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Properties of Pectinesterase and Endo-D-Polygalacturonase Coimmobilized in a Porous Glass Support

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ABSTRACT

Derivatives of pectinesterase and polygalacturonase, both individually immobilized and coimmobilized, were obtained and characterized. Homologous soluble systems were also studied to establish differences between the effect of the immobilization process and the presence of the other enzyme. Immobilization or coimmobilization did not change the optima pH or temperature for the enzymes. However, optimum ionic strength was displaced toward higher values for immobilized pectinesterase, while for polygalacturonase immobilization resulted in a wider range for activity. K_m value remained nearly unchanged for pectinesterase, and decreased for polygalacturonase. The V_{max} value decreased with the immobilization process for the two enzymes, except for polygalacturonase immobilization in presence of pectinesterase. Soluble pectinesterase activity showed a competitive inhibition by polygalacturonic acid (K_i=0.44 mg/mL). Either immobilization or presence of polygalacturonase rendered the enzyme insensitive to the inhibitory effect. Thermal stability of pectinesterase was not improved after immobilization. On the contrary, the thermal stability of endo-D-polygalacturonase was improved slightly by presence of pectinesterase, and in a greater extent by immobilization. Individually immobilized and coimmobilized pectinesterase activities kept 90 and 60%, respectively, of their initial values after more than one year stored at 3–5°C. The two endo-D-polygalacturonase derivatives showed the same activity decay pattern along 10 mo storage at

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3–5°C. The two immobilized pectinesterase derivatives showed similar operational stabilities during continuous operation. The presence of pectinesterase remarkably increased the operational stability of the immobilized endo-D-poly galacturonase.

Index Entries: Pectic enzymes; pectinesterase; endo-D-polygalacturonase; pectin degradation; bienzyme coimmobilized systems.

INTRODUCTION

Pectinesterase (PE) is a demethoxylating pectic activity that liberates pectic acid. This latter product can be further depolymerized by endo-D-polygalacturonase (PG), causing a viscosity decrease of pectin solutions (*1,2*). Immobilization of these enzymes presents several advantages based upon their use in continuous juice clarification processes (*3–6*). A PE plus PG coimmobilized system has been stated to be a more effective system than that consisting of separately immobilized enzymes (*7–10*). Besides the effect of PE on PG in reducing the viscosity of pectin solutions (*2*), an activity-enhancing effect of the presence of the latter enzyme over PE activity in a coimmobilized system was observed (*10*).

In previous papers, the coimmobilization process was optimized with respect to some parameters that affect the activity of the enzymes involved (*9*), and the efficiency of this bienzyme system over the one that uses separately immobilized enzymes checked (*10*). Recently, optimum reaction conditions for the coimmobilized PE system were studied in our laboratory, and the results compared to those obtained for the soluble individual enzyme (*11*). However, the behavior of each one of the enzymes could change both owing to the presence of the other pectic enzyme and the fact of being immobilized. So, it is interesting to carry out a comparative study of the behavior of each one of the enzymes activities both when they are single and when they are together, and both in the soluble and immobilized forms. Thus, three soluble systems: soluble PE (SPE), soluble PG (SPG), and soluble PE + PG (CoSE in general, CoSPE or CoSPG when talking specifically about cosoluble PE or PG activities, respectively), and three immobilized (IMPE, IMPG, and CoIME/CoIMPE/CoIMPG) were studied.

It is worth mentioning the theoretical basis of these kinds of studies, where on one side PE cannot hydrolyze any methylester bond along the PE substrate (polygalacturonic acid, PGA), and on the other side PG can hydrolyze neither the glycosidic linkages of the PE substrate (highly methoxylated pectin, HMP), nor the glycosidic linkages of the PE reaction product until the esterification degree of HMP is reduced by PE to a value lower than 45%. Therefore, PE behaves as an inert protein for PG (when using PGA as substrate), and the same is true for this latter on PE during the initial stages of the reaction (when using HMP as substrate).

