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Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support

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Abstract

Pectinase was immobilized on an activated agar-gel support by multipoint attachment. The maximal activity of immobilized pectinase was obtained at 5 °C, pH 3.6, with a 24 h reaction time at an enzyme dose of 0.52 mg protein/g gel, and the gel was activated with 1.0 M glycidol. These conditions increased the thermal stability of the immobilized pectinase 19-fold compared with the free enzyme at 65 °C. The optimal temperature for pectinase activity changed from 40 to 50 °C after immobilization; however, the optimal pH remained unchanged. The immobilized enzyme also exhibited great operational stability, and an 81% residual activity was observed in the immobilized enzyme after 10 batch reactions.

Keywords: Multipoint attachment; Pectinase; Immobilization; Stabilization; Activated agar-gel

1. Introduction

Pectic oligosaccharides have various biological functions such as plant growth promotion (Suziki et al., 2002) and antimicrobial activity (EL-Nakeeb & Yousef, 1970; Li, Yamauchi, & Kato, 1997), and these functions depend upon the degree of polymerization (DP) of oligomers. In general, pectic oligosaccharides are produced by chemical (Hortikiss, Lecrinier, & Hicks, 2001) or enzymatic (Spiro et al., 1993) hydrolysis of pectin. But, chemical hydrolysis yields large amounts of monosaccharides as a byproduct due to the difficulty in controlling the progression of the reaction. During enzymatic hydrolysis by pectinase, it is difficult to control the quality and DP of pectic oligosaccharides in batch production. Continuous production of pectic oligosaccharides via hydrolysis of pectin by pectinase is also difficult due to the low stability of the enzyme. Therefore, it is necessary to create a bioreactor based on immobilized pectinase for the production of pectic oligosaccharides.

To date, pectinase has been immobilized on various supports, such as nylon (Lozano, Manjón, Romojaro, Canovas, & Iborra, 1987), ion-exchange resins (Kmínková & Kućera, 1983), silk (Zhu, Lin, & Wang, 1998) and gamma-alumina (Pifferi, Spagna, Nava Rincon, & Setti, 1993); however, sufficient improvement of pectinase immobilization efficiency and stability has not been achieved.

The fact that enzyme-support multipoint attachment can exert an important effect on the stability of an immobilized enzyme derivative is commonly stated (Gupta, 1991; Klibanov, 1982). This stabilization strategy has been used to greatly improve the stability of a number of enzymes with only small losses in activity (e.g., lipase, Otero, Ballesteros, & Guisán, 1991; β-galactosidase, Guisán et al., 1993). In our previous study (Li, Wang, Li, Zhao, Guo, & Zhang, 2007), pectinase was immobilized onto alginate support using glutaraldehyde as a coupling agent, which yielded relatively high level of residual activity (66%). In the present study, we used multipoint attachment to immobilize pectinase on an agar-gel support to compare the

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immobilization efficiency in both methods, and find a suitable strategy in pectinase immobilization for applying in the industrial production of pectic oligosaccharides.

2. Materials and methods

2.1. Materials

Commercially available pectinase (EC 3.2.1.15; protein content, 14 mg/ml) was supplied by DSM Food Specialties (Shanghai, China) with a molecular weight of approx. 38 kDa and an isoelectric point of ca. 3.6. Agar powder was purchased from Tianjin fine Chemicals (Tianjin, China). Pectin was extracted from haw fruit as described (Li et al., 1997). All other reagents were analytical grade.

2.2. Activation of agar-gel support

The activated glyoxyl agar-gel support was prepared by etherification of 6% agar-gel with glycidol and further oxidation of the resulting glyceryl agar-gel by $NaIO_4$ based on the method of Guisán (1988). In brief, agar-gel (20 g) cut into 1 mm^3 cubes was admixed in 100 ml of 0.16 M NaOH containing 6 mg/ml $NaBH_4$ as an antioxidant, and then glycidol (0.5–6.0 M) was added to the mixture. The mixture was stirred for 18 h at 25 °C, and then the activated agar-gel was washed with water. The glyceryl agar-gel was suspended in 100 ml of 35 mM $NaIO_4$ and stirred for 1 h at 25 °C. The activated agar-gel was washed with water and stored in distilled water at 4 °C.

