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Preparation, characterization and catalytic behavior of pectinase covalently immobilized onto sodium alginate/graphene oxide composite beads

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ABSTRACT

Pectinase was immobilized onto sodium alginate/graphene oxide beads *via* amide bonds by using *N,N′*-dicyclohexylcarbodiimide/*N*-hydroxysuccinimide as the activating agent. The immobilized pectinase was characterized by Fourier transform infrared spectra and scanning electron microscopy analyses. Immobilization conditions were optimized by Box–Behnken design and the response surface method. The activity of the immobilized pectinase prepared under optimal conditions reached 1236.86 ± 40.21 U/g, with an enzyme activity recovery of 83.5%. The optimal pH of free pectinase was 4.5, while that of immobilized pectinase was shifted to 4.0. The optimal temperature of immobilized pectinase was increased to 60 °C, which was 10 °C higher than that of free form. Furthermore, the immobilized pectinase possessed a superior thermal stability and storage stability to those of free pectinase. Reusability studies indicated that the immobilized pectinase retained 73% of initial activity after six times cycles. Due to these good properties, such immobilized pectinase may find application in food industry.

1. Introduction

Pectinases (E.C.3.2.1.15) are a heterogeneous group of related enzymes that hydrolyze pectic polysaccharides of plant tissues into small molecules such as galacturonic acid. They have been widely used for clarification of fruit and vegetable juices, and wines (Rajdeo, Harini, Lavanya & Fadnavis, 2016). The problems encountered in the application of pectinase are enzyme recovery and recycling. In principle these issues can be counteracted by the use of immobilized enzymes (Fang, Chen, Zhang & Chen, 2016).

To date, pectinases have been immobilized onto various supports such as silica-coated chitosan (Lei & Bi, 2007), oxidized pulp fiber (Wu, He, Zhao, Qian & Li, 2013), agar–agar (Rehman, Aman, Zohra, & Ul Qader, 2014), entrapped in polyvinyl alcohol sponge (Esawy, Gamal, Kamel, Ismail & Abdel-Fattah, 2013) and alginate Rehman et al., 2013). Enzyme immobilization is usually carried out by four main methods: (i) physical adsorption, (ii) encapsulation, (iii) cross-linking, and (iv) covalent binding (Marchis, Cerrato, Magnacca, Crocellà & Laurenti, 2012). Covalent immobilization can eliminate or significantly reduce the leaching of protein (Mosafa, Shahedi & Moghadam, 2014).

Thus, exploiting novel supports and related immobilization strategy has been an attractive subject for enzyme engineering. Among such supports, the natural polymer sodium alginate (SA) has shown a range of advantages in the immobilization of enzymes due to its unique colloidal properties include thickening, stabilizing, suspending, film forming, gel producing and emulsion stabilization (Fayaz, Balaji & Girilal et al., 2009; Nie et al., 2015). SA has been successfully used as a support for the immobilization of a range of enzymes, such as levan-xylanase (Jampala et al., 2017), pectinase (Li et al., 2007), and alpha-amylase (Kumar, Muthukumar, & Garg, 2012). However, compared with conventional polymers, neat SA has some disadvantages, such as strong hydrophilic character, poor mechanical properties and low thermal stability (Alboofetileh, Rezaei, Hosseini, & Abdollahi, 2013).

Modification of polymers with inorganic materials is an effective protocol to improve their properties such as thermal conductivity and mechanical strength (Han, Yan, Chen, Li & Bangal, 2011). Graphene oxide (GO) has a large theoretical specific surface area and a large number of different types of oxygen functionalities, such as carboxyl, epoxy, and hydroxyl groups. Thus, GO can be easily activated for the conjugation with biological molecules (Ding et al., 2017). Indeed, GO has been illustrated as an ideal support for the immobilization of enzyme (Bolibok, Wiśniewski, Roszek, & Terzyk, 2017; Zhou et al., 2012).

