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Immobilization and stabilization of pectinlyase on synthetic polymers for application in the beverage industry

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Abstract

Fruit juice depectinization can be achieved by means of immobilized pectinlyase (PL, EC 4.2.2.3). PL immobilization on three synthetic polymers, i.e. Eupergit C, Nylon 6 activated with glutaraldehyde and XAD7 activated with trichlorotriazine was tested. Satisfactory activity results were obtained only with activated Nylon 6 and XAD7 (110 and 335 U/g, respectively). As the operational stability of these matrices was low, stabilization was performed by the structure enzyme rigidification through the subsequent cross-linking with glutaraldehyde.

Keywords: Fruit juices; Immobilized enzymes; Pectic enzymes; Pectinlyase; XAD7

1. Introduction

Pectinlyase (PL, EC 4.2.2.3) is a pectic enzyme which has received growing attention for its potential application in the food processing industry [1]. Depectinization for the production of fruit juices (apples, pears, berries, crowberries, oranges, grapes, tropical fruits, etc.) is currently carried out using two pectic enzymes in sequence: pectinesterase (PE, EC 3.1.1.11) and polygalacturonase (PG, EC 3.2.1.15), which involve de-esterification and depolymerization of the pectin molecules, respectively [2,3]. Some disadvantages of using these enzymes, are the release of methanol and the formation of colloidal precipitates in the system, between the de-esterified pectin and the endogenous Ca^{2+} [4]. The PL obviates these inconveniences, as it is the only enzyme capable of depolymerizing the pectins directly. Its immobilization, rather than that of PG and PE, and its application in continuous industrial processes, therefore, could offer considerable advantages. There are, however, several difficulties: commercial preparations of PL with a high degree of purity and low price are not available on the market, the purified enzyme is only slightly stable, PL's chemical–physical characteristics (composition, amino acid sequence, etc.) are little known, the literature concerning PL immobilization is scarce [5–9], and finally their use in industrial processes at low added value limits the choice of supports and of immobilization methods.

After having examined various enzyme preparations used by the Italian food industries, a commercial preparation purified by adsorption on bentonite was chosen as the PL source [10].

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Several criteria were adopted for choosing the supports, atoxicity (food grade), low cost and ready availability, easy use in industrial processes, chemical inertia (under conditions of use), non-biodegradability, and at last the ability of the support to react either directly or via a simple and inexpensive activation method. On the basis of these criteria, three macromolecular organic matrices were employed: Eupergit-C containing oxirane groups, already used in a previous paper [5] and compared to Nylon 6 activated with glutaraldehyde, and XAD7 [11], an acrylic ester resin hydrolysed in basic conditions and activated with tricholotriazine. Finally, an increase of stabilization was obtained by the subsequent cross-linking with glutaraldehyde.

2. Materials and methods

2.1. Materials

The enzymatic preparation Cytolase PCL5 (PCL5, batch 45, Genecor) was employed as the pectinlyase (PL) source after purification by means of adsorption on bentonite (BDH).

Apple pectin (Fluka) with a starting esterification grade of 72–75% was further esterified up to 94% by the procedure described by Kohn et al. [12]. For protein determination, bovine serum albumin (BSA, Pentex) was employed as standard and Coomassie® Brilliant Blue G-250 (Serva) as a colorimetric agent.

The following polymers were used as supports for immobilization: Eupergit C and Amberlite XAD7 (Rohm and Haas), and Nylon 6 (Serva), all with a granulometry between 77 and 177 μm. 50% Glutaraldehyde (GA, Fluka) and trichlorotriazine (TCT, Fluka) were used as activating agents.

Other reagents employed included: 3-methyl-2-benzothiazolinone hydrazone chlorohydrate (MBTH, Carlo Erba, Italy), *N,N*-dimethyl formamide (Carlo Erba), and iron trichloride (Merk). All the other reagents not specifically mentioned were supplied by Carlo Erba.