MATERIALS AND METHODS

Materials

PE from tomato (EC 3.1.1.11, mol wt 23,000, 650 U/g), PG from *Rhizopus sp.* (EC 3.2.1.15, mol wt 22,600, 455 U/g), *p*-phenylenediamine, sodium borohydride, and polygalacturonic acid (PGA) were all purchased from Sigma Chem. Co. (St. Louis, MO). Glycophase G™ controlled-pore glass was from Pierce (Rockford,IL; 200–400 mesh, 46 nm nominal pore size). Synthetic highly-methoxylated pectin (HMP, mol wt 34,000; degree of esterification, DE, 94%) was a gift from R. Kohn, Bratislave. Propanol, chromatographic grade, and the remaining reagents were from Merck, Darmstadt, Germany, used without further purification.

Immobilization of Pectic Enzymes

The glass support was activated by periodic acid oxidation and derivatized to arylamine-glass by linking *p*-phenylenediamine. The enzymes (0.5 mg/mL of PE and/or 0.5 mg/mL of PG) were covalently bound after a nitrous acid activation step of the *p*-phenylenediamine-derivatized support. Details of the immobilization procedures have been previously published elsewhere (*9,11,12*).

Assay of Soluble and Immobilized Activities

PE activity was tested by continuous titrimetry (with 0.05*M* NaOH) of the appearing carboxylate groups by using HMP as substrate (*13*). To a quantity of 3 mL of 7 mg/mL substrate, dissolved in 0.05*M* NaCl solution, pH 7.5, and incubated at 40°C, either 50 μL of an enzyme solution (having 22.6 μg of PE, either single or with PG) or 100–120 μL of an homogeneous suspension of IMPE or CoIMPE (150 mg of glass support up to a volume of 2 mL in 0.05*M* NaCl) were added. One unit of PE activity was taken as the amount of enzyme that liberates 1 μmol of H^+/min at 40°C and pH 7.5. A linear response was obtained up to 55 or 90 μg of protein (in absence or not of PG, respectively) or up to 7.5 mg of immobilized derivatives (either IMPE or CoIMPE).

To follow the operational stability of the PE derivatives, chromatographic determination of methanol was used. IMPE or CoIMPE derivatives were packed in a column (1 cm internal diameter and 2 cm bed-height), thermostated at 40°C, and fed with 7 mg/mL HMP dissolved in 0.05*M* NaCl adjusted to pH 7.5, at 1 mL/min flow rate. To 990 μL of the packed-bed reactor effluent a vol of 10 μL of propanol (used as internal standard) was added, and the mixture centrifuged at 2500*g* for 10 min. Aliquots (3 μL) from the supernatants were injected in a gas chromatograph (Konik KNK 2000, fitted with FID detector, a Hewlett-Packard 3380A integrator, and a Chromosorb 101 glass column, column, injector, and detector temperatures being 95, 150, and 200°C, respectively).

PG activity was measured by a modified Nelson-Somogyi method (*14*) using PGA as substrate. To a volume of 10 mL of 5 mg/mL substrate dissolved in 0.1*M* acetate buffer, pH 5.0, containing 0.05*M* NaCl and incubated at 40°C, either 100 μL of PG solution (0.56 mg/mL) or 100 μL of an homogeneous suspension of IMPG or CoIMPG (150 mg of glass support up to a vol of 2 mL in the same buffer) was added. Aliquots (1 mL) were usually withdrawn every 5 min, diluted with 150 μL of 2*N* HCl, centrifuged (2500*g* for 5 min) and, finally, the Nelson-Somogyi method applied to 0.5 mL of the supernatant. One unit of PG activity was taken as the amount of enzyme liberating 1 μmol of reducing groups/min at 40°C and pH 5.0. Assay linearities were attained in all the ranges used.

Appropriate blanks were used in all the activity determinations that were run under severe conditions (high temperature, extreme pH, or ionic strength values) to account for nonenzymatic degradation of the substrates. Operational stability of IMPG and CoIMPG derivatives was tested, the derivatives being packed in a column (1 cm internal diameter, 2 cm bed-height), thermostated at 40°C, and fed with 5 mg/mL of PGA dissolved in 0.1*M* acetate buffer, pH 5.0, containing 0.05*M* NaCl. Aliquots (1 mL) of the effluent were diluted with 150 μL of 2*N* HCl and the Nelson-Somogyi method applied to 0.5 mL of the resultant mixture.

