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The use of lysozyme to prepare biologically active chitooligomers

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Abstract

Two types of crustacean commercial chitosans (CS1, CS2) were dissolved in lactic acid solutions, hydrolysed by lysozyme and finally fractioned by methanol solutions into two parts containing chito-oligomers (CS-O1, CS-O2). The antioxidant power and antimicrobial properties of both fractions were studied and compared with non-hydrolysed CS1 and CS2. The antioxidant properties were determined by the ferric ion reducing antioxidant power (FRAP) method while the bioactive properties were evaluated against a strain of Listeria monocytogenes. CS-O obtained from the solid fraction of the chito-oligomers solid fractions treated with 90% methanol showed the highest reducing power. Microbiological tests showed that CS-O exhibit higher antilisterial activity than CS.

Keywords: *antilisterial activity, antioxidant, chito-oligomers, chitosan, lysozyme.*

1. Introduction

High risk of foodborne pathogens creates psychrotrophs, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*. Psychrotrophic microorganisms are capable to growth even at ≤ 7 °C. Fatality rate of foodborne listeriosis in comparison to foodborne salmonellosis is higher of 20-30% according to FAO/WHO report[1]. *Listeria monocytogenes* represent 28% of total food related deaths, when *Staphylococcus aureus* only 0.8%[2]. Ready to eat products, meat and meat products, raw milk, ripening chesses made from unpasteurized milk, poultry, vegetables and salads with fresh vegetables and also sea food during storage are exposed to *L. monocytogenes* contamination. Traditional methods of bacteria growth inactivation, such as: temperature, preservatives, pH, water activity are often not enough or impossible to use in view of organoleptic changes in regional food. Bioactive substances such as: bacteriocins (nisin), plant oils and extracts, some polysaccharides (chitosan, cellulose derivative), enzymes (lysozyme) can be also used in food preservation[3]. Possibility to increase the inactivation of *L. monocytogenes*, aerobic bacteria, yeasts and fungi in smoked salmon coated with a coating based on whey protein isolate and lysozyme was already noted[4]. Better antimicrobial properties against *L. monocytogenes* and barrier films produced with the addition of HPMC were obtained by chitosan addition[5].

Chitosans (CS) are a family of amino-polysaccharides, obtained after partial deacetylation of chitin mainly received from crustaceans or fungal strains. Chitosans consist of N-glucosamine (short notation: D) and N-acetyl-glucosamine (short notation: A) units linked by 1,4-glycosidic bonds. Some properties of chitosans are of outmost importance for food preservation or biomedical engineering; they are generally enhanced upon decreasing the acetylation degree meaning that such properties are intrinsically related with the polymer's amino groups that are cationic in acidic-neutral media[6]. Increasing the amount of 2-amino-2-deoxyglucose units in chitosans facilitates the solubility of this polymer in acidic aqueous media. Chitosans have primary aliphatic amine groups that can be protonated by selected acids, the pKa of the chitosan amine being 6.3.[7] This is the main reason why microbiological properties of chitosans are studied in aqueous acidic solutions such as acetic[8,9], lactic[10], ascorbic[11], glutamic[12] and hydrochloric[13] acids. Those acids improve bactericidal effect of chitosans, because they present this kind of properties themselves[14-16]. Organic acids at low pHs damage extracellular membrane, enzymatic system, proteins, DNA structure and cell deformation that lead to the death of microorganisms[17]. The antimicrobial effects of chitosans could be related to the impact of ammonium groups on some bacteria strains. Chitosan oligomers and low molecular weight chitosans are soluble in water and they present antitumor[18], antifungal[19], antimicrobial properties[20] and immunostimulating effects[21]. CS and CS derivatives display also antioxidant activity, due to the presence of hydroxyl and amino groups in CS chains[22-23]. CS antioxidant effects depend on the molecular weight[24]; they increase when the chains are shorter[25].