2.2. PL purification

The PCL5 enzymatic preparation was purified according to a method described in previous papers [10]. Four grams of bentonite were added under stirring to 30 ml of 0.07 M citric–phosphate buffer (C–P) at pH 5.0 and sonicated for about 20 min at 25°C. 70 ml of 0.07 M C–P at pH 5.0 and 100 ml of PCL5 were subsequently added to the suspension thus obtained. After the pH value had been adjusted to 5.0, the suspension was stirred for about 2.5 h, and then centrifuged at 2600*g* for 20 min. The enzymatic solution was treated again for 2.5 h with 4.0 g of bentonite mixed with 50 ml of 0.07 M C–P at pH 5.0 before being centrifuged once more. The enzymatic solution was then diluted twice with water and brought to a pH of 3.2–3.4 by adding 1 M HCl, after which it was placed in a refrigerator at 3°C overnight. The precipitate, essentially made up of extraneous proteins, pectins, mucilages and brown pigments, was separated by centrifugation at 5500*g* for 60 min at 3°C, and the purified PL containing solution was then used for immobilization. Protein determination was made by the procedure described by Bradford [13].

2.3. Nylon 6 activation

2 ml of 2.5% GA solution in 0.07 M C–P buffer at pH 3.5 were placed in 10-ml tubes containing 10 mg of Nylon 6 (Ny6). The preparation was allowed to react under slight stirring for 2 h at 25°C.

After centrifugation, the amount of GA in the solution was determined by Bersthorn's hydrazone method, without the oxidation step [14]. Two ml of standard GA solution (0.01–0.2%) or of an appropriately diluted sample were placed in a 20-ml flask and made to react at 100°C with 2 ml of 0.4% MBTH. After 3 min the mixture was rapidly cooled in an ice bath. 5 ml of 0.4% $FeCl_3$ were then added and the solution was brought to volume with acetone. After 15 min at 25°C absorbance of the standard solution or of the sample was read at 635 nm against the blank, which had been

obtained by replacing the GA solution with an equal volume of the same solvent in which the GA had been dissolved.

Ny6 activated with GA (Ny6-GA) was washed several times with 10 ml of water until GA was totally removed, GA disappearance being verified by reading the absorbance at 235 nm.

2.4. XAD7 activation

XAD7 was washed several times with water and methanol and then left to dry in an oven at about 55°C, after which 20 g of dry XAD7 were suspended in 200 ml of a 2.5 M solution of NaOH containing 15% isopropanol. Hydrolysis was performed by reflux under stirring for about 24 h. The support thus hydrolysed was then washed several times with water until neutral pH was reached. Finally, it was washed with methanol and once more dried in the oven.

In order to determine the carboxylic groups formed, 10 ml of *N,N*-dimethyl formamide were added to about 0.5 g of hydrolysed XAD7 and the solution was left to equilibrate for 1 h under stirring at 25°C. Three drops of a 1% Thymol Blue solution in ethanol were then added and titration finally performed with 0.05 M sodium methoxide dissolved in a benzene/methanol solution (7:3 v/v).

4 ml of 1.2% TCT solution dissolved in dioxane were placed in 10-ml tubes containing 10 mg of hydrolysed XAD7. After activation in a rotary carousel for at least 20 min at 25°C, the support (XAD7–TCT) was washed twice, first with 10 ml of dioxane and then with 10 ml of water.

2.5. PL immobilization and stabilization

Sixty to one hundred units of purified PL, brought to the desired pH by the addition of 0.1 M HCl or NaOH, were placed in 10-ml tubes with 10 mg of Eupergit C and of activated supports (Ny6–GA and XAD7–TCT). After about 16 contact hours in a rotary carousel (about 40 rpm) at 4°C, the tubes were centrifuged. The enzymatic activity of the supernatants was directly assayed, while that of the residues was assayed after having washed them with 10 ml of 0.5 M NaCl and twice with 10 ml of 0.05 M C–P at the pH of the enzymatic assay.