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GO is more stable than polysaccharides. Furthermore, hydrogen bonds can form between SA and GO due to their structural properties. Thus, the complexation of SA with GO can be expected to enhance the thermal stability of SA and improve its mechanical properties (Terzopoulou, Kyzas & Bikiaris, 2015). Indeed, the improvement of the thermal stability and the mechanical strength of the GO/SA composite film when compared to the SA film has been reported (Liu, Li, & Li, 2017; Hu et al., 2016).

In the present work, pectinase was effectively immobilized on SA/GO beads via amide bonds using *N*,*N*′-dicyclohexylcarbodiimide (DCC)/*N*-hydroxysuccinimide (NHS) as the coupling agents. The prepared immobilized pectinase was characterized by Fourier transform infrared spectra (FT-IR) and scanning electron microscopy (SEM) analysis. The kinetic behavior, pH and temperature profiles together with thermal stability of the immobilized pectinase are reported.

2. Materials and methods

2.1. Materials

SA, pectinase, pectin, DCC, NHS and graphite (99.95% metals basis, ≥325 mesh) were purchased from Aladdin (Shanghai, China). All other chemicals used were of analytical reagent grade.

2.2. Preparation of graphene oxide (GO)

GO was prepared according to the method described in a literature (Chang et al., 2015). Graphite (2.0 g) was added into a mixture of concentrated H_2SO_4 (3ml), $K_2S_2O_8$ (1.0 g) and P_2O_5 (1.0 g). The mixture was stirred at 80 °C for 4.5 h before cooling down to room temperature. It was diluted with deionized water (200 ml) and allowed to stand overnight. The oxidized product was separated by vacuum filtration, washed with deionized water and dried in air. The pre-oxidized graphite powder was then re-oxidized by Hummers method (Botas et al., 2013). Pre-treated graphite powder (1.0 g) was added to a mixture of concentrated H_2SO_4 (23 ml) and $NaNO_3$ (1.0 g) at 0 °C, and completely mixed for 1 h. $KMnO_4$ (6.0 g) was added gradually with vigorous stirring and cooling on an ice-bath. The resulting mixture was stirred at 35 °C for 4 h, and then diluted with 95 ml of deionized water, keeping the temperature below 90 °C. Deionized water (280 ml) was then injected into the mixture, followed by the dropwise addition of 30% H_2O_2. The color of mixture changed to brilliant yellow. Finally, the resulting product was collected by centrifugation, washed with dilute HCl solution and water successively until the pH value of the upper layer suspension was close to 7. The GO samples were collected and dried in a vacuum desiccator.

2.3. Preparation of SA/GO composite beads

SA/GO composite beads with different mass ratio of GO to SA (0:100, 2:100, 4:100, 6:100, 8:100) were prepared by using a $CaCl_2$-hardening method with some modifications (Fei, Li, Han & Ma, 2016). Aqueous dispersions of GO (0, 0.04, 0.08, 0.12 and 0.16 g/100 ml) were prepared by suspending in 100 ml water with ultrasound for 5 h. Then, SA powder (2g) was slowly added to the suspension with stirring for 3 h. The mixture was sonicated for 30 min to allow the complete blending. The resulting mixture was then added drop by drop into $CaCl_2$ solution (2.0 M, 45 ml) using a syringe, and allowed to stand at 4 °C overnight. After washing with deionized water six times and drying in a vacuum oven at 50 °C until the constant weight, the SA/GO composite beads were obtained.

2.4. Immobilization of pectinase onto SA/GO beads

Prior to the immobilization of pectinase, SA/GO beads were activated by DCC/NHS according to a reported method (Kemikli, Kavas, Kazan, Baykal & Ozturk, 2010). DCC (1.03 g) and NHS (0.575 g) were dissolved in *N*,*N*-dimethylformamide (10 ml), and SA/GO beads (1 g) with different mass ratios of GO to SA (0:100, 2:100, 4:100, 6:100 and 8:100) were added. The reaction mixtures were stirred at room temperature for 12 h, triethylamine (1 ml) was then added dropwise. After completion of the reaction, the mixture was filtered and the beads were washed with ethanol/cyclohexane (50 ml/50 ml) to remove unreacted reagents. Finally the SA/GO beads were dried under vacuum at 45 °C for 12 h.

