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Evaluation of critical formulation parameters influencing the bioactivity of β-lactamases entrapped in pectin beads

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Abstract

The bioactivity of β-lactamases upon entrapment in calcium-pectinate beads was evaluated. Non-amidated (NAP) and amidated pectin (AP) beads were prepared according to the ionotropic gelation method using calcium chloride ($CaCl_2$) as gelling agent, washed and dried at 37 °C in an oven for 2 h. Both enzyme activity and protein content were determined as well as bead calcium content. NAP allowed a better encapsulation of the protein than AP. Increasing both $CaCl_2$ concentration and bead residence time in the gelation medium led to a significant loss of β-lactamase activity. The drying process of beads also lowered the enzyme activity. Moreover, bead calcium content increased as the $CaCl_2$ concentration augmented. Being very hygroscopic, the excess of $CaCl_2$ correlates with an increase of moisture content in beads that affects enzyme activity. After elimination of free calcium from beads, it was shown that a small amount is needed to form the Ca-pectinate network and that the activity of β-lactamases is preserved in these conditions. Therefore, the bioactivity of encapsulated β-lactamases in pectin beads mainly depends on formulation parameters such as pectin type, $CaCl_2$ concentration, washing and drying processes.

Keywords: Pectin beads; β-Lactamases; Colonic drug delivery; Bioactivity; Peptide/protein delivery; Microencapsulation

1. Introduction

Colon delivery represents an interesting approach for delivery of peptides and proteins, either for local release or for enhancing absorption of these molecules by the intestinal epithelium (Mackay et al., 1997). Indeed, protease activity in the colon is lower than in the upper gastrointestinal tract (GIT) and the residence time of delivery systems is very long (Rubinstein et al., 1997; Haupt and Rubinstein, 2002; Bourgeois et al., 2005). Moreover, colon delivery can overcome the two barriers that hamper the oral administration of peptides and proteins: poor stability in the small intestine and, if absorption is needed, poor transport across biological membranes.

Bacterially-triggered delivery systems display great potentialities for site specific targeting of peptides and proteins to the colon (Sinha and Kumria, 2003). In this case, drug delivery can be achieved by exploiting microbial enzyme activity predominantly present in that location (McFarlane and Cummings, 1991). Therefore, a large number of polysaccharides, that are degraded by colonic enzymes only, could enter the composition of a suitable carrier for colonic delivery (Hovgaard and Brondsted, 1996; Sinha and Kumria, 2001). Among them, pectin, a naturally occurring polysaccharide found in plant cell walls, has been used as the main component of colonic drug delivery systems (Ashford et al., 1993; Rubinstein et al., 1993). Pectin is composed of linear chains of α(1-4)-D-galacturonic acid residues whose carboxylic groups are partially methoxylated. Some of the carboxylic groups may also be converted to carboxamide, producing amidated pectin. Therefore, pectin is characterized by its degree of esterification (DE) as well as its degree of amidation (DA). In addition, pectin is non-toxic, not digested by gastric or intestinal enzymes and almost totally degraded by pectinolytic enzymes produced by the colonic microflora. Low methoxylated pectin (DE < 50%), amidated or

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not, can form a gel in presence of divalent cations such as calcium. Through this mechanism, pectin has been used to prepare Ca-pectinate gel beads by ionotropic gelation with calcium ions (Aydìn and Akbuga, 1996). These mild conditions of encapsulation should be ideal to entrap sensitive molecules such as peptides or proteins (Sriamornsak, 1998; Musabayane et al., 2000; Kim et al., 2003). However, no study has seriously addressed the problem of protein encapsulation and stability in these beads.

In the present study, β-lactamases, β-lactam antibiotic-inactivating enzymes, were encapsulated within Ca-pectinate beads in order to be delivered to the colon. β-lactamases released in the colon should locally inactivate residual antibiotics either not absorbed in the small intestine or excreted by the bile. The presence of residual antibiotics in the colon leads to a disruption of the colonic microflora and consequently to the reduction of colonization resistance (Nord and Edlund, 1990). Therefore, the colonic delivery of β-lactamases might protect colonic microflora and help to fight against the spreading of resistant bacterial strains in the environment due to intensive use of antibiotics (Van der Waaij and Nord, 2000).

Taking advantage of the fact that it was possible to easily measure the bioactivity of β-lactamases, we have checked, in the present study, how the main parameters involved during bead formation could or not modify this activity. These parameters are the type of pectin, the calcium concentration, the residence time of beads in calcium chloride solution and finally, the washing and drying conditions.

