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Properties of Pectinesterase Immobilized on Glycophase-Coated Controlled-Pore Glass

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ABSTRACT

Pectinesterase was immobilized on a porous glass support, which surface was covered with glyceryl residues. The parameters of the immobilization were characterized with respect to the coupling method used as well as the support pore size. Chemical modification level resulted an important parameter in determining the activity of the immobilized derivative. Attachment of the enzyme through thiol groups gave the best results, whereas a nominal pore size of 20 nm seemed to be the most suitable for the demethoxylating activity of the enzyme on citrus pectin. Optimum conditions for activity as well as the inhibition constant for polygalacturonic acid did not change on immobilization, as the Michaelis constant did. Fluorescence spectra revealed a partial unfolding of the enzyme tertiary structure when immobilized.

Index Entries: Pectinesterase; immobilization; pectins; glycophase-glass support; immobilized pectinesterase characterization; chemical modification; fluorescence spectra.

INTRODUCTION

Pectin is a generic name for high molecular weight polysaccharides present in higher plants that are responsible of the consistency, turbidity, and appearance of fruit juices. From the last years, pectin degradation by

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pectic enzymes constitutes a common industrial practice to achieve clarification (*1*). The main pectic enzymes, pectinesterase and polygalacturonase, may be used either individually or sequentially to clarify, since the depolymerizing enzyme, polygalacturonase, operates only if pectic substances have a 60% or lower esterification degree. Given the advantages of using immobilized enzymes (*2*), several attempts have been carried out in order to obtain highly-active and stable insoluble derivatives of these enzymes (*3–13*). Pectinesterase seemed to be more sensitive to the effects of immobilization than polygalacturonase. In fact, the best derivative of tomato pectinesterase obtained to date shows only 7.5% of the specific activity of the soluble enzyme (*12*). When a fungal pectinesterase was immobilized by adsorption, the specific activity attained 25% of that of the soluble enzyme (*12*); although fungal enzymes seem to be more resistant than those from plant, this fact points to the importance of chemical modification on the enzyme activity. Additionally, the adsorption method of immobilization is unsuitable for the future application of immobilized pectinesterases, since the enzyme is easily lost when acting on viscous pectin solutions. No studies have been carried out in order to establish whether the unsuccessful results obtained after the enzyme covalent coupling are owing to the nature and characteristics of the support employed or to the effect of the chemical modification on the enzyme. In this later sense, all the authors used free amino groups to attach the enzyme to the support, with one exception, who used tyrosyl residues (*12*). Thus, tomato pectinesterase has been immobilized onto Glycophase-coated controlled-pore glass by four different coupling methods in order to involve different aminoacyl sidechains of the protein in the support-enzyme linkage. The enzyme activity dependence on different pore size was also evaluated and the best derivative obtained characterized.

EXPERIMENTAL

Materials

Pectinesterase (65 U/mg) from tomato, citrus pectin (60% esterification degree), 1-ethyl-3(3-dimethylamino-propyl)-carbodiimide-HCl, β-mercaptoethylamine, ethylenediamine, *para*-phenylenediamine, and sodium borohydride were all purchased from Sigma Chem. Co. Glycophase G™ controlled pore glass was from Pierce (Rockford, IL) (200–400 mesh particle size and 4, 10, 20, and 46 nm nominal pore sizes). All the remaining reagents were from Merck, analytical grade, used without further purification.

METHODS

Pectinesterase Immobilization

The enzyme was immobilized onto the glass support by previously described methods (*14*). In brief, the following procedure was used: The support was activated by treatment with 6 m*M* periodic acid. To the alde-

hyde-glass so obtained, either the enzyme (1 mg/5 mL of 0.1 *M* phosphate buffer pH 7) was directly coupled or a further derivatization step was performed. This later was accomplished by linking to the previous aldehyde-glass β-mercaptoethylamine (resulting in a thiol-glass) or ethylenediamine (resulting in an amino-glass) or *p*-phenylenediamine (resulting in an arylamine-glass) all 10 m*M*. To these later derivatized glasses, the enzyme (1 mg/5 mL of 0.1 *M* phosphate buffer pH 7) was coupled after ferricyanide or carbodiimide or nitrous acid, respectively, activation steps. The immobilized enzyme derivatives were, in all cases, washed and stored in 0.1 *M* phosphate buffer pH 7 at 3–5°C.

