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Encapsulation of an *Agrobacterium radiobacter* extract containing D-hydantoinase and D-carbamoylase activities into alginate–chitosan polyelectrolyte complexes Preparation of the biocatalyst

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ABSTRACT

Alginate–chitosan polyelectrolyte complexes (PECs) have been used for the first time as a suitable matrix for coimmobilisation of enzymes to reproduce a multistep enzymatic route for production of D-amino acids. Encapsulation of a crude cell extract from *Agrobacterium radiobacter* containing D-hydantoinase and D-carbamoylase activities into the PECs with negligible leakage from the formed capsules was accomplished. All results in this study indicate that the preparation of the biocatalyst (preparation method and chitosan characteristics) play a key role in the biocatalyst's properties. The most suitable biocatalysts were prepared using a chitosan with a medium molecular weight (600 kDa) and a degree of deacetylation of 0.9. For all of the preparation conditions under study, an encapsulation yield of around 60% was achieved and the enzymatic activity yields ranged from 30 to 80% for D-hydantoinase activity and from 40 to 128% for D-carbamoylase activity relative to the activities of the soluble extract. All of the biocatalysts were able to hydrolyze L,D-hydroxyphenylhydantoin into p-hydroxyphenylglycine with yields ranging from 30 to 80%.

1. Introduction

D-Amino acids are important molecules in the synthesis of chemicals such as semi-synthetic antibiotics, pesticides and hormones [1]. The D-p-hydroxyphenylglycine (p-HPG) is a side chain of β-lactam antibiotics, such as amoxicillin and cefadroxil. This D-amino acid can be produced in a hydantoin-transforming reaction starting from a racemic mixture of p-hydroxyphenylhydantoin (L,D-HPH) [2]. In basic media, L-HPH spontaneously racemises to D-HPH so a theoretical 100% conversion can be achieved. In the first step, D-hydantoinase (EC 3.5.2.2) converts D-HPH into N-carbamoyl-D-p-hydroxyphenylglycine (C-pHPG). In the second step, the intermediate product is converted into the final product p-HPG by the enzyme N-carbamoyl-D-amino acid amidohydrolase (D-carbamoylase; EC 3.5.1.77). A schematic representation of the process is shown in Fig. 1.

It has been reported that several microorganisms such as *Agrobacterium*, *Pseudomonas* and *Arthrobacter* contain both enzymatic activities, which are required for the conversion of hydantoin to p-HPG [3,4]. Not much data about the physico-chemical characteristics of both enzymes is available in the literature. It seems that both D-hydantoinase and D-carbamoylase enzymes are multimeric proteins. D-hydantoinase from *Agrobacterium* sp. is a tetrameric enzyme with a native molecular weight of 250 kDa and an isoelectric point of 6.5 [5]. On the other hand, D-carbamoylase from *Agrobacterium tumefaciens* and *Agrobacterium* sp. are dimers with molecular weights of 67 and 84 kDa and isoelectric points of 5.8 and 5.5, respectively [6,7]. Due to the low thermo-stability and sensitivity to the oxidative process of D-carbamoylase, the enzyme cannot be used in industry [8]. In commercial pHPG synthesis, the hydrolysis of C-pHPG is carried out by diazotation. During this chemical process, a high reaction temperature, long reaction times and many separation steps are needed. Moreover, large amounts of waste are generated. Therefore, there is a need to develop an industrially feasible and environmentally friendly enzymatic method to replace diazotation.

There are many techniques available that may be used to improve the enzyme's features such as chemical modification, protein engineering, use of additives and enzyme immobilisation [9]. In the particular case of enzyme immobilisation, with proper design of the system, it is possible to improve the enzyme activity, stability and selectivity [10,11]. Moreover, the use of immobilised biocatalysts may permit its reuse (if the immobilisation has increased sufficiently the enzyme stability) as well as a simplification of the design of the reactor and easier control of the reaction [12–14]. Several techniques to immobilise enzymes have been described (adsorption, covalent linking, encapsulation, etc.), each of them

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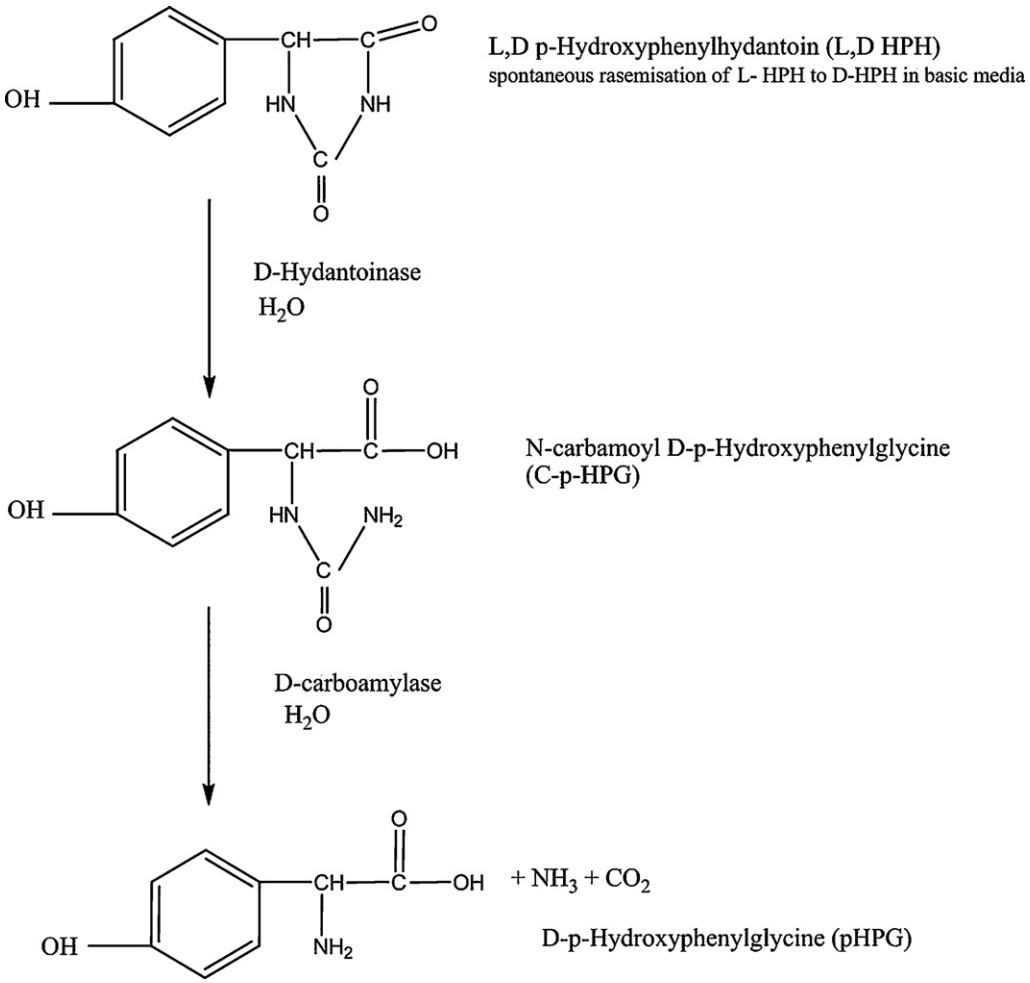

Fig. 1. Schematic representation of the biocatalyst process.

with their advantages and disadvantages, and their use will depend on each particular system [15,16].