Some of the CS properties, such as antioxidant activity or solubility, can be improved by chemical or enzymatic modifications of the biopolymer. Chemical hydrolysis by acids is the most common method used to produce chitosan oligomers (CS-O) in industrial scale[26,27]. But this option has some disadvantages, such as environmental pollution and low yields[27]. Oxidative[28] and radiative degradation[29] can also produce good results in CS-O

production. Modification of chitosan chains by enzymes such as chitosanase[30,31], pepsin or papaine[32] is known in literature. Most of the specific enzymes with high activity towards chitosan depolymerisation, such as chitosanases, are unavailable in bulk for commercial exploitation[33]. Chitosan depolymerisation by nonspecific enzymes may be interesting because they are easily obtained, which means lower production costs. Lysozyme (E.C. 3.2.1.17) is one of the cheapest enzymes omnipresent in nature. It was found in human and animal secretions, body fluids and tissues, but the highest concentration and main commercial source comes from hen egg albumen. Extensive studies have been devoted to their structure, catalytic mechanism, relationship between structure and activity, phylogeny, immunology and genetics[34]. It catalyses hydrolysis of β (1-4) glycosidic linkages between N-acetylmuramic acid and N-acetyl D-glucosamine in the cell walls of polysaccharides, including bacteria[35] and also chitosan[36-40]. Due to its lytic properties, lysozyme was applied as an antimicrobial substance. Lysozyme is mainly effective against Gram (+) bacteria cells. Inhibition effect of this enzyme can be also extended to growth inhibition of Gram (-) bacteria by using membrane-permeable compounds with lysozyme or using lysozyme with other substances[41]. Application of lysozyme in the production of protective coatings based on chitosan, extended its impact on *Escherichia coli* and *Streptococcus faecalis* bacteria[42]. In addition, the enzyme mixture has a strong effect with chito-oligomers by inhibiting the growth of such bacteria as *Escherichia coli*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Staphylococcus aureus* in refrigerated minced meat[43].

The aim of the present study was to obtain chito-oligomers from crustacean commercial chitosans (CS1 and CS2) with high antioxidant and antilisterial activity as the result of lysozyme treatment.

2. Experimental

2.1 Materials

A low molecular weight chitosan (CS1), prepared from crab shells, was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) with a deacetylation degree (DA) *ca* 86%. Another chitosan from crab shells, CS2 (30 kDa, DA 85%,), was obtained from France Chitine (Marseille, France). Lysozyme from hen egg white (EC 3.2.1.17) was purchased from Ovopol (Nowa Sól, Poland) with an activity 2000 U/mg.

2.2 Enzymatic hydrolysis of chitosans

CS stock solutions were prepared at a concentration of 4% (w/v) by diluting CSs in lactic acid. A stock of the enzymatic solution was prepared by dissolving lysozyme in distilled water at a concentration of 2% (w/w). After centrifugation and filtration to remove insoluble residues, the enzyme solution (125 mL) was added to chitosan stock solutions (125 mL) to obtain 1% of lysozyme in CS solutions. The pH was adjusted to 5.5 by adding an aqueous soda solution (2 M). The lysozyme kinetics reaction was defined as the amount of enzyme required to produce 1 mM of glucose per hour, using the DNS method described further.

Chitosan solutions were introduced, under magnetic stirring, into a water bath at 29 °C, 5 minutes before enzyme addition, to have the best conditions for enzyme activity. Samples (30 mL) were taken after 0.5, 1, 2, 3, 5, 18 and 24 hours of incubation times. The reaction was stopped by boiling for 30 minutes in a water bath and activity of enzyme was measured according to method described by Deaschel[44]. After deactivation solution was filtered to remove denatured lysozyme and the pH was adjusted to 6.5 with an aqueous soda solution (2 M). Experiments were repeated 3 times.

The reaction kinetics was determined by measuring the reducing-sugar ends using 3,5-dinitrosalicylic acid (DNS) as reagent and D-glucose as standard. DNS reagent solution contains 3,5-dinitrosalicylic acid (1 g), 2 M NaOH (20 mL) and potassium sodium tartrate (3 g) in distilled water (100 mL) according to Miller[45]. The same volumes of samples and DNS reagent were mixed and then boiled for 8 minutes. After cooling, the solution was centrifuged to eliminate the solid residue and the absorbance of the clear solution was measured at 540 nm using an UV-vis Lambda 18 Perkin-Elmer spectrometer (pathway: 1 cm). A standard calibration curve was established using D-glucose solutions (0 – 10 mM) at pH = 5.5.

