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Covalent Immobilization of a Yeast Endopolygalacturonase in Calcium Alginate

Inmovilización covalente de una endopoligalacturonasa de levadura en alginato de calcio

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Resumen

En este trabajo se estudió la inmovilización covalente de una endopoligalacturonasa (endo-PG) de levadura en alginato de calcio. El soporte se activó por dos vías: mediante la adición de glutaraldehído (GA) y la oxidación con peryodato (PO), respectivamente. La activación con GA incorporó al soporte 5,8 mol de grupos carbonilo/mol de residuo de ácido urónico, mientras que en la reacción de PO se oxidó el 0,13 % de los glicoles vecinales. Los rendimientos de unión covalente y la estabilidad del gel disminuyeron con el incremento de la razón enzima/soporte; los mejores resultados correspondieron al alginato activado con GA. La pérdida de actividad enzimática durante la inmovilización fue > 90 %, lo cual es característico de la unión covalente. En todas las variantes se obtuvo una alta actividad enzimática inmovilizada, de unas 200 U/g. Se concluyó que la activación del alginato con GA y PO constituyen alternativas satisfactorias para la inmovilización covalente de la endo-PG en este soporte.

Palabras clave: endopoligalacturonasa, alginato, inmovilización de enzimas, peryodato, glutaraldehído.

Abstract

In this work, the covalent immobilization of a yeast endopolygalacturonase (endo-PG) in calcium alginate was examined. The carrier was activated by two different ways: glutaraldehyde addition (GA) and periodate oxidation (PO), respectively. During GA activation 5,8 mol of carbonyl groups/ mol of uronic acid residue were incorporated on the support whereas in the PO reaction only 0,13 % of vicinal glycols were oxidized. Covalent union yield and gel stability diminished when enzyme/support ratio was increased, corresponding the best results to the GA-activated alginate. The lost of enzymatic activity during immobilization was > 90 %, which is characteristic of covalent union. In all variants a high immobilized enzymatic activity was reached, around 200 U/g. It was concluded that the alginate activation, either with GA or PO, are feasible alternatives for the covalent immobilization of endo-PG on this support.

Keywords: endopolygalacturonase, alginate, enzyme immobilization, periodate, glutaraldehyde.

Introduction

The use of enzymes to industrial scale often demands its immobilization, with the aim to increase its stability and to facilitate its reuse, as important issues for the development of an economically feasible process. The covalent immobilization constitutes the most attractive option to assure a bigger useful life of the immobilized biocatalyst.

The election of suitable carrier is a relevant issue which must be considered when an enzyme is immobilized. Alginate, a polysaccharide extracted from brown seaweeds, has been commonly used by these purposes, due to its low cost, low toxicity, biocompatibility /1, 2/ and its ability to form beads by ionotropic gelation /3/ in the presence of bivalent or tervalent cations such as Ca^{2+} or Al^{3+}, which ionically cross-link carboxylate groups in the uronate blocks of

alginate, giving it a gel-like character. However, almost all studies on enzyme immobilization using alginate as support have employed the entrapment method, which is inappropriate when enzyme substrates are high molecular weight polymers /4/, as occur with those ones of PGs. Other problem concerning to the entrapment is the tendency for the entrapped proteins to leach out of gel over a period of time, as mentioned by several authors /5, 6/. Immobilization by covalent linkage on alginate appears to be a good alternative to overcome these problems.

PGs are broadly used enzymes in the industry of the juice fruits and vegetable processing. Endo-PGs form an industrially important subgroup, because they promote a rapid decrease in the pectin molecular weight and hence in the solution viscosity, with minimum release of reducing sugars. These enzymes have recently gained a special interest concerning its applications in obtaining of pectin fragments with biological activity, which have found several applications in agriculture, plant biotechnology, pharmaceuticals and functional foods /7-9/.

Taken in account the practical relevance of endo-PGs, polymeric nature of its substrates and physical-chemical properties of alginate, this work was aimed at the study of the covalent immobilization of a yeast endo-PG on calcium alginate, employing either the bifunctional reagent glutaraldehyde (GA) or the (meta)periodate oxidation (PO) as ways for support activation.

• Materials and Methods

Enzyme

It was used the endo-PG of the *Kluyveromyces marxianus* CCEBI 2011 yeast strain, which was prepared according to Serrat *et al*. /10/, but omitting the step of ionic exchange chromatography.

