antimicrobial agents with different mechanisms of action is highly required. Cationic antimicrobial peptides or proteins (AMPs) whose killing mechanism is due to the interaction with the cytoplasmic membrane are promising candidates [7]. Intensive research is currently devoted to understand the effects of AMPs on intact cells using electron microscopy techniques to reveal the damage caused by these molecules on the bacterial morphology and membranes [8].

Another approach to obtain new cationic proteins is through the intentionally tailored chemical modifications of native proteins. Esterification can neutralize the negatively charged carboxyl groups of the aspartyl and glutamyl residues on protein molecules, transforming their net charge into positive. The obtained positively charged proteins

were proved antimicrobially active [9] and inhibited the growth of *L. monocytogenes* and *S. Enteritidis* in raw milk [10]. But this bacterial inhibitory action was not sufficiently qualified. Hence, the objective of the current research was to specify and characterize the extent and mode of action of these antimicrobial cationic proteins against these main pathogens (*L. monocytogenes* and *S. Enteritidis*) using standard media while identifying the main constituents responsible for this action.

Materials and Methods

Preparation of Cationic Proteins

Total Cationic Proteins

Soybean (*Glycine max* L. cultivar Giza 21) and chickpea (*Cicer arietinum* L. cultivar Giza 1) seeds were obtained from Agricultural Research Center, Cairo, Egypt, and used for extracting protein according to the procedure of Johnson and Brekke [11]. Total nitrogen was determined in soybean protein isolate (SP) and chickpea protein isolate (CP) using micro-Kjeldahl method according to AACC [12] and multiplied by the conversion factor 6.25 to get the total protein content. Protein was esterified with methanol according to the procedure of Sitohy et al. [13], and the esterification extent was quantified by the color reaction with hydroxylamine hydrochloride [14]. The resultant modified proteins were denoted as MSP (methylated soybean protein) and MCP (methylated chickpea protein).

Cationic Protein Subunits

The MSP was fractionated into its two main subunits (methylated 11S and methylated 7S) based on the procedure of Nagano et al. [15] with slight modifications. The MSP was dispersed in distilled water (1:15 w/v), adjusted to pH 10 with 2 M NaOH, stirred at room temperature for 2 h and centrifuged at $10,000\times g$ for 20 min at 4 °C. Dry NaHSO₃ was added to the supernatant (0.98 g NaHSO₃/L). Two fractions (expectedly methylated 11S and methylated 7S) were precipitated by successive lowering of the pH to 8 and 7.6, respectively, and isolated by centrifugation at $6,500\times g$ for 15 min at 4 °C after overnight incubation at 4 °C. The formed precipitates were suspended in distilled water, adjusted to pH 7.0 with 2 M HCl, dialyzed 3 days against distilled water and freeze-dried.

Native PAGE

Protein samples were dissolved (5 mg/mL) in a buffer (pH 6.8) containing 0.25 M Tris base and 50 % glycerol and

analyzed by native PAGE according to Laemmli [16] in 3 and 8 % acrylamide for the stacking and resolving gels, respectively. The electrode buffer (pH 8.3) was composed of 0.125 M Tris base and 0.96 M glycine.

Urea-PAGE

Native (SP and CP) and esterified (MSP and MCP) legume proteins as well as the isolated fractions (methylated 11S and methylated 7S) were analyzed by urea-PAGE in 3 and 10 % stacking and resolving gels, respectively, according to Williams and Evans [17].

Agar Well-Diffusion Assay

Listeria monocytogenes Scott A and *S. enterica* subsp. *enterica* serovar Enteritidis strain PT4 used in this study were kindly obtained from Prof. George Nychas, Laboratory of Food Microbiology and Biotechnology, Department of Food Science and Technology, Agricultural University of Athens, Athens, Greece. Cationic proteins (MSP and MCP) were tested for antimicrobial activity against *L. monocytogenes* and *S. Enteritidis* by the conventional agar well-diffusion assay [6] using penicillin as a positive control.

Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of the tested substances against *L. monocytogenes* and *S. Enteritidis* was assayed by the conventional broth dilution assay [18]. Negative control (sterilized distilled water) and positive control (penicillin) were carried out simultaneously.

Crystal Violet Assay

The alteration in membrane permeability was determined by crystal violet assay [19]. Exponential phase cultures of *L. monocytogenes* and *S. Enteritidis* strains were grown in MHB to a concentration of 1.05×10^9 CFU mL⁻¹ after overnight incubation at 37 °C. Bacterial cells were collected after centrifugation at $4,500\times g$ at 4 °C for 5 min, washed thrice and re-suspended in peptone buffer solution (pH 7.4) at the same original volume. The tested substances were added to the cell suspension at 0, 50 and 100 µg/mL except control and incubated at 37 °C for 30 min. Bacterial cells were harvested after centrifugation at $9,300\times g$ for 5 min and re-suspended in peptone buffer solution (pH 7.4) containing 10 µg/mL of crystal violet, incubated at 37 °C for 10 min and then centrifuged at $13,400\times g$ for 15 min. The OD₅₉₀ of the supernatant was measured using JENWAY 6405 UV/visible spectrophotometer (UK). The percentage crystal violet uptake was calculated by the following formula:

$$\frac{\text{OD of the sample} \times 100}{\text{OD of crystal violet solution}}$$