Protein Determination

Immobilized and coimmobilized protein was determined by the difference between the amount added and that recovered after coupling and subsequent washings using a modified Lowry's method (*15*).

RESULTS AND DISCUSSION

Immobilization and Coimmobilization of Pectic Enzymes

Immobilization was performed onto a Glycophase-coated glass support whose pore size had been previously demonstrated to be the best for the expression of the two different activities involved (*9*). Three different kinds of derivatives were obtained: one with immobilized pectinesterase (IMPE), one with immobilized endo-D-polygalacturonase (IMPG), and another having coimmobilized pectinesterase and endo-D-polygalacturonase (CoIME in general, and CoIMPE or CoIMPG when talking specifically about the PE or PG activity, respectively, of the coimmobilized derivative). The coimmobilization process was identical to the individual immobilizations, except for the presence of both PE and PG enzymes. Table 1 summarizes the standard immobilization parameters. Activities were measured specifically for each one of the enzymes, under the same conditions for all the systems considered, according to *Materials and Methods*, and no overall clarifying activity (PE+PG) was measured.

Table 1
Standard Parameters of Immobilization

Derivative	Efficiency,[a] %	Immobilized protein,[b] mg/g	Specific activity,[c] U/mg	Derivative activity,[d] U/g
IMPE	12.5	78.3	13.0	237.6
IMPG	12.6	92.9	2.7	34.0
PE	26.2		21.7	253.4
CoIME		11.8		
PG	96.2		2.4	28.0

[a]Efficiency: Ratio of activity units of the derivative to activity units removed from the initial solution (%).
[b]Immobilized protein: Amount of immobilized protein (mg) per dry weight of support (g).
[c]Specific activity: Units of activity per immobilized protein (mg).
[d]Derivative activity: Units of activity per dry weight of support (g).

The presence of PG in the derivative increased the measured PE activity as it shows the increase in the PE immobilization efficiency (Table 1). As a consequence, the specific PE activity increased to a higher value than that obtained in the individual derivative, even though the amount of immobilized protein considered for specific activity calculations corresponds to the derivative protein content of PE+PG. Thus, PG acts as an activity enhancer for the PE activity, an effect that cannot be explained on the basis that the second enzyme removes the first enzyme reaction product, inhibiting the first enzyme, since PG cannot degrade pectins having an esterification degree higher than 45%, and additionally IMPE or CoIMPE are not inhibited by their reaction products (*10*). For PG, the amount of immobilized protein and the efficiency of the immobilization process were similar to both in presence and absence of the PE demethoxylating activity. However, the CoIMPG derivative activity decreased with respect to the individually immobilized system, because of the lower quantity of immobilized protein contained in it.

Characterization (reaction optimum conditions and stabilities) of these immobilized enzymatic activities was carried out in systems in which the same enzyme units (either PE or PG) were placed, and the results compared to those shown by soluble systems containing the same activities. Thus, the following soluble systems were assayed: soluble PE, soluble PG, and soluble PE+PG. These studies were intended to investigate what effects could modify the pectic enzymes activity: either the presence of the other enzyme or the immobilization process itself. In fact, for PG, PE seemed to behave as in inert protein, because of the low PG activity alteration. However, it is of importance to study the effect of the presence or absence of PE on some of the parameters from all the soluble and immobilized systems.