Encapsulation of enzymes in alginate gels is characterised by the very mild conditions in which the immobilisation procedure is carried out and by its low cost and ease of use [17]. Moreover, it is possible to immobilise several enzymes at the same time. The coimmobilisation of enzymes presents several advantages. First, it reduces the diffusion process of the intermediate substrate from one enzyme to another, thus increasing the efficiency of the process. Second, the use of a single support reduces the cost of the immobilisation process (fewer reagents, less energy and time). However, since there is no covalent interaction between the enzymes and the capsules, no "rigidification" is expected. The rigidification involves a conformational change in the enzymes that, in some cases, may improve their stability [18]. Although no "rigidification" occurs during encapsulation, other stabilisation processes can occur. First, the encapsulation protects the enzymes against external interfaces such as air bubbles and, in this way, an "operational stabilisation" is observed, although no conformational changes occurs [15]. Second, the encapsulation may prevent multimeric enzyme dissociation and thus maintain the activity, since dissociation is known to be an inactivation process [14]. Finally, the creation of a highly hydrophilic shell around oxygen-sensitive enzymes has proven to be quite effective in the stabilisation of enzymes, due to a salting-out effect that reduces the presence of oxygen in the enzyme's surroundings [19].

Alginate capsules have been used as a matrix for the immobilisation of lipase, glucose oxidase, tannase, tyrosinase and for the coimmobilisation of glucose oxidase and catalase among others [20–24]. In a previous paper, a crude cell extract from *Agrobacterium rb* containing D-hydantoinase and D-carbamoylase was encapsulated in calcium–alginate capsules to produce p-hydroxyphenylglycine (p-HPG) [25]. The first step of the synthesis is a reversible reaction. Moreover, the C-pHPG is an acidic molecule and during its production, a reduction of the reaction media pH occurs, even in the presence of buffers. Under these conditions, a decrease in the rate of hydrolysis is observed. When both enzymes are coimmobilised, the C-p-HPG is transformed "in situ" into p-HPG and an increase of the reaction yield is expected, since the equilibrium of the first reaction is shifted to the production of C-p-HPG, as this molecule is consumed by the second reaction. Moreover, the reduction of the pH is controlled by the elimination of the C-pHPG as well as by the production of NH_3 as a by-product of the second reaction. However, the use of alginate as a matrix for the encapsulation of the extract shows several disadvantages such as low stability in calcium chelating buffers, easy microbial contamination during storage at 4 °C and the release of proteins [25,26]. To overcome these disadvantages, alginate–chitosan polyelectrolyte complexes (PECs) were selected as a matrix to encapsulate the crude extract.

The mixing of solutions of polyanions and polycations leads to the spontaneous formation of interpolymer complexes upon the release of the counterions. The driving force behind complex formation is mainly the gain in entropy due to the liberation of the low molecular counterions. The degree of conversion determines whether the ionic sites of the components are completely bound by the oppositely charged polyelectrolytes or whether low molecular counterions partly remain in the complex. Hydrogen bonding or

hydrophobic interactions may play an additional role [27]. The reaction of polyelectrolyte complex formation between alginate and chitosan is shown in the following equation:

$$(R\text{–}NH_3{}^+CH_3COO^-)_a + (R'\text{–}COO^-Na^+)_b \leftrightarrow (R\text{–}NH_3{}^+R'\text{–}COO^-)_{x-} + (R\text{–}NH_3{}^+CH_3COO^-)_{x-a} + (R'\text{–}COO^-Na^+)_{x-b}. \quad (1)$$

where $R\text{–}NH_3{}^+$, $R'\text{–}COO^-$ represent the charged chitosan and alginate chains, CH_3COO^-, Na^+ are the counterions and a, b are the number of cationic and anionic groups in the solution.

PECs have been previously used to immobilise enzymes [28–30], even those with low molecular such as carbonic anhydrase (30 kDa) [29]. However, to our knowledge, this is the first time that this system is used to coimmobilise enzymes.

Alginate mixed chitosan capsules and alginate coated with chitosan capsules can be easily prepared [31]. Alginate mixed chitosan capsules are prepared in a one-step procedure, by simply dropping an alginate solution containing the extract into a chitosan solution containing calcium ions. Calcium–alginate capsules coated with chitosan are prepared in a two-step procedure. First, calcium–alginate capsules are prepared by dropping alginate into a calcium chloride solution and the calcium–alginate capsules are then transferred into a chitosan solution.

Chitosan is described as a family of linear polysaccharides consisting of varying amounts of β (1 → 4) linked residues of N-acetyl-2 amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose residues. Due to their natural origin, chitosans cannot be defined as a unique chemical structure, but as a family of polymers which presents a high variability in their chemical and physical properties [32]. In this paper, several chitosan samples have been tested to determine the effect that the molecular weight and the degree of deacetylation of the chitosan have on the properties of the biocatalyst.

2. Materials and methods

2.1. Materials

Chemicals. Sodium alginate (medium viscosity), the bicinchoninic acid protein assay kit (Kit num. BCA-1) and N-acetyl-glucosamine were supplied by Sigma. Commercial chitosans (CS-1 and CS-2) were purchased from Primex (Norway). Chitosan from shrimp shells (CS-4) was prepared in the laboratory as described elsewhere [33]. Methanol (HPLC-Gradient) was supplied by Panreac. Other chemicals were of analytical grade.

Biological material. Crude extracts from over-expressed *Agrobacterium radiobacter* were prepared as previously described [25]. The protein concentration in the extract was 53 mg/ml.

2.2. Chitosan depolymerisation

Low molecular weight chitosan (CS-3) was prepared from a commercial sample (CS-1) by chemical depolymerisation [34]. Briefly, the chitosan was dissolved in HCl 0.1 M (0.68%, w/v). The depolymerisation reaction started with the addition of 1.5 ml of KNO_2 solution (33.36 mM) to 40 ml of the chitosan solution previously incubated at 65 °C. After 30 min, a mixture of acetone/water (80:20) was added to stop the reaction and to precipitate the chitosan. The chitosan was recovered by centrifugation (4500 rpm, 10 min), washed with acetone and air-dried.

2.3. Chitosan characterisation

The molecular weight of the chitosan samples (viscometric-average molecular weight) was calculated from the viscosity of diluted solutions (<1%, w/v) with a capillary viscometer (Ubbelhode type) and using the Mark–Houwink relationship:

$$[\eta] = K(Mv)^a,$$

where $[\eta]$ is the intrinsic viscosity in dl/g, $K = 0.181$, $a = 0.93$ (0.1 M acetic acid and 0.2 M NaCl) [35]. The acetylation degree was calculated by UV absorbance using N-acetyl-glucosamine [36]. The results are shown in Table 1.

Table 1
Chitosan characteristics.

Sample	η (dl/g)	Mw (kDa)	DD (%)
CS-1	5.70 ± 0.54	816 ± 89	0.90
CS-2	3.58 ± 0.36	495 ± 53	0.89
CS-3	0.62 ± 0.06	75 ± 8	0.92
CS-4	3.69 ± 0.15	519 ± 39	0.79

2.4. Enzymatic extract encapsulation

Alginate–chitosan mixed capsules (one-step procedure) and alginate capsules coated with chitosan (two-step procedure), were prepared by using the method described by Gaserod et al. with some modifications [31].

2.4.1. Alginate–chitosan mixed capsules

A specific amount of the cell extract was mixed with a sodium alginate solution (1–2.5%, w/v) and stirred at 4 °C. This mixture was extruded dropwise through a 22 gauge needle into a chitosan solution which contained $CaCl_2$ 50 mM. The ratio between the alginate-extract solution and the chitosan–$CaCl_2$ solution was 1:4. The capsules were cured in the solution for 1 h at 4 °C. Afterwards the solution was filtered out and washed thoroughly in deionised water. The chitosan solution was prepared by dissolving chitosan (0.15%, w/v) in acetic acid (1%, v/v). The pH of the chitosan solution was raised to pH 6.2 with NaOH 10 M. The wet capsules were stored in deionised water at 4 °C until further studies. A number of the capsules were freeze-dried and stored at 4 °C until further studies. Capsules without extract were also prepared.

