Catalysis Letters (2022) 152:28–42
https://doi.org/10.1007/s10562-021-03631-7

Preparation of a Calcium Alginate-Coated Polypyrrole/Silver Nanocomposite for Site-Specific Immobilization of Polygalacturonase with High Reusability and Enhanced Stability

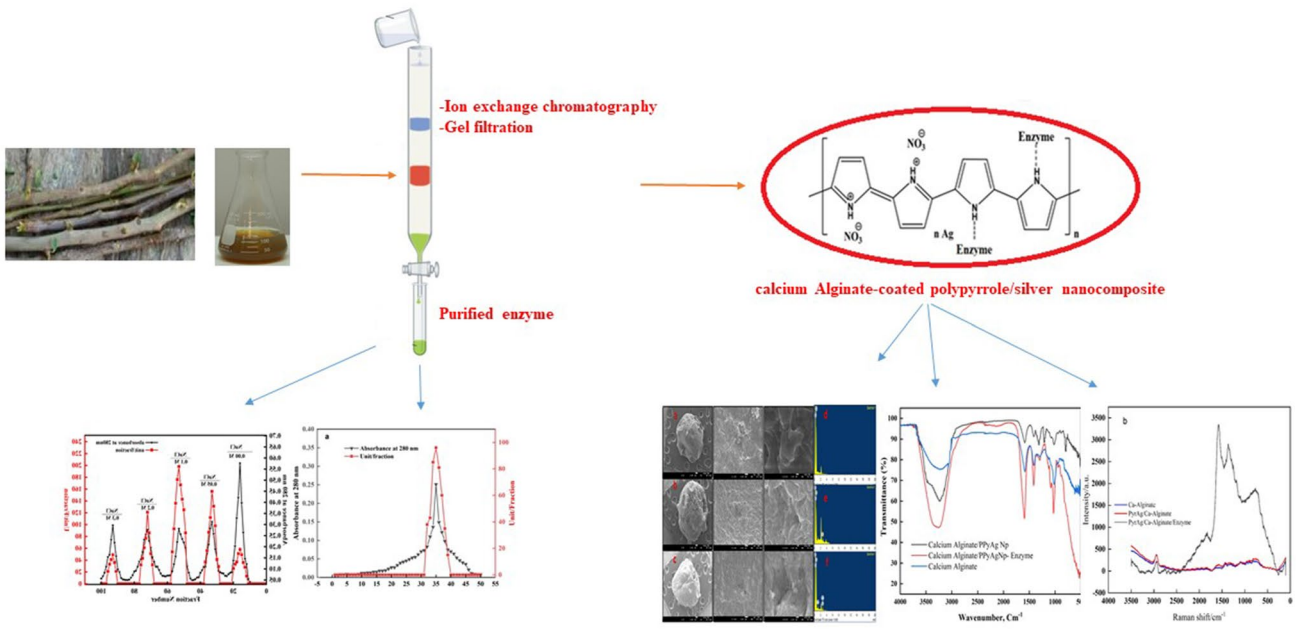
Yaaser Q. Almulaiky[1,2] · Sami A. Al-Harbi[3]

Received: 11 February 2021 / Accepted: 10 April 2021 / Published online: 19 April 2021

Abstract

Polygalacturonase (PG) catalyses the hydrolysis of pectin substances and is commonly used in the textile and food industries. Herein, PG was purified from Arabian balsam using three techniques (ammonium sulfate precipitation, ion exchange chromatography, and gel filtration) with a recovery of 11.2% and tenfold purification. The molecular weight of the purified PG was estimated to be 75.5 kDa using a Sephadex G-150 column. To improve the stability and reusability of the purified enzyme, a novel method to immobilize PG through calcium alginate-coated polypyrrole/silver nanocomposite was described. The immobilized PG was characterized by FTIR, TGA, SEM, EDX, and Raman spectroscopy. The immobilization efficiency was 84.4%. Excellent long-term storage stability of the immobilized PG was demonstrated with 83% of the initial activity preserved after 60 days. The immobilized PG was highly reusable, showing high activity (91% and 68%) after five and ten cycles. The immobilized PG showed improved stability to temperature and pH relative to that of the free enzyme. The K_m and V_{max} were determined to be 0.368 mg/mL and 5.33 µmol/mL for the immobilized PG and 0.667 mg/mL and 7.38 µmol/mL for free PG, respectively. Improved storage stability, catalytic efficiency (V_{max}/K_m), and reusability of the immobilized PG make it ideal for biotechnological and industrial applications.

Graphic Abstract



Extended author information available on the last page of the article

Keywords Polygalacturonase · Arabian balsam · Immobilization · Purification · PPyAgNp/Ca-alginate · Reusability

Abbreviations

FTIR Fourier transform infrared spectroscopy
TGA Thermogravimetric analysis
SEM Scanning electron microscopy
EDX Energy-dispersive X-ray spectroscopy