2.3 Fractionation of CS-O by methanol/water solutions

Chitosan hydrolysates after 24 h of enzymatic treatment were precipitated by methanol/water (v/v) solutions at three different concentrations: 70, 80 and 90%. The solutions were centrifuged to separate the precipitate and the supernatant. Each precipitate was washed with its corresponding methanol concentration (70, 80 or 90%), giving samples called "insoluble fractions". The supernatant and washing aqueous methanol solutions were combined and concentrated under vacuum, leading to samples called "soluble fraction".

2.4 Thin layer chromatography (TLC) of CS-O

The separation of CS-O was performed on silica gel plates (Merck 60 F 254), with a mobile phase constituted by a mixture of n-propanol/water/concentrated ammonia 7/2/1 (v/v/v) as described by Cabrera and Van Custem[46]. Spot visualization was obtained by spraying a solution of ninhydrin in ethanol (0.2%, w/v) on the plates followed by heating at 110 °C for 1 minute.

2.5 Antioxidant properties measurements

The antioxidant properties of CSs and CS-Os were determined by the ferric ion reducing antioxidant power (FRAP) method, which is based on the reduction of the Fe(III)-tripyridyltriazine (TPTZ) complex into the Fe(II)-TPTZ form[47]. The increase in absorbance was measured at 593 nm. FRAP reagent contains acetate buffer [100 mL, 300 mM prepared from sodium acetate trihydrate (3.1 g), glacial acetic acid (16 mL) and distilled water (1 L)], TPTZ [10 mL, prepared by dissolution at 50 °C in a water bath of TPTZ (0.031 g) in 40 mM HCl (10 mL)], $FeCl_3$, 6 H_2O [0,054 g in distilled water (10 mL)]. Samples (100 μL) were mixed with FRAP reagent (3 mL) and absorbance was read after 0 and 4 minutes. Standard calibration solutions were prepared with ferrous sulphate (0-1 mM).

2.6 Antimicrobial properties against Listeria monocytogenes

L. monocytogenes strain, selected from a private collection (Unité Sécurité Microbiologique des Aliments, ENSCBP, IPB, University Bordeaux, France), was grown in Difco 62 176 tryptose broth (Fisher Scientific Bioblock, Illkirch, France) at 37 °C for 18 h. The inoculum (1 mL) was diluted with tryptose broth (9 mL) and then with sterile distilled water to get approximately 10^8 Colony Forming Units (CFU/mL). Microbial inoculum (0.1 mL) was deposited on the tryptose/agar medium and left to dry in the flow hood at 25 °C for 30 minutes. Chitosan-based solutions (1 mL) were deposited on the surface of inoculated Petri dishes and distributed evenly. After drying for 2 hours in a flow hood at 25 °C, the plates were then incubated for 24 hours at 37 °C. Growth controls were incubated with both chitosans solution and with 1% (v/v) lactic acids (pH=5.5). After incubation, bacterial numeration (colony forming unit, CFU) was made and the bacterial growth was expressed as log CFU/mL.

2.7 Statistical analyses

All experiments were triplicated. Statistical ANOVA tests were made on all the data (kinetics, reducing capacity, antilisterial effect) with a significance level defined at $p \leq 0.05$.

3. Results and Discussion

3.1 Production and characterization of chitosan hydrolysates by lysozyme

3.1.1 Kinetics of enzymatic hydrolysis of chitosans

The amount of reducing sugar ends from the enzymatic hydrolysis of CS1 and CS2 solubilized in aqueous lactic acid solution versus time reaction is presented in Figure 1. Both curves have similar patterns, which could be divided into three parts. In the early reaction steps, a dramatic increase in the reducing ends was observed. Then, the curves showed a slow increase in the concentration of reducing sugars to reach a maximum after 16 hours for CS1 and 20 hours for CS2. Thereafter, a slow, but statistically significant ($p < 0.05$) decrease was noted.