Protein Determination

Immobilized protein was determined from the difference between the amount added and that recovered after coupling and several washings. Protein determinations were carried out by a modification of the Lowry method (*15*).

Pectinesterase Activity Assay

Activity measurements were carried out by continuous titration of the appearing carboxylate groups (*16*). A 0.5% (w/v) pectin solution in 0.05 *M* NaCl was used as substrate, the reaction medium being thermostatized at 40°C. Continuous stirring was applied to homogenize the mixture, especially when the immobilized enzyme derivative was present. One unit of activity was defined as the amount of enzyme that produces one micromole of hydrogen ions per minute under standard assay conditions.

Fluorescence Measurements

Fluorescence measurements were performed in a Perkin-Elmer MPF-44B spectrofluorimeter, equipped with a differential corrected spectra unit Hitachi DCSU-2. All the spectra were corrected and performed at 25.0±0.1°C, with 6 nm excitation and emission slits. Protein concentration in samples ranged from 10 to 21 μg/mL. Soluble enzyme was dissolved in water, whereas the immobilized enzyme and support alone were suspended in ethylenglycol to avoid precipitation. Appropriate blanks were checked for ethylenglycol fluorescence, and the amount of solid particles was chosen as low as possible to minimize the light scattering caused by the glass beads.

RESULTS AND DISCUSSION

Effect of the Coupling Method and Pore Size on the Immobilized Pectinesterase Activity

Pectinesterase was immobilized through four different aminoacyl side-chains. Direct coupling of the enzyme to the periodic acid-activated glass mainly involves the free amino groups of the protein (*17*). An analogous

Table 1
Effect of Coupling Method on Pectinesterase Immobilization

Support	IPR[a]	RAR[b]	AY[c]	SA[d]	DA[e]
Aldehyde-glass	15	82	1.0	1.1	0.8
Thiol-glass	82	95	3.2	3.5	14.0
Amino-glass	45	98	0.2	0.2	0.7

[a]IPR: Immobilized Protein Ratio: Ratio (as percent) of the protein removed from the solution to the total used for immobilization.
[b]RAR: Removed Activity Ratio: Ratio (as percent) of the activity removed from the solution during the immobilization process to the total units of soluble enzyme offered for immobilization.
[c]AY: Activity Yield: Ratio (as percent) of immobilized enzyme specific activity to that shown by the soluble enzyme.
[d]SA: Specific Activity: U/mg of immobilized protein.
[e]DA: Derivative Activity: U/g of immobilized derivative.

degree of selectivity was obtained when the thiol enzyme groups were linked to β-mercaptoethylamine-derivatized glass, forming a mixed disulfide bridge (*18*). The use of carbodiimides and supports containing amino groups results in the protein attachment through aspartyl and glutamyl free carboxylates (*19*). Less selectivity is shown by diazo coupling to the arylamine-glass; phenolic sidechain of tyrosyl residues is the main group participating in the linkage, although lysyl and arginyl sidechains can also be involved (*19*).

Tomato pectinesterase is the enzyme more actively immobilized in the literature, and given the lack of commercially available pure fungal enzyme preparations, it was used in this study. Initially, the enzyme was immobilized on a 4 nm pore-size glass support to make negligible the internal diffusional effects; neither the enzyme nor the substrate could enter the structure of the support. Under such circumstances, the derivatives showed enzymatic activity (Table 1) except when coupled to arylamine-glass. When the enzyme was linked to diazotized aminoaryl-supports (Enzacryl AA), the lack of pectinesterase activity was attributed, by the only authors who used this immobilization method (*12*), to the involvement of hystidyl and tyrosyl residues within the active site of the enzyme (*20*). However, this fact might not be the cause. The arylamine-glass-pectinesterase derivative showed activity when the substrate was changed from citrus pectin to a low molecular weight (38,000 daltons) and highly methoxylated (94% ED) pectin (*21*). Thus, the particular structure of the substrate is important in determining the expression of activity and, in this sense, no author has tried to establish the dependence of pectinesterase activity on esterification and/or polymerization degree of the pectic substrate.