1 Introduction

Enzyme technology is increasingly used to meet various human needs. Pectinolytic enzymes are widely used in industry to improve fruit juice production, fruit texture, and enzymatic fruit peeling [1–3]. Enzymes have been used in the scouring of cotton and textiles as well as in the fermentation of coffee and tea [4, 5]. Pectinases are classified as de-esterifying and depolymerizing enzymes, depending on the degradation mechanism. De-esterifying enzymes are used to de-esterify the methoxy group of pectic acid, while depolymerizing enzymes cleave α-1,4 glycosidic bonds in pectin either by trans-elimination (pectin lyases) or by hydrolysis (polygalacturonases) [6, 7]. The biological decomposition of pectin is one of the most essential biomass degradation mechanisms [8]. Pectinolytic enzymes are generally known to hydrolyse pectinolytic substances [9]. The long and recalcitrant molecule pectin, which often serves as a structural polysaccharide in the middle lamella and cell walls of plants, is hydrolysed by these enzymes [4]. Polygalacturonase (pectinase, EC. 3.2.1.15 is one of the most common enzymes in fungi [10, 11], bacteria [12] and plants [13], and it hydrolyses the α-1-4-glycoside bond between residues of galacturonic acid to release D-galacturonic acid oligomers. This enzyme makes up 25% of enzyme sales in the food industry worldwide [4]. Notwithstanding their excellent catalytic characteristics, native enzymes as biocatalysts always have some disadvantages, such as poor stability under intensive operating conditions, difficulty in the recovery of products, and non-reusability in industrial processes [14]. While important advances have already been made in biotechnology, native biocatalysts face several challenges, such as a high cost, low stability, and poor reusability [15, 16]. To counteract these difficulties, enzyme immobilization has been used to improve the catalytic properties of the enzymes to prevent denaturation and to make them economically viable for several applications [17–20]. The use of immobilization to increase the stability and recyclability of enzymes has been shown to be beneficial [21]. Alginate, one type of polysaccharide, is generally used as a biopolymer in a variety of biomedical applications, including cell culture, drug release, tissue engineering, and enzyme or metal stabilization for catalysts [22, 23]. Due to their mild reducibility, excellent biocompatibility and low cost, alginate hydrogels have proven to be good stabilizers and synthesis templates for metal nanoparticles such as Ag, Ni, Co, and Fe [24, 25]. In this study, polygalacturonase was purified from Arabian balsam and immobilized on PPyAgNp/Ca-alginate. Physico-chemical characterization of purified and immobilized enzymes was performed. To the best of our knowledge, this is the first time that PG has been purified from Arabian balsam, immobilized, and assessed in terms of reusability and stability characteristics.

2 Materials and Methods

2.1 Chemicals and Techniques

2.1.1 Chemicals

Sephadex G-150, polygalacturonic acid (PGA), pyrrole, silver nitrate, sodium alginate, and DEAE-Sepharose were purchased from Sigma-Aldrich (USA). Other solvents used in our study were of analytical grade.

2.1.2 Plant Collection

Commiphora gileadensis also known as "Arabian balsam", was used in our study and grows widely in Saudi Arabia especially in Makkah. Plant specimen collection was performed in April, 2020.

2.2 Polygalacturonase Purification

2.2.1 *C. gileadensis* Stem Peel Extract

Ten grams of plant stem peel specimens were collected and crushed into fine pieces using a mortar. The stem peel pieces were soaked in Na-acetate buffer (20 mM, pH 5.5), and crushed in a mortar again until totally smashed. The whole extract was filtered, the filtrate was centrifuged for 10 min at $1118 \times g$ and the supernatant was pipetted into a clean and dry container to be used in further steps.

2.2.2 *C. gileadensis* Polygalacturonase Precipitation

The protein fraction containing polygalacturonase was separated from the *C. gileadensis* peel extract by the ammonium sulfate precipitation method. Different weights of solid ammonium sulfate, to obtain 20 to 80% saturation, were

added to the peel extract in a stepwise manner to dissolve and separate the polygalacturonase at 0.0 °C. The activity of the polygalacturonase was examined gradually to achieve the highest enzyme activity upon ammonium sulfate saturation. Highly active polygalacturonase was refined at 40% saturated ammonium sulfate and then centrifuged at 1118 × *g* for 20 min. The precipitated polygalacturonase was separated and resuspended in a few millilitres of Na-acetate buffer (20 mM, pH 5.5) for further enzyme purification. The suspended sample was dialyzed in the same buffer for 12 h to remove the excess ammonium sulfate and then centrifuged at 1118 × *g* for approximately 20 min. The partially purified polygalacturonase was refrigerated at −20 °C to be used in ion-exchange chromatography (IEC).

2.3 Purification of Polygalacturonase by Ion Exchange and Gel Filtration

The partially purified polygalacturonase fraction of the *C. gileadensis* peel extract obtained from the previous step was poured into a column containing DEAE-Sepharose as the stationary phase equilibrated with Na-acetate buffer (20 mM, pH 5.5) at room temperature. Then, the column was washed with a gradient of sodium chloride (0.0–0.3 M) dissolved in Na-acetate buffer to elute the polygalacturonase. Every eluted fraction of the NaCl gradient was analysed for enzyme activity according to the protein peak at 280 nm. Eluted fractions with high polygalacturonase activities were collected and then lyophilized. The lyophilized polygalacturonase was dissolved, poured into an equilibrated Sephadex-150 column with Na-acetate buffer (20 mM, pH 5.5), and eluted with the same buffer at a flow rate of 30 ml/h.

2.4 Protein Concentration Measurements

The concentration of protein was evaluated and standardized to bovine serum albumin according to the method of Bradford [26].

2.5 Polygalacturonase Activity Determination

PGA was used as a substrate in the colorimetric determination of polygalacturonase activity [27]. The enzymatic reaction was performed in a 0.5 ml final volume containing an appropriate quantity of purified or immobilized enzyme, 2% PGA, and Na-acetate buffer (50 mM, pH 5.5), and then incubated at 37 °C for 1 h, followed by the addition of DNS (0.5 ml). The reaction mixture was boiled for 10 min in a water bath then cooled immediately under tap water reach to room temperature. The color intensity was measured at 560 nm. The purpose from adding the DNS was to evaluate the D-galacturonic acid oligomers produced from PGA hydrolysis that yielded a stable colour with DNS [28]. As the enzyme activity increased, the PGA was consumed, and the color intensity increased.

