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Anti *Listeria monocytogenes* activity of the lactoperoxidase system (LPS) using encapsulated substrates

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ANTI LISTERIA MONOCYTOGENS ACTIVITY OF THE LACTOPEROXIDASE SYSTEM (LPS) USING ENCAPSULATED SUBSTRATES

Milk contains several non-immunological proteins that have antimicrobial properties. The LPS is one of the most extensively studied systems. The Lactoperoxidase (LPO) catalyses the oxidation of thiocyanate ion (SCN^-) by hydrogen peroxide to the antimicrobial agent hypothiocyanite ($OSCN^-$). However, the major inconvenient of using this system is the need for mixing the components. Consequently, the goal of this work is to form a capsule containing the entire system (enzymes + substrates). At this stage of the study, the core of the capsule containing glucose and thiocyanate has been achieved. Antimicrobial efficiency was tested by the method of inhibition circles using *Listeria monocytogenes* ATCC 15313 as target strain. Results show that LPO and GOD can use encapsulated substrates to produce antimicrobial agent. Increase of substrates concentrations improves antibacterial activity. Inhibitory effect is saturate due to diffusion limitations of hypothiocyanite. This result provides some indications on the maximum distance will attend the capsule when applied on food product.

KEY WORDS: Lactoperoxidase - Glucose oxydase - Encapsulation - Acacia gum - *Listeria monocytogenes.*

Listeria monocytogenes, ubiquitous bacteria in nature, occurs in soil, vegetation and water, is often found in food-processing environments. As consequence of its wide distribution, its ability to survive for long periods under adverse conditions and to grow rapidly at refrigeration temperatures, *L. monocytogenes* is recognised as an important foodborne pathogen.[1] It has often been detected in a variety of foods, and had caused a number of large-scale outbreaks of listeriosis in the USA, Canada, and in Europe.[2] The lactoperoxidase system (LPS), naturally present in raw milk, is one of the most extensively studied antimicrobial system to battle against food contaminants. It consists of lactoperoxidase (LPO, hydrogen peroxide oxidoreductase, E.C. 1.11.1.7), thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2). The LPO catalyses the oxidation of SCN- by H_2O_2 with the production of short-lived antimicrobial oxidation products such as the hypothiocyanite anion ($OSCN^-$).[3 4] To increase the duration of LPS inhibition, H_2O_2 is produce enzymaticaly by glucose oxidase / glucose association.[5] Action spectrum of LPS is now well known and its efficiency against *Listeria monocytogenes* make it very interesting for industrial applications. The use of this system as temporary preservative for raw milk in developing countries is now generalising.[6] However, the major inconvenient is the need for mixing the components before using. That why encapsulation could be a

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good option to make the use of LPS easier. Some studies have been done about entrapment of the both enzymes, lactoperoxidase and glucose oxidase, in liposomes but never the whole system.[7-9] So we proposed to form a capsule containing the entire system: lactoperoxidase, glucose oxidase, glucose and thiocyanate. The biggest problem of this approach is the stabilisation of potassium thiocyanate and glucose mixture. Glucose is very sticky material and KSCN is a very hygroscopic substance that is necessary to protect from water vapour to avoid a resolubilisation and eventually loss. Acacia gum has been choose as inert matrix to stabilising the mixture. A core containing the gum, glucose and thiocyanate has been made by spray drying. Different concentrations of substrates have been used up to a final ratio substrates / polymer equal 50 %. To the best of our knowledge, in press study was relative to the antimicrobial activity due to the peroxidation of iodine containing substrates (I^- or IO_3^-) by LPO.[10] Therefore, antibacterial action of the system was also tested using I- instead of SCN^-

Materials and methods

Bacterial cultures

Listeria monocytogenes.—ATCC 15313, an avirulent strain from the American Type Culture Collection (Rockville, USA), was used as target strain. Stock culture was maintained at 4°C in slants on Trypcase-Soy Agar, 0.6% yeast extract (TSAYE). Strain was transferred from stock cultures into a Trypcase-Soy broth (TSBYE) and incubated at 37° for 24 h. A second transfer was made into TSBYE, which was similarly incubated.

Preparation of preservatives

The LPS was composed of two enzymes : LPO (BioSerae, Montolieu, France) and GOD (BioSerae, Montolieu, France) and three substrates : KSCN (Prolabo, Paris, France), KI (Merck, Darmstadt, Germany) and glucose (Prolabo, Paris, France).

Enzymes solutions.—Both LPO and GOD solutions were prepared 100-fold concentrated in distilled water, then, sterilised by filtration through 0.22 µm filters (Millipore Corp., Bedford, MA, USA). One ml of each solution was added to 98 ml TSAYE at 50°C. The final concentrations of LPS components in solution were LPO 35 mg/l (142 U/mg) and GOD 1 mg/l (45 U/mg).

TABLE I.—*Compositions of powders tested.*

Multiplying factor of initials concentrations	S → KSCN g/l	SH → Glucose g/l	SI → KI g/l
1	0.04	0.2	0.04
10	0.4	2	0.4
50	2	10	2
100	4	20	4
200	8	40	8
400	16	80	16

S: classical system with glucose and KSCN; SI: substituted system with glucose and KI; SH: H_2O_2 control system with only glucose.