2. Materials and methods

2.1. Materials

Low methoxylated non-amidated pectin (NAP) (Unipectine^{TM} OF 400, DE from 27 to 32%) and low methoxylated amidated pectin (AP) (Unipectine^{TM} OG 175C, DE from 22 to 28% and DA from 19 to 23%) (Mw from 120 to 140 kDa) were gifts from Degussa Texturant Systems (Boulogne-Billancourt, France). Penicillinase (28 kDa) (3175 international units (IU) of benzylpenicillin substrate/mg of protein) from *Bacillus cereus*, a mixture of β-lactamases I and II, was purchased from Sigma–Aldrich (Saint-Louis, USA). Nitrocefin, a chromogenic cephalosporin used for the evaluation of β-lactamase activity, was provided by Oxoid (Basingstoke, England). Calcium chloride was obtained from Acros Organics (Geel, Belgium). Phosphate buffer saline (PBS), hydroxyethylpiperazine–ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA) and Bradford reagent were purchased from Sigma–Aldrich (Saint-Louis, USA). Sodium chloride was obtained from Merck Eurolab, VWR (Strasbourg, France).

2.2. Preparation of pectin beads

Calcium pectinate beads were prepared by ionotropic gelation according to a modification of a procedure previously described (Bodmeier et al., 1989). A 6% (w/v) pectin solution was prepared by dissolving non-amidated pectin (NAP) or amidated pectin (AP) in distilled water. Penicillinase was then added to the pectin solution to a final concentration of 0.12 mg of protein/mL corresponding to 385 IU of benzylpenicillin/mL. This solution was dropped, using a peristaltic pump and a nozzle of 0.8 mm inner diameter, into a calcium chloride ($CaCl_2$) solution at a concentration varying from 2 to 8%, under stirring and at room temperature. By contact with calcium ions, pectin droplets instantaneously formed gelled beads. These beads were allowed to stand in the $CaCl_2$ solution for a given time (from 20 min to 24 h), separated by filtration, washed by distilled water. Beads were then dried at 37 °C for 2 h, in an oven at atmospheric pressure. The duration of drying was based on dehydration kinetics showing that a plateau for water elimination was reached at 2 h. In some cases, an additional procedure of washing by distilled water was conducted on beads before drying, in order to eliminate the excess of free calcium retained in beads. For this purpose, 60 undried beads were dipped into 80 mL of distilled water three times during 5 min each.

2.3. Characterization of pectin beads

The effects of formulation parameters on bead characteristics such as the type of pectin (NAP or AP), the concentration of $CaCl_2$ solution (2, 4, 6 and 8% (w/v)), the residence time of beads in $CaCl_2$ (20, 30 min and 1, 2, 4, 8 and 24 h), the drying and the washing processes were investigated.

2.3.1. Particle diameter

The mean diameter of 30 dried beads was determined using an optical microscope (BH2 Olympus, Japan). The size of an individual bead was calculated using a pre-calibrated eyepiece micrometer. Results are expressed as the mean diameter (μm) ± standard deviation. Mean diameters of NAP and AP beads were determined as a function of both residence time and $CaCl_2$ concentration.

2.3.2. Scanning electron microscopy

Typical external structure of Ca-pectinate beads was investigated by scanning electron microscopy (SEM). Dried beads prepared with AP and 6% of $CaCl_2$ were coated with a 6 nm platinum/palladium layer under vacuum (Cressington 208 HR, Eloise, France) and were examined using a scanning electron microscope (LEO 9530, Gemini, France) at an accelerating voltage of 3 kV.

2.3.3. Determination of protein content and β-lactamase activity in beads

In order to determine their content in protein and active β-lactamases, 10 beads were dissolved in 15 mL of Hepes/NaCl (10 mM/145 mM) pH 7.4 buffer containing 1% EDTA (solution A). The content of protein (active and inactive β-lactamases) was determined in the solution A using a protein assay based on a complex formation between a dye, Brilliant blue G (Bradford reagent), and β-lactamases, which could be quantified by a spectrophotometric method at 595 nm (Lambda 11 spectrometer, Perkin-Elmer, USA). Furthermore, the content of active β-

lactamases in beads was determined by an enzymatic assay using a chromogenic cephalosporin, nitrocefin. The degradation of nitrocefin by β-lactamases causes a shift in the absorption maximum of nitrocefin from 390 to 486 nm. Absorbance of degraded nitrocefin was proportional to the active β-lactamases present in the medium. Determination of the activity of β-lactamases in beads was performed as follow: 100 μL of nitrocefin reagent (500 μg/mL) was diluted with 775 μL of Hepes/NaCl containing 1% of EDTA and 125 μL of the solution A was added. Samples were incubated for 40 min at 37 °C and the absorbance of each sample was then determined at 486 nm (Perkin-Elmer Lambda 11). The entrapment efficiencies (EE) of the protein or the bioactive enzyme were calculated according to the following equations:

$$EE_{protein}(\%) = \frac{\text{actual protein amount in beads (μg)}}{\text{initial protein amount in pectin solution (μg)}} \times 100$$

$$EE_{activity}(\%) = \frac{\text{actual β-lactamase activity (I.U.)}}{\text{initial β-lactamase activity (I.U.)}} \times 100$$

Entrapment efficiencies were determined in triplicate and the results were expressed as the mean $EE_{protein}$ (%) or $EE_{activity}$ (%) ± standard deviation.