2.6 Assessment of Enzyme Molecular Weight

The molecular weight of the purified polygalacturonase was determined by gel filtration using Sephadex G-150. The column was equilibrated using a gel filtration Markers Kit for Protein molecular weights (12,000–200,000 Da).

2.7 Immobilization of Polygalacturonase on PPyAgNp/Calcium Alginate Beads

PPyAgNp was synthesized by oxidative polymerization of pyrrole in the presence of silver nitrate as an oxidant according to a previously reported method [29]. Then, 100 mg of PPyAgNp nanocomposite was added to 100 units of purified enzyme dissolved in 10 mM Tris–HCl buffer (pH 7). Immobilization via ionic bonds was achieved by shaking at 90 rpm overnight at room temperature. Then, 10 mL of 2% (w/v) sodium alginate solution was added to the mixture and sonicated for 10 min. The mixture was carefully aspirated with a sterile syringe and dropped into a container containing 2% (w/v) $CaCl_2$ solution. After 1 h, the beads were removed from the $CaCl_2$ solution and washed with deionized water. The PG activity (units/g support) and immobilization efficiency (%) were determined using the following equations:

$$\text{PG activity (units/g support)} = \frac{\text{Immobilized enzyme activity}}{\text{g support}}$$

$$\text{Immobilization efficiency(\%)} = \frac{\text{Immobilized enzyme activity}}{\text{Initial enzyme activity}} \times 100$$

2.8 Morphological Characterization

An FT/IR-4600 spectrometer was used to obtain the attenuated total reflectance Fourier infrared (ATR–FTIR) spectrum of the immobilized enzyme. Raman spectroscopy of the PPyAgNp/Ca-alginate beads before and after immobilization was performed on a Raman: DXR (Thermo Scientific, USA) with a 532 nm laser as an excitation source. Thermogravimetric analysis (TGA) of the samples was accomplished using an SDT Q600 V20 9 Build 20 analyser. PPyAgNp/Ca-alginate beads with/without enzyme were gradually heated (20 °C min) until reaching a maximum temperature of 800 °C. The morphological features of the PPyAgNp/Ca-alginate beads before and after immobilization were measured by field emission scanning electron microscopy (FESEM, JEOL JSM 7600F FEG-SEM) coupled with

energy dispersed X-ray spectroscopy (EDX). All samples were mounted on carbon tape over copper stubs and sputtered for 10 s with platinum prior to observation.

2.9 Immobilized Polygalacturonase Reusability and Storage Stability Assessment

The reusability of the immobilized PG was assessed under the optimum reaction conditions. The immobilized enzyme was filtered out of the reaction mixture and then suspended in Na-acetate buffer (50 mM, pH 5.5). After that, the immobilized PG that remained active after filtration was suspended again in the reaction mixture and filtered. The activity measured after the first suspension was considered 100% and used as a control. The activities of all other subsequent reused enzymes were calculated compared to the control. To calculate the storage stability of the enzyme, the purified and immobilized PG was kept for approximately 60 days at 4 °C. Then, the enzyme activity was assessed every ten days under the optimum reaction conditions.

2.10 Physicochemical Characterization of the Enzyme

2.10.1 Determination of the Optimum pH

Different pH ranges were used to determine the enzyme's optimal pH. Acetate buffer (50 mM) with pH values ranging from 4.0 to 6.0 and Tris–HCl buffer with pH values ranging from 6.5 to 9.0 were used in our experiments.

2.10.2 Determination of the Optimum Temperature and Thermal Stability

The optimum temperature at which the enzyme achieved the highest activity was determined by exposing the purified and immobilized PG to a series of temperatures (30–90 °C) and then measuring the enzyme activity. All other conditions were held constant throughout the experiments. To evaluate the thermal stability of the purified and immobilized PG, the samples were incubated at a series of temperatures (50–80 °C) for 10, 20, 30, 40, 50, and 60 min. After 24 h, an enzyme assay was carried out under standard assay conditions.

2.10.3 Kinetic Behavior Evaluation

Purified and immobilized enzyme were incubated with different concentrations of PGA substrate, and their kinetic behaviour was measured by plotting Line-weaver–Burk plots and then calculating the K_m and V_{max} values.

2.11 Statistical Analysis

To examine the analysis of statistical significance of differences in the results, we have used SPSS 18 to analyze the data. Analyzed using Wilcoxon Signed Ranks Test, we estimated that there is a statistically significant difference between relative activity (%) free enzyme and relative activity (%) immobilized enzyme across different conditions.