The aldehyde content in the activated agar-gel support was determined to be equivalent to the amount of periodate consumed (Guisán, 1988). The periodate that was not consumed in the activation of the gel was measured by titration with IK (Nevell, 1963).

The surface density of aldehyde groups in the activated gel support varied from 22 to 60 μmol/g gel, corresponding to the variation of glycidol concentration from 0.5 M to 6.0 M.

2.3. Enzyme immobilization

Activated agar-gel support (10 g wet weight) was suspended in 60 ml of either 0.1 M phosphate (pH 2.0–8.0) or borate buffer (pH 9.0–10.0) containing adequate amounts (1.4 or 5.2 mg) of enzyme protein and stirred gently for 1–28 h at 5 or 25 °C. After a specified contact time, the gels were transferred to 60 ml borate buffer, (pH 10.0), $NaBH_4$ was added directly to give a final concentration of 1 mg/ml, and the suspension was gently stirred for 30 min at 25 °C to constitute a suitable end-point of the enzyme-support interactions (Guisán & Blanco, 1987). The gel support was recovered by filtration and washed with 0.1 M phosphate buffer (pH 7.0) and distilled water until the absorbance of the washings was less than 0.01 at 280 nm. The resulting immobilized pectinase on the gel support was stored at 4 °C.

2.4. Assay of enzyme activity and determination of pectin hydrolysis

The activity of free and immobilized pectinase were determined by monitoring the increase in the amount of reducing sugar. One ml free, or 3 g (wet wt.) immobilized pectinase was added to 100 ml haw pectin solution (10 mg/ml, w/v) prepared with 0.02 M phosphate buffer (pH 3.0), and the enzyme reaction mixture was incubated at 40 °C for exactly 10 min. The reducing sugar was quantified using the Somogyi-Nelson method improved by Hatanaka and Kobara (1980). One unit was defined as liberating 1 μmol of galacturonic acid from pectin per minute.

Determination of pectin hydrolysis by free and immobilized pectinase was followed by assaying the amount of reducing sugars (Hatanaka & Kobara, 1980) under assay conditions.

2.5. Operational stability of the immobilized pectinase

The operational stability was assessed by carrying out the hydrolysis of 1% (w/v) haw pectin (prepared with pH 3.5 acetate buffer) at 50 °C while stirring at 120 rpm; this procedure was performed in consecutive cycles while repeatedly reusing the enzyme. After each 4 h cycle, the immobilized particles were washed with acetate buffer (pH 3.5) and water. Each incubated solution was shaken for 10 min to measure the increase in amount of reducing sugar for the determination of the enzyme activity.

3. Results and discussion

3.1. Effect of reaction pH, contact time and temperature for pectinase immobilization

To optimize the immobilization procedure, a number of variables that might influence the interaction between the enzyme and support were investigated using a low enzyme loading (0.14 mg protein/g gel) to avoid substrate diffusion problems in the activity assays. The optimal immobilization conditions selected from these trials were a reaction pH value of 3.0–4.0 (Fig. 1), a reaction temperature of 5 °C, and a reaction time of 24 h (Fig. 2) with 1.0 M glycidol activated support (the surface density of aldehyde groups, 40 mol/g gel). The residual activity of enzyme immobilized at low pHs (3.0–4.0) were higher than that at high pHs (5–10). This seemed to conflict with the suggestion that enzyme immobilization by multipoint attachment method will occur better at alkaline pH value, which was reported by Mateo et al. (2005). The detailed mechanisms need further researches, the two reasons however might be taken into consideration, one of which might associate to the enzyme nature, from literatures (Lin, Yang, Zhu, Wang, & Chen, 2006; Yang, Ma, & Li, 2005) and our experiments (data not shown), free pectinase used in this experiment was stable at narrow pH range (3.0–4.5) and would lose most activity in alkaline solution; the other reason might be that