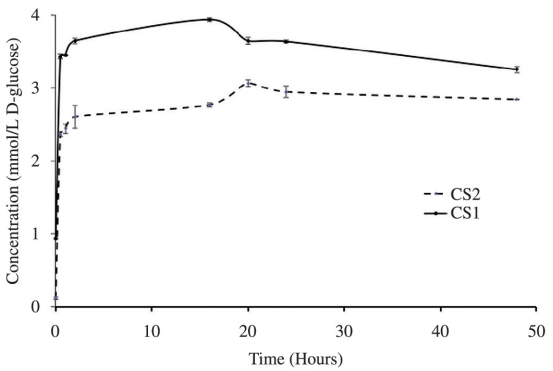

Figure 1. Enzymatic hydrolysis kinetics of the crustacean chitosans CS1 and CS2 solubilized in aqueous lactic acid solution after 0.5, 1, 2, 3, 5, 18, and 24 h reaction times (**I** – standard deviation).

Non-linear kinetics of chitosan hydrolysis has already been reported[48,49]. Changes in chitosan viscosity were observed during enzymatic reactions[50,28]. The lowest viscosities corresponding to the lowest molecular weights of chitosan fractions were obtained after approximately 4 hours of hydrolysis. Fen et al.[47] had the highest concentration of reducing sugars after 7 days of chitosan reaction with chitosanase; after this time, a marked decrease in the chitosan reducing ends amount was noticed. On the other hand, susceptibility of chitin to degradation by lysozyme increasing with an increase in its deacetylation degree (DA). The maximum degradation was reached at about 50% deacetylation of chitosan; then, a continuous decrease was observed until 97% DA of chitosan[37].

The curves given in Figure 1 can be interpreted as a combination of two competing phenomena: hydrolysis of chitosans by the enzyme (rapid kinetics) and interactions between N-acetylated fractions of hydrolysed chitosans and lysozyme (slow kinetics). At the beginning of the reaction, hydrolysis of chitosans is predominant (first part of the curves); as N-acetylated chitosan fractions are created, the competition between lysis and binding interactions occurs (middle part of the curves); when there are no more possibilities of hydrolysis, binding interactions can function alone and the reducing ends decrease slowly (third part of the curve). As the DA is equal to both CS1 and CS2, the lower content of reducing sugars for CS2 is likely due to its higher molecular mass.

3.1.2 Thin layer chromatography of chito-oligosaccharides (CS-O)

analysis of hydrolysates showed different spots on TLC plates, which were related to the methanol concentrations used in the separation process (Figure 2). As CS1 and CS2 do not present any fragments before enzymatic hydrolysis (results not presented), the spots are evident of CS-O products, which proves that chitosan was degraded by lysozyme. According to Varum et al.[39], lysozyme hydrolyses mainly chitosan by cleavage of glycosidic bonds of the type -AA | AA- and -AA | AD- whereas -AD | AA- and -DD | AA- are not cleaved or at a very low rate. The absence of N-acetylglucosamine (NAG) in the hydrolysis products indicates that CS-O1 and CS-O2 do not have acetyl units as terminal groups. The higher concentration of methanol in the fractionation process of chitooligomers the higher the amount of separated hydrolysis products.

Similar TLC analyses[45,51] with the same eluent mixture (n-propanol/water/ammoniac: 7/2/1 v/v) reveal that this method allows to separate chito-oligomers soluble in methanol with a degree of polymerization (DP) less than 6, whereas CS-O with higher DP cannot be analysed. The lower the DP, the higher the migration on the plate[45]. The separation of the CS-O1 and CS-O2 chito-oligomers with 70% and 80% methanol gives similar number of CS-O fractions with lower DP, whereas the addition of 90% leads to separation of more oligomers with higher masses.