Table 1 Purification scheme for the Arabian balsam PG

Step	T. units*	T. proteins mg	Specific activity Units/mg protein	Fold purification	Recovery 100%
Crude extract	2588	15.24	169.8	1	100
$(NH_4)_2SO_4$ precipitation					
40% (PG)	1691	6.35	266.3	1.57	65
Ion exchange chromatography (PG)					
0.0 M NaCl (PG 1)	165	1.91	86.4	0.51	6.4
0.05 M NaCl (PG 2)	373	1.13	330	1.94	6.7
0.1 M NaCl (PG 3)	**564**	**0.84**	**671.43**	**3.95**	**21.8**
0.2 M NaCl (PG 4)	215	0.79	272.15	1.60	8.3
0.3 M NaCl (PG 5)	144	0.88	163.6	0.96	5.5
Gel filtration chromatography (PG 3)					
(PG 3A)	**291**	**0.17**	**1711.8**	**10**	**11.2**

Bold values indicate that the isoenzyme (PG 3) that collected from IEC was selected to next step in purification process (gel filtration)

*One unit of enzyme activity was defined as the amount of enzyme that liberated one μmol of maltose per min under standard assay conditions

3 Results and Discussion

3.1 Polygalacturonase Purification

In this study, PG from the Arabian balsam plant was purified through ammonium sulfate $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography (DEAE-Sepharose column), and gel filtration (Sephadex G-150 column). The purification results are summarized in Table 1. In the initial purification step, the crude extract of PG was precipitated by $(NH_4)_2SO_4$, and different fractions were collected from 20 to 80%. The PG activity was measured in all collected fractions, and significantly higher PG activity was detected in the fraction obtained by 40% $(NH_4)_2SO_4$ precipitation. Compared to the crude extract, the PG obtained from 40% $(NH_4)_2SO_4$ precipitation showed an increase in specific activity from 169.8 U/mg to 266.3 U/mg, with a recovery of 65% and 1.57-fold purification. Therefore, the fraction obtained by 40% $(NH_4)_2SO_4$ precipitation was subjected to a DEAE-Sepharose column. Ammonium sulfate precipitation is a technique that is still commonly used to partially purify target proteins. For example, ammonium sulfate precipitation was used for purification of peroxidase and α-amylase from *Commiphora gileadensis* [30, 31] and peroxidase from haricot beans [32] and horseradish cv. Balady [33]. Since certain proteins have few hydrophilic areas, they can accumulate and precipitate at low ammonium sulfate concentrations (20–40% saturation). The protein, on the other hand, has significantly more hydrophilic areas, so it can stay in solution until the ammonium sulfate concentration is significantly higher (50–80% saturation) [34]. In the second step of purification, as shown in Fig. 1, five peaks with PG activity were eluted with 0.0, 0.05, 0.1, 0.2, and 0.3 sodium chloride and designated PG 1–5. PG3 showed an increase in specific activity to 671.43 U/mg, with a recovery of 21.8% and 3.95-fold purification. Therefore, fraction PG3 was subjected to Sephadex G-150 in the third step of purification. Figure 2a shows the elution profile. In this step, only one peak (PG3A) was observed, indicating that the purified enzyme was obtained successfully. The PG3A fraction exhibited specific activity of up to 1711.8 U/mg, with a recovery of 11.2% and tenfold purification. The molecular weight of the purified PG was estimated to be 75.5 kDa using the Sephadex G-150 column, as shown in Fig. 2b. Some polygalacturonase was stated to have a molecular weight between 69.7 and 110 kDa, which may be due to variations in amino acid sequence or glycosylation [35]. For example, the molecular weight of PG was 69.7 kDa for *Aspergillus giganteus* [36], 70 kDa for *Botrytis cinerea* [37], 92 kDa for *Penicillium viridicatum* [38], and 110 kDa for *Bacillus paralicheniformis* [39].

3.2 Polygalacturonase Immobilization

Purified PG has wide potential for use in the food industry. Therefore, purified PG was immobilized on polypyrrole/silver (PPyAgNp) via ionic bonds at pH 7 and entrapped into calcium alginate beads. Because polygalacturonase contains disulfide and/or thiol and amino acid groups within

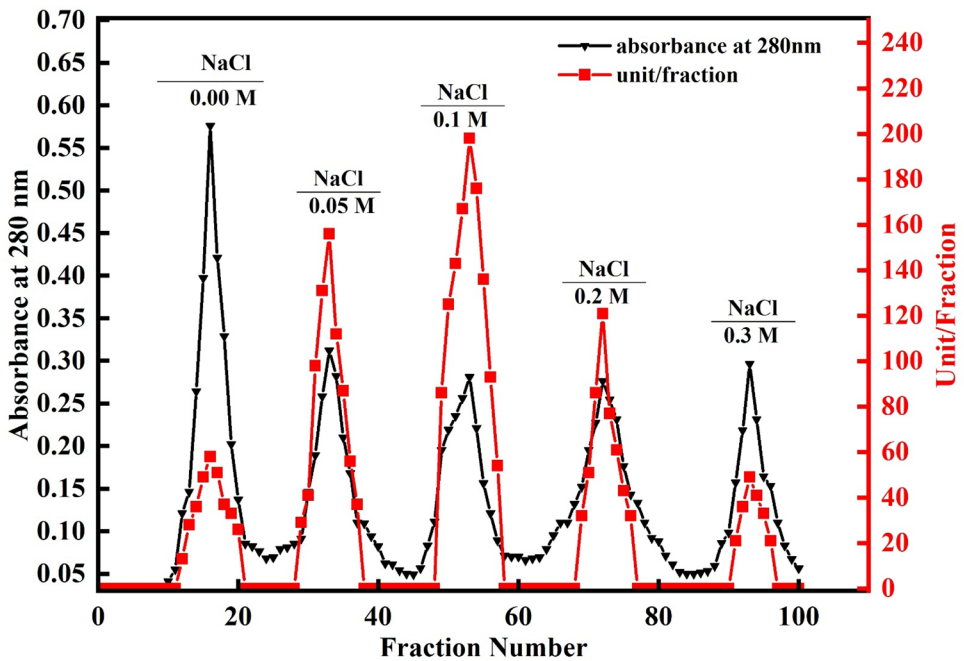


Fig. 1 A typical elution profile for the chromatography of PG using a DEAE Sepharose column