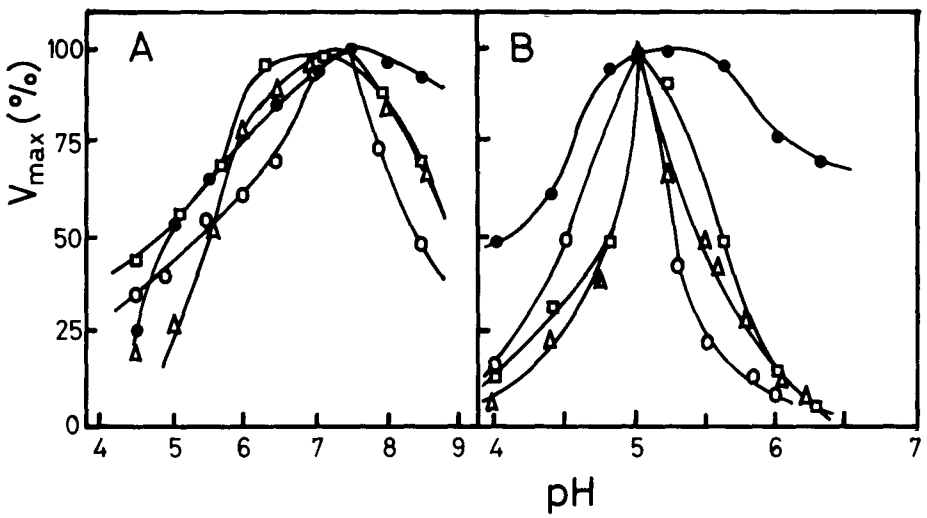

Fig. 1. pH-activity profiles for pectinesterase (A) and endo-D-polygalacturonase (B) in soluble (○), cosoluble (●), immobilized (△), and coimmobilized (□) forms.

pH Dependence of the Pectic Enzymes Activity

The behavior of PE and PG activities vs pH for all the systems (SPE, SPG, CoSE, IMPE, IMPG, and CoIME) was tested. The optimum pH for PE or PG activities did not change noticeably (Fig. 1). However, the curves for the CoSE systems were broader than those shown by any other system under study, even the immobilized ones. The lack of influence of the immobilization proces on the optima pH of the pectic enzymes seems to be a general rule (*16–25*). However, within the pH range for its optimum activity (6.0–7.5), PE activity increased in the following order: soluble < cosoluble < immobilized < coimmobilized. This result seems to emphasize an additive effect of the PG presence and the immobilization process on the PE activity.

Saline Effects on the Pectic Enzymes Activities

Because of the polyelectrolyte nature of the respective substrates, saline effects are important when measuring the activity of the pectic enzymes. For the PE activity, the IMPE and CoIME derivatives showed their maximum activities at values of NaCl concentrations higher than those for the soluble systems (0.1*M* vs 0.05*M*, respectively), and immobilization allowed the enzymes to retain more activity at high NaCl concentrations (0.2–0.3*M*). The results were coincident with those shown when KCl or sodium acetate were used as the saline media, thus pointing to a single phenomenon of ionic strength that has also been reported elsewhere (*17,26,27*).

Table 2
Optimum Ionic Strength Buffer
Plus NaCl Concentration for PG

System	I $M \times C^2$
Soluble	0.150
Cosoluble	0.150
Immobilized	0.125
Coimmobilized	0.175

When testing the PG activity, the immobilized derivatives showed a broader range of NaCl concentrations than the solubles ones for which activity could be measured. However, the results did not point out to a reported PG activation (*18*). Immobilization both increased the activity and broadened the activity vs NaCl concentration curve for PG, and these effects were enhanced in presence of PE. When considering the overall ionic strength, owing to buffer plus NaCl concentrations, for the PG activity slight differences on the optimum value could be seen in all the systems under study (Table 2).

In the literature survey, an activating effect of $CaCl_2$ (at m*M* concentration) on the PE activity has been reported, this effect being attributed to a decrease of the enzyme inhibition by carboxylate groups as a result of their blockage by calcium ions, as well as to a better substrate dispersion in the medium (*26,28*). Here, for $CaCl_2$ concentrations above 0.5 m*M*, a decrease in the PE activity, both in absence and presence of PG, was observed, this decrease being higher than that for NaCl, as reported in the literature (*29,30*). Calcium chloride inhibited the PG activity in all the systems considered, the inhibition being severe for the soluble systems at $CaCl_2$ concentrations higher than 0.5*M*. This inhibitory effect was previously reported (*18–20,24,25*) and explained on the basis of formation of insoluble calcium pectate. However, the inhibitory effect was less evident when PG was immobilized, specially in presence of PE.