Table 1 shows that chemical modification of free amino and carboxylate groups gave poorly active derivatives, although it should be borne in mind that amino groups have been usually used to attach the enzyme to different supports in the literature (*8–13*). Additionally, during coupling,

Table 2
Effect of Support Pore Size on Immobilization Parameters[a]

Pore size, nm	IPR	RAR	AY	SA	DA
4	82	95	3.2	3.5	14.0
10	88	97	8.4	9.1	40.0
20	85	96	11.6	12.6	54.3
46	87	98	8.1	8.8	38.0

[a]The meanings of IPR, RAR, AY, SA, and DA are those explained in Table 1.

the uncoupled soluble enzyme lost partially its activity; this result might reflect some kind of specific interaction with the aldehyde-glass support, since the coupling medium (0.1 *M* phosphate buffer pH 7) was the same in all the immobilization procedures carried out. Furthermore, although the degree of derivatization was similar in all supports used, the amount of coupled protein to the aldehyde-glass was the lowest one. Thus, amino groups seem not to be the most suitable pectinesterase groups to be used for covalent immobilization of the enzyme.

Formation of a mixed disulfide bridge between a thiol-glass and the enzyme resulted in the best method to immobilize the enzyme, thus emphasizing the importance of chemical modification on the immobilized pectinesterase activity expression. In spite of the high activity shown by the immobilized derivative, its specific activity was a fraction of that shown by the soluble enzyme. Obviously, the presence of the support must impose severe steric restrictions on the interaction between the enzyme and the high molecular weight substrate.

Support pore size is a parameter of great importance in determining the number of protein-support bonds, stability of the immobilized enzyme, diffusional limitations, and accesibility of the substrate to the enzyme, especially if the substrate is of high molecular weight, as pectin is. Thus, four well-differenced pore sizes were tested (Table 2), 20 nm being the one that allowed maximum activity on citrus pectin. The decrease in activity observed when using 46 nm pore size should be attributed to internal diffusional limitations. Given the Stokes radius of the pectinesterase molecule (4.4 nm) it should be immobilized into the 46 nm pore size support in a more internal location than in the 20 nm pore size support; thus the internal diffusional limitations should be higher, considering the large size of the substrate (290,000 for citrus pectin); so, a lower level of activity was measured on the 46 nm pore size derivative. The thiol-glass (20 nm pore size)-pectinesterase derivative was then characterized.

pH-Activity Profiles

Figure 1 shows the pH-activity behavior of pectin-esterase, both soluble and immobilized in thiol-glass. Optimum pH was similar because of the absence of proton partitioning owing to the neutral nature of the sup-