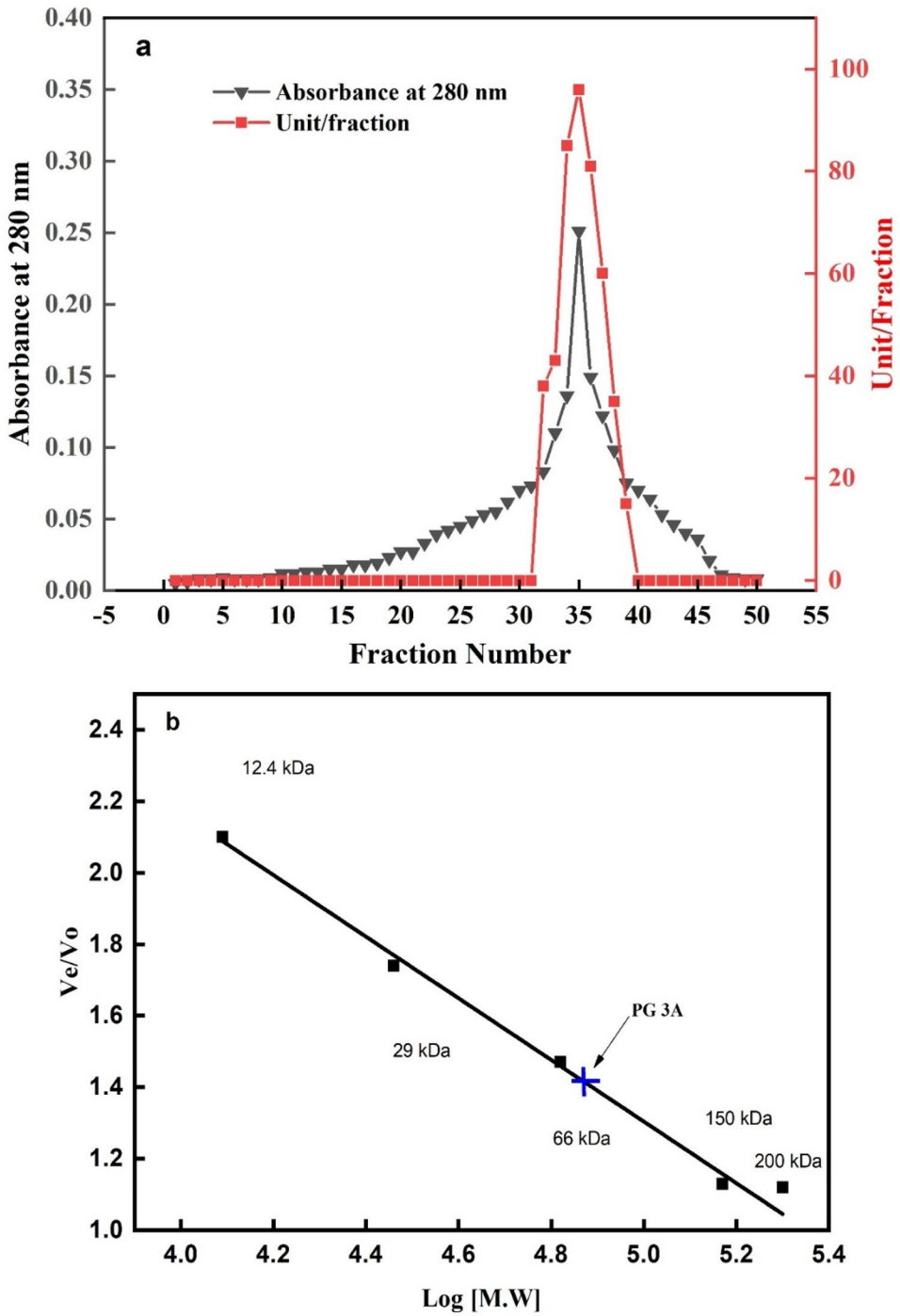


Fig. 2 a Chromatography of the polygalacturonase PG3 DEAE-Sepharose fraction using a Sephadex G-150 column (**a**), The molecular weight value for polygalacturonase PG3 was calculated from the calibration curve of the Sephadex G-150 column. Standard proteins: cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine albumin (66 kDa), 4) alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). The void volume was determined with dextran blue (2000 kDa) (**b**)

its chemical structure, it was therefore assumed that a PPy and AgNp composition would provide a good matrix for enzyme immobilization due to the positive charges on PPy that help to bind the enzyme via ionic bonds and the propensity of AgNp to bind to disulfide and/or thiol groups [29, 40]. The PG activity was 35.17 units/g support, and the immobilization efficiency was 84.4%. In a previous study, α-amylase was immobilized onto PPyAgNp/Fe_3O_4 and calcium alginate/Fe_2O_3 with immobilization efficiencies of 75% and 54.5% [30, 41]. In this study, the use of the PPyAgNp/Ca-alginate matrix increased the immobilization efficiency due to two types of interactions between the enzyme and the supporting matrix: ionic bonding and entrapment.

