Green Synthesis of Porous Spherical Reduced Graphene Oxide and Its Application in Immobilized Pectinase

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ABSTRACT: Pectinase is widely used in juice production, food processes, and other fields. However, owing to poor stability, free pectinase is difficult to separate from a substrate after hydrolysis and cannot be reused, and thus its industrial use is limited. Immobilized pectinase can solve these problems well. We prepared a carrier material of immobilized enzyme, which is called porous spherical reduced graphene oxide (rGO) with a rich pore structure, large specific surface area, strong hardness, and good biocompatibility to enzyme. Then, we evaluated the performance of the porous spherical rGO immobilized pectinase and characterized its structure by IR, XRD, and SEM. Using this material as a carrier of immobilized enzyme improves the load and catalytic activity of the enzyme. After 10 times of continuous use, the porous spherical rGO immobilized enzyme still maintained its initial relative enzyme activity at around 87%, indicating that immobilized pectinase had a stronger cycling stability, and its thermal stability, acid−base tolerance, and storage stability were superior to those of free pectinase. The results were compared with those of other studies on immobilized pectinase. The relative activity of pectinase immobilized by porous spherical rGO was at a high level after 10 consecutive uses. Overall, the spherical rGO is an excellent immobilized enzyme carrier material.

1. INTRODUCTION

Pectinase is widely used in food processing, medicine production, paper making, textile and feed production, environment conservation, and other fields and is the most widely used type of biocatalysts in various industries.1,2 However, free pectinase has poor stability during hydrolysis and is easily deactivated, and the reaction process is uncontrollable. Moreover, it is difficult to separate from a reaction substrate, cannot be reused, and increases the production cost.3 These disadvantages hinder its use in industrial production. Enzyme immobilization is an effective method for solving these problems.4 The nature of a carrier determines the efficiency of an immobilized enzyme. An ideal immobilized enzyme carrier requires a large specific surface area, improves the load of enzymes, and has good biocompatibility. It has lasting enzyme activity and strong mechanical properties resulting in stability.5 Given that it can be easily separated from a substrate, substrate contamination is prevented. A highly efficient immobilized enzyme can be easily separated from a reaction system and thus simplifies the purification process, facilitates the continuous and automatic operation of enzyme-catalyzed reactions, and significantly reduces production cost.

Carbon materials are considered to have great development potential due to their advantages, such as good environmental stability, large specific surface areas, and uniform pore structure distribution. These materials include carbon fibers,6 carbon hollow microspheres,7 carbon nanotubes,8 graphene oxide (GO),9 and reduced graphene oxide (rGO).10−12 They have a wide range of practical applications in various fields, such as energy storage,13 catalysis,14 sensing, and separation.15 rGO has obvious practical value in electrocatalysis,16 catalyst loading,
sewage treatment,\textsuperscript{17} and dye adsorption\textsuperscript{18} owing to its good adsorption property.

With the progress of the reduction reaction, the oxygen-containing functional groups decrease continuously, which make it harder for rGO to bind to the reaction substrate and effectively prevent agglomeration caused by electrostatic effect in the reaction process. Moreover, rGO is the hardest material discovered at present. It has excellent mechanical properties, porosity, large specific surface area, and relatively stable characteristics, which make it have a broad application prospect in subsequent industrial production.\textsuperscript{19} However, at present, the synthesized rGOs mostly exist in the form of light flakes in the catalytic reaction, floating on the surface of the solution and unable to fully combine with the reaction solution, which is not conducive to the catalytic reaction.\textsuperscript{20,21}

For these problems, external and internal structures can be changed to a certain extent to spherical structures with abundant pores, large specific surface areas, high enzyme loads, fast separation speed, high separation efficiency, and strong mechanical properties.

In this paper, a porous spherical rGO was prepared through a green reduction reaction using ascorbic acid as the reductant based on the preparation of GO in the previous stage in this laboratory. The prepared rGO showed structures with abundant pores, large specific surface areas, high enzyme loads, fast separation speed, and high separation efficiencies. It was used as a carrier material to immobilize pectinase. Moreover, the reusability, catalytic performance, carrying capacity, and stability of an immobilized enzyme were evaluated through a series of studies. The porous spherical rGO is a promising material for repeated enzymatic hydrolysis reactions.