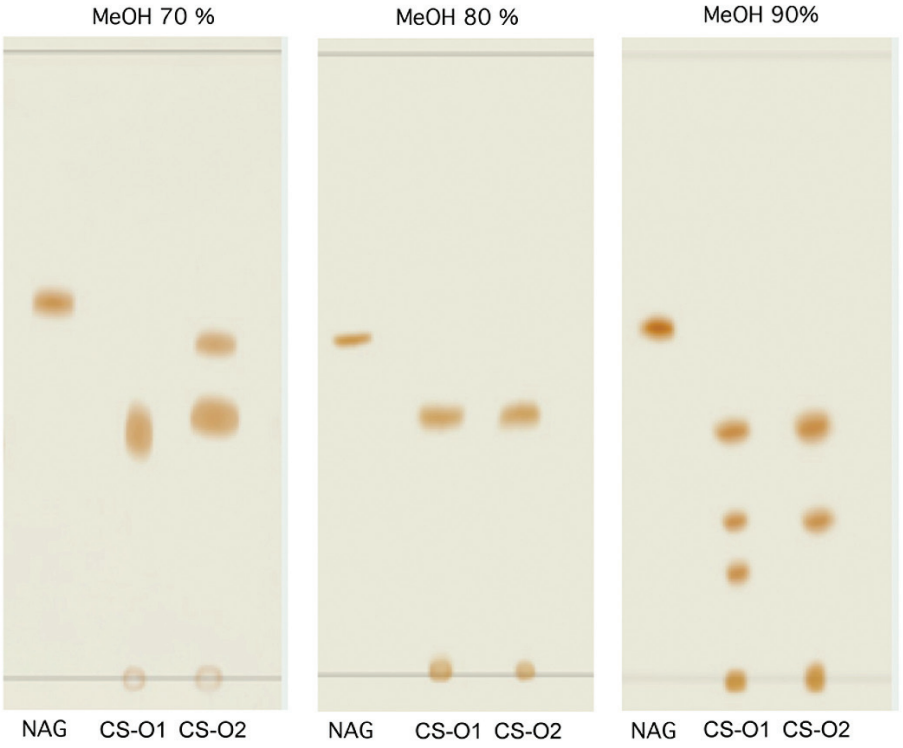

Figure 2. Thin layer chromatography analysis of methanol soluble CS-O resulting from chitosan CS1 and CS2 hydrolysis with lysozyme.

3.2 Antioxidant properties

From CS1 and CS2 after lysozyme hydrolysis for 24 hours, two fractions (soluble and insoluble) were obtained after precipitation of CS-O with methanol/water mixtures. The antioxidant activity of the solid parts was measured after partial dissolution in aqueous lactic acid solutions. The insoluble part was discarded. The results of antioxidant activity of the CS-O are presented in Figure 3 according to the methanol/water proportions. Examined CS1 and CS2 reference samples reduced ferric ions at concentration, respectively 447.5±53.5 and 536.6±28.6 µM ($p<0.05$). In comparison with chito-oligomers of soluble fraction CS-O1 and CS-O2, it seems that original chitosans CS1 and CS2 present better antioxidant properties, but still worse than insoluble fraction of both CS-O1 and CS-O2.

Figure 3 indicates that CS-O2 generally have a better antioxidant activity than CS-O1 ($p<0.05$) which has longer polysaccharide chain. In general, antioxidant activity of chitosans and their derivatives increases as their molecular weights and their acetylation degrees decrease[52]. These parameters modify the intra- and intermolecular hydrogen bonding in chitosans, which control the access to amino groups in C-2 position, which is mainly responsible for their antioxidant activity [43[REMOVED NOTEREF FIELD]]. The higher the methanol concentration, the higher the reducing capacity for both CS-O liquid and solid fractions ($p<0.05$). Nevertheless, there was a noticeable unexplained exception of CS-O1 liquid fraction, which shows opposite effect ($p<0.05$). Moreover, it should be noted that the solid fractions have a higher reducing capacity than the liquid ones ($p<0.05$). This observation might be due to both the presence of additional lactic acid and dissolution of lower mass fractions. TLC analyses (*vide infra*) and observation by Cabrera and Van Cutsem[45] on CS-O fragments, with an identical fractionation method, indicate that supernatants of CS-O contain shorter chains than the solid parts. Moreover, it is known that the solubility of chitosan and its derivatives is largely influenced by the molecular weights[53]. Some fractions with small molecular weights of the solid part can be separated by dissolution in water or acid mediums. When the molecular weight of chitosans decreases, the water solubility of the polysaccharides increases[52] and the low molecular weight fractions contribute more to the antioxidant potential of the chitosans[54,55].