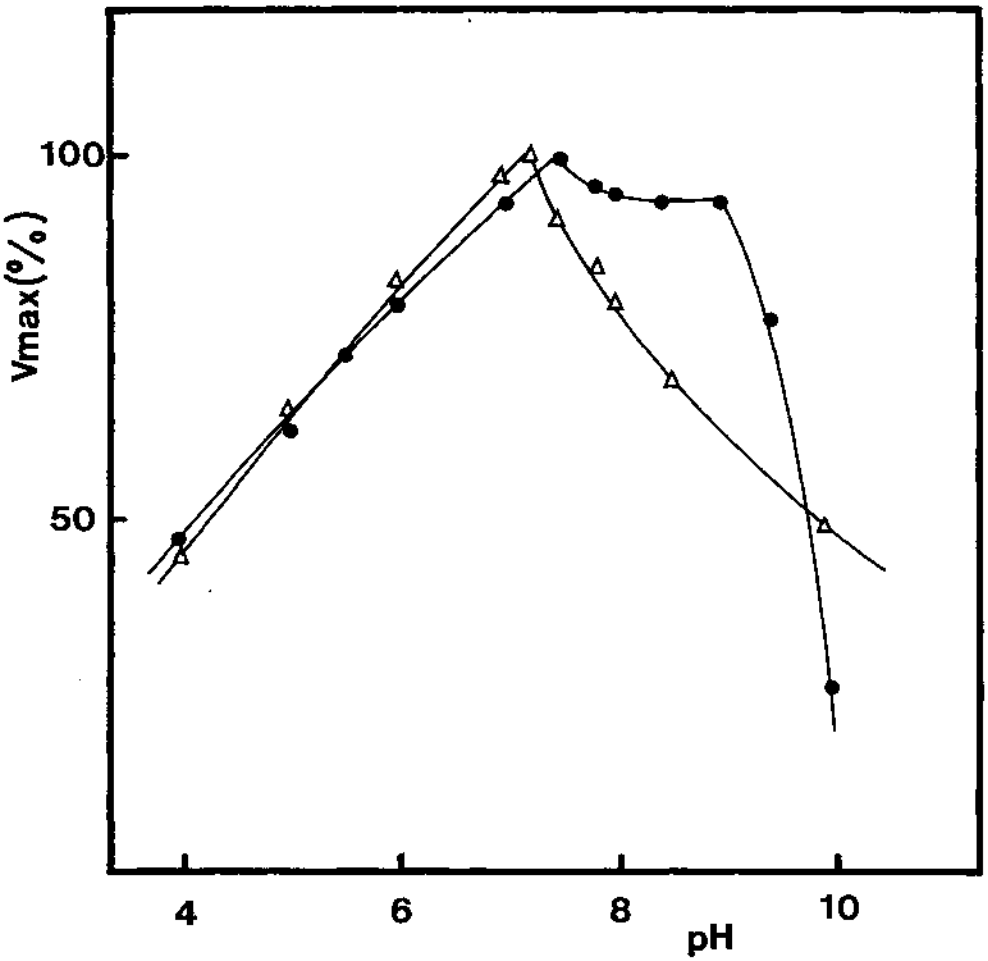

Fig. 1. pH-Activity profiles of (●) soluble and (Δ) immobilized pectinesterase, as determined on citrus pectin in 0.05 *M* NaCl at 40°C. Corrections were made to account for the partial ionization of galacturonic acid below neutrality.

port used (*17*). Appropriate corrections to the initial rates were made to account both for base-catalyzed methyl-ester hydrolysis at high pH (22) and partial dissociation of the resulting uronic acid at low pH (a $pK_a = 3.75$ was used).

Effect of Temperature on Activity

Optimum temperature for activity of the immobilized derivative was the same as for the soluble enzyme, 60°C (Fig. 2). However, in the following all activity, assays were carried out at 40°C to avoid unspecific hydrolysis of methyl-ester groups of pectins at temperatures above 50°C (22). The activation energy slightly decreased on immobilization (from 31.7 to 27.9