Substrates capsules.—Powders have been obtained by spray drying (Niro Minor) of arabic gum solutions at 10% with substrates. Glucose concentrations range from 200 mg/l to 80 g/l and LPO substrates (KSCN or KI) from 40 mg/l up to 400 times more (16 g/l). Under these conditions, ratio substrates / gum vary from 0.2 to 50%. Three types of powders have been obtained: (i) “S” type with glucose and thiocyanate corresponding to classical system, (ii) "SI" type with glucose and iodide corresponding to substituted system, (iii) “SH” type with only glucose to control H2O2 action (Table I).

Effects of capsules on bacterial growth

Listeria monocytogenes.—ATCC 15313 as target strain. Absorbency of an overnight culture of the strains was measured on a spectrophotometer (Shimadzu UV 160-A) to determinate inoculum volume to obtain initial population of about 10^4 colony forming units (CFU) for one millilitre. One ml of each enzyme solution was then added to TSAYE media at 50°C. Culture without LPS served as a growth control. Antimicrobial efficiency was tested by the method of inhibition circles. This method is a variation of the disk assay.[11] Fifty milligrams of each powder were applied on an agar plate containing the target strain, the lactoperoxidase and the glucose oxidase.

Incubated 24 h at 4°C, the active compound diffuses through the agar setting up a concentration gradient. A no growth zone around this area indicates inhibition, which is the measure of activity. Antibacterial efficiency was assimilated to the diameter of this inhibition zone. Each experiment carried out in triplicate is placed at 37°C.

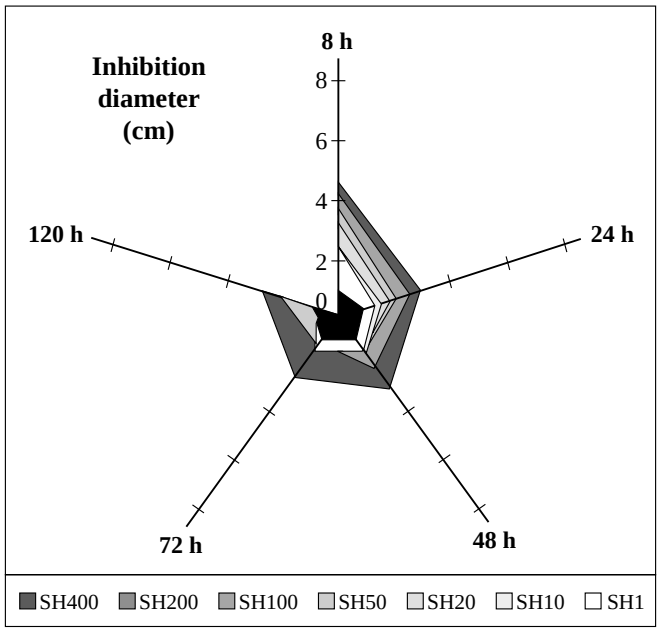

Fig. 1.—Evolution of inhibition diameter against *Listeria monocytogenes* (N_0=10^4 UFC/ml) with increaing concentrations of glucose and different incubation times at 37°C. The black zone on the centre of the graph corresponds to powder deposit zone.

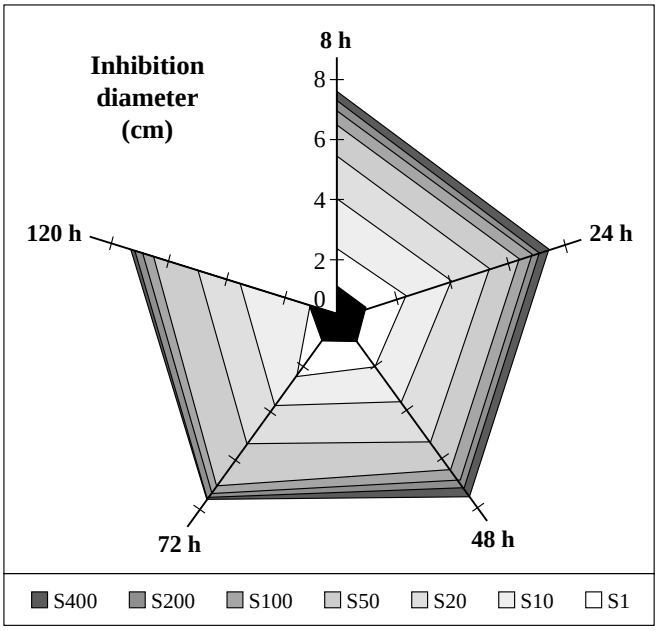

Fig. 2.—Evolution of inhibition diameter observed against *Listeria monocytogenes* (N_0=10^4 UFC/ml) with increaing concentrations of glucose and different incubation times at 37°C. The black zone on the centre of the graph corresponds to powder deposit zone.