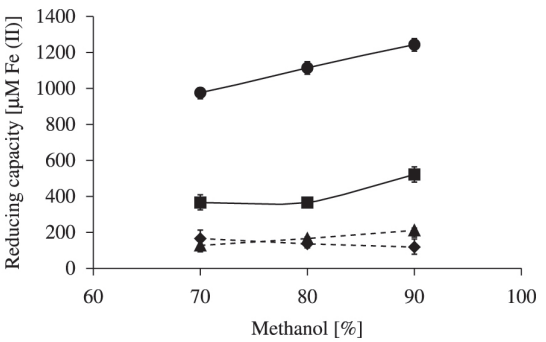

Figure 3. Reducing capacity of COS1 chito-oligomers (solid fraction (■), soluble fraction (♦)) and of COS2 chito-oligomers (solid fraction (●), soluble fraction (▲)) versus methanol/water mixtures (**I** – standard deviation).

Table 1. Antimicrobial activity of CS1 and CS2 crustacean chitosan and CS-O1 and CS-O2 hydrolysates fractions (0.5% w/v) dissolved in lactic acid (1%, v/v) against the growth of *L. monocytogenes*.

	Type of fraction	Concentration of methanol [%]	Log CFU/mL
CS-O1	Soluble	70	9.80±0.37
		80	10.49±0.08
		90	10.48±0.15
	Insoluble	70	0.00
		80	0.00
		90	0.00
CS1			11.31±0.01
CS-O2	Soluble	70	9.75±0.08
		80	10.81±0.08
		90	10.70±0.04
	Insoluble	70	0.00
		80	0.00
		90	0.00
CS2			11.01±0.05
Growth Control			12.42±0.08

3.3 Antimicrobial activity against L. monocytogenes

The antimicrobial effectiveness of chitosans CS1 and CS2 and chito-oligomers CS-O1 and CS-O2 against Gram (+) bacteria *L. monocytogenes* is shown in Table 1. Insoluble CS-O fractions of crustacean CS1 and CS2 strongly inhibit the bacteria growth in each investigated case. However, the liquid fractions have not shown strong activity against Gram–positive bacteria. Nevertheless, the effect of the soluble fractions for both chitosan hydrolysates was significantly different ($p<0.05$) between 70% and 80% methanol concentrations (and between 70% and 90%), but not significantly different ($p>0.05$) between 80% and 90%. As a result, a slightly higher anti-listerial activity was observed with the fraction obtained with the lower concentration in methanol, for both CS-O1 and CS-O2.

According to Jeon et al.[21], water-soluble chitosans exhibit better antimicrobial properties than CS-O obtained from them. Other authors suggested that low molecular weight chitosans (5 – 20 kDa) present better functional–biochemical impact than chitosans with higher masses[32]. Also particle size of the used crab shells in chitosan production may have influence on its physicochemical and functional properties[56]. In this study, higher inhibitions of microbial growth were observed with chito-oligomers of insoluble fraction, compared to water-soluble fractions ($p<0.05$). This antilisterial effect of chito-oligomers might be improved by the lactic acid addition in tested solutions. Lactic acid possesses antimicrobial properties what was tested and reported in many food products[57].

4. Conclusions

The present study demonstrates that lysozyme is a good source of hydrolysis of crustacean chitosan because it provides higher antimicrobial and antioxidant properties than not modified chitosans. Usage of the non-specific enzyme caused non-linear kinetics of hydrolysis process, what suggest potential problems with controlling this process. Higher masses of produced chito-oligomers can be obtained in separation process with higher methanol concentration. More efficient reducing capacity was presented by insoluble fraction of CS-O2 than by not hydrolysed CS1 and CS2 and both fractions of CS-O1. Antioxidant feature may have possible usage in pharmaceutical production of dressings. Also significant implication is seen for use of chitosan oligomers as a preservative in coating or film component to prevent health hazards related to the consumption of contaminated food products.

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