2. RESULTS AND DISCUSSION

2.1. Structural Characterization of Porous Spherical rGO. In this study, GO was synthesized using the Hummers—Offeman method, and porous spherical rGO was obtained through the thermal reduction of ascorbic acid. Pectinase was loaded on the porous spherical rGO mainly through physical adsorption and covalent binding.

Morphology was characterized using field emission scanning electron microscopy (FESEM; Hitachi S-4800). The morphology of the GO, porous rGO, and porous spherical rGO at different reaction times are shown in Figure 1. As shown in the graph, reaction time has an obvious effect on the morphology of the porous spherical rGO. During the thermal reduction process of ascorbic acid, a large number of bubbles were generated in the solution under the action of heat flux. The strong bubble interference force promoted the interaction between the rGO flakes, resulting in a certain degree of self-assembly, forming a loose and porous 3D structure, and greatly improving the adsorption performance.\textsuperscript{22} By controlling the action time of heat flow, good mechanical properties and adsorption properties were achieved by adjusting the aperture, size, and 3D structure. This method not only increases the load...

![Figure 1. SEM images of the (a) GO, (b) porous rGO 1 h, (c) porous rGO 2 h, (d) porous rGO 3 h, (e–g) porous spherical rGO.](https://dx.doi.org/10.1021/acsomega.0c05078)
of the enzyme but also improves its mechanical strength and makes it more reusable.\(^6\)

The unreduced GO showed a typical flaky morphology (Figure 1a). When the reduction reaction time was 1 h (Figure 1b), the rGO flakes began to self-assemble initially, and small stacks appeared on the surface. When the reduction reaction time was 2 h (Figure 1c), the size of the product increased, and the 3D structure was not obvious after self-assembly. When the reduction reaction time was extended to 3 h, the 3D structure of the product became increasingly obvious, and irregular pores appeared (Figure 1d). When the reduction reaction time was extended to 4 h (Figure 1e), the unique 3D porous spherical microstructure was clearly observed. In the magnified microstructure (Figure 1g), flexible rGO flakes partially overlapped or coalesced, resulting in rich porous structures within them. The morphology and pore structure of the self-assembled rGO were changed mainly by controlling heat flow during boiling. Large heat flows led to persistent bubble nucleation on the surface, which formed self-assembled graphene as water evaporated.

The molecular structure and composition of bonds were examined by Fourier transform infrared spectroscopy (FT-IR; Bruker Vector 22, 4000–500 cm\(^{-1}\)). As shown in Figure 2,

![Figure 2](image)

**Figure 2.** FT-IR spectra of the GO, porous spherical rGO, and graphite.

the typical peaks of GO were observed at 3381 cm\(^{-1}\) (O–H stretching vibration), 1728 cm\(^{-1}\) (C=O stretching vibrations), 1620 cm\(^{-1}\) (C=C stretching of aromatic rings), 1368 cm\(^{-1}\) (–OH stretching vibrations), 1223 cm\(^{-1}\) (C–O–C vibrations of epoxy groups), and 1053 cm\(^{-1}\) (C–O stretching vibrations).\(^{23}\) At increased reduction reaction times, the peak intensities of the above oxygen-containing functional groups for rGO decreased gradually.

The crystal structure was examined by X-ray diffraction (XRD; Ultima IV, 40 kV, 150 mA, Cu Kα). As shown in Figure 3, compared with GO, which showed a sharp peak at 2θ of 11.8°, the porous spherical rGO showed a broad weak characteristic peak at 2θ of 26°, which was caused by the decrease in the interlayer spacing of rGO.\(^{24}\) This result demonstrates that the oxygen-containing functional groups were effectively eliminated, and the rGO sheets underwent long-range restacking after reduction by ascorbic acid without annealing.

**2.2. Structural Characterization of Porous Spherical rGO Immobilized Pectinase.** In order to fully explain the results of pectinase immobilization, we performed FT-IR comparison between porous spherical rGO and porous spherical rGO immobilized pectinase (FT-IR; Bruker Vector 22, 4000–500 cm\(^{-1}\)). As shown in Figure 4, the typical peaks of porous spherical rGO immobilized pectinase were observed at 1654 cm\(^{-1}\) (–C=–N stretching vibration), 1542 cm\(^{-1}\) (–C=O stretching vibrations), and 1140 cm\(^{-1}\) (C–O–C asymmetrical stretching vibration, an acyl transition state).\(^{25–27}\) This fully elucidates the specific amino structure of the enzyme molecule introduced by immobilization.