2.4.2. Alginate capsules coated with chitosan

Calcium alginate capsules loaded with the extract were prepared as previously described [25]. Briefly, an alginate solution (1–2.5%, w/v) containing the crude extract was dropped into a 50 mM $CaCl_2$ solution and the capsules were cured for 30 min. After that, the capsules were transferred to a chitosan solution and cured for 1 h. This solution was prepared as previously described. The wet capsules were stored in deionised water at 4 °C until further studies. A number of capsules were freeze-dried and stored at 4 °C until further studies. Capsules without extract were also prepared.

2.5. Encapsulation efficiency

The concentration of protein inside the capsules was determined directly. A complete batch of capsules was disrupted by incubation in 0.1 M citrate buffer pH 9.0 for 18 h. The protein content of the dissolved capsules was measured by micro-BCA assay with BSA as the standard protein [37].

$$EE = \left(\frac{\text{amount of protein inside capsules}}{\text{amount of protein added to alginate solution}}\right) \times 100.$$

2.6. Protein release

A given amount of wet or freeze-dried capsules was incubated in 0.1 M Tris–HCl buffer pH 8 at 40 °C. Aliquots were withdrawn at

predetermined time intervals and the protein concentration was measured by using the micro-BCA assay.

2.7. Activity assay

D-Hydantoinase and D-carbamoylase activities of the encapsulated extract were determined by analysing the amounts of p-HPG and C-pHPG formed in standard reactions. The standard reaction for D-Hydantoinase activity determination started with the addition of a soluble or encapsulated extract to a L,D-HPH solution (10 g/l) in 0.1 M Tris–HCl at pH 8.0, previously incubated at 40 °C for 10 min.

After 60 min a sample was taken from the reaction mixture, centrifuged and diluted 1:5 with HCl 0.1 N to stop the enzymatic reaction.

The standard reaction for D-carbamoylase activity determination started with the addition of a soluble or encapsulated extract to a C-pHPG solution (2 g/l) in 0.1 M Tris–HCl buffer at pH 8.0, previously incubated at 40 °C for 10 min.

After 120 min, a sample was taken from the reaction mixture, centrifuged and diluted 1:5 with HCl 0.1 N to stop the enzymatic reaction.

In both cases, preliminary experiments revealed that the activity of both enzymes was linear up to 120 min in the prevailing experimental conditions.

The pHPG and C-pHPG formed were analysed by HPLC using a Waters 625 LC system equipped with a photodiode array detector (λ = 265 nm) and Millennium software. A reverse phase column Lichosphere 100 RP 18 5 μ was used. The mobile phase consisted of 20 mM Na_2HPO_4-H_3PO_4 pH 2.5/methanol (95/5), the flow rate was 0.7 ml/min and 50 μl of reaction solution was injected.

D-Hydantoinase activity in the soluble/immobilised extract was calculated as the total amount (μmol) of D HPH converted to C-pHPG plus p-HPG per gram of protein (soluble or immobilised) over a 1 h reaction period. D-Carbamoylase activity in the soluble/immobilised extract was calculated on the basis of the amount of pHPG produced (μmol) per gram of protein soluble/immobilised over a 2 h reaction period.

p-HPG production yield was defined as the ratio between the amount of p-HPG per gram of encapsulated protein produced by the encapsulated extract to the amount of p-HPG per gram of soluble protein produced by the soluble extract.

2.8. Activity yield

This is defined as the ratio of the activity of the immobilised enzyme to the activity of the soluble enzyme placed in contact with the alginate solution.

$$\mathrm{IY} = \frac{(\text{immobilised enzyme activity(U/gencapsulated protein)}}{\text{soluble enzyme activity(U/gsoluble protein)}} \times 100.$$

2.9. Characterisation of alginate–chitosan capsules

2.9.1. Morphological characterisation

The surface of wet capsules was examined by environmental scanning electron microscope (ESEM, Phillips XL30). The shape and surface characteristics of the freeze-dried capsules were examined by scanning electron microscopy (SEM). Randomly selected capsules were freeze-dried, sputter-coated with Au/Pd using a vacuum evaporator and examined with a scanning electron microscope (Jeol JSM-6400, Tokyo, Japan) at 10 kV accelerating voltage. The diameter of the capsules was measured by using a Mitutoyo digimatic micrometer (UK)/Ltd.

Table 2
Average diameter of the alginate–chitosan PECs loaded with cell extract (N = 50).

Alginate (%)	Chitosan	Diameter[a] (mm)	Diameter[b] (mm)
1	CS1	2.22 ± 0.10	2.49 ± 0.06
2	CS1	2.68 ± 0.11	2.88 ± 0.09
2.5	CS1	2.72 ± 0.13	2.89 ± 0.09
1	CS2	2.10 ± 0.13	2.35 ± 0.09
2	CS2	2.63 ± 0.08	2.82 ± 0.09
2.5	CS2	2.70 ± 0.20	2.89 ± 0.07
1	CS3	1.95 ± 0.12	2.07 ± 0.07
2	CS3	2.43 ± 0.08	2.38 ± 0.08
2.5	CS3	2.56 ± 0.10	2.49 ± 0.09
1	CS4	2.09 ± 0.13	2.32 ± 0.09
2	CS4	2.48 ± 0.07	2.59 ± 0.10
2.5	CS4	2.53 ± 0.10	2.51 ± 0.08

[a] Calcium–alginate capsules coated chitosan.
[b] Alginate–chitosan mixed capsules.

2.9.2. Capsules Stability—'burst assay'

The mechanical stability of alginate–chitosan PECs capsules was determined using a burst assay as described by Thu et al. [38]. This method is based on the swelling of alginate gel capsules in water. Depending on the strength of the membrane, the capsule will burst when the swelling pressure exceeds a certain value. Alginate–chitosan capsules (N = 100) were transferred to petri dishes and deionised water was added. After a week at room temperature the number of broken capsules was counted. Capsules without extract were used as a control.

2.9.3. Equilibrium water uptake

The freeze-dried capsules were accurately weighed (W_0) and then immersed in water. The weight of the swollen samples was measured against time, after the excess surface water had been removed by means of filter paper, until no more weight was gained. The water uptake was calculated using the following expression:

$$W = \frac{W_{eq} - W_0}{W_0}.$$

where W_{eq} and W_0 are the sample weights at equilibrium and in the dry state, respectively.

3. Results

In the present work, a crude cell extract from *Agrobacterium rb* was encapsulated in alginate–chitosan polyelectrolyte capsules by two different methods. In the one-step procedure, alginate–chitosan mixed capsules were prepared by dropping an alginate-extract solution into a chitosan solution containing calcium ions. In the two-step procedure, alginate capsules coated with chitosan were prepared by coating calcium–alginate capsules loaded with the extract with chitosan.

The variables under study in the present work consist of the method of preparation, alginate concentration and chitosan characteristics (molecular weight and degree of deacetylation).

3.1. Morphological characterisation

In all cases, alginate–chitosan PECs capsules loaded with the cell extract were slightly turbid and nearly perfect spheres. As can be seen in Table 2, the average diameter ranged from 2 to 3 mm and only depended on the alginate concentration while being unaffected by the chitosan characteristics or the preparation method.