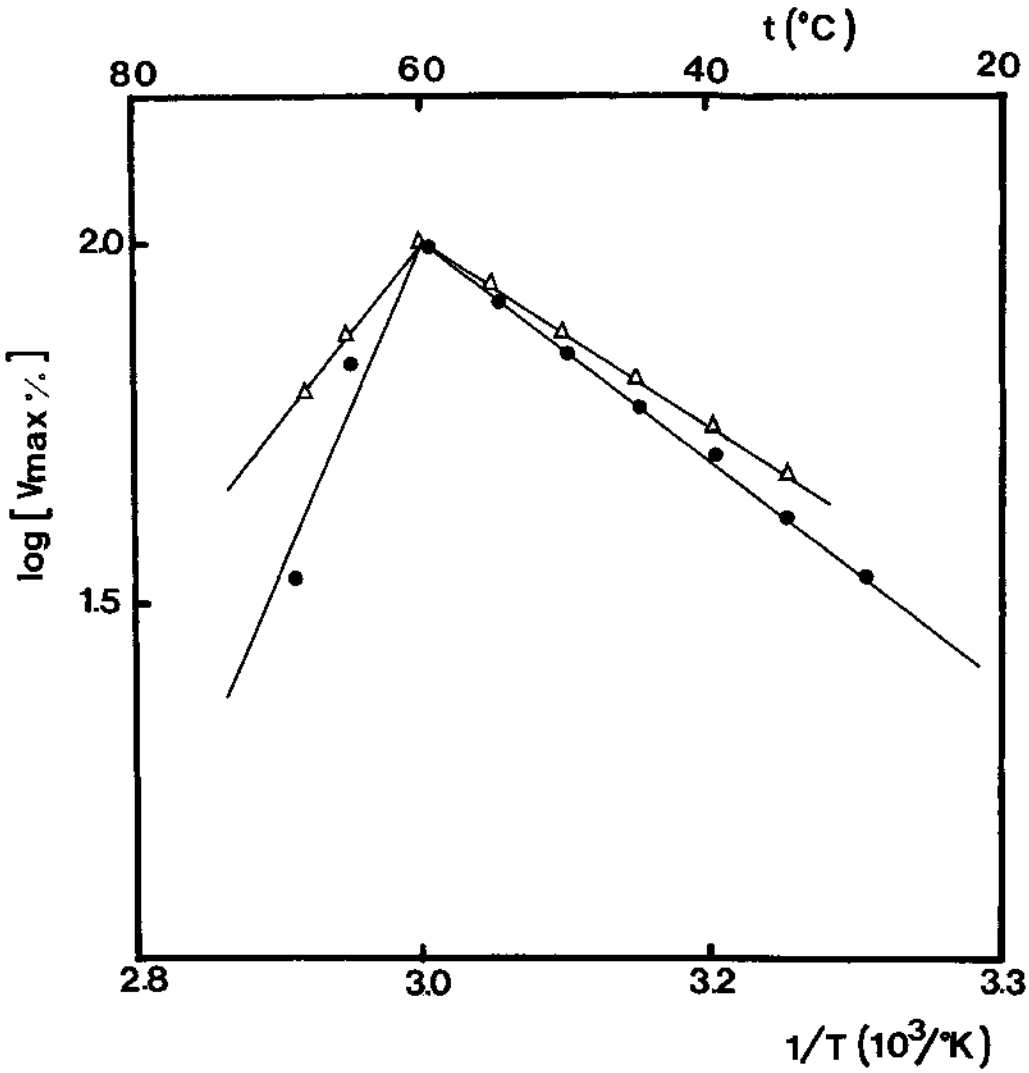

Fig. 2. Arrhenius plots of the dependence of (●) soluble and (Δ) immobilized pectinesterase on temperature. Assays were carried out at optimum conditions, except for temperature of the reaction media.

kJ/mol), thus allowing a higher catalytic efficiency of pectinesterase when immobilized. No such determinations have been made in the literature.

Effect of Ionic Strength

The activity of pectinesterase was clearly dependent on this parameter. Both soluble and immobilized enzymes attained a maximum activity at 0.05 *M* NaCl (Fig. 3). No similar comparison has been previously made, different authors assaying the immobilized enzyme at the same ionic