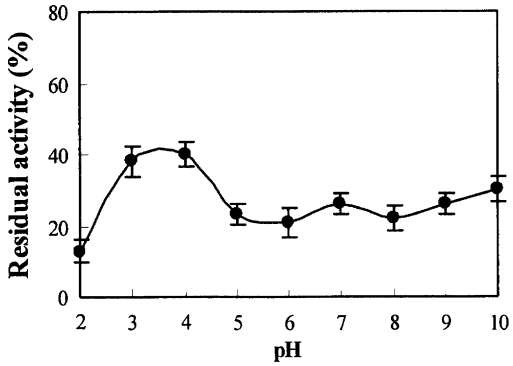

Fig. 1. Effect of reaction pH on pectinase immobilization. The residual activity was calculated as the ratio of the activity of the enzyme immobilized on the support to the total activity of free enzyme prior to immobilization. The data plotted correspond to the mean ± SD of three replicates. The agar-gel was activated with 1.0 M glycidol, and the immobilization was performed with the enzyme dose of 0.14 mg protein/g gel at 5 °C for 24 h.

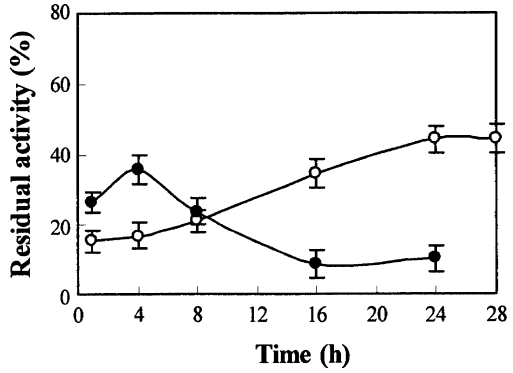

Fig. 2. Effect of reaction time and temperature on pectinase immobilization. The residual activity was calculated as described in Fig. 1. The data plotted correspond to the mean ± SD of three replicates. The immobilization temperature was 5 °C (open circles) or 25 °C (closed circles). The agar-gel was activated with 1.0 M glycidol, the enzyme dose was 0.14 mg protein/g gel, and the immobilization reaction was performed at pH 3.6.

pectinase was very sensitive for conformational changes on immobilization caused by relatively intense chemical modification with multi-linkages at alkaline conditions. To verify the results obtained here, the immobilized pectinase was washed by phosphate and acetate buffers (pH 3.0–7.0) with different ionic strengths (0.02–1.0 M NaCl), the residual activity however showed no significant changes. Moreover, the immobilization efficiency (expressed as residual activity) of pectinase on non-activated agar-gel was only 10%, and it increased to 40% on activated agar-gel (Fig. 1) at the conditions mentioned above. These results indicated that pectinase immobilized on activated agar-gel was the result of multi-covalent bonds.

3.2. Effect of glycidol and enzyme concentration on pectinase immobilization

Fig. 3 shows the effects of enzyme dosage and glycidol concentration on pectinase immobilization efficiency. The

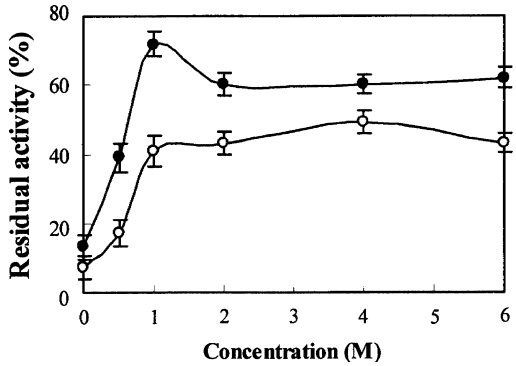

Fig. 3. Effects of enzyme dosage and glycidol concentration on pectinase immobilization. The residual activity was calculated as described in Fig. 1. The open circles represent dose of 1.4 mg protein/g gel and the closed circles, 5.2 mg protein/g gel. The data plotted correspond to the mean ± SD of three replicates. Immobilization was performed at 5 °C, pH 3.6 for 24 h.