Alginate–chitosan PECs capsules, as well as calcium–alginate capsules, were observed under ESEM. A representative sphere for

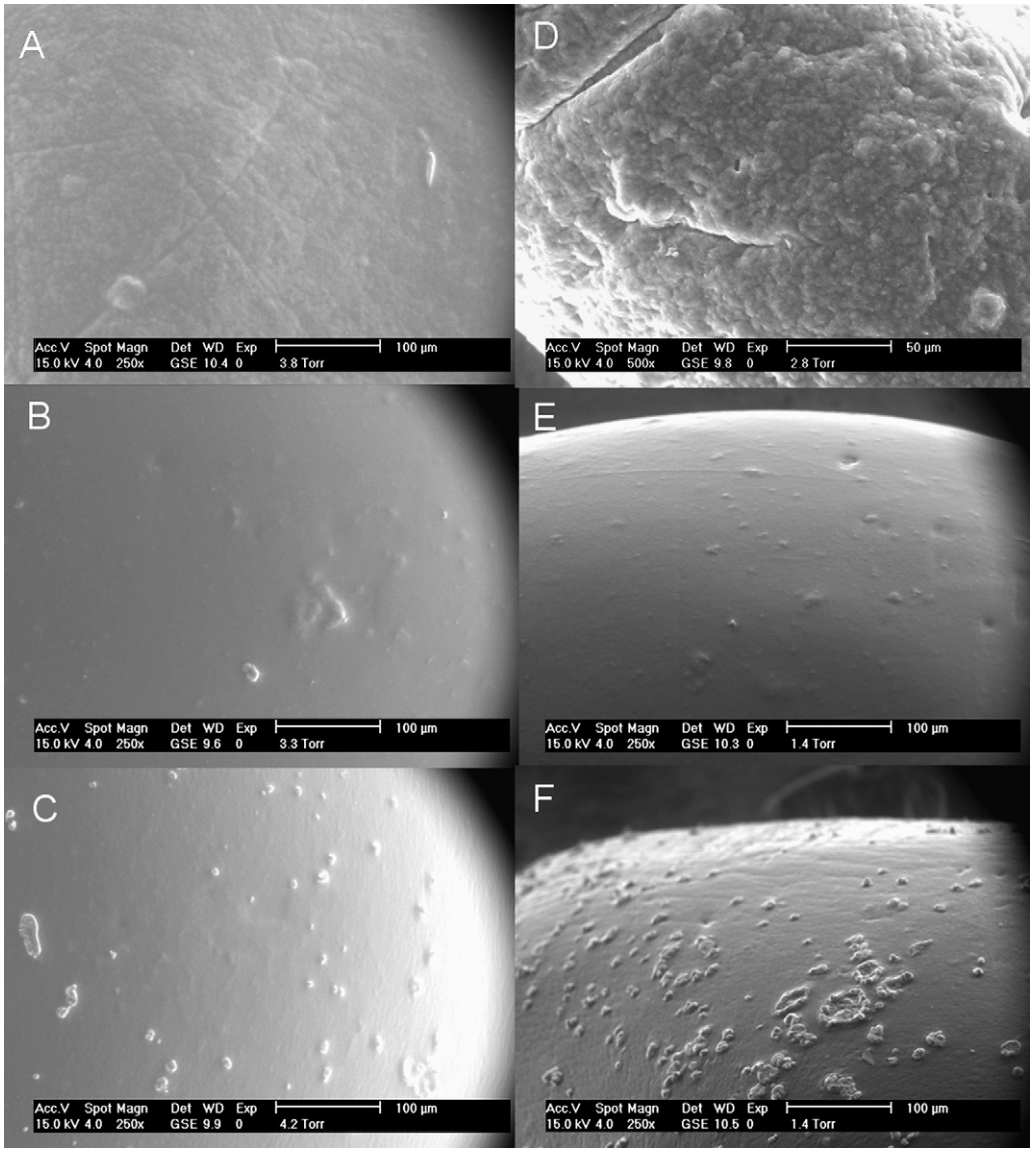

Fig. 2. ESEM micrographs of wet (A) or dehydrated (D) calcium–alginate capsules, wet (B) or dehydrated (E) calcium–alginate capsules coated with chitosan, wet (C) or dehydrated (F) alginate–chitosan mixed capsule (alginate 2.5% and chitosan CS-1).

each preparation method is shown in Fig. 2(A–C). Since with this technique no treatment is carried out to observe the capsules, their original structure is observed. Calcium–alginate capsules, coated with chitosan and calcium–alginate capsules, showed a similar smooth surface while alginate–chitosan mixed capsules showed spots over the smooth surface. Capsules were dehydrated by reducing the pressure inside the microscope and a very different behaviour was observed. Calcium–alginate capsules shrunk and the typical surface of a dehydrated polymer was observed. On the other hand, the presence of chitosan reduced the shrinkage of the capsules and an increase of the solid particles over the surface was observed (Fig. 2D–F).

The capsules were freeze-dried and the structure and surface of the capsules were studied under SEM. As can be seen in Fig. 3, after the freeze-drying process the capsules collapsed and their diameter was reduced by around 50%. A detail of the surfaces of the capsules is shown in Fig. 4. Calcium–alginate capsules coated with chitosan showed a homogeneous rough surface and the presence of precipitates on the surface was observed. On the other hand, alginate–chitosan mixed capsules showed two types of surfaces, a rough surface very similar to the one previously described and a smooth surface similar to the previously reported in the calcium–alginate capsules [25].

The inner structure of the freeze-dried capsules is shown in Fig. 5. For both methods of preparation, a liquid core surrounded by a thin polymer membrane (around 15 μm estimated by SEM micrographs) was observed. This morphology is the same one previously reported when calcium–alginate capsules were studied [25].

3.2. Mechanical properties

The mechanical stability of the capsules is one of the most important requirements for the application of the biocatalyst. The mechanical stability of both loaded and non-loaded capsules was determined by using a “burst assay”. Non-loaded capsules were stable throughout the assay. This was independent on the alginate concentration, chitosan characteristics or preparation method. On the other hand, the stability of the loaded capsules depended on the preparation method as well as on the chitosan molecular weight (Table 3). When both methods of preparation are compared, it is clear that alginate–chitosan mixed capsules presented a higher stability than the chitosan coated ones. The latest only showed good