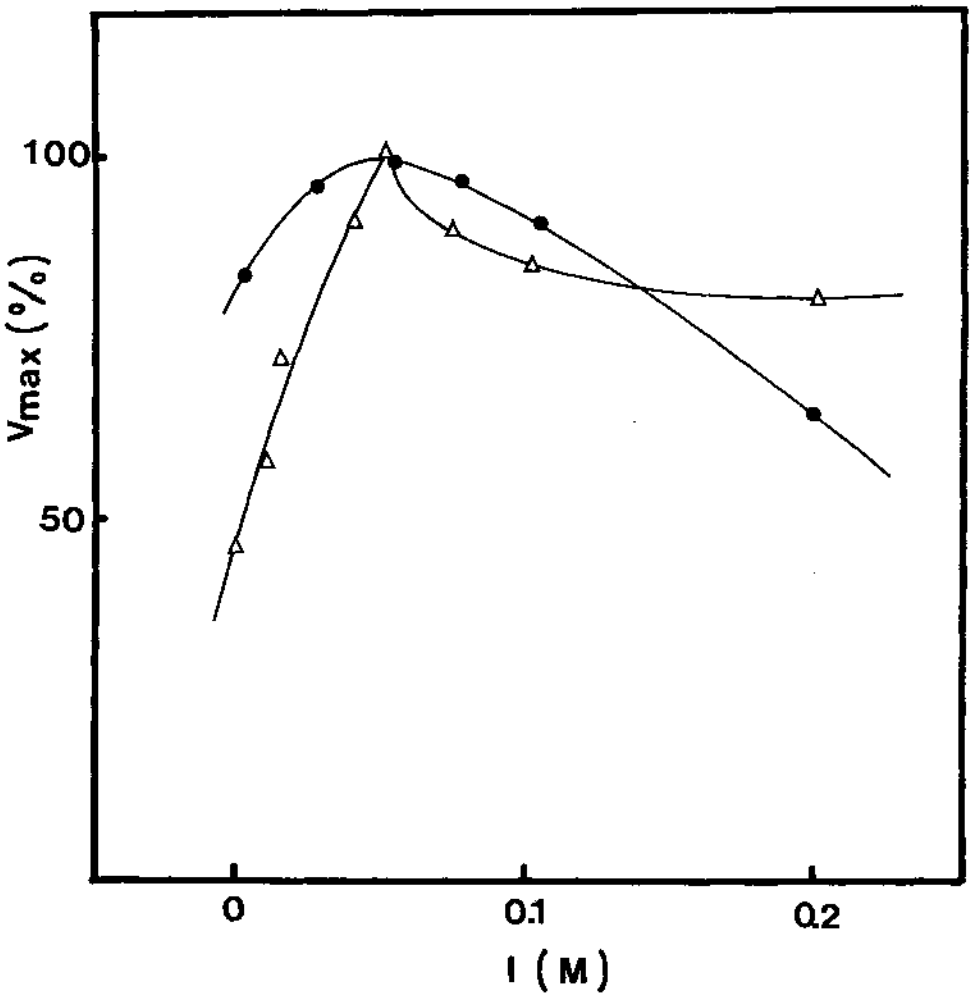

Fig. 3. Effect of the ionic strength given by NaCl on the activity of (●) soluble and (Δ) immobilized pectinesterase.

strength as the soluble (*9,12*). The effect of sodium chloride was unspecific, because the same results could be attained when either potassium chloride or sodium nitrite were used.

Kinetic Constants

The enzyme, both soluble and immobilized, followed a Michaelis-Menten behavior when using citrus pectin as a substrate. Hanes-Woolf analysis of the experimental data allowed to calculate the kinetic constants shown in Table 3. The enzyme immobilization resulted in a decrease of the Michaelis constant for citrus pectin. One of the reasons for such changes would be a change in the conformation of the enzyme on immobilization. In fact, the fluorescence spectrum of immobilized pectinesterase shows a

Table 3
Kinetic Parameters of Pectinesterase

Enzyme form	V_{max}, U/mg	K_m, mg/mL	K_i, mg/mL
Soluble	108	0.92	0.18
Immobilized	11	0.49	0.16

displacement of the emission maximum to higher wavelengths (Fig. 4), thus indicating a conformational change of its structure to a conformational state in which the tryptophanyl residues moved to a more hydrophylic environment (*23*).