Temperature-Activity Profiles

The optimum temperature values for PE and PG activities did not change noticeably in all the systems studied (Table 3), and were similar to those reported for these pectolytic enzymes from similar sources (*18,21,22*). As particular details, CoIMPE showed activity over a temperature range broader than for any other PE system, IMPE displayed an optimum temperature slightly higher than SPE (60 and 54°C, respectively), and optimum temperature for PG decreased after immobilization (55 and 45°C, respectively), as it has been previously reported (*29,30*).

Table 3
Optimum Temperature, Activation Energy, and Temperature Parameter Values for PE and PG

	T, °C		Ea, kJ/mol		Q_{10} 50°C/40°C	Q_{10} 40°C/30°C
System	PE	PG	PE	PG	PE	PG
Soluble	54	55	25.9	27.2	1.05	1.05
Cosoluble	60	50	24.7	36.4	1.10	1.10
Immobilized	60	45	27.6	27.6	1.10	1.10
Coimmobilized	40–45	55	28.8	29.3	1.05	1.10

Table 4
Kinetic Parameters and First-Order Effectiveness Factor of PE and PG on HMP and PGA, Respectively, as Substrates

	Vm, U/mg		Km, mg/mL		ϵ	
System	PE	PG	PE	PG	PE	PG
Soluble	87.0	4.3	1.0	0.5	—	—
Cosoluble	43.5	2.2	1.0	0.5	—	—
Immobilized	17.5	3.4	0.8	0.2	0.4	0.9
Coimmobilized	22.5	5.7	0.9	0.4	0.4	0.8

These changes seemed to be related to both the type of support and method of immobilization, as reported (*17,26,27,31*), as well as to the presence or absence of PE (45°C for IMPG and 55°C for CoIMPG). The catalytic efficiency for the immobilized systems was similar to that for the soluble ones, as it was the E_a and Q_{10} (activity ratio at two temperatures differing ten degrees) values (Table 3), that are only slightly different to others reported (*18,29,30,32*).

Kinetic Parameters

Eadie-Hofstee plots allowed to calculate the kinetic parameters of PE and PG acting on HMP and PGA, respectively. Straight lines were obtained for the soluble systems, and immobilization caused the plot to appear S-shaped. Extrapolation of the slopes according to Engasser and Horwath (33) allowed to calculate the apparent kinetic parameters for the immobilized systems (Table 4), as well as the value of the effectiveness factor at low substrate concentrations (ϵ).

The V_{max} values decreased after immobilization, as it would be expected when considering the diffusional restrictions imposed by the matrix. This was not the case for CoIMPG, making it difficult to draw an

explanation for these data. However, it has to be considered that the figures shown represent specific activity, and that means that the values for the single-enzyme and double-enzyme systems cannot be compared because of the fact that these latter systems contain protein that does not contribute to one of the activities measured, in contrast with the single-enzyme systems.

The K_m values decreased after immobilization, a fact already reported for some immobilized pectinases (*34,35*) and explained on the basis of some partitional effects within the microenvironment of the enzymes, in spite of having used an uncharged support. An additional reason could be a result of a conformational change of the enzymes, as reflected by the intrinsic fluorescence spectra (*29,30*).

Remarkable internal diffusional limitations were observed from the ϵ values worked out from the Eadie-Hofstee plots analysis (*33*). The value of ϵ was closer to the unit for the immobilized PG systems than for the PE immobilized derivatives. This result could arise either as a consequence of the low PG activity in the derivative as compared to the PE activity, or because of a more external location of the PG enzyme with respect to PE within the pore of the support, or both.

Effect of Polygalacturonic Acid on the Pectinesterase Activity

PGA behaved as a competitive inhibitor only for the single-enzyme SPE activity, the inhibition constant value being 0.44 mg/mL (*10*). The presence of PG or the immobilization process rendered PE insensitive to PGA inhibition, either because the aminoacyl residues involved in PGA recognition were also involved into PE-PG protein-protein interactions, or into linkages to the support, or because a conformational change of the immobilized enzyme to a state insensitive to PGA inhibition occurred (a conformational change of PE upon immobilization has been reported [*29,30*]. Additionally, the K_i value calculated from the Dixon plot for SPE was the highest value registered among those in the literature (*2,17,29, 30,36*), although citrus pectin, not highly methoxylated pectin, was used as substrate in the literature studies.