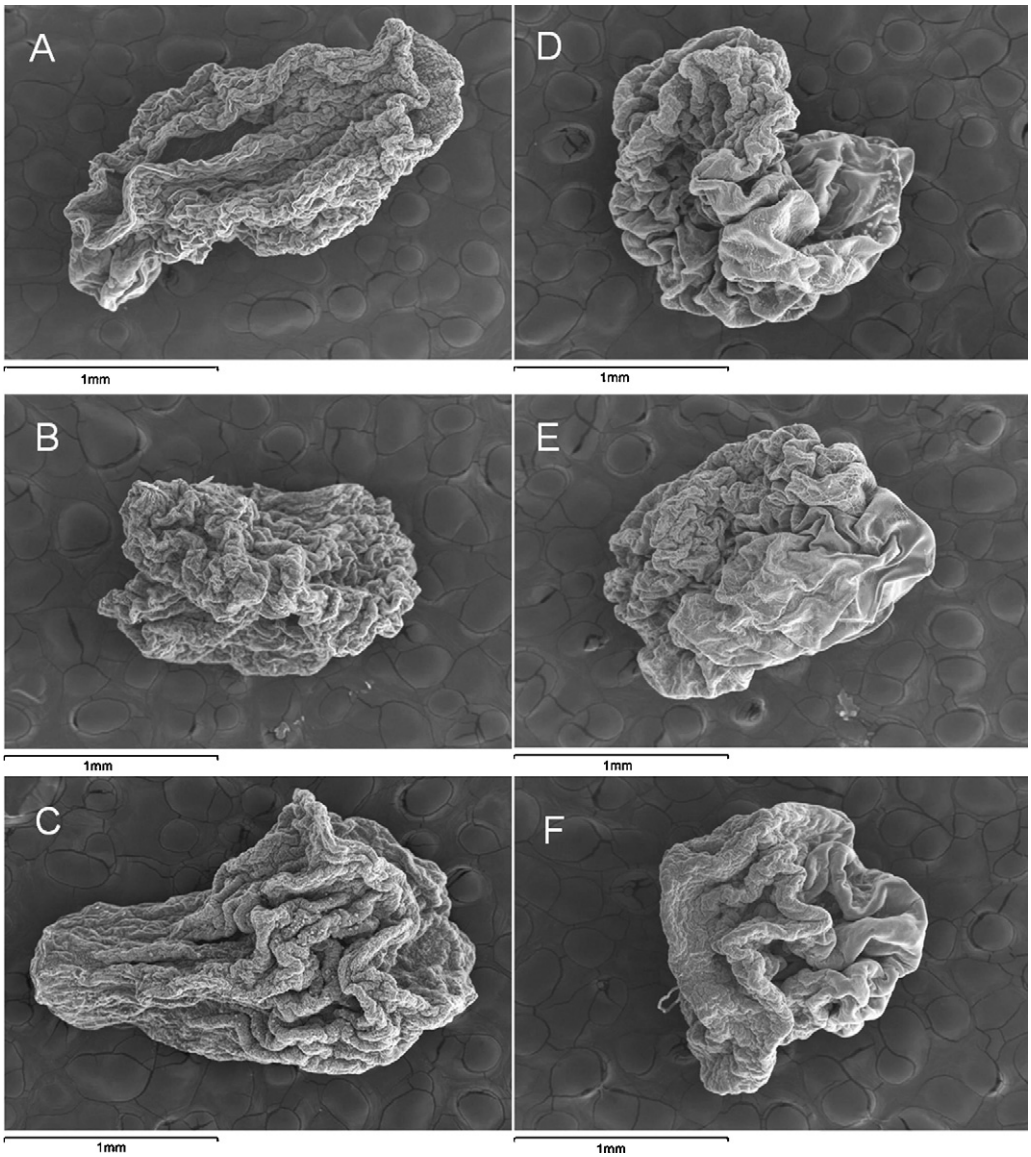

Fig. 3. SEM micrographs of freeze-dried capsules. In all samples alginate 2.5% was used. Calcium alginate capsules coated with chitosan (A) CS-1, (B) CS-2 and (C) CS-3. Alginate mixed chitosan capsules (D) CS-1, (F) CS-2 and (E) CS-3. Scale bar = 1 mm.

Table 3
Mechanical stability of the alginate–chitosan PEC capsules loaded with extract. Burst assay ($N = 100$).

Alginate (%)	Chitosan	Stability (%)[a]	Stability (%)[b]
1	CS1	100	100
2	CS1	50[c,d]	100
2.5	CS1	30[c,d]	100
1	CS2	100	100
2	CS2	100	100
2.5	CS2	100	100
1	CS3	10[c,d]	100
2	CS3	7[c,d]	100
2.5	CS3	0[c,d]	100
1	CS4	100	100
2	CS4	100	100
2.5	CS4	100	100

[a] Calcium–alginate coated capsules.
[b] Alginate–chitosan mixed capsules.
[c] Differences due to the preparation method.
[d] Differences due to the chitosan Mw.

stability when the medium molecular weight chitosan was used. One thing to notice is the decrease in stability as the alginate concentration increases. This effect is even more noticeable when the high molecular weight chitosan was used.

The measurement of the equilibrium water uptake is an indirect way to evaluate the mechanical stability of the capsules. The capsules tend to absorb water (free or bulk water) in order to fill the void regions of the polymer network until the equilibrium state. This phenomenon is provoked by the relaxation of the polymer network in the presence of osmotic pressure. The swelling occurs until the osmotic pressure equals the forces of the crosslinking bonds that maintain the structure of the polymer network stable. When these two forces are equal, no further water gain from the capsules is observed. The amount of water gained depends on the strength of the crosslinking forces. When these forces are higher less water is expected to be gained.

As can be seen in Table 4, a direct relationship between capsule stability and equilibrium water uptake values was found.

Capsules coated with the low molecular weight chitosan showed the highest equilibrium water uptake values (Table 4) which is in accordance with the high swelling of the calcium–alginate capsules

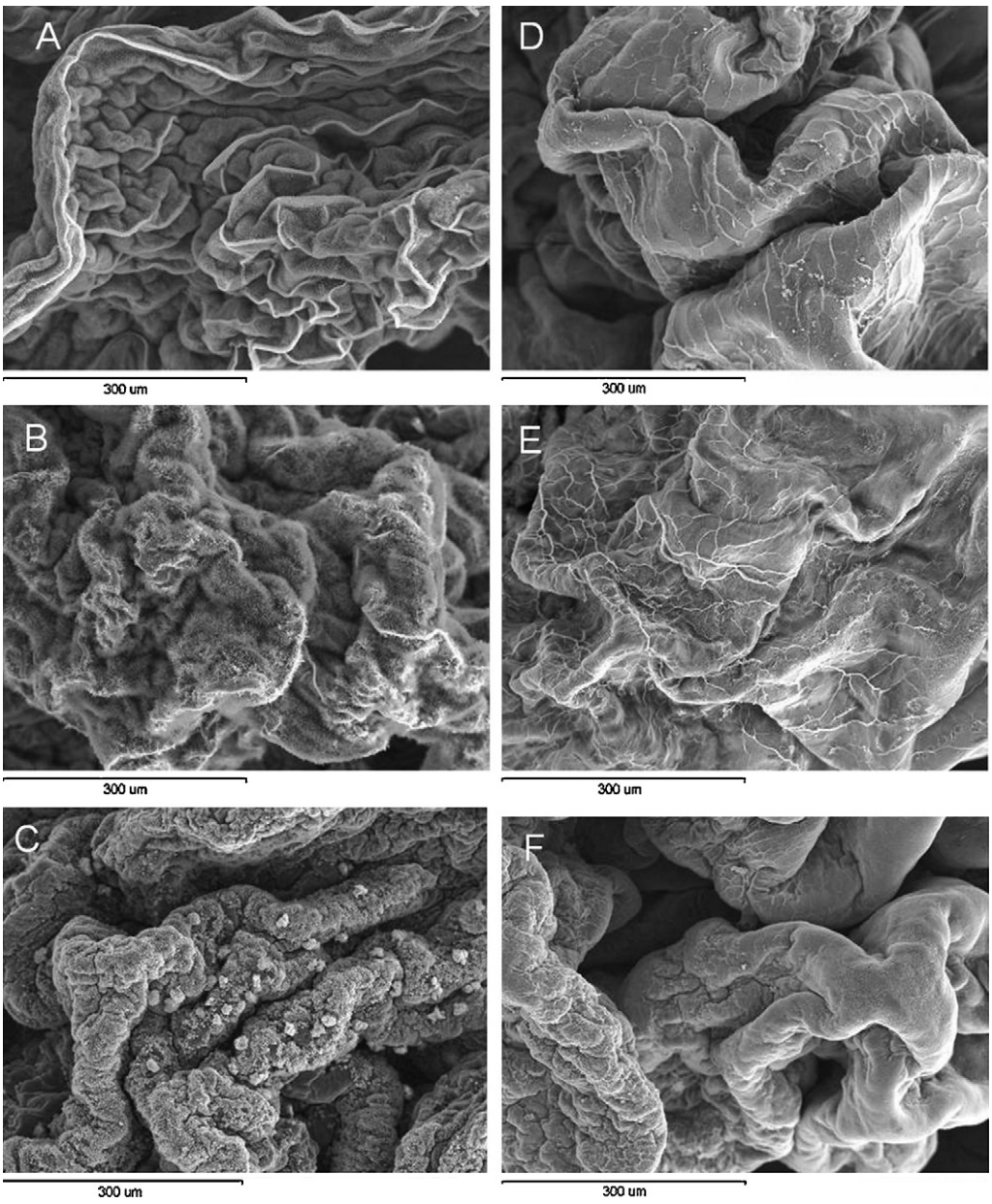

Fig. 4. SEM micrographs of freeze-dried capsules, detail of the surface. In all samples, alginate 2.5% was used. Calcium alginate capsules coated with chitosan (A) CS-1, (B) CS-2 and (C) CS-3. Alginate mixed chitosan capsules (D) CS-1, (F) CS-2 and (E) CS-3. Scale bar 300 μm.

coated with low molecular weight chitosan reported by McKnight et al. [39].