Cell Lysis Assay

Exponential phase cultures of *L. monocytogenes* and *S. Enteritidis* strains were grown overnight at 37 °C to 1.05×10^9 CFU mL⁻¹. An aliquot (1.5 mL) of the last bacterial suspension was centrifuged at 4,500×g for 15 min at 4 °C. Bacterial cells were washed thrice and re-suspended in peptone buffer solution (pH 7.4) at the same original volume. The tested substances (0, 50 and 100 µg/mL) were added to the cell suspensions and incubated at 37 °C for 30 min before separating the bacterial cells at 13,400×g for 15 min and recording OD₂₆₀ absorbance as an indicator of the released UV-absorbing materials and cell lysis [20].

$$\frac{\text{Number of intact cells (control)} - \text{Number of intact cells (treatment)} \times 100}{\text{Number of intact cells (control)}} \quad (1)$$

SDS–PAGE of Bacterial Proteins

Overnight-grown cultures of *L. monocytogenes* and *S. Enteritidis* strains (1.05×10^9 CFU mL⁻¹) were treated with the cationic proteins (0–500 µg/mL) for 24 h at 37 °C, prepared for SDS–PAGE according to [19] and electrophoresed in 12 % SDS–PAGE according to Laemmli [16].

Transmission Electron Microscopy (TEM)

Listeria monocytogenes and *S. Enteritidis* were grown in tryptone soy broth (TSB, Biolife, Italy) and incubated at 37 °C to reach a maximum level of 10^9 CFU mL⁻¹ which was subsequently diluted to 10^8 CFU mL⁻¹ with peptone solution (0.1 % containing 0.85 % NaCl). The cationic proteins and penicillin (100 µg/mL) were added to the cell suspensions except control and incubated at 30 °C for 4 h. Bacterial cells were fixed in glutaraldehyde (2.5 % in 0.1 M phosphate buffer, pH 7.4) for 1 h, rinsed thrice with 0.1 M phosphate buffer (pH 7.4) for 10 min and post-fixed with 1 % osmium tetra-oxide at 4 °C for 2 h. The washing step was repeated, and then cells were dehydrated sequentially using 30, 50, 70 and 95 % acetone for 15 min each and finally with 100 % acetone three times for 30 min each. Subsequently, cells were treated twice with propylene oxide at 4 °C for 10 min and sequentially infiltrated with a mixture of propylene oxide: Durcupan's

ACM epoxy resin (3:1, 1:1 and 1:3) for 45 min. Polymerization of the resin to form specimen blocks was performed in an oven at 60 °C for 72 h. The specimen blocks were sectioned with a diamond knife in a Reichert Ultracut R ultramicrotome (Leica, Wetzlar, Germany). Thin sections (70–80 nm) were placed on 300 mesh copper grids, stained in uranyl/ethyl alcohol (1:1) for 15–20 min and then washed three times with saline solution for 2 min. A drop of Reynold's lead citrate was added before examination using Transmission Electron Microscope (JEOL-TME-2100F, Japan). The number of intact cells and deformed cells were visually counted in 10 fields, and the averages were calculated. The rate of bacterial mortality and the rate of cellular deformation were calculated according to the formulas 1 and 2, respectively.

$$\frac{\text{Number of deformed cells (treatment)} \times 100}{\text{Number of intact cells (treatment)}} \quad (2)$$

Statistical Analysis

All experiments were performed in triplicate, and results were expressed by the mean ± standard deviation. Statistical analysis software (SAS version 9.1, SAS Institute, Inc., Cary, NC, USA, 2003) was used to perform ANOVA and least significance difference (LSD) analysis using general linear models (GLM) procedure. The level of significance was $p < 0.05$.

Results and Discussion

Chemical Characterization

Total protein contents in methylated soybean protein and methylated chickpea protein (MSP and MCP) were nearly in the same range: 91 and 92 % (g protein/100 g dry weight), respectively, while the extent of methylation was 83 % in both, that is, ca. 83 % of the free carboxylic groups of Asp and Glu residues were transformed into methyl carboxylate annulling their negative charges. As a consequence, MSP and MCP should have acquired net positive charges.

The native PAGE electropherograms of soybean and chickpea proteins before esterification (Fig. 1, lane 1 and 3) indicate two major bands of around 360 and 200 kDa corresponding to 11S and 7S subunits, respectively, in agreement with Nielsen et al. [21]. The two major fractions of soybean protein (11S and 7S) constitute approximately 80 % of the total protein with respective molecular masses of 360 and 150 kDa where 11S is composed of 6 constituent subunits, each of which consisting of an acidic and a basic polypeptide, linked together by a disulfide bond [21]. The molecular mass of the constituting subunits of 11S and 7S were in the range 20–34 and 50–75 kDa, respectively. The final modified soybean protein (MSP) prepared in this study is a population of cationic proteins (methylated 11S and methylated 7S) mainly with high and wide molecular mass range (200–360 kDa) but composed of smaller subunits. After esterification, the migration extents of the corresponding bands (lane 2 and 4) were slower referring to increased molecular masses as a result of methyl group grafting into the two legume proteins.

The migration in urea-PAGE into cathode direction indicated that MSP and MCP (lanes 2 and 4) were much faster than their respective native proteins (lanes 1 and 3) referring to bigger positive charges. Esterification with different alcohols leads to the blocking of free carboxyl groups, thus raising the net positive charge and rendering the modified proteins more basic [22]. The similar migration rates of the two modified proteins refer to similar basicity which highly depends on the extent of esterification (83 %) and similar amino acid composition.