In the stabilization trials, 5.0 ml of 0.01% GA solution at pH 3.5 (0.01 M C–P buffer) were added to the PL immobilized on the various supports at 4°C and with a contact time of 15 min.

2.6. Determination of PL activity

The activity was determined according to the procedure already described in a previous paper [5]. Three ml of 1.1% esterified pectin dissolved in 0.05 M C–P buffer at the established pH were added under magnetic stirring at 25°C to 2.0 ml of PL in solution or of immobilized PL suspended in the same buffer in a 10-ml tube. In the case of the immobilized enzyme, the tubes were centrifuged. For the blank test the order of the reagents was reversed, i.e. the acid was first added to the enzyme, followed by the pectin solution. The sample solution was read against the blank solution at 235 nm. The increase in absorbance was due to the double conjugate bond of the $\Delta^{4,5}$ unsaturated uronide formed during the reaction. One unit of the enzyme is defined as the amount which catalyzes an increase of 0.555 in absorbance at 235 nm in 1 min at 25°C and at optimal pH [15].

2.7. Characterization of the free and immobilized PL

The following parameters were determined for the free enzyme and for the enzyme immobilized on various supports: optimum pH (between 3.5 and 7.5) at 25°C; optimum temperature (between 20 and 90°C) at optimal conditions; stability over time at pH 3.5 and 25°C; and finally, K_m and V_{max} (with pectin solutions, between 0.025 and 1.1%).

2.8. Definition of yields

Adsorption yield (AY) and immobilization yield (IY) are defined as follows:

$$AY = U_{ads}/U_{eq} \times 100,\ IY = U_{act}/U_{ads} \times 100$$

where U_{ads} are the adsorbed enzyme units, U_{eq} the equilibrated enzyme units, and U_{act} the active immobilized enzyme units.

3. Results and discussion

3.1. PL immobilization

Table 1 shows the results of PL immobilization on supports featuring different chemical–physical structures.

Eupergit C is a macroporous polymer (180 m^2/g) consisting of methacrylamide and *N,N'*-methylenebismethacrylamide, and containing oxirane groups (about 800 $\mu M/g$) capable of covalently reacting with the nucleophilic groups of the enzyme.

Nylon 6, a linear aliphatic polyamide featuring a low surface area (less than 20 m^2/g), was activated with glutaraldehyde (about 1000 μM of CHO groups/g) (Ny6–GA).

The aminic and presumably amidic groups of nylon 6 are capable of reacting above all with the aldehydic groups of GA (Schiff base). A reaction with the double bonds (Michael adduct) of GA originating from aldolic condensation has also been suggested. This latter mechanism, however, may be safely ruled out in our case given the acid pH employed for support activation [16]. PL with its aminic groups subsequently reacts with the bonded GA by the same mechanism (Schiff base).

XAD7 is a macroporous support (450 m^2/g) derived from polyacrylic ester. In order to make it chemically reactive, it was preliminarily hydrolysed in basic conditions so as to form oxydryl and carboxyl groups (about 660 $\mu mol/g$). The matrix was subsequently functionalized with TCT (XAD7–TCT), at least one of the Cl atoms of which may be replaced with an aminic group of the enzyme [17].

The supports examined exhibited fairly good adsorption yields (AY), and except for XAD7–TCT, a low catalytic response (IY and Uact) (Table 1). Even though the physical and chemical characteristics of the supports are very different one from the other, several considerations may be made. In the case of Eupergit C and XAD7, adsorption may have been facilitated by their high surface area as well as by the number and density of their reactive centres. In the case of Nylon 6, however, the latter factor may be the most important as this support features a low surface area. This assumption is further borne out by the fact that, when this support is not activated, its adsorption yield is lower.