2.3.4. Determination of calcium content in beads

In order to determine the amount of calcium retained by beads, those were dissolved in Hepes/NaCl (10 mM/145 mM) pH 7.4 buffer containing 1% EDTA (10 beads/5 mL). After beads dissolution, the amount of calcium released was determined by atomic absorption spectroscopy (AAS). Calcium content in NAP and AP beads was determined as a function of both the concentration of $CaCl_2$ in the preparation solution and the residence time of beads in this solution. Moreover, the influence of the washing process on the amount of calcium retained by beads has also been investigated. After elimination of free calcium as described above, the amount of calcium was then determined in the beads and in the washing medium. All results were determined in triplicate and expressed as the calcium amount (mg/bead) ± standard deviation.

2.3.5. Determination of weight-loss of beads during the drying process

The weight-loss of NAP and AP beads during drying was determined by thermogravimetric analysis measuring particle weights before and after the drying process. Weight-loss of beads was expressed as a percentage according to the equation:

$$\text{weight loss}(\%) = \frac{mt_0 - mt_{eq}}{mt_0} \times 100$$

with mt_0 and mt_{eq} representing the weight of undried and dried beads, respectively.

Percentage of weight-loss during drying was determined in triplicate as a function of the concentration of $CaCl_2$ solutions used as bead gelation medium. This percentage was also determined for each $CaCl_2$ concentration after elimination of the free calcium by a washing process.

3. Results

3.1. Particle diameter

The mean diameter of the beads made either from NAP or AP is represented in Table 1. Diameters were measured as a function of the concentration of $CaCl_2$ in the gelation medium and as a function of the residence time of beads in the $CaCl_2$ solution. For a given residence time, an increase of the $CaCl_2$ concentration generally led to an augmentation of bead mean diameter. However, an increase of the residence time in $CaCl_2$ resulted in no significant change in the diameter of beads. Furthermore, the mean diameter of beads made from NAP appeared to be generally higher than the diameter of beads made from AP.

3.2. Scanning electron microscopy (SEM)

From the SEM pictures it appears that dried AP beads prepared with 6% of $CaCl_2$ are characterized by an ovoid shape and a smooth surface (Fig. 1A). Detailed SEM examination of dried bead (Fig. 1B), showed exterior surface of the beads to be covered with a network of small cracks and fissures. Same observations were made with NAP beads (data not shown).

3.3. Determination of protein content in beads

Modification of both concentration and residence time in $CaCl_2$ solution during bead formation did not modify the encapsulation of total protein in NAP or AP beads. $EE_{protein}$ remained stable around 75% for NAP beads and around a lower value of 45% for AP beads. Moreover, during the drying process, encap-

Table 1
Influence of $CaCl_2$ concentration and residence time on NAP and AP bead mean diameter

	[$CaCl_2$]			
	2%	4%	6%	8%
Residence time in $CaCl_2$				
NAP beads				
20 min	1175 ± 47	1157 ± 31	1274 ± 65	1409 ± 58
30 min	1201 ± 33	1230 ± 33	1298 ± 62	1346 ± 76
1 h	1164 ± 24	1185 ± 36	1223 ± 55	1341 ± 54
2 h	1139 ± 34	1205 ± 36	1239 ± 24	1121 ± 37
4 h	1147 ± 17	1154 ± 23	1346 ± 30	1215 ± 35
8 h	1140 ± 36	1211 ± 22	1315 ± 40	1346 ± 48
24 h	1168 ± 21	1189 ± 27	1247 ± 32	1319 ± 52
AP beads				
20 min	961 ± 17	1088 ± 22	1199 ± 28	1262 ± 48
30 min	959 ± 15	1089 ± 21	1161 ± 27	1243 ± 32
1 h	965 ± 16	1027 ± 28	1173 ± 51	1170 ± 48
2 h	1017 ± 18	1120 ± 41	1151 ± 33	1141 ± 34
4 h	992 ± 18	1070 ± 23	1120 ± 20	1226 ± 22
8 h	953 ± 28	1088 ± 22	1168 ± 43	1228 ± 22
24 h	993 ± 21	1040 ± 27	1159 ± 25	1023 ± 31

Results were expressed in μm ± standard deviation.