3.3 FT-IR Analysis

The FT-IR spectra of calcium alginate, PPyAgNp/Ca-alginate, and PPyAgNp/Ca-alginate with the enzyme are

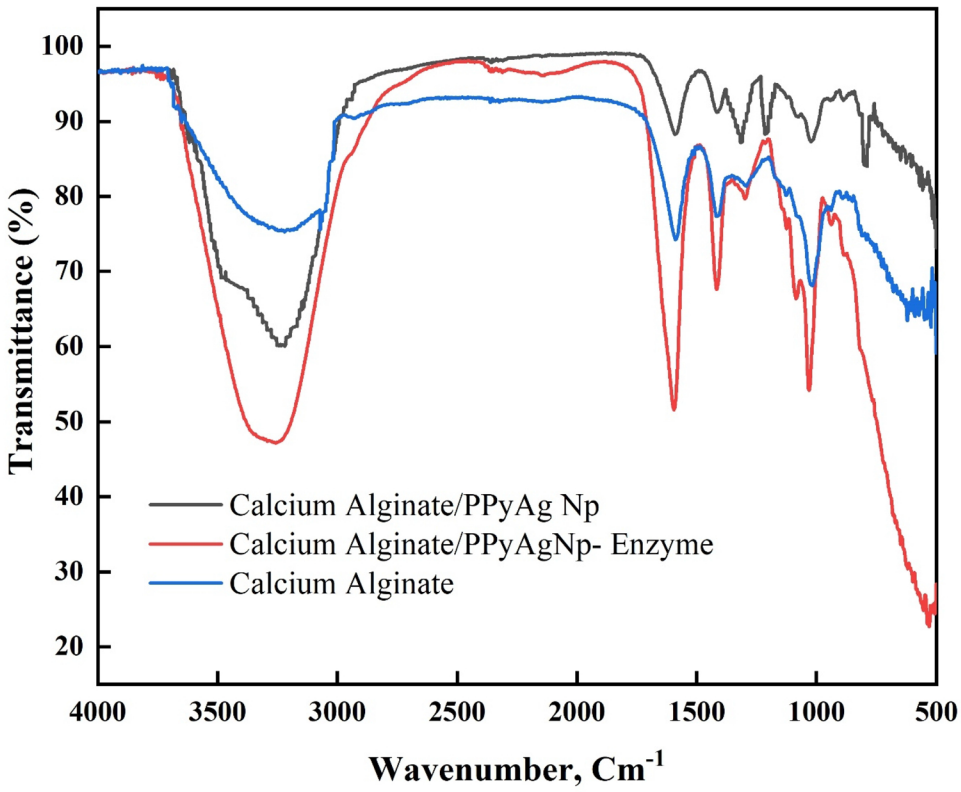


Fig. 3 FTIR spectra of calcium alginate beads, calcium alginate beads coated with polypyrrole/silver nanocomposites, and PG immobilized onto calcium alginate beads coated with polypyrrole/silver nanocomposites

shown in Fig. 3. The peak at 3251 cm^{-1} corresponds to the O–H bond stretching vibrations of the alginate. The peak at 1595 cm^{-1} corresponds to the symmetric stretching vibrations of the $COO-$ group. The peaks at 1460 and 1591 cm^{-1} correspond to the stretching vibration of the C–C and C–N bonds of the pyrrole ring, respectively. The peak at 1214 cm^{-1} corresponds to the breathing vibration of the pyrrole ring. The peak at 1319 cm^{-1} may result from the absorption assigned to doped NO^{3-} in PPy or the interactions between AgNp and PPy. The peak at 795 cm^{-1} corresponds to C–H wagging. The broad peaks observed after immobilization at 3300 cm^{-1} and 1538 cm^{-1} were attributed to stretching of the OH and NH groups present in the PG enzyme. Amide bands I and II appear at 1600 cm^{-1} (CO stretch, amide I) and 1415 cm^{-1} (NH bend, amide II), respectively. The peak at 1027 cm^{-1} corresponds to the stretching vibrations of the C–O bond of the glycosidic linkage [30, 41, 42].

3.4 Raman Spectroscopy Analysis

Raman spectroscopy was further used to confirm the immobilization of the enzyme on PPyAgNp/Ca-alginate because the binding of the enzyme inside PPyAgNp/Ca-alginate would have an effect on the induced polarizability and the light dispersion characteristics of this matrix. The combination of IR and Raman spectroscopies is a more potent approach for sample analysis [43]. In Fig. 4a, the black spectrum corresponds to the enzyme with PPyAgNp/Ca-alginate. The intensities of the bands based on the concentration of the enzyme are compared to those of the supporting material. The significant vibrational modes of the enzyme are at 750, 1193, 1375, 1593, and 1882 cm^{-1}. The bands of PPyAgNp/Ca-alginate are consistently lower in intensity than the vibrational bands of the enzyme. On the other hand, in Fig. 4b, it seems that the significant vibrational modes of calcium alginate are attributed to carboxyl (COO-) groups, whose vibrational bands are observed at 805 and 888 cm^{-1}, and hydroxyl (OH-) groups, whose vibrational bands are observed at 1625 cm^{-1}. Additionally, significant vibrational modes of polypyrrole are observed at 735, 810, 957, 1047, 1356, 1442, and 1550 cm^{-1}. These results are agreement with the result of Liu and Hwang [44].