Good stability was achieved with Ny6–GA, while for the other polymers stability was about one, half-life being virtually comparable to that of the free enzyme. Compared to the other supports, Ny6–GA features a lower surface area and a higher number of reactive centres (aldehydic groups) so that the density of bonds for each enzyme molecule is higher. This means that the enzyme's protein structure is more blocked and therefore less subject to irreversible unfolding.

Moreover, as can be seen in Fig. 1, notwithstanding the differences between the support tested, the maximum activity of all the supports was found out at the same immobilization pH value, close to the p*I* of the PL (about 3.6) [18–20]. At this pH, intra-and inter-molecular interactions of the enzyme molecules are at a maximum, so that molecules protect themselves from inactivation phenomena during support fixation.

Table 1
Yields (AY and IY), activity (U_{act}) and stability of the PL immobilized on various polymer supports

Support	Activation	AY	IY	U_{act} (U/g)	Stability [a] (pH 3.5, 25°C)
Eupergit C	–	19.0	3.2	61	1.0
Nylon 6	GA	17.0	6.5	110	4.5
XAD 7	TCT	15.4	31.4	335	1.3

[a] Stability is defined as the ratio between the half lives ($t_{1/2}$) of immobilized and free PL, under storage conditions of pH 3.5 and 25°C.

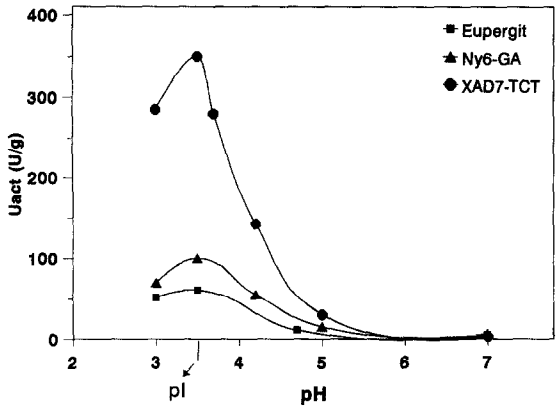

Fig. 1. Activity (U_{act}) of the immobilized PL as a function of immobilization pH.

Table 2
Chemical and physical characteristics of free PL and of PL immobilized on various polymer supports

Enzyme	pH_{opt}	T_{opt} (°C)	K_m (mM)
Free PL	6.1	65	0.16
PL on Eupergit C	5.6	75	1.50
PL on Nylon 6-GA	6.5	75	0.20
PL on XAD 7-TCT	6.4	73	1.41

3.2. *Characterization of free and immobilized PL*

As can be seen in Table 2, the optimum pH of immobilized PL as compared to free PL, undergoes variations towards the acid zone in the case of Eupergit C and towards the alkaline zone in the case of Ny6–GA and XAD7–TCT. These results can probably be accounted for by the different surface chemical structure of the various supports [21].

In the supports which behave as polycations, such as the Eupergit C containing amidic groups, a crowding of negative charges and of hydroxyl ions occurs at the surface and therefore in the micro environment of the immobilized enzyme. The micro-environment thus becomes more alkaline than the external solution so that an apparent shift of the optimum pH to the acid zone is observed. In contrast, a matrix which behaves as a polyanion causes a shift of the optimum pH towards the alkaline zone. This is probably the case with the hydrolysed XAD7 containing oxydryl and carboxyl groups.

As compared to free PL, the optimum temperature of immobilized PL is higher (8–10°C). In fact, the formation of bonds, covalent and non-covalent between the enzyme and the support reduces the degrees of freedom of the protein molecular structure of the enzyme and protects it from the denaturation caused by high temperature.