residual activity of pectinase after immobilization was low at a lower concentration of glycidol (<1.0 M); and a higher concentration of glycidol (>1.0 M in the case of 5.2 mg protein/g gel; >4.0 M in the case of 1.4 mg protein/g gel) would not increase the residual activity of immobilized pectinase, although the enzyme protein was approximately 100% attached to the gel support when glycidol concentration was over 1.0 M. This might depend on the amount of covalent-linkages between enzyme and support; however, excessive covalent-bonds would result in the conformational changes to decrease the enzyme activity. A maximum residual activity of 72% was obtained with 1.0 M glycidol when the enzyme dose of 0.52 mg protein/g gel was used for immobilization. This immobilization efficiency was similar to the high level (74%) for pectinase immobilized on synthetic polymers (Lei & Bi, 2007), and was higher than the efficiency obtained using a pectinase-alginate systems (66% by Li, Wang, Zhao, Guo, & Zhang, 2007 and 56% by Roy, Sardar, & Gupta, 2003). On the other hand, similar results for the efficiency of immobilization also could be found in other enzymes bonded on same glyoxyl-agarose (agar-gel) support by multipoint attachment (e.g. 60% in chymotrypsin, Pedroche et al, 2007; 70% in cyclodextrin glycosyltransferase, Ferrarotti et al., 2006). Our result was in reasonable agreement with these literatures. Thus, the highly activated agar-gel could be a suitable support for pectinase immobilization to yield high activity recovery from free enzyme. Moreover, the agar-gel support was less expensive, safer and more readily available than the synthetic support.

3.3. Optimal pH and temperature of immobilized pectinase

The pH profiles for free and immobilized pectinase activity were shown in Fig. 4. The activities of both forms of pectinase were similar as pH varied from 2.0 to 7.0; however, the influence of the pH below 4.5 on free enzyme seemed to be relatively higher than that on immobilized

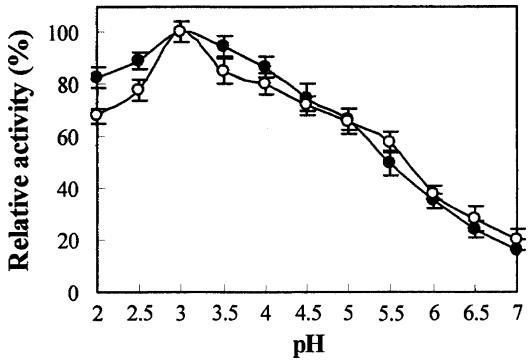

Fig. 4. Effect of pH on the activity of free and immobilized pectinase. The open circles represent free enzyme and the closed circles, immobilized enzyme. The enzyme was immobilized with 1.0 M glycidol, 0.52 mg protein/g gel, at 5 °C, pH 3.6 for 24 h. The enzyme activity at the optimum pH was considered as 100%. The data plotted correspond to the mean ± SD of three replicates.

enzyme, the optimal pH range was slightly broader after immobilization. This result differed from our previous observation that pectinase immobilized on alginate shifted its optimal pH towards the alkaline range (Li et al., 2007). The pH profile of pectinase implied that the microenvironment of the immobilized enzyme on glyoxyl-agar-gel might have been buffered and immobilized enzyme was less affected by the acidity of the solution, which might due to the structural and conformational stability of enzyme caused by multipoint attachment.

The temperature range at which the immobilized pectinase retained more than 80% of its initial activity (30–55 °C) was wider than that for free enzyme (30–45 °C); the optimal temperature of enzyme after immobilization slightly broadened and increased by 10 °C, and it was more stable than the free enzyme above 40 °C (Fig. 5). These results would be due to the stabilization by the multipoint binding between pectinase molecule and the support gel, and even at a higher temperature the immobilized pectinase could retain its active structure compared to free form. The temperature-activity curve coincided with the results of pectinase immobilized on a chitosan support (Li, Zhong, Xiao, Ge, & Guo, 2002) and formate dehydrogenase immobilized on the same activated agarose support (Bolivar et al., 2006). However, these results differed from our previous study in which the optimal temperature of 40 °C for pectinase immobilized on the alginate support remained unchanged (Li et al., 2007). The shift in the temperature for the maximum catalytic activity indicated a greater rigidity of the multi-attached immobilized pectinase molecule that was more resistant to unfolding at higher temperatures than the free form.