3.3. Encapsulation efficiency and release assays

The encapsulation efficiency was around 60% and was unaffected by the alginate concentration, chitosan properties or preparation method. In order to evaluate the potential reuse of the biocatalysts or their use in a long term biotechnological process the release of proteins from the capsules was studied. In all cases, the release from wet capsules over a 24 h period was lower than 5%. The freeze-dried capsules showed a release of about 10% at the zero-hour time point and no additional release was observed during the assays. Probably, during the freeze drying process the structure of some capsules was partially damaged and the proteins were released into the medium. This result is in good agreement with the ability of these systems to avoid protein release [29].

3.4. Enzymes activities and bioconversion reaction

The enzymatic activities as well as the catalytic capability of the biocatalysts to produce p-HPG from D,L-HPH were tested.

The results of the biocatalysts prepared by the two-step procedure (calcium alginate capsules coated with chitosan) are shown in Table 5. At low alginate concentration (1%, w/v), D-hydantoinase activity was almost unaffected by chitosan molecular weight or degree of deacetylation showing activity yields of around 70%. On the other hand, D-carbamoylase activity showed a maximum activity yield (90%) when the medium molecular weight chitosan was used and this result did not depended on the degree of chitosan deacetylation. Finally, p-HPG production increased when the chitosan molecular weight was lowered (58% vs. 73%). On the other hand, a decrease of 10% in the p-HPG production was observed when the chitosan with a low degree of deacetylation was used.

At medium alginate concentration (2%, w/v), D-hydantoinase activity increased as chitosan molecular weight increased (50% vs.

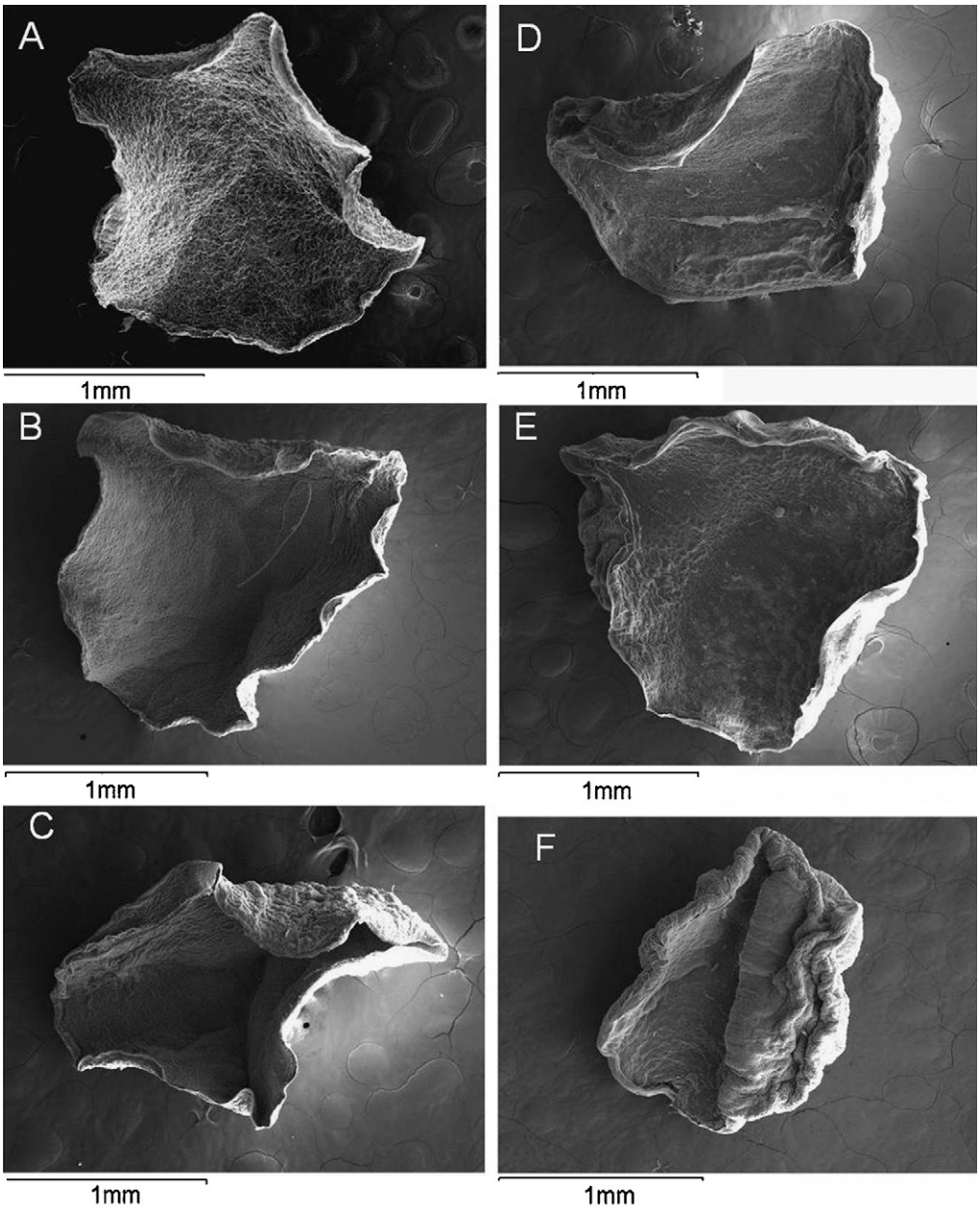

Fig. 5. SEM micrographs of freeze-dried capsules. Inner structure of the capsules. In all samples alginate 2.5% was used. Calcium alginate capsules coated with chitosan (A) CS-1, (B) CS-2 and (C) CS-3. Alginate mixed chitosan capsules (D) CS-1, (F) CS-2 and (E) CS-3. Scale bar = 1 mm.

65%). No effect attributable to the degree of chitosan deacetylation was observed. On the other hand, D-carbamoylase activity decreased as the chitosan molecular weight decreased (from 115 to 40%). D-Carbamoylase activity was also reduced by using the low deacetylated chitosan. The highest p-HPG production (70%) was achieved by using the chitosan sample with medium molecular weight and high degree of deacetylation (CS-2).

Finally, at the highest alginate concentration (2.5%, w/v), D-hydantoinase activity decreased as chitosan molecular weight and degree of deacetylation were reduced. The best biocatalyst (Chitosan CS-1) showed an activity yield of 77%. On the other hand, D-carbamoylase showed low activity yields (34–54%) in all cases. The highest p-HPG production (80%) was achieved by using the chitosan with the medium molecular weight and high degree of deacetylation. A remarkable reduction of p-HPG production was observed when the chitosan with the lower degree of deacetylation was used (from 80 to 40%).