The morphology and internal microstructure of porous spherical rGO and porous spherical rGO immobilized pectinase were analyzed by FESEM. As shown in Figure 5, the surface of the porous spherical rGO had a clear lamellar structure and obvious pore structure. By contrast, after loading with pectinase, pores on the porous spherical rGO surface were significantly reduced and presented a granular-layered structure, indicating that pectinase was fixed to the porous spherical rGO (Figure 5b).

As can be seen from Figure 5, porous spherical rGO has a rich pore structure and large pore size. Because the particle size of the enzyme molecule is about 2–50 nm, we adjusted the pore size of porous spherical rGO so that the enzyme molecule can be better immobilized on the carrier. During the preparation of porous spherical rGO, heat flow will directly affect the size of the bubbles generated in the solution, the strong bubble interference force promoted the interaction between the rGO flakes. Therefore, we adjusted the pore structure of the porous spherical rGO by controlling heat flow,\(^22\) so as to obtain a structure with an appropriate pore structure and good mechanical properties, which can be better
applied in the subsequent immobilization process. In the immobilization process, a large amount of pectinase can be loaded. However, the pectinase molecules adsorbed only by static electricity are easy to fall off in the subsequent use. In

Figure 5. SEM analysis of (a) porous spherical rGO and (b) porous spherical rGO immobilized pectinase.

Figure 6. The effect of immobilized parameters on enzyme activity: (a) pH; (b) incubation time; (c) enzyme concentration; (d) glutaraldehyde concentration; and (e) temperature.
order to improve this problem, glutaraldehyde was used as a cross-linking agent to make it bond with the amino group in the enzyme molecule and the remaining oxygen-containing group on porous spherical rGO, thus making the adsorption more stable. FT-IR data have verified that the above bond reactions occurred. It was further proved that the enzyme molecules were bonded to the porous spherical rGO to form a more stable structure (Figure 4).

2.3. Optimization of the Immobilization Conditions.
The time on immobilization, pH, reaction temperature, and concentration of pectinase were included in the analysis of the immobilization parameters.

As shown in Figure 6a, under the conditions of a temperature of 45 °C, incubation time of 4 h, enzyme concentration of 5.0 U/L, and glutaraldehyde concentration of 0.05 mg/mL, the effect of the pH was investigated from 3.0 to 6.0, and the optimal amount of pH was 4.0. When pH was greater than 4.0, protein molecules were denatured to a certain extent with the gradual increase of solution acidity, resulting in the loss of enzyme biological activity.28,29

As shown in Figure 6b, when the optimal pH was 4.0 and the other experimental conditions were the same as Figure 6a, the effect of incubation time was investigated from 0.5 to 5.0 h. With the extension of immobilization time, the enzyme load in the carrier material increased gradually in the early stage of adsorption, and thus relative enzyme activity increased gradually. After more than 4 h, the enzyme molecules in the pores of the carrier were unable to make complete contact with the substrate because of over accumulation, leading to gradual decrease in enzyme activity.30

As shown in Figure 6c, when the optimal incubation time was 4.0 h, the other experimental conditions were the same as Figure 6b. The effect of the enzyme concentration was investigated from 4.2 to 5.8 U/L, and the optimal amount of enzyme was 5.0 U/L. The enzymatic activity increased substantially while enzyme concentration increased from 4.2 to 5.0 U/mL, further increasing the consistence did not lead to further growth in terms of enzymatic activity, this may be due to the carrier material space adsorption supersaturation, and thus relative enzyme activity gradually had no change.31