Fractionating the methylated soybean MSP (83 % esterification extent) produced methylated 11S and methylated 7S with lower esterification extents of 80 and 75 %, respectively (data not shown), probably as a result of the de-esterification process during subjection to alkaline conditions (pH 10) in the fractionation process. The migration of these two separated fractions in urea-PAGE indicated slightly lower migration rates than MSP (Fig. 1, lanes 2A and 2B). Methylated 7S indicated further lower

migration rate than methylated 11S in accordance with the difference in the esterification extent and with the fact that 11S is originally more basic than 7S.

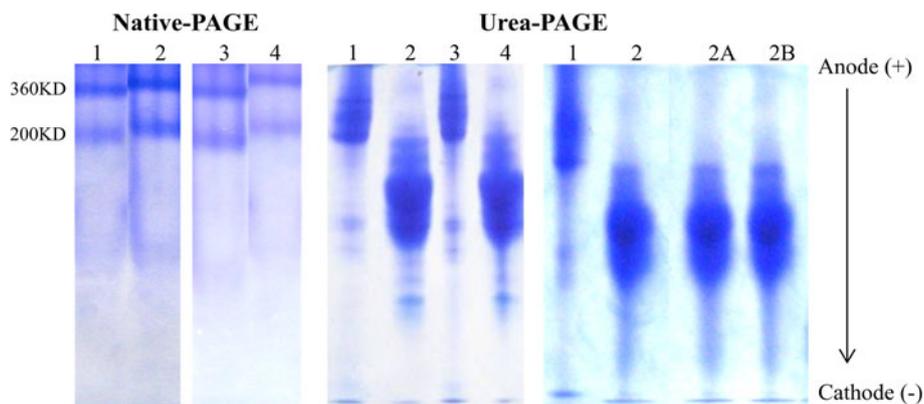
Magnitude of Anti-*Listeria* and Anti-*Salmonella* Action

Agar well-diffusion assay (Fig. 2) indicated concentration-dependent inhibition zone diameters for MSP and MCP where the minimum inhibitory concentration (MIC) against the two studied bacteria was 100 µg/mL. A concentration-dependent antibacterial effect was also observed when adding the cationic proteins (0–500 µg/mL) to the two studied bacteria in MHB and incubating at 37 °C for 24 h (Fig. 2). The substance concentration corresponding to 50 % inhibition of *Listeria* (IC₅₀ %) was 17 µg/mL against 16 µg/mL for penicillin, that is, they are not significantly different ($p < 0.05$). The IC₅₀ % of MSP and MCP on *S. Enteritidis* was 15 µg/mL against 85 µg/mL for penicillin, that is, they are significantly ($p < 0.05$) more potent than penicillin.

The growth curves of *L. monocytogenes* and *S. Enteritidis* at 37 °C reached a maximum number of cells within 6–12 h (Fig. 3). A concentration of 100 µg/mL of MSP or MCP inhibited the growth of *L. monocytogenes* by about 95–96.5 versus 97 % for penicillin. After the same period, the growth of *S. Enteritidis* was inhibited by about 90–91 % by MSP and MCP versus 77–80 % by penicillin, that is, they were significantly ($p < 0.05$) more effective than penicillin. The anti-*Salmonella* action of penicillin was diminishing with time and maintained only 59 % of bacterial inhibition against 90 % by MSP and MCP after 24 h of incubation at 37 °C.

Based on the results of the agar well-diffusion assay and bacterial growth curves, the anti-*Listeria* actions of MSP and MCP are nearly within the same level, leading to the conclusion that the antibacterial action is mainly due to the chemical modification and more specifically to the acquired cationic nature quite similar in the two products. This is particularly true since the native forms of these two

Fig. 1 Native and urea-PAGE of soybean and chickpea proteins before (1 and 3) and after (2 and 4) methylation as well as urea-PAGE of the fractionated methylated subunits of soybean proteins: methylated 11S (2A) and methylated 7S (2B)