3.4. Thermal and storage stability of immobilized pectinase

The immobilized pectinase retained almost 100% of activity after storage for 30 days at 4 °C. Furthermore, compared to the free enzyme at 65 °C, the immobilized enzyme exhibited higher thermal stability (Fig. 6), which was represented as a 19-fold stabilization factor (expressed as the ratio of half-lives of immobilized and free enzyme). This stability level was higher than that of pectinase immobilized on alginate support, which quickly lost all activity under the same conditions (Li et al., 2007). At 50 °C, both free and immobilized enzymes were stable within 24 h; however, the immobilized enzyme exhibited more than 3.5-fold the relative residual activity compared to the free form after a 72 h incubation (Fig. 6), which was 10% higher than that measured for pectinase immobilized on alginate support under the same conditions (Li et al., 2007). The thermal stability was also higher than that of pectinase covalently immobilized on silica gel (Rao, Kembhavi, & Pant, 2000) or on Con A–Seralose 4B support by the bioaffinity layering method (Sardar & Gupta, 2005). This thermal stability behavior might be due to the protection of the immobilized enzyme from conformational changes and lower flexibility of immobilized form, due to the multi-attachments to the support (Tardioli, Zanin, & Moraes, 2006). It was said by

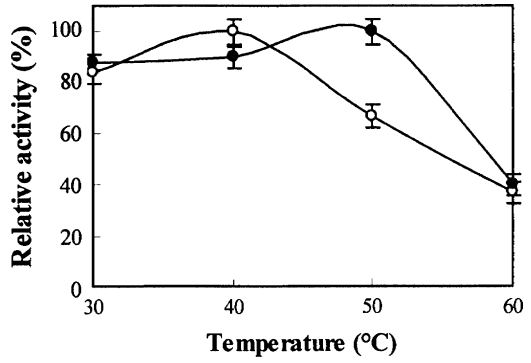

Fig. 5. Effect of temperature on the activity of free and immobilized pectinase. The open circles represent free enzyme and the closed circles, immobilized enzyme. The enzyme was immobilized with 1.0 M glycidol, 0.52 mg protein/g gel, at 5 °C, pH 3.6 for 24 h. The enzyme activity at the optimum temperature was considered as 100%. The data plotted corresponds to the mean ± SD of three replicates.

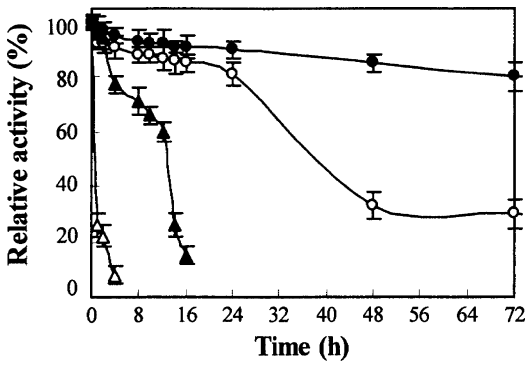

Fig. 6. Thermal stability of free and immobilized pectinase at 50 and 65 °C. Circles represent 50 °C, and triangles represent 65 °C. The open symbols represent free enzyme, and the closed symbols represent immobilized enzyme. The enzyme was immobilized with 1.0 M glycidol, 0.52 mg protein/g gel, at 5 °C, pH 3.6 for 24 h. The data plotted correspond to the mean ± SD of three replicates.