Carrier preparation and activation

Calcium alginate gel was obtained by ionotropic gelation with calcium ions /11/ from 0.5-2 % (w/v) sodium alginate (from *Laminaria hiperborea*; BDH) solutions. Activation with GA was performed in acid medium according to Spagna *et al*. /12/. In brief, one hundred alginate beads (ca. 0,8 g) are added into 15 mL of 50 mM citrate buffer; pH 3,5; containing 2 % (w/v) GA (Fluka). Then, the mixture was shaken (160 rev/min) during 1,5 h at 25 ºC.

Alginate oxidation with periodate (Sigma) was conducted using 1:5 and 1:25 oxidant/uronic acid residues molar ratios. These relations were estimated from reaction stoichiometry (figure 1). The reaction was carried out in the dark at 25 ºC, during 60 minutes. The reaction was stopped by the addition of an equimolar amount of propylene glycol. The degree of oxidation was evaluated by determining the aldehyde groups using the Somogyi-Nelson method /13, 14/, and it was expressed as percent of vicinal glycols oxidized. A GA solution, which was previously standardized by the bisulfite method /15/, was used as standard.

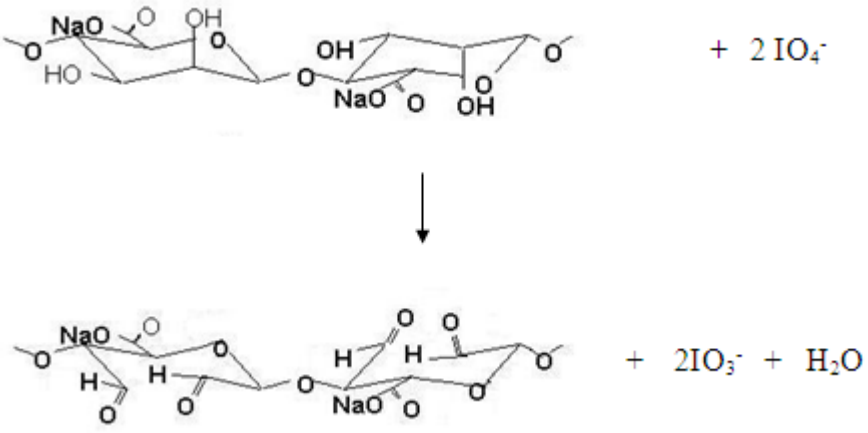

Fig. 1 Chemical equation of oxidation-reduction reaction between sodium alginate and periodate.

Enzyme immobilization

The reaction was performed in 50 mM acetate buffer (pH 5) and 10 mM phosphate buffer (pH 7) for GA-activated and PO-activated carriers, respectively. In any case the reaction mixtures were maintained in agitation (60 rev/min) during 16 h at 25 ºC. Enzyme/ support ratios of 10 U/mg and 70 U/mg were used. The covalent union yield (Y_{CU}) was defined as the enzyme fraction which has been linked on the support as regards the total introduced in the assay, whereas the yield of active immobilized enzyme (Y_{AI}) was calculated as the fraction of active enzyme as regards the total linked on the support.

Enzyme activity determination

The PG enzymatic activity was determined using 0,5 % (m/v) polygalacturonic acid (Sigma) in 50 mM acetate buffer as substrate. The assay was conducted by addition of 100 uL of enzyme (five beads by immobilized biocatalyst) to 400 uL of substrate, both previously incubated at 37 ºC, followed of incubating the reaction mixture at this temperature during 10 minutes. The reaction was stopped by addition of alkaline cupper reagent (Somogyi-Nelson assay for reducing sugars). One unit of PG activity was defined as the amount of enzyme that produces an increasing in the reducing power of the reaction mixture of one micromole by minute. The reducing power was quantified according Somogyi-Nelson method /13, 14/, using galacturonic acid (Sigma) as standard.

Determination of the enzyme accessibility to the carrier

This assay was conducted by means of molecular exclusion chromatography. For this, a glass column (20 · 2 cm) was carefully packed with calcium alginate beads and equilibrated with five volumes of 50 mM acetate buffer (pH 5) containing 100 mM NaCl, to prevent non-specific protein adsorption. Then, 1 mL of enzyme (ca. 500 µg) was applicated onto column and eluted at the same buffer with a flow of 1,2 mL/h. 1 mL-fractions were collected and the protein content was determined according Lowry method /16/, using albumin from bovine serum (BDH) as standard, as well as polygalacturonase activity. Dextran blue and phenol red were used as standards for column calibration.