Moreover, as compared to free PL, immobilized PL exhibits a lower affinity towards the substrate

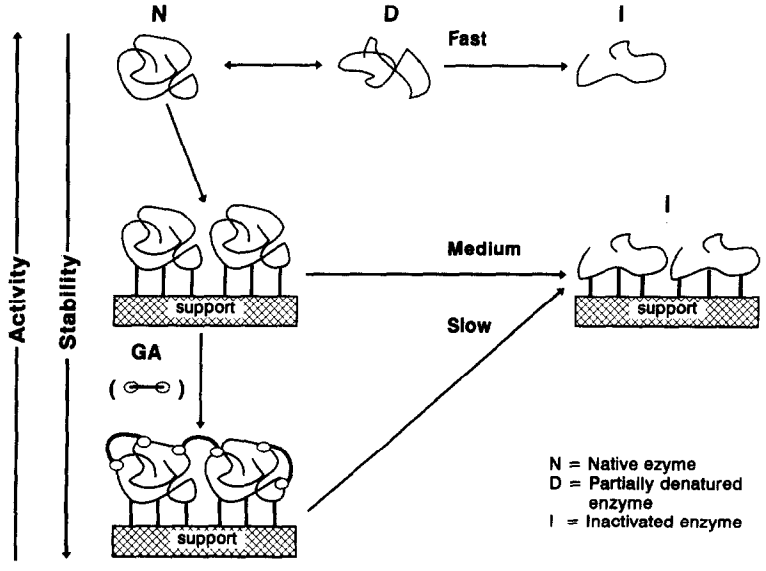

Fig. 2. Strategy for PL immobilization/stabilization.

Table 3
Activity (U_{act}) and stability of the PL immobilized on various polymer supports after cross-linking with glutaraldehyde

Activated support	Cross-linking	U_{act} (U/g)	Δ% [b]	Stability [a] (pH 3.5, 25°C)	Δ% [b]
Eupergit C	GA	35	−43	1.9	+90
Nylon 6-GA	GA	30	−73	5.0	+10
XAD 7-TCT	GA	200	−40	2.3	+77

[a] See notes of Table 1.
[b] Variations with respect to the results shown in Table 1.

(higher K_m values). One of the reasons which may explain this finding is the large molecular dimension of the pectic substrate (larger than 600 Å), which reduces its accessibility to the active site of the immobilized enzyme (steric hindrance).

3.3. Stabilization

According to the Mozhaev theories [22] on the irreversible inactivation mechanisms of the enzymes, protein unfolding is probably the most important phenomenon, for the immobilized PL stored in mild conditions (pH 3.5 and 25°C); while the dissociative phenomena can be ruled out as the PL does not seem to possess either a quaternary structure or a coenzyme [18].

An attempt to improve the stability of the PL immobilized on various supports was made by reducing its unfolding rate. For this purpose, the rigidity of the secondary and tertiary protein structure of the immobilized enzyme molecules was increased through subsequent cross-linking with a bifunctional agent (Fig. 2) [23–25]. Unfortunately, there are no precise criteria for the choice of the cross-linking agents. As can be seen in Table 3, although glutaraldehyde was able to increase PL stability, it also caused a decrease in its activity. This latter finding is probably due to the reduction in the degrees of freedom of the enzyme molecules, which also restricts its conformational adaptability towards the substrate. Moreover, cross-linking reactions may also involve amino acids which are essential for the catalysis accomplishment, as these are present in the active site or close to it.

4. Conclusion

Our findings showed that the catalytic response (IY and U_{act}) obtained with XAD7 activated with trichlorotriazine (XAD7–TCT) is very good and higher than that reported in the literature [5–9]. Moreover, stability was found to become fairly good only after the subsequent cross-linking with glutaraldehyde.

The PL immobilized on Nylon 6 activated with glutaraldehyde (Ny6–GA), despite featuring an activity which was about 1/3 of the one reported for XAD7–TCT, exhibited even greater stability.

If one also considers the higher optimum temperature of these immobilized enzymes, as compared to that of the free one, both Ny6–GA and XAD7–TCT could therefore be employed in industrial processes.

Our findings have shown that Eupergit C is the least suitable for application, as its activity and stability were seen to be lower.

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