The results of the biocatalysts prepared by the one-step procedure (alginate mixed chitosan capsules) are shown in Table 6. In general, D-hydantoinase activity of the extract encapsulated by the one-step procedure was similar to the one previously described for the biocatalyst encapsulated by using the two-step procedure. This finding did not depend on alginate concentration or chitosan molecular weight. However, a remarkable reduction of the activity was observed when the chitosan with a low degree of deacetylation was used (activity yields of around 25–30% were observed).

In contrast, D-carbamoylase activity showed a great dependence on the preparation method when the high and the medium molecular weight chitosans (CS-1 and CS-2) were used. At low and medium alginate concentration, D-carbamoylase had its maximum activity when the medium molecular weight chitosan was used (CS-2). These biocatalysts showed higher activity than the soluble extract (activity yields of around 120%). At high alginate concentration, D-carbamoylase activity decreases as the chitosan molecular

Table 4
Mechanical stability of the alginate–chitosan PEC capsules non-loaded with extract. Water uptake assay ($N = 10$).

Alginate (%)	Chitosan	Water uptake (g water/g polymer)[a]	Water uptake (g water/g polymer)[b]
1	CS1	37 ± 2[c]	27 ± 2
2	CS1	35 ± 2[c]	20 ± 1
2.5	CS1	29 ± 1[c]	19 ± 1
1	CS2	34 ± 1	31 ± 1
2	CS2	21 ± 2	19 ± 2
2.5	CS2	21 ± 1	18 ± 1
1	CS3	90 ± 5[c,d]	35 ± 3
2	CS3	60 ± 2[c,d]	35 ± 1[d]
2.5	CS3	56 ± 2[c,d]	25 ± 2[d]
1	CS4	33 ± 1	30 ± 1
2	CS4	20 ± 1	19 ± 2
2.5	CS4	19 ± 1	17 ± 2

Water uptake = $(W_{eq} - W_0)/W_0$ where W_{eq} and W_0 are the sample weights at equilibrium and in the dry state, respectively, of non-loaded capsules.
[a] Calcium–alginate coated capsules.
[b] Alginate–chitosan mixed capsules.
[c] Differences due to preparation method.
[d] Differences due to the chitosan Mw.

Table 5
Biocatalytic properties of the extract encapsulated in calcium–alginate capsules coated with chitosan ($N = 3$).

Alginate (%)	Chitosan	D-Hydantoinase activity (μmol/min g)	D-Carbamoylase activity (μmol/min g)	Activity ratio	p-HPG (%)
1	CS1	216 ± 11	8.7 ± 0.5	0.040	58.2 ± 3.2[a]
2	CS1	171 ± 7	18.5 ± 1.2	0.108[a]	44.3 ± 2.9[a]
2.5	CS1	262 ± 18	8.6 ± 0.4	0.033	69.3 ± 3.7
1	CS2	245 ± 19	14.3 ± 0.9[a]	0.058[a]	70.6 ± 3.9
2	CS2	222 ± 18	17.1 ± 1.3	0.077[a]	70.2 ± 3.6
2.5	CS2	239 ± 19	9.4 ± 1.0	0.039	79.8 ± 4.0
1	CS3	251 ± 19	9.5 ± 0.9	0.039	73.6 ± 3.6
2	CS3	211 ± 18	6.3 ± 0.5[a]	0.029[a]	61.7 ± 3.3
2.5	CS3	188 ± 14[a]	5.5 ± 0.5[a]	0.029	66.6 ± 3.4
1	CS4	245 ± 17	15.0 ± 0.8[a]	0.061	60.1 ± 3.3
2	CS4	239 ± 20	12.2 ± 1.0	0.051[b]	55.8 ± 3.4[b]
2.5	CS4	102 ± 10	7.9 ± 0.9	0.077[b]	39.2 ± 2.9[b]

Soluble D-hydantoinase activity = 342 ± 10 μmol/min g. Soluble D-carbamoylase activity = 16.1 ± 0.6 μmol/min g. Activity ratio D-carbamoylase/D-hydantoinase = 0.047.
p-HPG yield = (p-HPG formed from L,D-HPH by the encapsulated extract (μmol/min g of encapsulated protein)/p-HPG formed from L,D-HPH by the soluble extract (μmol/min g of soluble protein) × 100.
[a] Differences due to the chitosan Mw.
[b] Differences due to the chitosan degree of deacetylation.

weight decreases. The best biocatalyst showed an activity yield of 130%. Interestingly, D-carbamoylase activity was not as negatively affected by the low degree of deacetylation of the chitosan as D-hydantoinase activity was.

Finally, in general, p-HPG production was also similar to the one described previously for the one-step procedure biocatalyst when samples prepared by using chitosan CS1, CS2 or CS3 were compared. On the other hand, a reduction of the production was observed when the chitosan with a low degree of deacetylation was used (chitosan CS-4).

4. Discussion

In a previous paper, a crude cell extract of *Agrobacterium rb* was encapsulated into calcium–alginate capsules. In order to improve the properties of the biocatalyst, the crude cell extract has been encapsulated in alginate–chitosan polyelectrolytes prepared by two different methods.

As can be seen from the ESEM and SEM micrographs (Figs. 1–3) the preparation method affects the structure and surface of the capsules. The collapse of the capsules after the freeze-drying process indicates that in both cases a heterogeneous distribution of the polymers through the capsules was achieved. That means that the concentration of the polymers inside the capsule is lower than on the border and thus the capsules collapse during their dehydration [40]. In fact, a liquid core surrounded by a thin membrane polymer was observed (Fig. 4). The density of the polymer network throughout the capsules can affect the enzymatic activities by increasing the diffusion rates of the substrates through the polymer membrane. Moreover, the enzymes can be encapsulated in two different ways in the same capsule. Enzymes can be encapsulated within the liquid core and they can also be entrapped in the structure of the membrane composed of a dense polyelectrolyte complex network. The enzymes inside the liquid core can move and rotate as freely as an enzyme in solution resulting in much easier accessibility and contact between enzyme and substrate than those included inside the polymer network. Moreover, the presence of an inhomogeneous gel core has also been related to a lower protein release [41]. Therefore, the morphology of the alginate–chitosan PECs seems very appropriate for their application as a matrix for the encapsulation of enzymes.

The behaviour of the capsules during the burst assay as well as their water uptake was also affected by the method of preparation as well as by the chitosan characteristics and alginate concentration.

The first point to be noticed is that alginate–chitosan PEC capsules showed higher water uptake values than those previously reported for the same extract encapsulated in calcium–alginate capsules [25]. Alginate–polycation membranes present a lower elasticity than the calcium alginate ones and therefore, alginate–polycation membranes render more swellable

Table 6
Biocatalytic properties of the extract encapsulated in alginate-mixed capsules ($N = 3$).