• Results and Discussion

Carrier preparation and activation

Table 1 show the main characteristic of alginate gels obtained for different sodium alginate concentrations. Calcium alginate gel obtained from 2 % (w/v) sodium alginate solution, by means of ionotropic gelation in presence of Ca^{2+} ions, consisted in uniform spherical and translucent beads of 3,23 ± 0,17 mm of diameter and 8,02 mg of average weight.

TABLE 1. MAJOR CHARACTERISTICS OF THE CALCIUM ALGINATE BEADS OBTAINED FOR DIFFERENT POLYSACCHARIDE CONCENTRATIONS

Gel concentration [% (w/v)]	Geometric shape/ texture	Unitary weight [a] (mg)	Bulk density ($g \cdot mL^{-1}$)	Diameter [b] (mm)
2	Spherical/hard	8,02	1,045 6	3,23 ± 0,17
1	Ovoid/hard	4,87	1,022 7	2,60 ± 0,25
0,5	Irregular/soft	1,74	1,038 0	ND

[a]Average from 50 units; [b]Average ± standard deviation; ND: non determined.

Alginate activation with bifunctional agent GA was performed in acid medium to favour nucleophilic addition, which yielded an incorporation of carbonyl groups on support of 5,8 moles by mole of uronic acid residue. This result is considerably higher than the theoretical expected value, which predict that to each mole of uronic acid residue correspond two moles of GA linked (one by each hydroxyl group). Various factors could be affecting the predicted result, such as GA polymerization /12/ or the existence of non-specific adsorption, being the later favoured by the formation of hydrogen bonds between carrier and GA.

To assess the alginate oxidation by periodate, three assays were carried out. At the first experience, a 1:5 molar ratio periodate/uronic acid residues was used. The Fehling´s qualitative test for aldehydes was used to evaluate the extension of the oxidation. This test resulted negative for native alginate, whereas a big positive response (abundant red precipitated of cuprous oxide) was observed in oxidized alginate, which suggested that the oxidation of vicinal glycols occurred at a large extension.

However, the gel obtained in this case showed a fine fibrous structure, very different of the characteristic spherical beads, which could be caused due to depolymerization occurred in presence of oxidant agent /17, 18/. By other hand, the oxidative cleavage of C2-C3 bond of the pyranosic ring by periodate lead to a increasing in polymer chain flexibility, which could prevent the adequate formation of the egg-box structures presents in the calcium alginate gels /3/.

The PO was repeated but employing a lower periodate/uronic residues molar ratio (1:25). Under these conditions a degree of oxidation of 0,13 % was reached, which was considered satisfactory. The beads formed from the oxidized carrier in presence of calcium ions had an ellipsoidal form and showed a lower mechanical resistance with respect to that obtained from native alginate.

Finally, the PO reaction was conducted directly on calcium alginate gel, using a 1:25 /uronic residues molar ratio. The degree of oxidation was similar to that reached for soluble alginate, but with the advantage that under these new conditions the beads conserved its common spherical form and hardness.

Enzyme immobilization

Catalytic activity of endo-PG on sodium alginate was evaluated previous to develop the immobilization assays, taken in account that structural units of alginate, b-D-manuronic and a-L-guluronic acids, are stereoisomers of the galacturonic acid, which is the constitutive unit of polygalacturonate, natural substrate of PGs. Once known that *K. marxianus* endo-PG was inactive on alginate, then the accessibility of the enzyme into the alginate beads was determined, using a gel filtration assay. The results are shown in the figure 2.