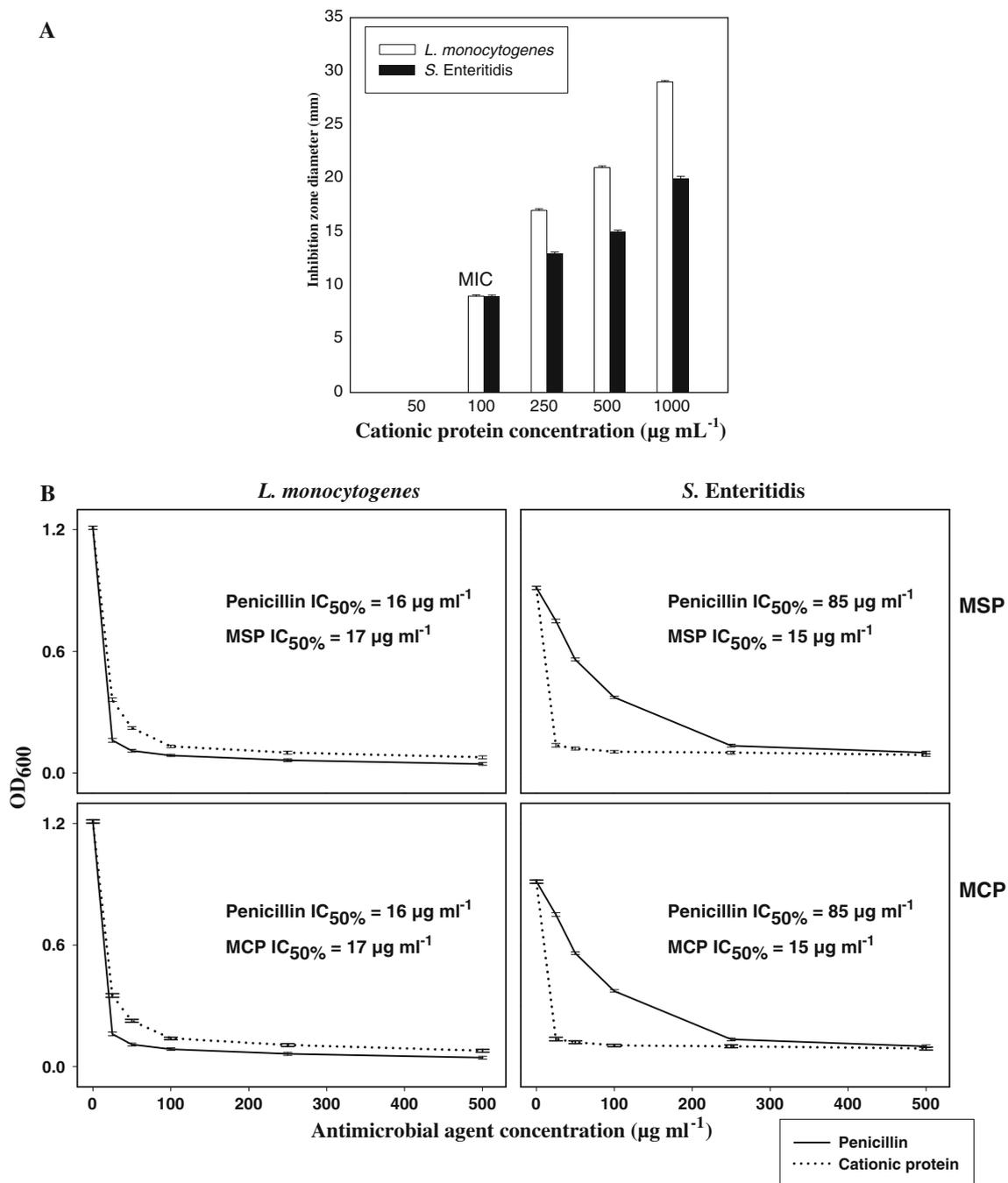


Fig. 2 Agar well-diffusion assay **a** of the antimicrobial action of cationic soybean protein (0–1,000 µg/mL) and broth tube dilution assay **b** of the antimicrobial activity of methylated soybean

chickpea proteins (MSP and MCP) (25–500 µg/mL) against *L. monocytogenes* Scott A and *S. Enteritidis* PT4

products did not produce any significant difference from the negative control. The observed action was nearly in the level range exerted by penicillin G reported mostly active toward Gram-positive bacteria [23], including *Listeria* spp.

The inferior level of IC₅₀ % of MSP and MCP against *S. Enteritidis* compared to penicillin refers to their higher antibacterial action against Gram-negative bacteria which

develop resistance to penicillin. This relatively low effectiveness of penicillin against the Gram-negative *Salmonella* is in accordance with Ridley et al. [24] as most *S. Enteritidis* isolates were reported resistant to antibiotics [25].

The acquired anti-*Listeria* and anti-*Salmonella* actions of the studied proteins are mainly due to the chemical