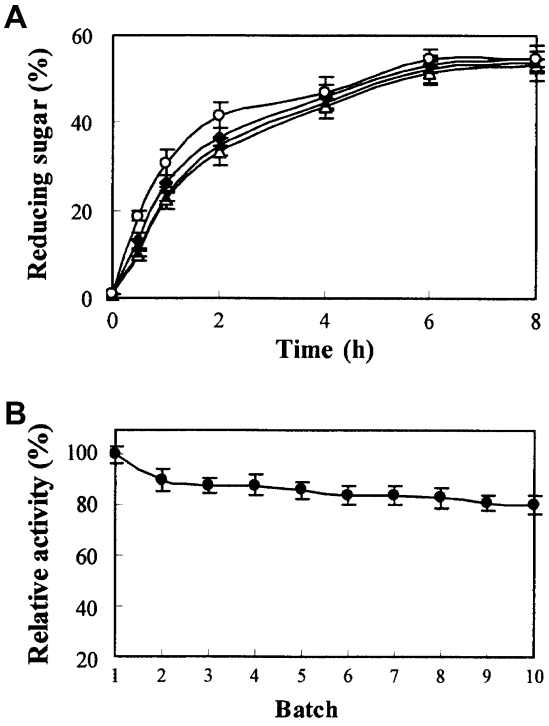

Fig. 7. Time course for pectin hydrolysis (A) and operational stability of immobilized pectinase (B). The enzyme was immobilized with 1.0 M glycidol, 0.52 mg protein/g gel, at 5 °C, pH 3.6 for 24 h. The open circles represent free enzyme, and the closed rhombus, squares and open triangles represent the first, second, and third runs of immobilized enzyme, respectively. The data plotted correspond to the mean ± SD of three replicates.

Guisán (1988) that the distortions of tridimensional structure of enzyme molecular produced as a consequence of the enzyme-support multi-interactions would be smaller than that produced by an amine-active group irreversible chemical reaction. From such literatures and our results obtained presently and previously (Li et al., 2007), it is likely that the conformation of pectinase molecular immobilized on glyoxyl-agar-gel with multi-linkages (between pectinase molecular and support) was more stable than that on glutaraldehyde activated support (alginate) as well as free form, at higher temperature.

3.5. Repeated hydrolysis of pectin and operational stability of the immobilized pectinase

Fig. 7A shows that the immobilized pectinase kept a relatively uniform velocity for the hydrolysis of pectin compared to the free enzyme. Following the first run, there was a slight decrease in the hydrolytic activity of immobilized pectinase; however, the time courses for hydrolysis in the second and third runs were almost identical, and enzymolysis was similar to that of the free enzyme (Fig. 7A). In the batch reaction repetitions, the activity of the immobilized enzyme remained stable throughout the experiment, and 81% activity was retained after consecutively repeating the reactions 10 times of 4 h at 50 °C (Fig. 7B). This value of 81% was similar to that obtained using the alginate support under the same condition (80% by Li et al., 2007), but the value was higher than that reported by Roy et al. (2003) for pectinase entrapped in alginate, which exhibited a 55% loss in activity after four batch reactions.

4. Conclusions

Agar-gel, used as a support, is easily handled and activated, and is also compressible, therefore, resistant to mild stirring devices. This study analyzed the optimal procedure for immobilization and stabilization of pectinase onto an activated agar-gel support. The optimal immobilization reaction parameters were 1.0 M glycidol for agar-gel activation, and the enzyme dose of 0.52 mg protein/g gel at 5 °C with a pH of 3.6 for 24 h. After immobilization, the optimal pH and temperature curves for enzymatic activity were slightly broadened. The immobilized enzyme exhibited higher thermal stability compared to the free form and also compared to pectinase immobilized on alginate support using glutaraldehyde as a coupling agent (Li et al., 2007). Furthermore, the immobilized pectinase exhibited a relatively uniform velocity and high operational stability for the pectin hydrolysis. Thus, this multipoint attachment strategy seemed to permit good results in terms of pectinase immobilization efficiency and stability. The results of this experiment can be used to develop a bioreactor for the production of pectic oligosaccharides and for juice defecation in industries.

Acknowledgements

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