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MULTIENZYMATIC SYSTEM ENCAPSULATION: APPLICATION TO THE LACTOPEROXIDASE SYSTEM

The use of enzyme often implicates a stabilisation to limit loss of activity and a suitable dry formulation to simplify handling. Freeze drying and spray drying are two classic methods but when complex systems are studied, it becomes difficult to achieve a correct formulation. In this work, the realisation of a dry microbead containing a multienzymatic system (2 enzymes, 2 substrates) is presented. The lactoperoxidase system associated with an enzymatic hydrogen peroxide generator was chosen as the model. The microbead, made by spray drying and spray coating, was characterised in terms of size, shape, chemical structure, flowability, encapsulation efficiency and antimicrobial activity. Different compositions of the coat were tested, as well as their influence on the bead properties.

The use of enzymes often implicates a stabilisation to limit loss of activity and a suitable dry formulation to simplify handling. Freeze–drying and spray–drying are two typical methods used but when complex systems are studied, it becomes difficult to achieve a correct formulation. In this work, the realisation of a dry microbead containing a multienzymatic system constituted of two enzymes and their substrates is presented. The lactoperoxidase system was chosen as a model. This system was composed of lactoperoxidase (LPO, EC 1.11.1.7), a basic glycoprotein present in mammalian secretions such as tears, milk or saliva, and thiocyanate (SCN^-). This system allowed thiocyanate (SCN^-) conversion into hypothiocyanite ($OSCN^-$) with H_2O_2. Hypothiocyanite is a very powerful antibacterial agent. It binds onto the SH groups of bacterial proteins, which leads to the reversible inhibition of most Gram positive bacteria such as Listeria monocytogenes and to the irreversible inhibition of Gram negative bacteria such as *Escherichia coli* [1]. The system needs hydrogen peroxide to be activated. Under natural conditions, some *Lactobacillus, Lactococcus* or *Streptococcus* can produce H_2O_2 under aerobic conditions [2]. In our work, the lactoperoxidase system was activated artificially with an enzymatic generator, an association of glucose oxidase (GOD) and glucose (Fig. 1).

The interest in using enzymatic generation instead of direct addition is the increase of antibacterial activity by the progressive liberation of hydrogen peroxide [3]. From the perspective of encapsulation of the LPS and its hydrogen peroxide generator, a dry core containing both enzyme substrates (glucose, thiocyanate) was produced. Due to the sticky behaviour of glucose and the hygroscopicity of thiocyanate, an inert polymer was added to stabilise the mixture. Acacia gum was chosen for its ability to control water uptake and its low viscosity at high concentrations allowing a good drying rate [4]. The microbead, made by spray drying and spray coating, was then characterised in terms of size, shape, chemical structure, flowability, encapsulation efficiency and antimicrobial activity.

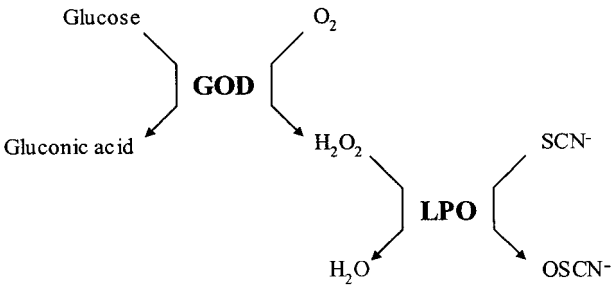

Figure 1. Overall reaction of hypothiocyanite formation by the lactoperoxidase system coupled with enzymatic generation of hydrogen peroxide

Different compositions of cores and coats were tested, as well as their influence on the bead properties. This paper will summarize the formulation properties and its efficiency against food contaminants such as *Listeria monocytogenes*.

MATERIAL AND METHODS

The beads were performed in two steps, first dry core production containing the substrates and second coating of enzymes around these cores.

Core production

The spray drying powder composition was: D–glucose (Sigma, France) 40 g/l, potassium thiocyanate (Labosi, France) 8 g/l and acacia gum (Colloids Natural International, France) 100 g/l. The drying was performed on a Niro Atomizer Minor with an inlet air temperature of 170°C, an outlet of 90°C, a rotary

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nozzle and a product flow of 5 Hz (pump motor frequency).