Results

Graphic representation has been choose for it illustration of inhibition areas. The black centre of each graph, between 0 and 1 cm, materialise the average diameter of powder deposit zone. In this specific area, it is very difficult to determinate bacterial growth. When inhibition zone correspond to less than 1 cm it is assimilated to a "no effect" action. After 120 h of incubation, if there is no bacterial colony in the inhibition zone, the effect is assimilated to a bactericidal operation, meaning that no bacteria is alive on this area. If bacteria growth restarts, we assume a bacteriostatic activity of the system.

Acacia gum alone shows no inhibitor effect against *Listeria monocytogenes* ATCC 15 313 (results not presented). So, inhibition diameters obtained in others cases correspond only to antibacterial agent production ($OSCN^-$, OI^- or H_2O_2).

Without substrate for lactoperoxidase, H_2O_2 accumulates by degradation of glucose by glucose oxidase. A maximum inhibition of 4.5 cm is observed after 8 h of incubation at 37°C and only for highest glucose concentration (Fig. 1). *L. monocytogenes* rapid-

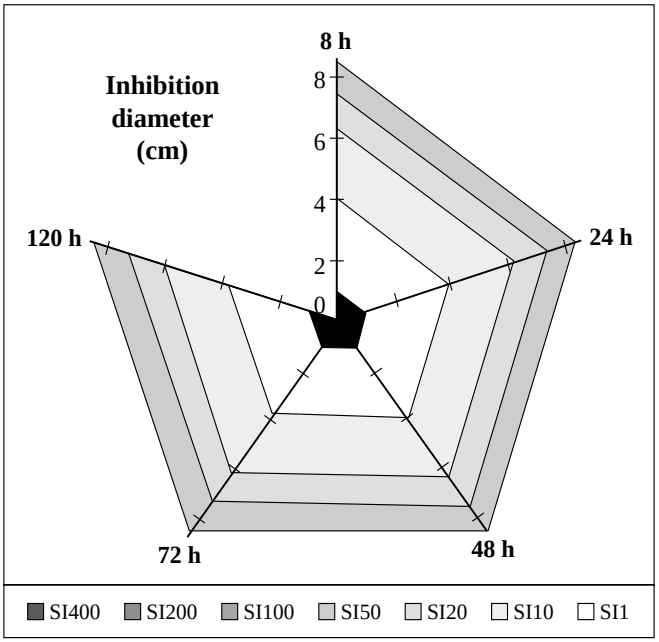

Fig. 3.—Evolution of inhibition diameter against *Listeria monocytogenes* (N_0=10^4 UFC/ml) with increaing concentrations of glucose and different incubation times at 37°C. The black zone on the centre of the graph corresponds to powder deposit zone.

ly re-growths in the inhibition area, it means that inhibitory effect is bacteriostatic. Antibacterial efficiency of hydrogen peroxide is in fact, very low compared to the ones obtained with complete systems (Fig. 2 and 3). As a result, the effects observed later are repercussion of antimicrobial agent production by LPO not a masked hydrogen peroxide action.

Figure 2 resumes the effect obtained with "classical system" using glucose and thiocyanate as substrates. For the lowest substrates concentrations, S1 and S10, a bacteriostatic effect is observed lasting up to 72 h at 37°C. After, the entire zone is recolonised by *L. monocytogenes*. Increase of glucose and thiocyanate concentrations leads to an increase of inhibition diameter and bacteriostasis transformation into bactericidal action. For low substrates concentrations, the inhibition zone is proportional to the substrates quantities applied. However, when multiplying factor of initials concentrations goes beyond one hundred times (S100, S200 and S400), the relation is no more linear. The distance to cover by substrates (glucose or thiocyanate) or products (hydrogen peroxide or hypothiocyanite) becomes too large. It is almost due to limitation of diffusion transport. This result provides some indications on the maximum distance will attend the capsule when applied on food product.

When potassium iodide is used instead of potassium thiocyanate, a bactericidal effect is obtained with basic concentrations (Fig. 3). This result is in accordance with those obtained in liquid cultures observed in literature.[10] LPS using SCN^- has almost only bacteriostatic effect against *Listeria monocytogenes*.[12 13] The substitution of SCN- by I- improves LPS activity against L. monocytogenes, which becomes bactericidal. When substrate concentration becomes higher, the inhibition diameter increases quickly and is maximum since SI50 (Fig. 3). So with substituted system, inhibition is effective on all box surface. This means that there is less limitation due to diffusion of glucose, hydrogen peroxide, potassium iodide or hypoiodite. Diffusion studies of KSCN in agar media allow us to say that the limiting factor for classical system is LPS product hypothiocyanite.

Conclusions

Lactoperoxidase and glucose oxidase can utilising encapsulated substrates and produce antibacterial agent active against *Listeria monocytogenes*. In liquid media, LPS allows control of *L. monocytogenes* development but only for short period. By using encapsulated substrates, extended effect is observed. Furthermore, this first part of the work has permitted to identify some product diffusion phenomena mostly with classical system using glucose and thiocyanate. Next studies will be done with the complete capsule. Enzymes and substrates will be coming closer together, which will limit diffusion limitations and improve antibacterial effect.

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