Alginate (%)	Chitosan	D-Hydantoinase activity (μmol/min g)	D-Carbamoylase activity (μmol/min g)	Activity ratio	p-HPG (%)
1	CS1	234 ± 13	10.2 ± 0.7	0.043	64.4 ± 5.3
2	CS1	239 ± 10[a]	15.7 ± 0.9	0.066[a]	71.3 ± 4.7
2.5	CS1	250 ± 9	20.5 ± 1.3[b,a]	0.082[b,a]	75.7 ± 4.8
1	CS2	273 ± 10[b]	19.2 ± 0.5[b,a]	0.070[b,a]	78.0 ± 5.0
2	CS2	268 ± 31	18.1 ± 0.9	0.067	74.4 ± 5.1
2.5	CS2	222 ± 15	6.3 ± 0.6[a]	0.028[a]	63.3 ± 4.7
1	CS3	234 ± 15	10.2 ± 0.9	0.043	76.1 ± 4.4
2	CS3	194 ± 16[b]	7.9 ± 0.7[b]	0.040[b,a]	66.7 ± 4.8
2.5	CS3	176 ± 11	5.5 ± 0.7	0.031	63.4 ± 4.8
1	CS4	86 ± 11[c]	13.3 ± 0.9[c]	0.154[c,a]	30.0 ± 4.2[c,a]
2	CS4	114 ± 9[c]	11.8 ± 0.9[c]	0.103[c,a]	34.0 ± 3.9[c,a]
2.5	CS4	102 ± 10[c,a]	9.5 ± 0.8[c]	0.093[c,a]	29.7 ± 4.0[c,a]

Soluble D-hydantoinase activity = 342 ± 10 μmol/min g. Soluble D-carbamoylase activity = 16.1 ± 0.6 μmol/min g. Activity ratio D-carbamoylase/D-hydantoinase = 0.047.
p-HPG yield = (p-HPG formed from L,D-HPH by the encapsulated extract (μmol/min g of encapsulated protein)/p-HPG formed from L,D-HPH by the soluble extract (μmol/min g of soluble protein) × 100.

[a] Differences due to preparation method.
[b] Differences due to the chitosan Mw.
[c] Differences due to the chitosan degree of deacetylation.

membranes [42]. When comparing both preparation methods, capsules prepared by the one-step procedure showed a lower water uptake and more stability in the burst assay than those prepared by the two-step procedure.

The strength and stability of the interpolymeric complexes depend on several parameters such as chemical composition, sequential structure, conformation, molecular weight of the alginate and the polycation and method of preparation [42].

For the two-step procedure, the binding of chitosan to the calcium alginate capsules depended on the molecular weight of the chitosan. According to their molecular weight, chitosans can diffuse throughout the alginate network. Low molecular weight chitosan diffuses freely into the alginate network rendering a homogeneous distribution of chitosan. As the molecular weight of chitosan increases, this process becomes more difficult or is even hindered if the pores of the alginate network are smaller than the size of the chitosan [41]. Capsules prepared with chitosan of high molecular weight showed a different behaviour with regard to the alginate concentration. As the alginate concentration increases the capsules can link to more chitosan due to the larger contact surface but, at the same time, the polymer network is more compact and thus the diffusion of chitosan throughout the alginate network is more difficult. Thus, chitosan is piled up onto the capsule surface rendering a less flexible membrane which is more liable to burst. The effect of the degree of deacetylation on the formation of the polymer complexes is not clear. Gaserod et al. have suggested that the increase of the acetylation of chitosan affects the conformation of the polymer chain rendering a more extended one with a lower diffusion coefficient [31]. However, Berth et al. did not observe this modification in the conformation of the polymer [43,44]. It has also been suggested that the decrease in the degree of acetylation of the chitosan reduces the charge density of the polymer and therefore the electrostatic interactions between the alginate and the chitosan are reduced [44].

In the case of the one-step procedure capsules, when dropping a solution of alginate into a chitosan solution, the amount of binding chitosan is lower than for those produced using the two-step procedure. In these capsules, the gelling reaction of alginate due to the presence of calcium ions in the chitosan solution competes with the precipitation reaction between alginate and chitosan. Due to the low chitosan concentration used in this study (0.15%) and the lower size of the calcium ions with regard to the chitosan chains, these capsules showed a very similar structure and behaviour to that of the calcium–alginate capsules.

When the crude cell extract was previously encapsulated in calcium–alginate capsules, no effect of the alginate concentration on the D-hydantoinase activity and pHPG production was observed, while an increase of the D-carbamoylase activity was observed as the alginate concentration increased [25]. However, in the present study, it was not possible to find a direct relationship between the method of preparation, the alginate concentration or the chitosan characteristics.

The first point to be noted is that D-hydantoinase activity and p-HPG production were almost unaffected by the method of preparation while D-carbamoylase activities were clearly affected by this parameter.

Chitosan degree of deacetylation neither affected capsule morphology nor mechanical stability, but differences in the enzymatic activities were observed. In general, lower activities were observed when the lowest DD chitosan was used. Since the reduction of the activity was more remarkable when the biocatalysts were prepared by the one-step procedure, it can be ascribed to the lower pH of the chitosan solution (5.6 vs. 6.2 since CS-4 chitosan precipitates at pH higher than 5.6). In the two-step procedure the enzymes are protected inside the calcium–alginate capsule, while in the one-step procedure, the capsule is formed in the chitosan solution.

With regard to the molecular weight of chitosan a very complex behaviour was observed. A point to keep in mind is that the amount of chitosan and its distribution along the alginate–chitosan capsule affects not only the porosity of the membranes but also their net charge. In these conditions a wide range of possibilities is opened. Depending on the membrane charge, partition effects of the substrate and products may occur. Moreover, the enzymes of the extract may interact with the membrane thus affecting the enzymatic activities. Even if interaction between the enzymes and the polymer membrane does not occur, the environment inside the capsule may change from one biocatalyst to another. Therefore, the enzymatic activities will depend on the sum of several effects: steric hindrance, diffusion restrictions and electrostatic interactions between the polymers and the enzymes.

An increase of D-carbamoylase activity (7-fold increase) has been reported after the encapsulation in calcium–alginate capsules of a crude cell extract from *A. tumefaciens* using low viscosity alginate [45]. However, our previous work on the encapsulation of the *Agrobacterium rb* extract using medium viscosity alginate did not report such increase in activity [25]. In the present work, some biocatalysts showed an increase of D-carbamoylase activity (around 1.3 times) compared to the soluble one. This enhanced activity can

be ascribed to several effects. First, a partial purification of the crude cell extract occurs during the encapsulation process, since low molecular weight proteins are released from the capsules due to their low size. Second, the highly hydrophilic and ionic environment inside the capsule reduces the presence of oxygen in the D-carbamoylase surroundings [19]. Finally, the ionic residues of the enzyme may establish interactions with the carboxylic groups of alginate as well as with the amine groups of the chitosan altering the protein conformation.

In general, we observed an increase in the activity ratio between D-carbamoylase and D-hydantoinase activity. Taking into account that the limiting step in the conversion of D,L-hydantoins into D-aminoacids is the second step [46], the increase of D-carbamoylase activity is of great interest in improving the enzymatic process.

Taking into account the stability of the biocatalysts, their ability to produce p-HPG and the activity ratio between D-activity and D-hydantoinase activity, one biocatalyst of each preparation method was selected as the most appropriate one. In both cases, chitosan of medium molecular weight and a degree of deacetylation of 0.9 was chosen as the most appropriate for preparation of the biocatalyst. The alginate concentration was fixed at 1 and 2% for alginate mixed chitosan capsules and calcium alginate coated capsules, respectively.Concluding remarks

Application of modern encapsulation techniques using polyelectrolytes complex-based (PECs) capsules may lead to a successful immobilisation of all available forms of biocatalysts such as enzymes, membrane fractions, particular systems as well as permeabilised and live cells.

In this paper, alginate–chitosan PEC's have been used for the first time as a suitable matrix for coimmobilisation of enzymes. In the best conditions, p-HPG production up to 80% was achieved. The biocatalysts showed a very complex behaviour and this study pointed to the key role of the chitosan characteristics in the properties of the biocatalyst.

Interestingly, these biocatalysts showed high D-carbamoylase activities, so these systems seem to be very appropriate when enzymes with poor stability are used.

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