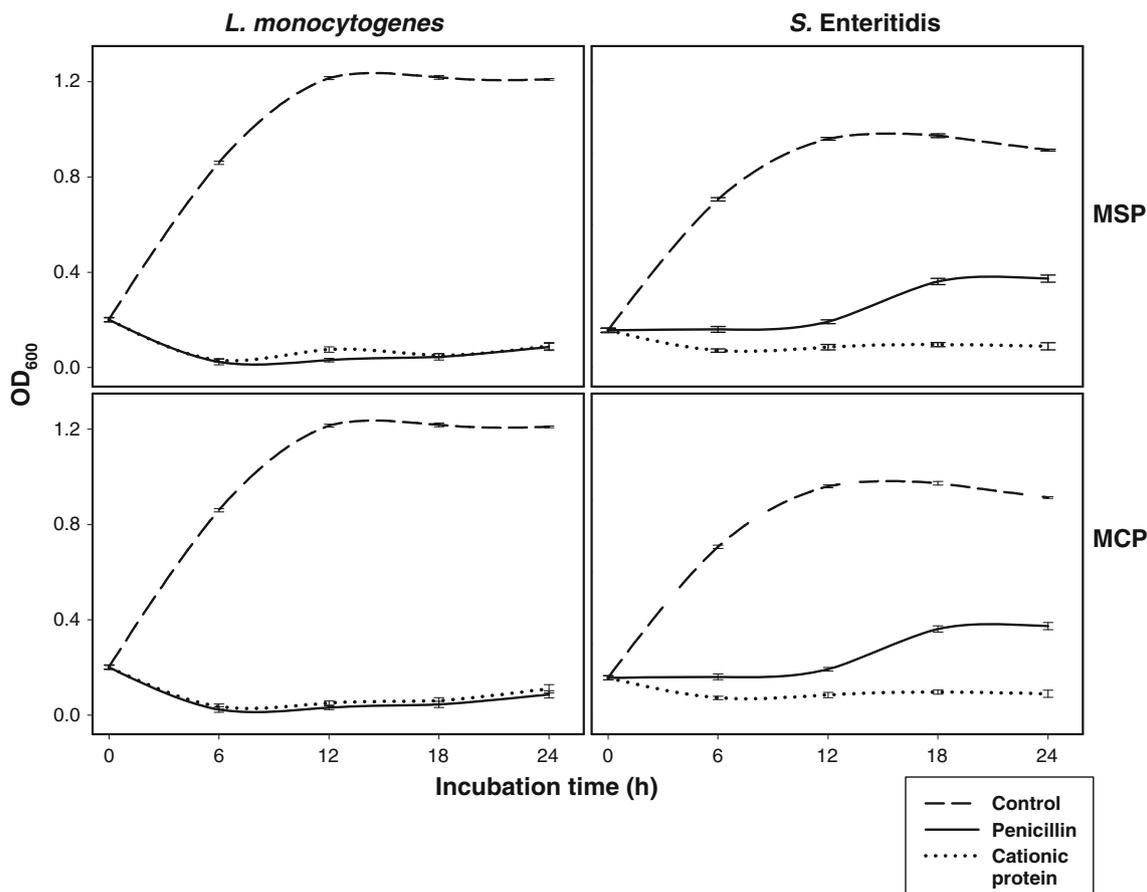


Fig. 3 Growth curve of *L. monocytogenes* Scott A and *S. Enteritidis* PT4 during 24 h at 37 °C as affected by cationic soybean or chickpea protein (MSP and MCP) (100 µg/mL)

modification in spite of the slight antibacterial activity of the native proteins. The basic component (11S) of soy protein was found to possess an outstanding antibacterial activity, but this activity is abolished when existing in a mixture with the acidic 7S subunit [26]. The current biotechnological approach could eliminate this antagonistic interaction between these two components by transferring 7S into basic protein turning the whole protein antimicrobially active.

TEM Image Analysis

The presence of MSP and MCP (100 µg/mL) in peptone buffer medium of *L. monocytogenes* ($OD_{600} = 0.5$ at the time of application) has evidently reduced the relative content of the intact cells after 4 h of incubation at 37 °C as revealed by transmission electron microscopy (TEM) presented in Fig. 4. Mortality rates of *Listeria* induced by MSP and MCP were 72.5 ± 4.2 and 66.25 ± 3.7 %, respectively, compared to 65.6 ± 3.9 % by penicillin. Bacterial cells escaping death were liable to different

manifestations of deformation. The ratio of the bacterial cells deformed by MSP and MCP were 60 ± 2.9 and 53 ± 2.3 % of the total intact cells, respectively, against 24 ± 1.8 % by penicillin. The cationic proteins induced deformation signs on *S. Enteritidis* including cell shrinkage, cell membrane wrinkles and pore formation and cellular emptiness, indicating generally that they rather targeted cell walls and cell membranes. Cellular membrane changes in *S. Enteritidis* were more associated with cationic proteins than with penicillin and were more evident than in case of *L. monocytogenes* probably due to their outer cell membrane directly exposed to the action of the substances. TEM images indicated also inhibiting effect on cell division as the bacterial cells treated with MSP failed to complete the division process (Fig. 5) and underdeveloped cell membranes (E2).

Cell Lysis and Permeation

The MSP treatment was associated with higher rates of crystal violet stain permeation in both *L. monocytogenes*

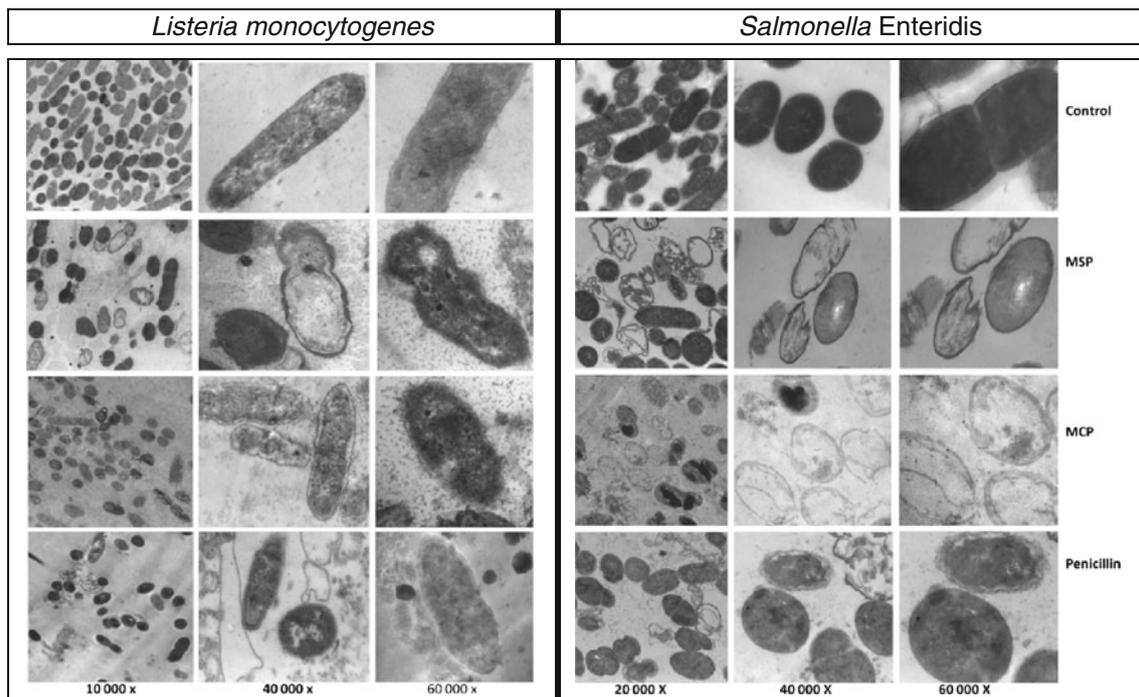


Fig. 4 Transmission electron microscopy (TEM) of *L. monocytogenes* Scott A and *S. Enteritidis* PT4 as affected by 100 $\mu\text{g}/\text{mL}$ of methylated soybean or chickpea protein (MSP and MCP) as compared to penicillin