3.5 Thermogravimetric Analysis

The thermal behaviour of calcium alginate, PPyAgNp/Ca-alginate, and PPyAgNp/Ca-alginate with the enzyme was studied by TGA from room temperature to 900 °C at a rate of 20 °C min^{-1} under a nitrogen atmosphere. These thermal estimations helped to evaluate the fabrication of Ca-alginate surfaces with PPyAgNp and the immobilization of the enzyme to PPyAgNp/Ca-alginate. For this purpose, the prepared compounds were subjected to TGA, and the

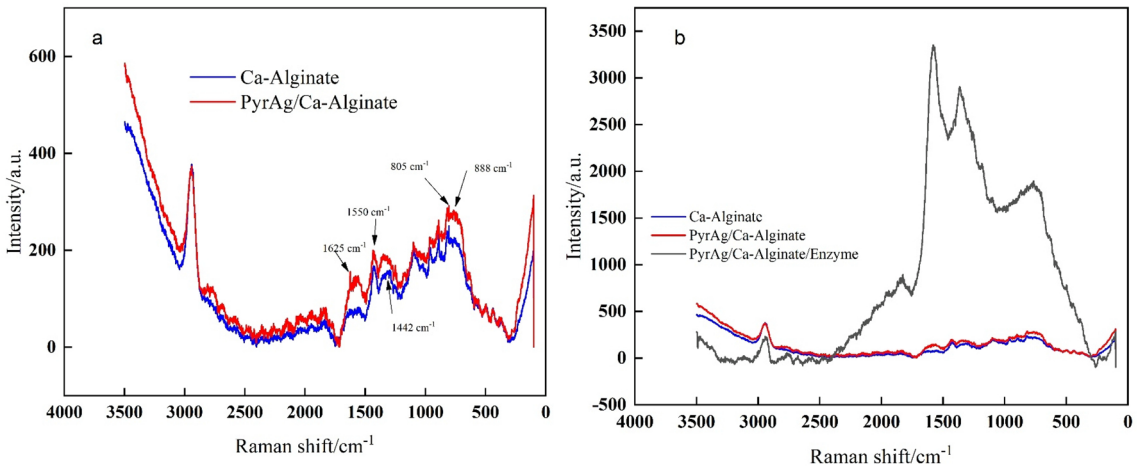


Fig. 4 Raman spectra of **a** calcium alginate and PPyAgNp/calcium alginate, **b** calcium alginate, PPyAgNp/calcium alginate, and PPyAgNp/calcium alginate with the enzyme

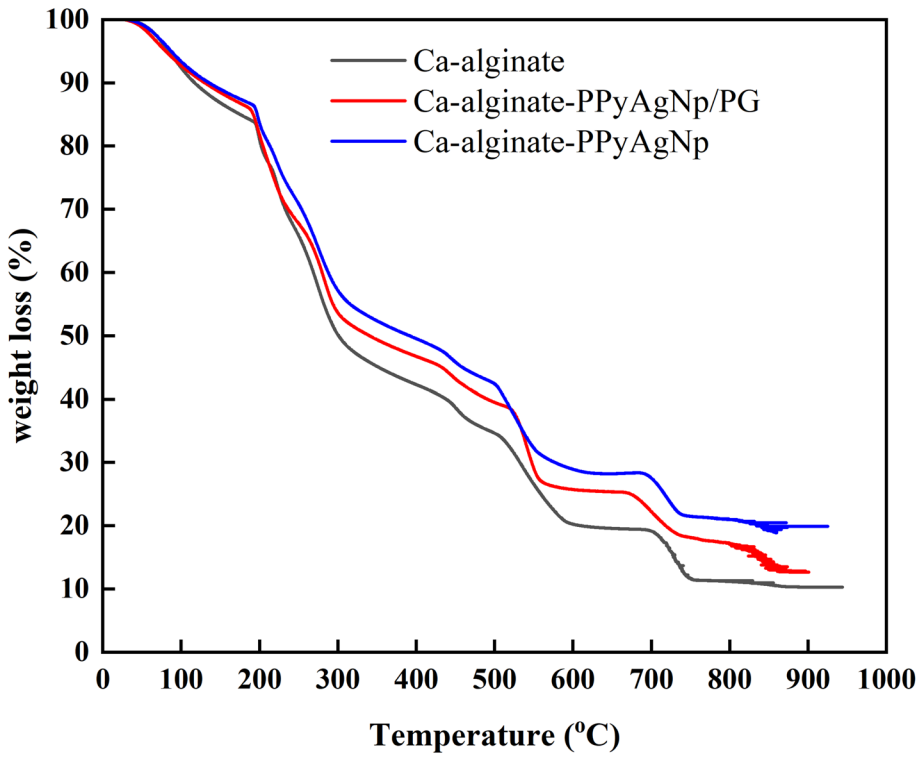


Fig. 5 Thermograms of calcium alginate beads, calcium alginate beads coated with polypyrrole/silver nanocomposites, and PG immobilized onto calcium alginate beads coated with polypyrrole/silver nanocomposites

thermograms obtained are shown in Fig. 5. A significant mass loss at 300 °C was observed for Ca-alginate, with a mass loss of 50%, while the same mass loss was observed for PPyAgNp/Ca-alginate and PPyAgNp/Ca-alginate-enzyme at 390 °C and 340 °C, respectively. This significant difference in the thermal behaviour of the studied support provides evidence that the enzyme has been successfully immobilized onto PPyAgNp/Ca-alginate.