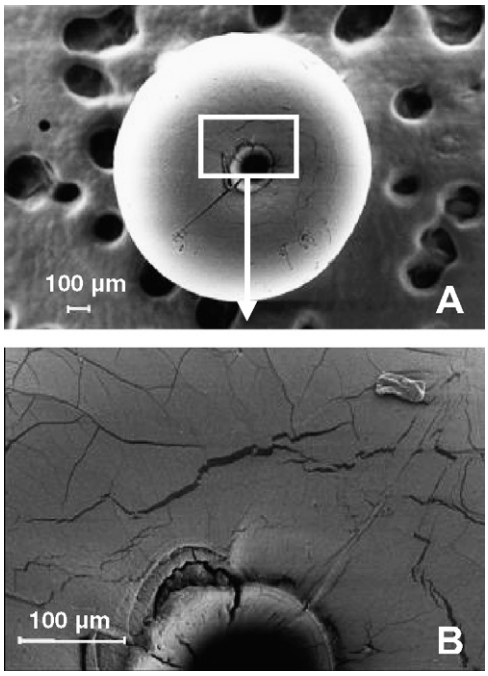

Fig. 1. SEM pictures of Ca-pectinate bead prepared with AP and 6% of $CaCl_2$ (A) and zoom on the bead surface (B).

sulation of the protein in both types of bead remained unchanged since all the amount of β-lactamase contained in the beads was recovered after drying.

3.4. Determination of β-lactamase activity in beads

β-Lactamases activity in beads was influenced by several parameters such as the concentration of the $CaCl_2$ solution, the residence time of beads in $CaCl_2$ the type of pectin and the drying process. Increasing $CaCl_2$ concentration in the gelation medium led to a reduction of $EE_{activity}$ of β-lactamases in beads (Fig. 2). In addition, the activity of encapsulated β-lactamases in beads was largely reduced as their residence time in $CaCl_2$ increased (Fig. 3).

Furthermore, the enzyme activity was diminished when beads were dried at 37 °C. For example, undried NAP beads prepared in an 8% $CaCl_2$ solution had an $EE_{activity}$ of β-lactamases of $84.9 \pm 2.5\%$ whereas the $EE_{activity}$ was of $32.0 \pm 1.0\%$ after drying, demonstrating the denaturing effect of this experimental step (Fig. 2A). Finally, the type of pectin used also modified the enzyme activity in beads since $EE_{activity}$ of β-lactamases was initially lower in AP beads than in NAP beads (Figs. 2 and 3).

3.5. Determination of calcium content in beads

$CaCl_2$ concentration in the gelation medium impacted on the amount of calcium retained in beads produced with a residence time of 20 min in $CaCl_2$. It can be easily observed in Fig. 4 that as the $CaCl_2$ concentration increased, the amount of calcium in both type of dried beads increased too. Moreover, except for beads prepared in 2% of $CaCl_2$, the amount of calcium was always greater in AP than in NAP beads. When measured as a

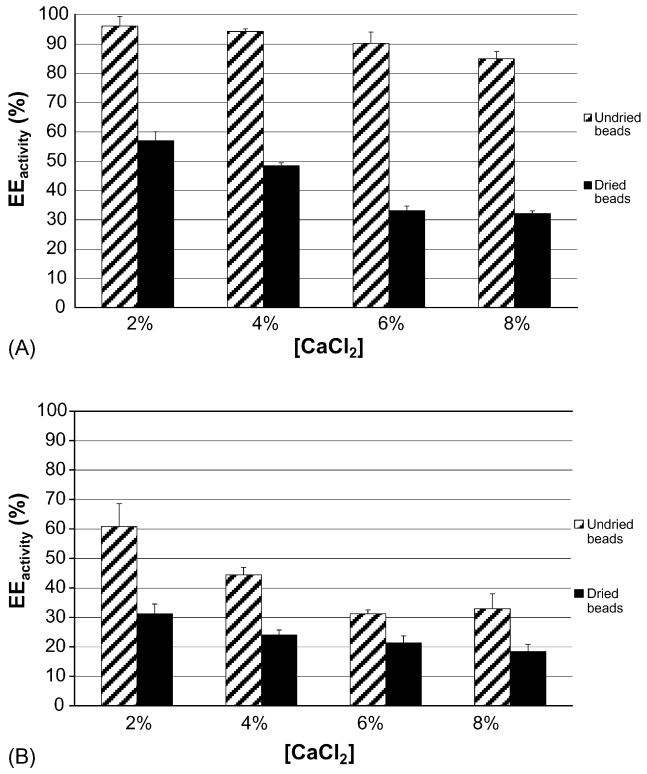

Fig. 2. Influence of $CaCl_2$ concentration and drying process at 37 °C on the encapsulation efficiency of active β-lactamases in NAP (A) and AP (B) beads (residence time in $CaCl_2$ = 20 min).