Enzyme coating

The lactoperoxidase and the glucose oxidase were coated with a GPCG1 spray coater (GLATT) using Wurster technology. Enzyme solutions were prepared in salted solution (0.9%) and phosphate buffer (0.1 M, pH 6.4) respectively for the lactoperoxidase and the glucose oxidase. The lactoperoxydase was sprayed first with an inlet temperature of 45°C and a product temperature of 26°C. The glucose oxidase was then sprayed under the same conditions and the beads were dried for 10 min.

Bead physical properties

The size distribution of the bead particles was studied by laser light scattering with a Malvern Mastersizer S (Malvern Instruments) in a wet basis. The particles were suspended in ethanol and the measurements were performed with a 300RF lens which allows size measurements between 0.05 and 900 μm. The results obtained are the diameters of the equivalent spheres expressed in volume. The values d_{10}, d_{50} and d_{90} were noted; those values mean that 10%, 50% or 90% of particles had a diameter smaller than this value. The size of the distribution was also calculated: span = $(d_{90} - d_{10}) / d_{50}$.

The particles were observed by scanning electronic microscopy (JEOL JSM T330 A). The samples were placed on a brass cylinder and coated with a gold palladium thin layer with a Fine–Coat Ion Sputter JFC 1100.

The rheological properties of the powders were measured with a Hosokawa micron powder tester (Model PT–N, Hosokawa) which provided seven mechanical measurements and three supporting measurements of the dry bulk powders. It complies with the Standard Test Method ASTM D6393–99 "Bulk Solids Characterization by CARR Indices" [5].

Antimicrobial activity

The antimicrobial activity was tested against *Listeria monocytogenes* ATCC 15313 with an adaptation of the inhibition circles method. Different quantities of beads were sprayed on the surface of the dry culture medium (TSAYE: trypcase soya 30 g/L, yeast extracts 6 g/L, agar medium 8 g/L) containing *Listeria monocytogenes* (10^4 UFC/ml).

RESULTS AND DISCUSSION

Microbead concept

The microbeads consist of a core containing the two substrates glucose and thiocyanate embedded in an acacia gum matrix and several coats of lactoperoxidase, glucose oxidase and control polymer (Fig. 2).

The conception of the microbead was studied in regards of its final application. The lactoperoxidase system is a very powerful antimicrobial agent and several studies were done about the use of the system to control the bacterial growth of fish contaminants [6]. The fish salting stage, during fish preparation, appears to be the best moment to include the system, that is why a dry formulation was chosen. The humidity of the product dissolved the beads and thus activated the system leading to total surface protection (Fig. 3).

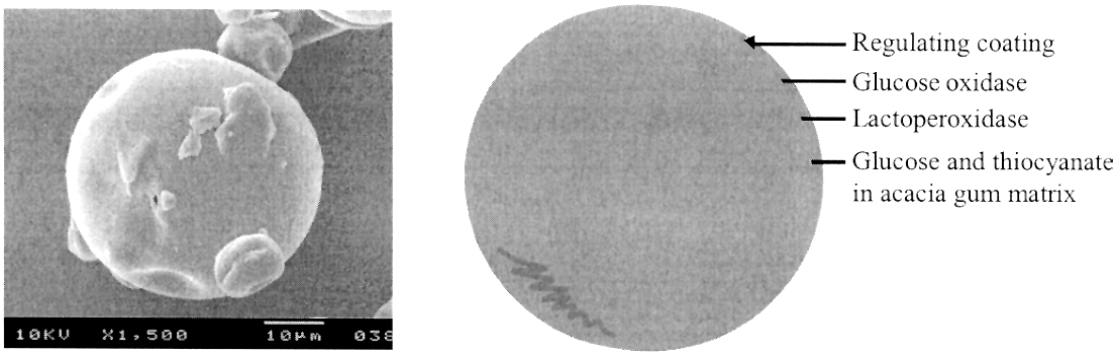

Figure 2. Microscopic view and schematic of the internal organisation of the microbead

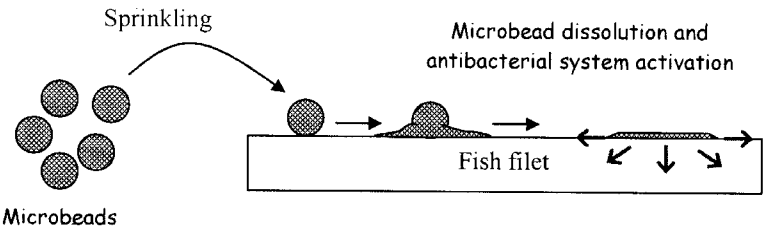

Figure 3. Scheme of microbead use and activation on a fish fillet

Microbead physical properties

The following table summarizes the size distributions of the cores and microbeads according to the type of regulation coating, acacia gum or carrageenan (Table 1).