and *S. Enteritidis* than penicillin evidently due to some membrane distortion, leading to higher rates of bacterial cell lysis (measured by OD_{260}) (Fig. 6). The MSP added to *S. Enteritidis* cellular suspensions ($\text{OD}_{600} = 1.2$) at 50–100 $\mu\text{g}/\text{mL}$ increased the stain permeation by about 82–86 % in a concentration-dependent mode compared to 75–78 % in case of penicillin, indicating its superiority over penicillin. The MCP gave similar results without significant differences ($p < 0.05$) from MSP (data not shown). Respective native proteins (SP and CP) were nearly ineffective at the low concentration (50 $\mu\text{g}/\text{mL}$) and showed very slight increase in permeation (8.5 %) at high concentration (100 $\mu\text{g}/\text{mL}$), confirming that the lysis is mostly due to the chemical modification.

Methylated 11S and methylated 7S separated from MSP were comparatively less effective than the parent substance probably due to less esterification extent and less positive charges as exhibited by urea-PAGE. Higher effectiveness of methylated 11S over methylated 7S may originate from higher positive charge and higher basic amino acid constituents as reflected by the pI of 11S and 7S (6.5 and 4.8, respectively). Additionally, methylated 11S is characterized by hydrophobic domains in its original structure [27] participating in its antibacterial action. In conclusion, all protein subunits participate in the antibacterial action,

particularly methylated 11S. The antibacterial action of prepared substance is evidently due to the chemical modification accentuating positive charge as well as the original or acquired hydrophobicity.

The evident and multiple signs of bacterial cell wall deformation indicate that the main mechanism of action of the cationic proteins may occur through direct interaction with the cell wall and cell membrane as mediated by their amphipathic natures. Cationic proteins possess both the hydrophilic and hydrophobic nature since esterification reaction cancels their negative charges, enhancing the hydrophobicity of the Glu- and Asp-rich domains and increasing the protein net positive charge. Less magnitude of cellular wall or membrane damage by penicillin may indicate the absence of equivalent direct action as it rather exerts its action through inhibiting bacterial cell wall synthesis [28]. The cationic proteins may directly interact with cell wall and cell membrane through electrostatic and hydrophobic bonding causing their deformation and disintegration. The electrostatic interactions may take place between the positively charged fragments of the cationic proteins and the negatively charged regions of cell wall or cell membrane emerging from teichoic acid component and phospholipid constituent, respectively [29], and the hydrophobic bonding may occur between the alike regions of the two reactants.