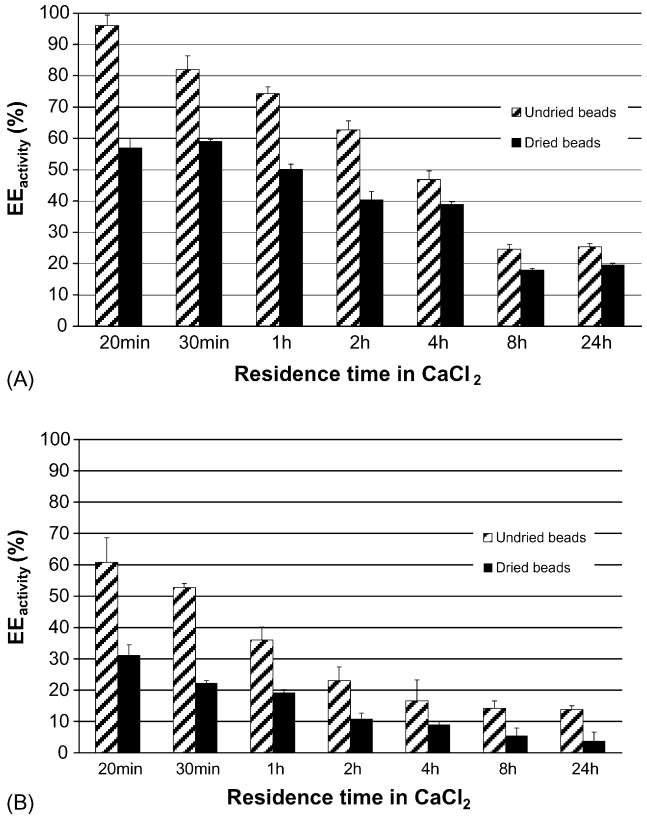

Fig. 3. Effect of $CaCl_2$ residence time on encapsulation efficiency of active β-lactamases in NAP (A) and AP (B) beads ([$CaCl_2$] = 2%).

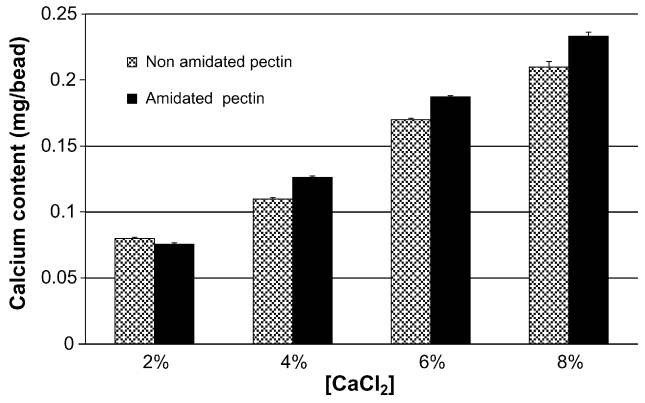

Fig. 4. Influence of $CaCl_2$ concentration on calcium-content in NAP and AP beads incubated during 20 min in $CaCl_2$.

function of residence time, calcium content in beads prepared with 2% of $CaCl_2$, increased rapidly during the first 20 min, after which it reached a plateau of approximately 0.08 mg/bead for both types of beads.

The amount of calcium retained in beads after elimination of free calcium by distilled water was considerably reduced (Fig. 5). Indeed, only a small amount of calcium is retained in beads since it actually represents approximately 10 % of the total amount of calcium quantified in Fig. 4. Moreover, when $CaCl_2$ concentration increased from 6 to 8%, the amount of free calcium, quantified in the washing medium, still increased