Table 1. Size distributions of the cores and microbeads according to the number of coatings

	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)	Span
Cores	14 ± 0.5	37 ± 1	84 ± 1	1.80
Beads	28 ± 0.3	61 ± 0.3	140 ± 1.7	1.82
Beads + acacia gum	26 ± 0.4	61 ± 0.7	151 ± 10	2.04
Beads + carrageenan	62 ± 1.1	158 ± 2.5	479 ± 21	2.63

In relation to the coating adding around the microbead, a large increase of the size can be obtained. This size increase is the best way to modify the flow properties of the powder and, consequently, make mixing with salt easier (Table 2).

Table 2. Flowability indexes of the powder formed by the cores alone, LPS beads, LPS beads with acacia gum coating (beads+AG) and with carrageenan coating (beads+Carr)

	Cores	Beads	Beads+AG	Beads+Carr
Flowability	42	42	59	60

The flowability index increased with the size (Table 2). The enzyme coating was not sufficient to improve the flow properties in spite of size increase (Table 1). This phenomenon is due to an increase in inter–particle interactions [7]. The addition of a small amount of acacia gum around the bead decreases the particle interactions without significant increase of size, which is sufficient to improve the flowability (Table 2). The rheological properties of the microbeads after carrageenan coating are just the same as after acacia gum coating. Nevertheless, carrageenan is interesting in the total control of fine particles leading to a healthy powder. It also modifies the dissolution rate of the microbeads. In this case, it becomes possible to control the activation parameters of the system (Table 3).

Table 3. Dissolution rates of the microbeads depending on the composition (AG: acacia gum coating; Carr: carrageenan coating)

Powder	Dissolution rate mg/L/min
Core	23
Beads	23
Beads + AG	22.6
Beads + Carr.	10.3

The dissolution rates of the cores, beads and after acacia gum coating are equal (Table 3). The total release of the active system was achieved in 30 min. Only, carrageenan coating decreased the rate of dissolution due to lower polymer solubility in water and microbead specific surface. This property is interesting to control the antibacterial activity of the system. The shell of carrageenan around the soluble core transforms the microbeads into microreactors. Enzymes and substrates are entrapped in the gel formed during rehydration and only the small molecules of the antibacterial product OSCN– spread.

Antibacterial activity of the encapsulated system

The antibacterial efficiency of the encapsulated LPA was tested against *Listeria monocytogenes* ATCC 15313. Different amounts of LPS bead powders were spread on a surface medium containing the strain (Fig. 4).

LPS beads inhibited *Listeria monocytogenes* (Fig. 4). No more growth was observed inside the inhibition zone after 120 h at 37°C. There was no significant size difference in the zones obtained with 10 and 50 mg of beads spread. This result suggests that the powder was too concentrated and that the size of the inhibited area was only controlled by diffusion phenomena. When coated beads were tested, the same result was observed and more clearly with carrageenan coating. To test this hypothesis, six mixtures of microbeads and acacia gum, with ratios ranging from 0:50 to 50:0 (w:w), were prepared and tested against *Listeria monocytogenes* (Fig. 5).