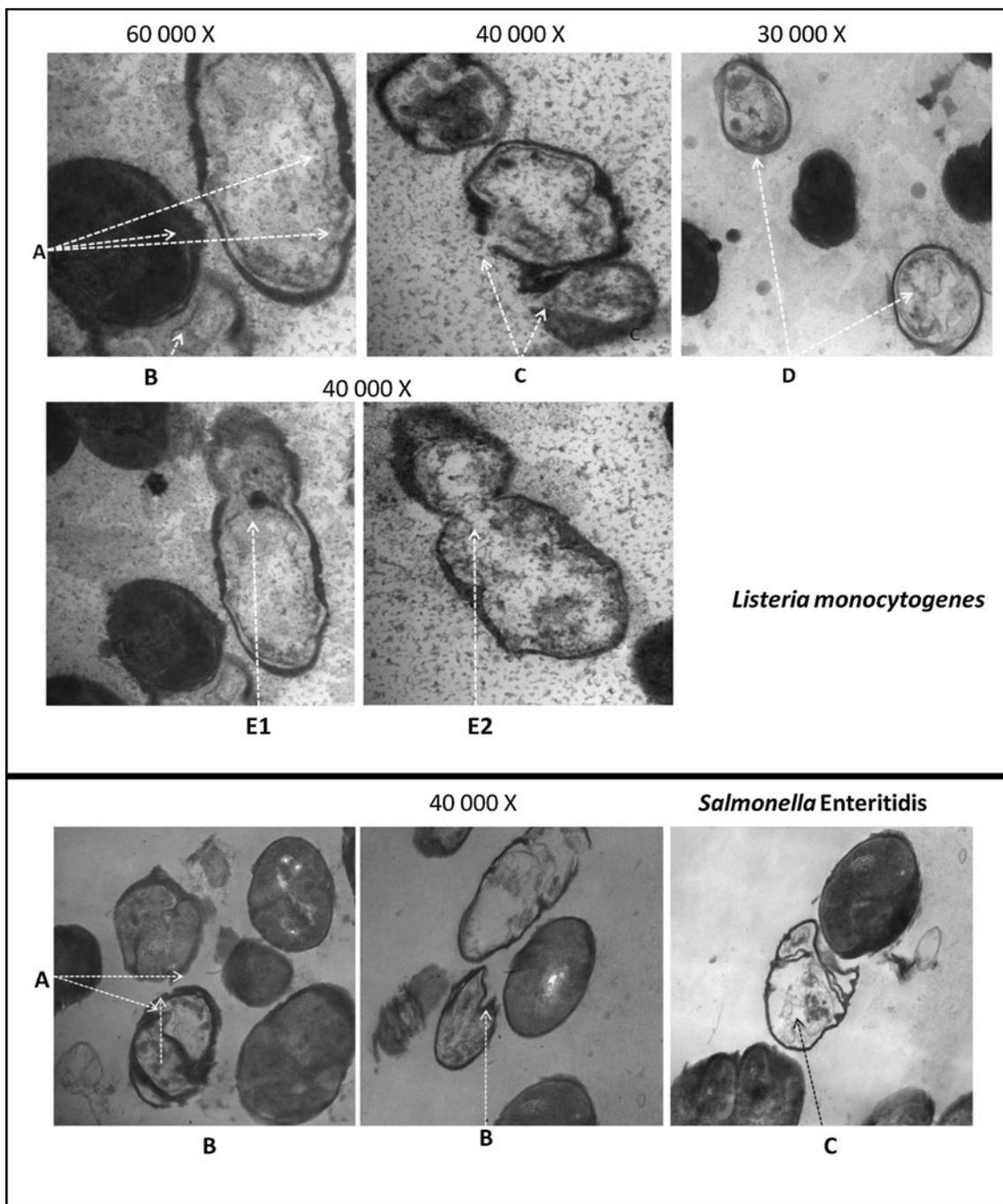


Fig. 5 Possible stages of antimicrobial action of methylated soybean or chickpea protein (MSP and MCP) on *L. monocytogenes* and *S. Enteritidis* PT4 as revealed by TEM. (A cellular membrane wrinkling,

B disintegration, C poring, D cell emptiness leading to ghost cells, E1 impaired binary division, E2 failed binary division)

Bacterial Proteins

Equal amount of bacterial cells were treated with MSP or penicillin and subjected to SDS electrophoresis profiling to investigate the possible specific effect on bacterial protein (Fig. 7). *L. monocytogenes* bacterial protein pattern was only affected by the high MSP concentration (100 $\mu\text{g}/\text{mL}$), indicating maximum diminution of protein bands

corresponding to 20–28 kDa. *S. Enteritidis* bacterial proteins were reduced at lower concentration of MSP (50 $\mu\text{g}/\text{mL}$), particularly those of 26–35 and 48 kDa. Penicillin effect on the bacterial proteins of the two species was much less remarked. These results lead to the conclusion that the cationic proteins could affect protein synthesis through inducing membrane damages which may constitute a signal altering gene expression.

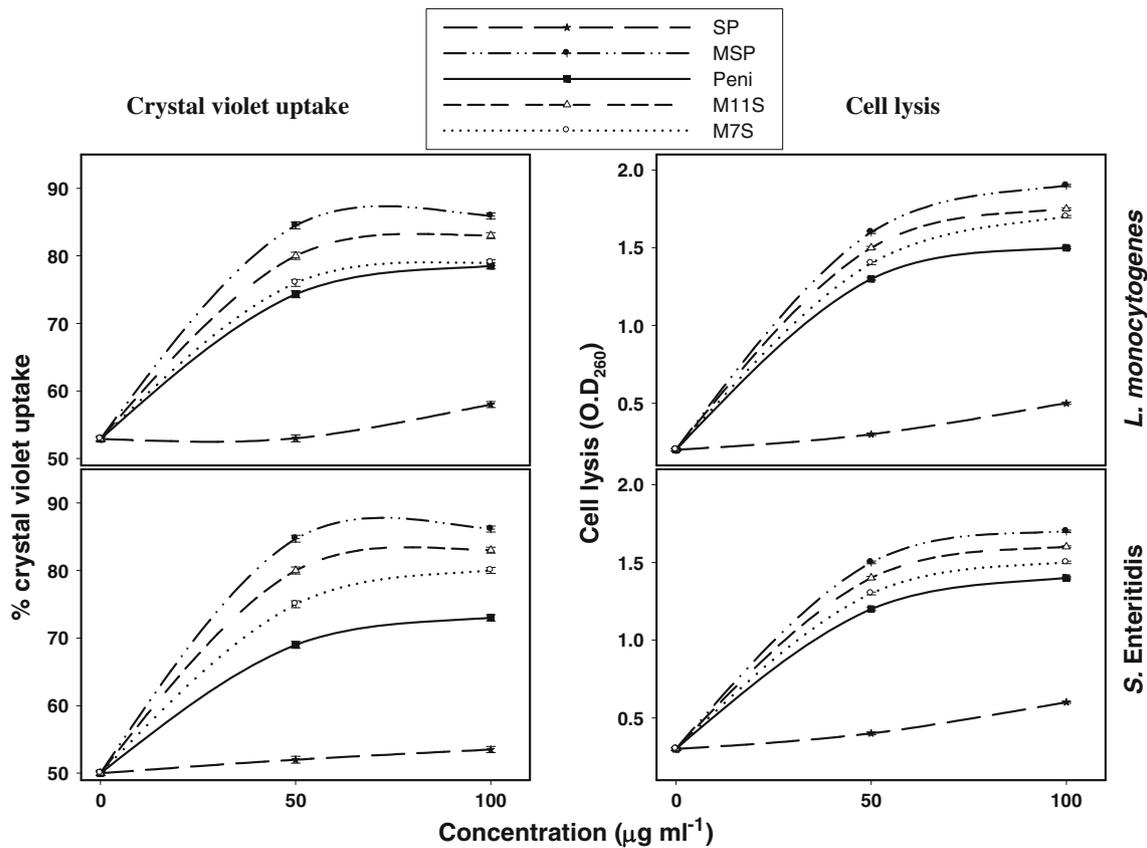
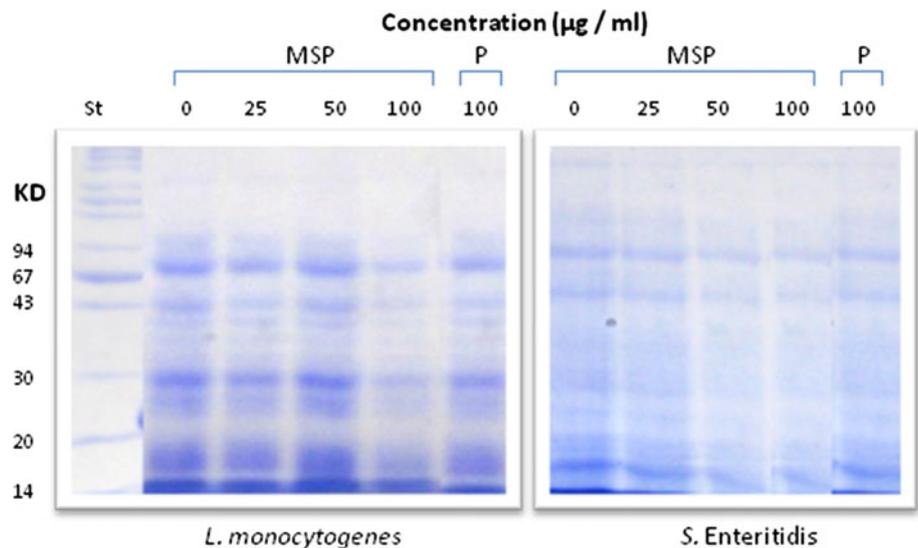