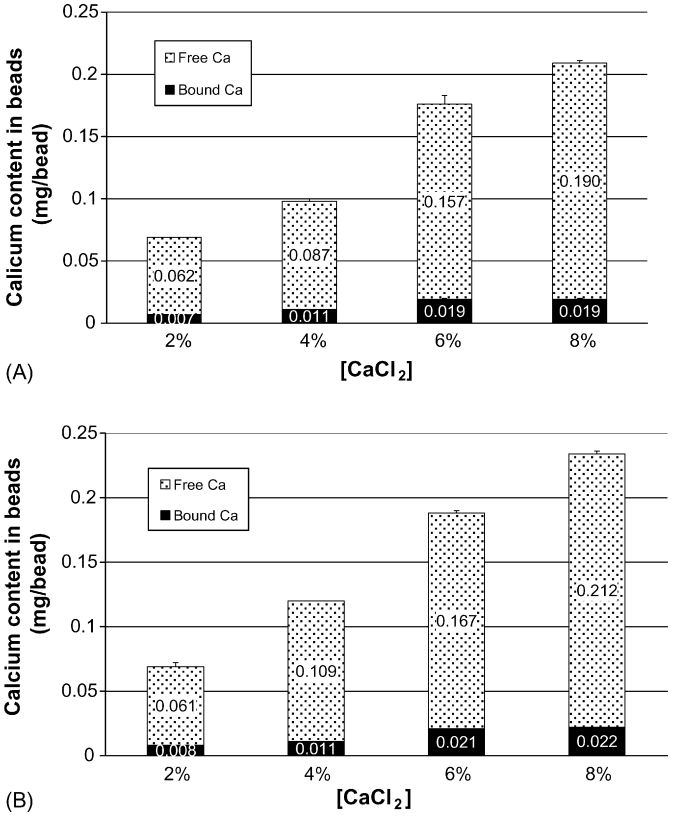

Fig. 5. Influence of washing process on calcium content in NAP (A) and AP (B) beads: determination of calcium content in beads after elimination of free calcium.

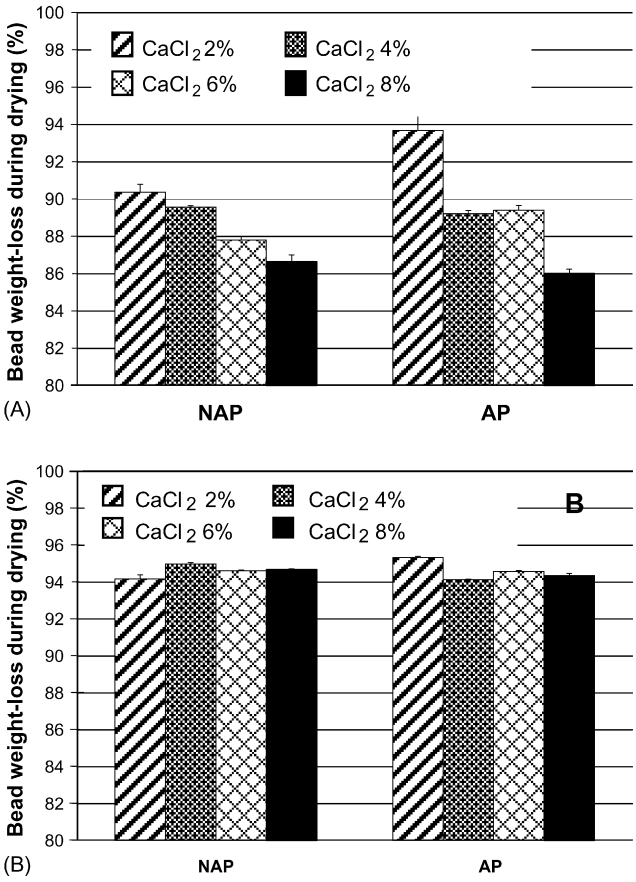

Fig. 6. Weight-loss of NAP and AP beads as a function of the concentration of $CaCl_2$ solution, before (A) and after (B) elimination of free calcium.

whereas the quantity of calcium retained in beads seems to reach a constant value. These observations were made for both type of pectin beads (Fig. 5A and B).

3.6. Determination of beads weight-loss during the drying process

The effect of $CaCl_2$ concentration and elimination of free calcium on the percentage of weight-loss during drying of NAP and AP beads is represented on Fig. 6. Before elimination of free calcium, bead weight-loss during drying decreased with increasing $CaCl_2$ concentrations. Therefore, the weight of dried beads was mainly influenced by $CaCl_2$ concentration since, in the case of AP beads, the weight-loss during drying varied from $93.7 \pm 1.2\%$ for 2% of $CaCl_2$ to 86.0 ± 0.2 for 8% of $CaCl_2$, demonstrating a rise of beads moisture content as the calcium concentration increased (Fig. 6A). However, when free calcium was eliminated from beads, their weight-loss sharply increased and was not significantly influenced by the modification of $CaCl_2$ concentration. Indeed, for 2, 4, 6 and 8% of $CaCl_2$, the percentage of weight lost during drying of NAP and AP beads remained constant with an average value of $94.8 \pm 0.2\%$ of the initial weight for NAP beads and $94.4 \pm 0.2\%$ for AP beads (Fig. 6B).