As shown in Figure 6d, when the optimal dosage of enzyme was 5.0 U/L, the other experimental conditions were same as Figure 6c. The effect of glutaraldehyde concentration was investigated from 0.01 to 0.06 mg/mL. In a range of 0.01—0.05 mg/mL, the relative activity of the enzyme increased with glutaraldehyde concentration. The possible reason is that the aldehyde group of glutaraldehyde bound to the amino group of the enzyme molecule to form a Schiff base and a stable structure. Above 0.05 mg/mL, relative enzyme activity decreased rapidly. This effect may be due to the high concentration of glutaraldehyde solution, which resulted in the alkylation of hydroxyl and carboxyl groups on the enzyme molecule and subsequent inactivation of the enzyme molecule.32

As shown in Figure 6e, when the optimal dosage of glutaraldehyde was 0.05 mg/mL, the other experimental conditions were same as Figure 6d. The effect of the temperature was studied from 30 to 60 °C, and the optimal amount of temperature was 45 °C. When it goes over 45 °C, the relative activity of pectinase gradually decreases, this may
be caused by the gradual change of enzyme spatial structure and the gradual loss of enzyme activity caused by temperature.33

2.4. Stability Experiments of Free and Immobilized Pectinase. To illustrate the performance of porous spherical rGO immobilized pectinase, we studied the relative activity of free and immobilized enzymes under certain conditions. The effect of pH on the relative activity of immobilized pectinase was assayed with free pectinase as control in a pH range of 3.0–6.0 (Figure 7a). The initial optimal pectinase relative activity was defined as 100%. The optimal pH value for the free and immobilized pectinases was 4.0. In addition, in a pH range of 3.0–6.0, the relative activity of immobilized pectinase was greater than that of free pectinase. It means that the scope of pH stability of immobilized pectinase is significantly expanded.34

The optimum temperature for free and immobilized pectinases is shown in Figure 7b. The optimum temperature for both types of pectinase was 45 °C. The relative activity of immobilized pectinase was higher than that of free pectinase in a temperature range of 40–60 °C. The results showed that immobilized pectinase has better thermal stability than free pectinase.5

The reusability of the immobilized pectinase is an important characteristic for potential industrial application. As shown in Figure 7c, immobilized pectinase maintained approximately 87% of its original relative activity after 10 consecutive operations. After repeated use, the activity decreased. The probable reason is that some enzyme molecules fell off from the carrier, resulting in the falling off of pectinase from the surface of the carrier.

The free and immobilized pectinases were stored at 4 °C for 30 days, and relative enzyme activity was measured daily for five days. The immobilized pectinase retained approximately 60% of its initial relative activity after 30 days, whereas the free pectinase only exhibited 20% of its initial relative activity. These results indicated that the immobilization with porous spherical rGO improved the storage stability of pectinase (Figure 7d).

2.5. Kinetic Parameters. The kinetic resolution experiments were conducted under the conditions of pH 4.0, temperature 45 °C, reaction time 4 h, enzyme concentration 5.0 U/L, and glutaraldehyde concentration 0.05 mg/mL. Under the above conditions, the enzyme activity of immobilized pectinase in porous spherical rGO was the highest (8572.63 U/mg), and the immobilization rate was 94.5% (calculated by eq 1).

Under the above conditions, according to eq 3 in Section 4.7, the \( K_m \) and \( V_{\text{max}} \) of free pectinase were 10.26 and 0.49 mg/mL/min, respectively, whereas those of immobilized pectinase were 13.82 and 0.25 mg/mL-min, respectively. The increase in \( K_m \) value after immobilization indicated the reduction of affinity between pectinase and the substrate. This result may be due to the steric hindrance effect of the immobilized carrier material on the active site of the enzyme molecule. The effect may have hindered enzyme activity to a certain extent. Although the effect of steric hindrance had a certain effect on the binding of the immobilized pectinase to the substrate, the immobilized pectinase was more stable than the free pectinase and can be reused.35 The decrease in \( V_{\text{max}} \) may be attributed to the inability of the substrate to bind to the active sites of the immobilized pectinase because of increased diffusion limitation.36

The activation energy \((E_a)\) of the free and immobilized pectinases was calculated using the Arrhenius formula. By using \(1000/T \) (K) as the x coordinate and \(\ln k\) (logarithm of % residual activity) as the y coordinate, the regression equations of the free and immobilized pectinases were \(y = 4.15x - 0.109\) and \(y = 3.05x - 0.369\), respectively (Figure 8). The slope of the equation was \(E_a/R\). Hence, we used this formula to determine \(E_a \) (eq 4 in Section 4.7). The result showed that the \(E_a\) of the free and immobilized pectinases shifted from 34.50 to 25.35 kJ mol\(^{-1}\), which led to a higher catalytic efficiency of the immobilized pectinase.