Fig. 6 Cell lysis (estimated by the bacterial supernatant absorbance at 260 nm) and cell permeation of *L. monocytogenes* Scott A and *S. Enteritidis* PT4 treated with methylated soybean (MSP) or its

subunits: methylated 11S (M11S) and methylated 7S (M7S) compared to penicillin (Peni) and native protein (SP)

Fig. 7 SDS–PAGE pattern of *L. monocytogenes* Scott A and *S. Enteritidis* PT4 treated with a cationic soybean protein (MSP) at different concentrations (0–100 µg/mL) in Müller–Hinton broth during 24 h of incubation at 37 °C as compared to penicillin (100 µg/mL)



Conclusions

Esterification of legume proteins turns them positively charged and hence exhibits outstanding anti-*Listeria* and

anti-*Salmonella* actions. This action turns the net charge of 7S from negative into positive while it intensifies the positive charge on 11S. This modification eliminates the negative interaction between these two subunits, allowing

the whole protein to exert antibacterial action. The current biotechnological technique can provide antimicrobially active cationic proteins. These prepared mixtures of cationic proteins can be invested in the antimicrobial applications without the need to use costly and time-consuming procedures for isolating the active protein component (11S). The antimicrobial action of the cationic proteins may be initiated by an electrostatic interaction between their positively charged regions and the negatively charged regions of cell wall or cell membrane accompanied by a hydrophobic interaction between alike regions of the two reactants. Oscillating random Brownian motion of protein macromolecules [30] attached to the cell walls and membranes may cause their stretching, producing big-sized pores, pore channels and cell wall and cell membrane disintegration, engendering higher cell permeability leading finally to cell emptiness, lysis and death. These cationic proteins can be efficiently used to counteract these two potent pathogens (*L. monocytogenes* and *S. Enteritidis*) in different food applications, particularly when these products are not associated with toxic hazards (results under publication elsewhere).

Potential allergenic implications may be associated with these modified novel proteins (MSP, MCP, M11S and M7S). Native soybean proteins are well known for their allergenicity [31] where their two major fractions (glycinin and β -conglycinin) were recognized as allergens [32] having many potential epitopes, for example, 15 epitopes in α -subunit of β -conglycinin [33]. Esterification of these native proteins may probably change their biochemical characteristics, for example, their susceptibility to proteolysis affecting their potential allergenicity. For example, esterification enhances the susceptibility of β -lactoglobulin to peptic hydrolysis as a result of a structural change exposing the potential peptic cleavage sites to the enzymatic action and as the esterified glutamate and aspartate groups are peptic targets [34]. This change may theoretically reduce the allergenic action of the esterified soybean proteins compared to their original forms. Proteolysis is often followed by a reduction in the number of epitopes and consequently by a decrease in allergenicity [35]. However, esterification was reported to turn milk proteins more resistant to tryptic [36], that is, it may enhance their allergenicity since digestion stability of food allergens is an indicator of potential allergenicity [37]. Alternatively, as esterification annuls the negative charges of the glutamate and aspartate residues on the surface of the protein molecules, resulting in more positively charged molecules with lower hydrogen-bonding capacity, the interactions between allergens (modified proteins) and antibodies are expected to be much attenuated leading to less allergenic effects. There are three different bonding forces between antibodies and antigens: van der Waals, hydrogen bonding and

electrostatic interactions [38]. The electrostatic interactions provide the exquisite specificity needed for antigen recognition [39]. However, annulling the negative charges of the glutamate and aspartate groups on the protein surface may increase the hydrophobicity enhancing the antibody–antigen interactions. So, the actual influence of these molecular changes on the allergenic potential effects of these products should be experimentally investigated before application as an antimicrobial food additive.

Acknowledgments The authors would like to thank Zagazig University for financially supporting this work.

Conflict of interest The authors encounter no conflicts of interests.

Ethical Standards There are no issues that may jeopardize ethical standards.

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