Table 2
Effect of the elimination of free calcium on characteristics of AP dried beads prepared with a 6% $CaCl_2$ solution

	Initial beads	After elimination of free Ca
Calcium content (mg/bead ± S.D.) ($n = 3$)	0.187 ± 0.001	Bead: 0.021 ± 0.000, Supernatant: 0.167 ± 0.002
Weight-loss during drying (% ± S.D.) ($n = 3$)	89.41 ± 0.26	94.59 ± 0.05
Active β-lactamases EE (% ± S.D.) ($n = 3$)	21.30 ± 2.37	86.49 ± 6.79

3.7. Effect of free calcium elimination on β-lactamase activity in dried beads

The effect of the washing process on the main characteristics of beads was made on those prepared with AP since the beads made from this polymer are more stable in the gastrointestinal tract (Wakerly et al., 1997). Results are summarized in Table 2 for a 6% $CaCl_2$ concentration. Table 2 underlined particularly the effect of washing on the activity of encapsulated β-lactamases after drying the beads. Indeed, $EE_{activity}$ of β-lactamases is largely increased when free calcium was eliminated from beads with distilled water. In this case, the drying process did not affect the enzyme since there was no activity loss before and after drying the beads.

4. Discussion

Design of calcium-pectinate beads, composed of low methoxylated (LM) pectin, for colon delivery of β-lactamases was achieved using the ionotropic gelation technique. The goal of the present study was to check if the preparation of such beads could affect the bioactivity of β-lactamases.

Bead mean diameter was around 1000 μm, however increasing $CaCl_2$ concentration in the gelation medium generally led to particles of larger size in relation with the increasing amount of calcium retained in the beads. This was explained by greater water retention in beads as the initial concentration of $CaCl_2$ increased, which was obviously due to the hygroscopic character of $CaCl_2$. Indeed, when free calcium was eliminated from beads, there were no differences in the mean diameter between particles prepared in medium with increasing concentrations of $CaCl_2$ (data not shown). Nevertheless, increasing residence time in $CaCl_2$, for a given concentration, led to no significant changes in the diameter of beads since they were saturated by calcium ions within the first hour. In these conditions, because the amount of calcium and residual water remained constant in beads, their mean diameter did not change. Finally, the mean diameter was generally smaller for AP than NAP beads. This may be due to the presence of amide groups on AP pectin chains, which reduces the hydrophilicity of pectin and increases its gel-forming ability (Kim et al., 1978). Moreover, an increased strength of amidated pectin gels was reported due to hydrogen bonding between amide groups (Thakur et al., 1997). These interactions allow consolidating cross-linking between AP pectin chains leading to the formation of a more compact Ca-pectinate network, which could explain the smaller size of AP beads compared to NAP beads. Regarding the amount of calcium retained by the beads, we have observed that, except for 2% $CaCl_2$, the total amount of calcium in AP beads is greater than in NAP beads. Ca-pectinate gel formation results from a specific interaction between calcium ions and blocks of galacturonate residues containing a majority of free carboxyl groups. Moreover, it was demonstrated that bonds between low methoxylated pectin and calcium ions are stable when there are at least seven consecutive carboxyl groups on each participating chain (Thakur et al., 1997). NAP (DE ∼ 30%) has approximately 70% of free carboxyl groups and then more blocks are present (region rich in free carboxyl groups) than AP pectin (DE ∼ 25% and DA ∼ 20%), which has approximately 55% of free carboxyl groups. Consequently, NAP is more susceptible than AP to present the seven or more consecutive carboxyl groups necessary to achieve "egg-box" structures. This difference results in an improvement of the reactivity of NAP chains to calcium ions. The Ca-pectinate network is therefore more homogeneous and consequently needs less calcium ions than pectin with fewer blocks such as AP. Furthermore, it has been observed that the final amount of calcium retained in beads was reached very rapidly after 20 min of incubation in $CaCl_2$ probably due to the quick formation of the Ca-pectinate network. Indeed, mechanism of gel formation has been described with as an initial rapid elaboration of dimers between calcium ions and two chains of pectin, which requires large amount of calcium ions (Morris et al., 1982; Thakur et al., 1997). Then, if more calcium is available, there is an aggregation of the initial dimers. This second step of the formation of the Ca-pectinate network needs less calcium ions than the previous and could therefore explain the very quick saturation of the calcium pectinate network by calcium after 20 min of incubation.