To reflect the performance of the immobilized pectinase prepared in this study, we compared the enzyme activity of porous spherical rGO immobilized pectinase with that of other immobilized enzymes. The results are shown in Table 1. When the studies were all about immobilized pectinase, the residual relative enzyme activity of other studies was lower than that of the immobilized pectinase prepared in this paper. However, the residual relative activity of Fe\(_3\)O\(_4\)-reduced graphene oxide immobilized laccase after 10 cycles was higher than that reported in the present study.37 Therefore, the performance of the immobilized pectinase prepared in this paper is better than that of the most immobilized enzymes.

![Figure 8. Arrhenius plots for the free and immobilized pectinases.](image)

| carrier                  | types of enzymes | cycles | residual relative activity | refs |
|--------------------------|------------------|--------|----------------------------|------|
| 1 porous spherical rGO   | pectinase        | 10     | 84%                        | current work |
| 2 UiO-66-NH\(_2\)@PMAA   | pectinase        | 8      | 81%                        | 31   |
| 3 Fe\(_3\)O\(_4\)@SiO\(_2\)–NH\(_2\)  | pectinase      | 7      | 64.4%                      | 1    |
| 4 calcium alginate       | pectinase        | 3      | 63%                        | 29   |
| 5 alginate/gelatin/calcium oxalate | pectinase | 10 | 40%                         | 2    |
| 6 alginate/calcium oxalate | pectinase   | 8      | 40%                        | 2    |
| 7 alginate/water         | pectinase        | 7      | 40%                        | 2    |
| 8 1,3,5-triazine-functionalized silica-encapsulated magnetic nanoparticles | xylanase | 10 | 55%                         | 38   |
| 9 Fe\(_3\)O\(_4\)-reduced graphene oxide | laccase | 10 | 92.6%                      | 37   |
3. CONCLUSIONS

In order to further improve the application value of immobilized pectinase in industrial production, we prepared a porous spherical rGO immobilized pectinase with excellent adsorption performance and investigated its properties. The optimal pH of immobilized pectinase was 4.0, concentration of glutaraldehyde cross-linking agent was 0.05 mg/mL, temperature was 45 °C, immobilization time was 4 h, and enzyme concentration was 5.0 U/L. Furthermore, the immobilized pectinase possessed thermal stability and pH tolerance that were superior to those of free pectinase. The immobilized pectinase relative activity remained at approximately 60% after 30 days of storage at 4 °C, whereas only 20% free pectinase remained. The residual relative enzyme activity of the immobilized pectinase was higher than that of free pectinase. This result shows that the immobilized pectinase has better storage stability than free pectinase. Relative activity remained 87% after 10 consecutive operations. Owing to these good properties, immobilized pectinase has potential applications to the food industry and shows promising application potential for enzymatic catalysis in the juice production industry.

4. MATERIALS AND METHODS

4.1. Chemicals. Flake graphite of 325 mesh size was provided by Shanghai Macklin Biochemical Co. LTD. Ascorbic acid was purchased from Tianjin BaiShi Chemical Co. LTD. Sulfuric acid, hydrochloric acid, potassium permanganate, and hydrogen dioxide were provided by Tianjin Zhiyuan Chemical Reagent Co. Ltd. All chemicals were of analytical grade and used without any purification. The ultrapure water was used throughout the experiments.

4.2. Preparation of GO and Porous Spherical rGO.

4.2.1. Preparation of GO. GO was obtained from graphite using the Hummers–Offeman method and improved Hummers–Offeman method.

4.2.2. Preparation of Porous Spherical rGO. Porous spherical rGO was obtained by the Shanshan Wang method and the experimental conditions were optimized. The steps were as follows: 30.00 mL of GO (1 mg/mL) aqueous dispersion was sonicated for 60 min. Ascorbic acid (0.1500 g) was then added and sonicated for another 10 min. The mixture was transferred to a four-neck flask and subjected to continuous stirring at 90 °C and different times (from 0.5 to 4 h). The precipitant was then collected and washed with deionized water and freeze-dried.