The above mentioned K_m decrease on immobilization is the only one reported. Other authors who calculated such parameters gave values that are 5–6-fold higher than that of soluble enzyme (*12*). This increase is usually related to high steric limitations caused by the support structure. And, obviously, the way in which the enzyme is attached to the support renders an orientation that is important in the magnitude of the steric hindrances.

The Hanes-Woolf plot was a straight line, reflecting a kinetic, not diffusional, control of the reaction (*24*), probably as a consequence of the low immobilized enzyme density and activity.

Inhibition Studies

Polygalacturonic acid behaved as a competitive inhibitor of the citrus pectin deesterifying activity of the immobilized pectinesterase (Fig. 5). Dixon plots allowed to calculate the inhibition constant that was similar to that of soluble enzyme (Table 3).

The reaction rate was not affected by methanol at concentrations as high as 0.5 *M*, a value largely higher than that produced by enzyme action under most favorable conditions.

Storage and Operational Stabilities

The immobilized enzyme derivatives retained 95–100% of their initial activity for at least 1 mo when stored at 3–5°C suspended in 0.05 *M* NaCl.

Operational stability was tested in a thermostated continuous stirred tank reactor (CSTR, 10 mL liquid capacity, 150 mg immobilized enzyme derivative), to which a glass pH electrode and the alkali port of a pH-stat were attached. Although not currently used for operational stability studies, the CSTR so equipped allowed an easy and continuous monitoring of the immobilized pectinesterase activity and avoided the problems derived from the use of a viscous substrate flowing through a packed-bed reactor. A 0.5% (w/v) citrus pectin solution was pumped through at a 20 mL/h