Pectinase was immobilized onto the activated SA/GO beads with different mass ratios of GO to SA using the following method. The activated SA/GO beads (1 g) were added to a pectinase solution (10 ml, 10 mg/ml in 0.25 M citrate buffer, pH 3.0). The mixture was shaken at a rate of 120 rpm at 30 °C in a water bath for 30 min. Then the beads were washed with distilled water until no enzyme was detected in the washing liquid. The immobilized enzyme was stored at 4 °C. All the preparations were made in triplicate.

According to a single factor test, DCC/NHS-activated composite material with a GO to SA ratio of 4:100 was used for the immobilization of pectinase in the following investigation (details were presented in Section 3.1, Fig. S1).

The immobilization conditions for pectinase onto the activated SA/GO beads, including four independent variables enzyme concentration (mg/ml), temperature (°C), time (min) and pH value, were further optimized using response surface methodology (RSM). On the basis of preliminary studies, a Box–Behnken design (BBD) was used to survey effects of independent variables at three levels on the dependent variable (activity of the immobilized pectinase). A total of 29 randomized experiments were designed (Table 1).

Table 1
Experimental design and results of Box-Behnken design.[a]

Run	Variable level[b]				Activity of immobilized enzyme[c]	
	A	B	C	D	Determined	Predicted
1	10.0	2.5	25	30	859.1 ± 22.68	855.50
2	10.0	3.0	30	30	1251.4 ± 41.22	1244.39
3	12.0	3.0	35	30	941.88 ± 36.21	956.49
4	10.0	3.0	30	30	1224.68 ± 45.32	1244.39
5	10.0	3.5	35	30	867.83 ± 33.11	873.24
6	12.0	3.5	30	30	885.1 ± 36.76	879.47
7	8.0	3.0	25	30	885.32 ± 28.99	879.24
8	8.00	2.5	30	30	789.91 ± 27.46	797.96
9	12.0	3.0	30	25	1015.56 ± 41.12	1006.76
10	12.0	3.0	25	30	952.58 ± 37.65	962.08
11	10.0	3.0	30	30	1238.64 ± 47.65	1244.39
12	10.0	3.0	35	25	982.11 ± 40.53	981.62
13	8.0	3.0	30	25	928.01 ± 39.72	920.02
14	10.0	3.5	30	25	895.62 ± 35.67	903.28
15	10.0	3.0	25	35	979.74 ± 29.89	982.65
16	10.0	2.5	30	35	899.18 ± 31.67	900.05
17	10.0	3.0	25	25	1005.44 ± 33.65	1001.95
18	8.0	3.0	30	35	921.84 ± 36.98	919.70
19	10.0	3.5	35	30	856.26 ± 25.66	849.92
20	10.0	3.0	35	35	986.22 ± 41.21	992.13
21	10.0	2.5	35	30	838.55 ± 30.31	826.44
22	10.0	3.0	30	30	1264.76 ± 45.62	1244.39
23	8.0	3.0	35	30	874.96 ± 39.64	873.99
24	10.0	3.0	30	30	1242.48 ± 47.65	1244.39
25	12.0	2.5	30	30	834.32 ± 25.87	837.60
26	8.0	3.5	30	30	754.64 ± 29.76	753.78
27	10.0	3.5	25	30	830.34 ± 31.63	831.11
28	10.0	2.5	30	25	875.67 ± 28.97	878.79
29	12.0	3.0	30	35	1021.25 ± 35.76	1008.30

Note:
[a] The activity of the free enzyme is 14,465 ± 39 U/g.
[b] A, B, C, and D represent enzyme concentration (mg/mL), immobilization pH, reaction temperature (°C), and reaction time (min), respectively.
[c] In U/g beads.