4.3. Immobilization of Pectinase onto Porous Spherical rGO. A certain amount of porous spherical rGO was added to a citric acid buffer, and different concentrations of enzymes and glutaraldehyde cross-linking agents were added. The immobilized reaction was carried out at a specific temperature and time, and unabsorbed pectinase was removed by washing with buffer three times. The immobilized pectinase was stored at 4 °C before use.

4.4. Immobilized Pectinase Activity Assay. The activities of immobilized pectinase were determined by the 3,5-dinitrosalicylic acid method. A total of one unit of pectinase activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of reducing sugar per minute under the described conditions.

4.5. Optimization of the Immobilization Conditions. A total of 0.0500 g of porous spherical rGO was added to 2.00 mL of citrate buffer (pH 3.0–6.0) containing different enzyme concentrations (4.2–5.8 U/L) and different concentrations of glutaraldehyde cross-linking agent 1.00 mL (0.01–0.06 mg/mL). Immobilization reaction was carried out at a specific temperature (30–60 °C) for a specific time (0.5–5 h). Subsequently, the beads were washed three times with a buffer solution for the removal of unabsorbed pectinases. The immobilized pectinase was stored at 4 °C before use. The immobilization yield was calculated using eq 1.4

\[
\text{yield of immobilization(%) = } \frac{\text{immobilized pectinase activity}}{\text{free pectinase activity}} \times 100
\]  

4.6. Stability Experiments of Free and Immobilized Pectinase. A total of 1.0000 g of immobilized pectinase was added to 2.00 mL of pectin solution of different pHs (pH 3.0–6.0). The immobilization reaction was carried out at a specific temperature (30–60 °C) for 30 min. The storage stability of the immobilized pectinase was evaluated by calculating the residual percentage of the enzymatic activity of each measurement stored at 4 °C for a time period. The recycling stability of immobilized enzyme was evaluated by measuring the activity of immobilized enzyme during recycling.

In all the stability experiments, the highest activity of each experiment under optimal conditions was used as a reference and defined as 100%. Relative activity was calculated using eq 2.

\[
\text{relative activity(%) = } \frac{\text{activity at specific condition}}{\text{activity at optimal condition}} \times 100
\]  

4.7. Kinetic Parameters. To study the rates of reactions catalyzed by enzymes and various factors affecting reaction rate, we use the kinetics of enzyme-catalyzed reactions for further investigation. The Michaelis–Menten kinetics (K_m) value signifies substrate affinity toward an enzyme. Substrate affinity is the ability of an enzyme to bind to a substrate and directly affects the catalytic efficiency of the enzyme. A high K_m value implies that an enzyme has low affinity toward its substrate. Maximum reaction velocity (V_max) is the maximum reaction velocity obtained at an infinite substrate concentration. V_max and K_m were used in calculating the enzymatic activities of free and immobilized pectinase at different concentrations of pectin solution (0.2–2.0 g/mL). To explore the influence of a substrate on the rate of enzymatic reaction, we evaluated this influence with classical Michaelis–Menten kinetics.

\[
V = \frac{V_{\text{max}}[S]}{K_m + [S]} \times 100
\]  

Where V (mg/mL-min) is the initial reaction rate, [S] (mg/mL) is the pectin concentration, V_max (mg/mL-min) is the maximum reaction velocity obtained at infinite substrate concentration, and K_m (mg/mL) is the Michaelis–Menten constant.

To elucidate the influence of other reaction conditions on enzymatic reaction, we use the Arrhenius equation for further investigation. It is an empirical formula for the relationship between reaction rate constant and temperature. The relationship between reaction rate constant at different temperatures and corresponding temperature can be obtained. The E_a values of the free and immobilized pectinases were calculated from

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the Arrhenius formula. It can be seen that the drawing of ln $k/T$ is a straight line with slope $E_a/RT$, as given in the following equation:\(^{(4)}\)

\[
k = Ae^{-E_a/RT}
\]

(4)

$R$ is the gas constant (8.314 J·mol\(^{-1}\)·K\(^{-1}\)).

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### Notes

The authors declare no competing financial interest.

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