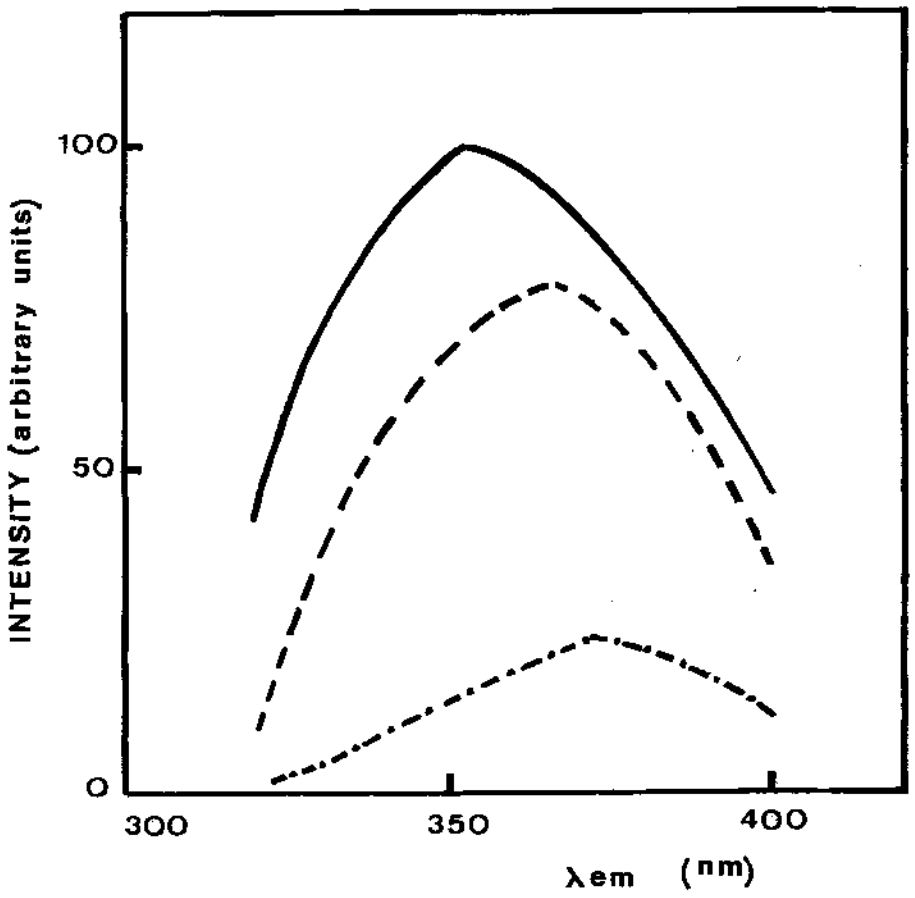

Fig. 4. Emission fluorescence spectra of (———) soluble pectinesterase in water, (— — — —) immobilized pectinesterase on thiol-glass, and (— · — · —) the thiol-glass support, these two later suspended in ethylenglycol. All the spectra were run at 25°C, the protein-containing samples having a similar amount of it.

flowrate, the conversion being directly measured by continuous titrimetry of the appearing carboxylate groups with 0.05 *M* NaOH. The immobilized enzyme was fully stable for 10 h, and a decrease in activity was observed afterward because of the progressive deterioration of the support matrix, which was clearly ground by the stirring bar.

SUMMARY

The influence of chemical modification on the immobilized pectinesterase derivatives activity has been shown. The enzyme is highly sensitive to the modified aminoacyl residue as well as to the immobilization

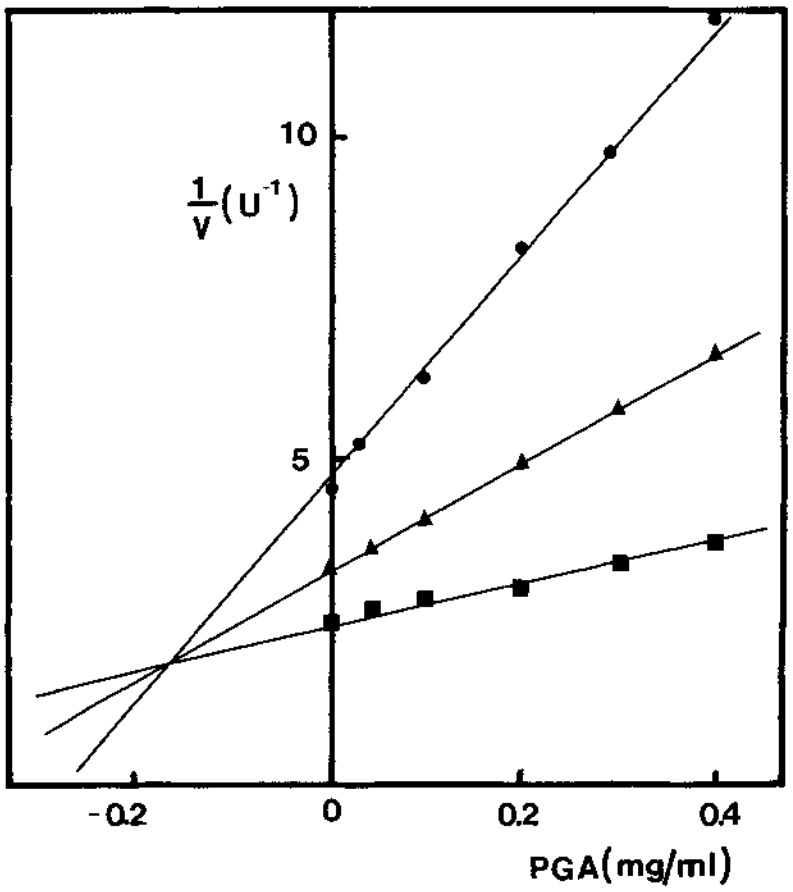

Fig. 5. Dixon plots of the inhibition pattern of polygalacturonic acid on immobilized pectin-esterase activity on (●) 0.5, (Δ) 1.0, and (□) 1.5 mg/mL citrus pectin as substrate.

process itself, probably owing to the steric limitations that the support imposes on the interaction of the enzyme with its macromolecular substrate. Hence, it is important to further the studies on pectinesterase immobilization in order to improve the attachment methods, considering also the chemical nature of the support used.

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