Instantaneous gelation of pectin allowed easy encapsulation of β-lactamases in Ca-pectinate beads. However, in the present study, it was observed that the modification of both concentration and residence time in $CaCl_2$ during bead formation did not modify the encapsulation of the total protein in NAP or AP beads. Nevertheless, a greater encapsulation of the protein was observed in NAP compared to AP. By taking into consideration the isoelectric point of β-lactamases which is around pH 8.5 (Connolly and Waley, 1983), electrostatic interactions could occur at the pH of gelation medium (pH 6) between positively charged basic amino acids such as arginine, histidine and lysine of β-lactamases and the negatively charged carboxylic groups of pectin (pK_a 3.5–4). Negatively charged carboxylic groups are more numerous in NAP than in AP. Therefore, NAP beads could entrap higher amount of protein than AP beads.

Determination of the encapsulation efficiency of bioactive β-lactamases has demonstrated that a loss of the enzyme activity occurred as the amount of calcium in beads increased. However, the incubation of β-lactamase with $CaCl_2$ induced only a slight modification of the enzyme bioactivity after 24 h (around 80% of the initial activity was preserved). Moreover, it was also

observed that the moisture content in beads is correlated to the amount of calcium. Therefore, it has been suggested that the denaturant effect of calcium might be related to the increase of water content due to the retention of an excess of $CaCl_2$ in beads. Indeed, regarding the calcium entrapped in beads, it was demonstrated that only a small amount actually contributed to the formation of the Ca-pectinate network, and that the majority of the quantified calcium was under a free form, probably free $CaCl_2$, remaining entrapped in Ca-pectinate beads inducing by its hygroscopic character the retention of water. The presence of a high degree of moisture could contribute to the degradation of encapsulated β-lactamases. Indeed, when free calcium was eliminated, the amount of residual water in beads was less important and remained constant as the concentration of the $CaCl_2$ in the gelation medium increased. Moreover, the elimination of free calcium from beads and the decrease of moisture content allowed preserving the activity of encapsulated β-lactamases. These experiments were only conducted on AP beads since particles made from this polymer were previously claimed to be more stable in the gastro-intestinal tract (Wakerly et al., 1997). Preserving β-lactamase activity was brought as an additional advantage to this property.

It was also shown that the loss of bioactivity was greater as the residence time of beads in $CaCl_2$ increased during the gelation process. This loss of enzyme activity could not be due to a release of the encapsulated proteins during the incubation in $CaCl_2$ since the amount of protein in beads remained constant or to an enhanced penetration of calcium since calcium in beads reached a plateau after 20 min of incubation. Therefore, it could be concluded that the denaturation of β-lactamase activity in beads is a kinetic process that is time dependent.

In the present study, it has been also observed that the drying process amplified the loss of enzyme activity although in solution the activity of the β-lactamase is not changed at 37 °C for 2 h. It has been previously demonstrated that a conformational change in the Ca-pectinate network takes place when the gels are dried to solid films (Morris et al., 1982). The transition was suggested to be a result of a conversion from the two-fold (2_1: two residues per turn of the helix), to the three-fold (3_1) helical conformation of the polygalacturonate chains of pectin. This 2_1 to 3_1 conversion might induce conformational change in the encapsulated protein, which occurs during the drying of beads inducing a loss of β-lactamase activity without affecting the rate of encapsulation. Reduction of β-lactamase activity in dried beads might also result from shear stress. It has previously been proposed that the cracks and fissures within the dried matrices of pectin beads, clearly visible in scanning electron micrographs, can be an artefact of electron microscopy sample preparation (Sriamornsak, 1999). However, a more likely explanation for the existence of these fractures is that in common with other gels, the cracks result from shear forces generated when evaporation of water caused shrinking of the gel (Powell et al., 1982; Scherer, 1990; Caddock and Hull, 2002). Evidence for the strength of these shear forces, is provided by the fact that dried beads yield larger proportions of degraded enzymes. In this study, it could have been possible to use freeze-drying instead of oven drying as many others describe lyophilisation as an interesting method to preserve proteins activity (for review see Roy and Gupta, 2004). However, this process renders the beads more fragile and therefore unsuitable as delivery system to the colon (Sriamornsak, 1999).

In final, the effect of calcium on β-lactamase stability was evaluated. From the data, a formulation that preserves β-lactamase can be obtained using a large concentration of Calcium (6–8%) only if free calcium is removed from beads and leaving the beads in the gelation medium for a short time.

5. Conclusion

This study has demonstrated that the bioactivity of the encapsulated β-lactamases depends on bead formulation and process parameters. The encapsulation of the protein is function of the type of pectin used (NAP or AP) but mostly the presence of a large amount of free calcium in beads considerably influences the activity of encapsulated β-lactamases. A drastic elimination of free $CaCl_2$ from Ca-pectinate network reduces moisture content in beads and avoids the risk of protein hydrolysis. Finally, the drying process of beads also modified the activity of encapsulated protein. However, such process and formulation parameters can be easily controlled in order to preserve the activity of encapsulated β-lactamases.

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