Thermal and Storage Stabilities

Plots of the logarithm of the activity vs incubation temperature gave two straight lines for all the systems under study. This biphasic thermal behavior reflected the existence of at least two different conformations of the enzymes, with different thermal deactivation constants. Table 5 reflects the calculated values for the first thermal deactivation constants (the kinetic constant controlling thermal enzyme denaturation) from the corresponding slopes.

Table 5
Thermal Inactivation Constant Values for PE and PG, $\times 10^3$ seg^{-1}

	Temperature, °C								
	40	45	50	55		60		65	70
System	PG	PG	PG	PG	PE	PG	PE	PE	PE
SE	0.04	0.2	1.1	2.7	0.1	14.3	0.3	0.9	3.3
CoSE	0.2	0.6	1.9	3.3	0.2	5.8	0.5	1.2	5.8
IME	0.3	0.9	1.6	3.1	0.1	3.5	0.4	1.8	4.9
CoIME	0.4	0.7	1.9	2.8	0.1	3.8	0.4	1.2	5.6

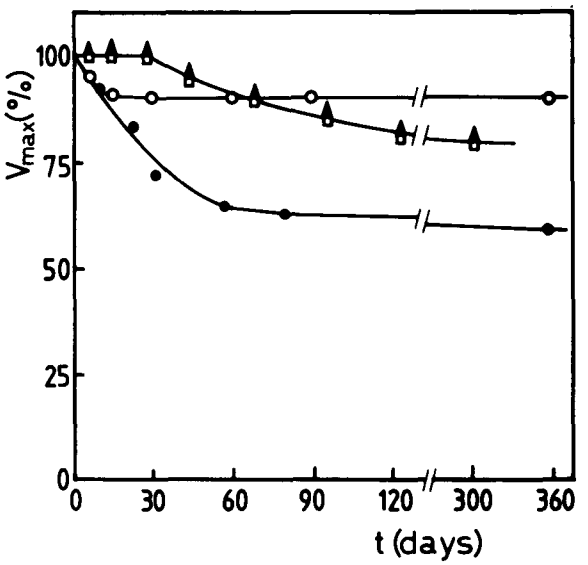

Fig. 2. Storage stability of IMPE (○), CoIMPE (●), IMPG (▲), and CoIMPG (□).

The PE activity was not stabilized by either the presence of PG or immobilization, while PG, on the contrary, was stabilized by the PE presence or immobilization. When stored at 3–5°C the immobilized PE activity was less stable in presence of PG, but this latter activity was not affected by the presence or absence of PE (Fig. 2), and the result was similar to that previously reported for other individually immobilized derivatives (*34,37,38*).

Operational Stability of the Immobilized Derivatives

A decay on the immobilized derivatives activity during continuous operation was observed. The results fitted to a first-order deactivation kinetics, so the plot of logarithm of activity vs time gave straight lines, whose slopes resulted to be the operational deactivation constants (or

Table 6
Operational Deactivation Constant
and Half-Life Time Values for PE and PG
Derivatives on HMP and PGA, Respectively

Derivative	K_d, $days^{-1}$	$t_{1/2}$, days
IMPE	0.121	5.72
IMPG	0.280	2.47
CoIMPE	0.101	6.86
CoIMPG	0.173	4.01

operational decay constants, the kinetic constants controlling the operational deactivation of the enzymes). From the operational deactivation constants the half-life times can be easily calculated.

The PE activity was slightly stabilized during continuous operation by the presence of PG, this being reflected by the relative values of the deactivation constants and half-life times, as shown in Table 6. The PG operational stability remarkably increased in presence of the demethoxylating activity, and the following sequence of stability could be written: IMPG < IMPE < CoIMPE < CoIMPG. It is worth mentioning that several derivatives of individually immobilized PG have shown operational stabilities higher than those found here (*37,39*), but it could be as a consequence of the use of impure enzyme preparations, containing PE in most of the cases, that increases the PG operational stability. In fact, a derivative containing pure-PG immobilized through thiol groups to a thiol-containing support showed an operational stability as low as that for IMPG (*29,30*).

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