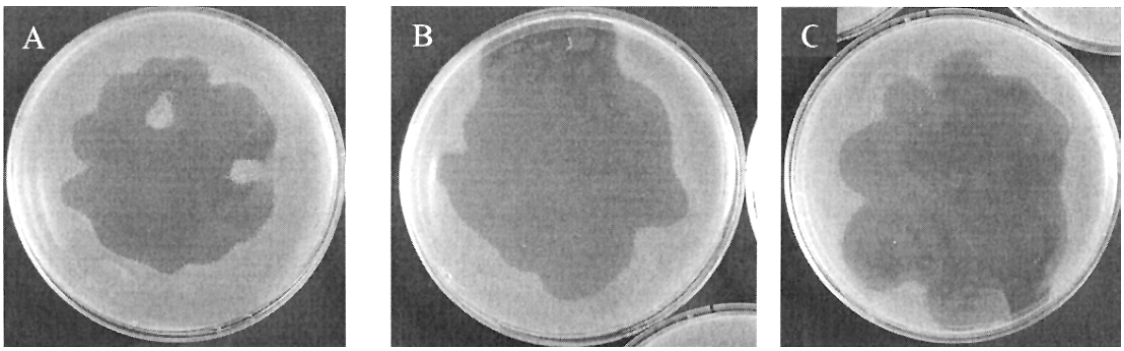

Figure 4. Inhibition zone observed with 10 (A), 30 (B) and 50 mg (C) of encapsulated LPS, sprinkled on a culture medium containing Listeria monocytogenes

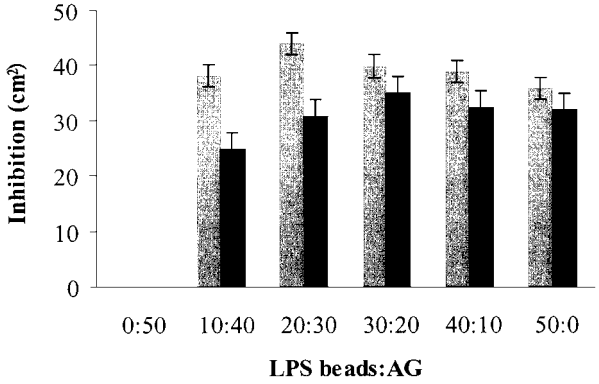

Figure 5. Inhibition areas obtained with 50 mg of powder composed of six blends of LPS beads: acacia gum (AG) ranging from 100% acacia gum to 100% microbeads

First, the results obtained confirmed that acacia gum alone had no antibacterial action and, therefore, that the inhibitory effect was due to LPS activation (Fig. 5). Second, the mixing of microbeads with this inert powder simplified the test procedure because sprinkling was easier with 50 than 10 mg. The results became more repeatable.

Moreover, it demonstrated that LPS microbeads have a powerful potential because only 10 mg were enough to control bacterial growth on 40 cm^2 meaning 0.25 mg microbeads/cm^2 (Fig. 5). According to the microbead composition, this bactericidal effect was obtained with only: 0.068 mg of glucose, 0.013 mg of thiocyanate, 0.0015 mg of lactoperoxidase and $7.5 \cdot 10^{-5}$ mg of glucose oxidase. These quantities are very interesting and are about 10 times smaller than those proposed in previous work done with immersion of the product in a LPS solution [8].

This paper demonstrates the interest in encapsulated LPS powder instead of solution spraying. The components are less concentrated leading to a less expensive product and the processes involved are simples. Moreover, the powder is easy to stock, handle and contains the right quantity of each component. In this case, it becomes more comfortable to use and decreases the hazard of mixing mistakes. The addition of a secondary coating after enzyme coating increases the particle size, but it also decreases the amount of dust. Under these conditions, the LPS powder presents a low sanitary risk.

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IZVOD

Enkapsulacija multienzimskog sistema: Primena kod sistema laktoperoksidaze

(Naučni rad)

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Primena enzima često implicira njihovu stabilizaciju u cilju smanjenja gubitka aktivnosti i pogodnog načina sušenja kako bi se olakšalo njihovim rukovanjem. Sušenje na niskim temperaturama smrzavanjem ili sprej tehnikom dve su najčešće korišćene metode ali kada se proučava kompleksan sistem, postaje teško da se postigne prava formulacija koja dovodi do efikasnog sušenja. U ovom radu je prikazano sušenje mikročestica koje sadrže multienzimski sistem (2 enzima i 2 supstrata). Kao model je upotrebljen sistem laktoperoksidaze koji se dovodi u vezu sa enzimatskim stvaranjem vodonik peroksida. Mikročestice, koje su dobijene tehnikama sprej sušenja i putem sprej nanošenja odgovarajućeg premaza (oblaganje mikročestice), karakterisane su u pogledu veličine, oblika, hemijske strukture, tečljivosti, efikasnosti enkapsulacije i antimikrobne aktivnosti. Testirani su različiti sastavi premaza kao i njihov uticaj na osobine čestica.

Key words: Enzymes • Encapsulation • Lactoperoxidase •
Ključne reči: Enzimi • Enkapsulacija • Laktoperoksidaza •