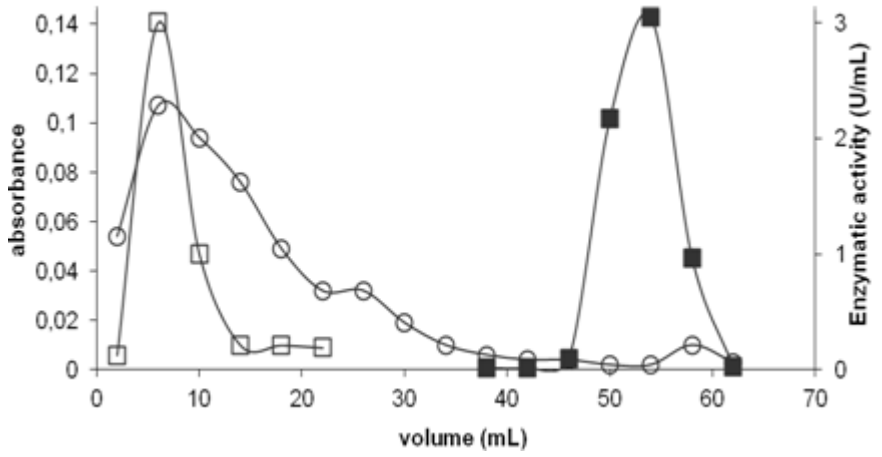

Fig. 2 Elution profiles of blue dextran (□), phenol red ( ) and enzyme (O) when are passed through packed column with calcium alginate bed 2 % (w/v).

The elution volume of enzyme was near to the exclusion volume, suggesting that the enzyme accessibility into the gel is low (ca. 15 % of the inner volume). This result is in agreement with Pedroza-Islas /19/ (2002), who have referred that calcium alginate gels are not permeable to molecules which molecular weight is over 10 000 Da. Thus, it is assured that the immobilized enzyme will be, essentiality, restricted to the external surface of the gel, which is convenient due to the endo-PG substrate is unable to enter into the gel as consequence of its high molecular weight.

A summary of the results obtained during immobilization of *K. marxianus* endo-PG on calcium alginate activated with GA and PO are shown in the table 2. As can be observed, Y_{CU} diminished when enzyme/support ratio was increased, which could be caused by steric hindrances as result of an high concentration of support-linked enzyme molecules. When enzyme/support ratio was 70 U/mg the alginate gel was turned unstable. This fact could be caused as consequence of an ionic exchange between positively charged enzyme molecules and calcium ions, which could cause distortion in the egg-box gel structure. This problem was satisfactorily solved performing the immobilization in presence of calcium ions at 50 mM of concentration, which confirmed the hypothesis earlier exposed. Undoubtedly, enzyme/support ratio constitutes an important variable to be considered in next studies conducted to the optimization of this procedure of immobilization.

TABLE 2. SUMMARY OF ENDO-PG IMMOBILIZATION ON CALCIUM ALGINATE ACTIVATED WITH GA AND PO

E/S, enzyme/support ratio; Y_{CU}, yield of covalent union; Y_{AI}, yield of active immobilized enzyme; EA_{inm}, immobilized enzymatic activity PO-pre/post, periodate oxidation pre-/post- gelation.

PO-activated alginate showed lower values of Y_{CU}, in agreement with its lower availability of actives groups (carbonyl) for enzyme coupling. The variant where alginate oxidation was carried out before gel formation showed the lowest Y_{CU} value. This fact suggest that an considerable proportion of carbonyl groups stayed arranged inside the gel, being thus unavailable for the reaction with amino groups of enzyme.

The lost of enzymatic activity during immobilization was considerable in all variants (Y_{AI} < 10 %), which is characteristic of covalent union. This is caused by several factors, such as conformational changes in enzyme, conformational or chemical changes in the active site, steric hindrances, and other ones /4/. The variant where the enzyme/support ratio was 70 U/mg and GA-activated alginate was used as carrier, Y_{AI} was barely of the 1 %. If is taken in account that density of enzyme molecules by surface unit is the highest in this variant, then could be expected a higher affectation to the enzymatic activity as consequence

of steric hindrances and conformational changes in enzyme.

All variants showed a high immobilized enzymatic activity, around 200 U/g, being reached the best value (400 U/g) in the variant where alginate was activated by PO after gel formation. The activity of immobilized biocatalyst can be considered satisfactory compared with other results, such as that obtained by Spagna *et al.* /12/ in covalent immobilization of a pectin lyase.

Conclusions

This study have shown that alginate activation with GA or PO are feasible alternatives to covalent immobilization of the K. marxianus endo-PG on this carrier, corresponding the best results to PO-activation, where enzymatic activity in immobilized biocatalyst is twice the obtained in other variants.

Several parameters, such as the enzyme/ support ratio and availability of active carbonyl groups onto support are issues that should be optimized in next studies. These results are an important first step in the development of enzymatic bioreactors based in K. marxianus endo-PG covalently immobilized, which could offer a wide spectrum of applications in food and agrochemical industries.

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