3.6 Field Emission Scanning Electron Microscopy Analysis

Figure 6 shows the surface morphologies of calcium alginate beads coated with polypyrrole/silver nanocomposites before and after immobilization with PG at various magnifications. The FESEM images demonstrate a disparity between the morphological characteristics of the beads with and without immobilized PG. Figure 6a shows that there were no observable pores on the surface of the control bead. Figure 6b

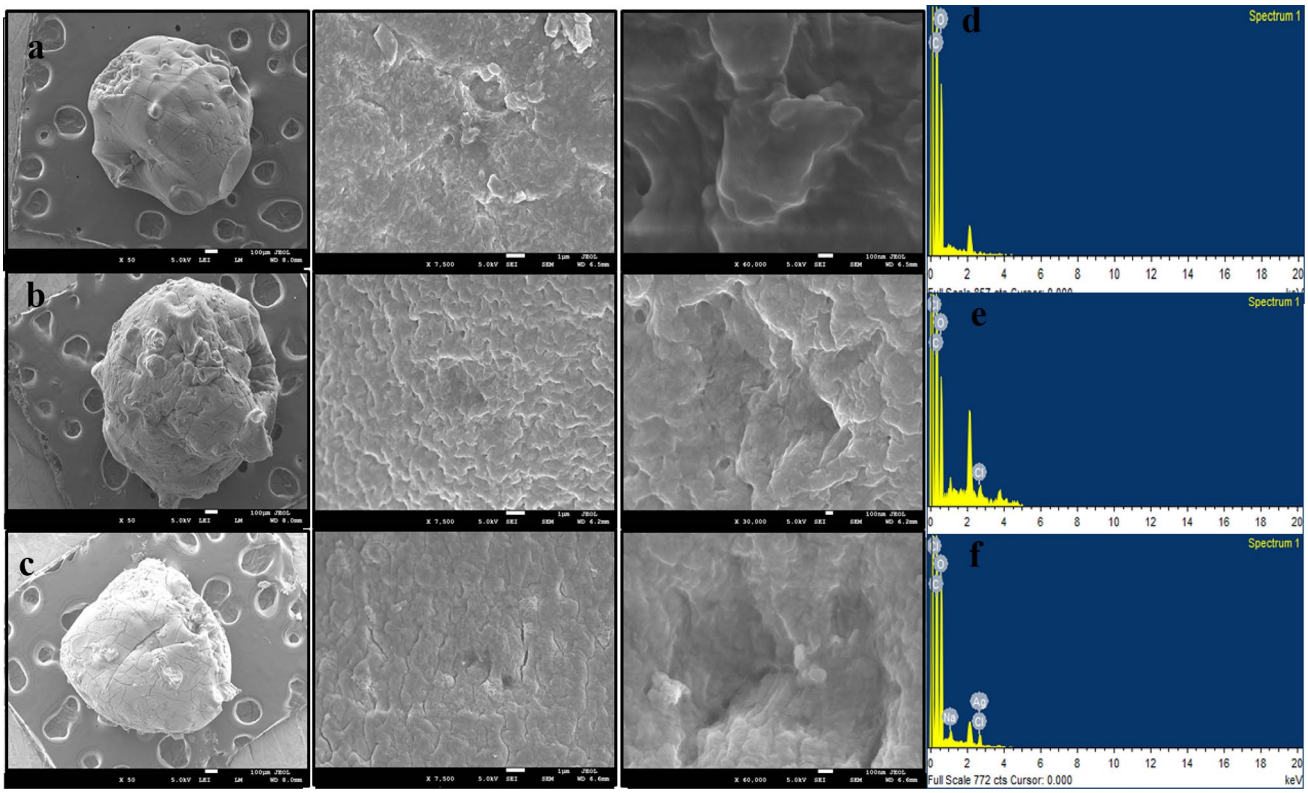


Fig. 6 High and low magnification FESEM images of **a** calcium alginate beads, **b** calcium alginate beads coated with polypyrrole/silver nanocomposites **c** immobilization of polygalacturonase onto calcium alginate beads coated with polypyrrole/silver nanocomposites, **d**, **e**, **f** the SEM–Energy-dispersive X-ray (EDX) spectra of calcium alginate beads, calcium alginate beads coated with polypyrrole/silver nanocomposites, and polygalacturonase onto calcium alginate beads coated with polypyrrole/silver nanocomposites, respectively

indicates that PPyAg nanoparticles were very uniformly distributed on the bead surface. Following immobilization of the enzyme, the bead surface coated with PPyAg was covered with dense PG particles displaying an irregular morphology and a rugged surface compared to the control bead, as shown in Fig. 6c. The presence of silver nanoparticles was proven using EDX, as shown in Fig. 6d, e, f.

3.7 Stability and Reusability of Immobilized Enzyme

The operational stability of immobilized PG has been investigated because of its significance in production cost reduction. The immobilized PG preserved 91% of its initial activity after 5 cycles and 68% after 10 cycles (Fig. 7a), indicating the high reusability of the PG immobilized on PPy-AgNp/Ca-alginate. The operating stability of the immobilized enzyme was higher than that of immobilized α-amylase in calcium alginate/Fe_2O_3 (43% activity remained after 10 cycles) [30]. Generally, over time, free enzyme activity decreases. Enzyme immobilization is intended to improve enzyme stability during storage. A comparison of the activity of free and immobilized PG during storage at 4 °C for 60 days shows that the reduction in the activity of immobilized PG was smaller than that of the free enzyme (17% vs. 74%) (Fig. 7b).

3.8 Physico-Chemical Characterization of Purified and Immobilized Enzymes

To study the effect of pH on the activity of free and immobilized PG, different pH values from 4.0 to 9.0 were applied; the results can be seen in Fig. 8a. The optimum pH for the free enzyme was determined to be pH 5.0, while that of the immobilized form was pH 5.5. At both low and high pH values, the immobilized PG activity was higher than that of the free form. Increasing the enzyme activity of immobilized PG relative to that of the free form can be attributed to decreased autolysis of the enzyme and possible stabilization of PG immobilized on a high-surface-area support material and confinement, resulting in improved substrate binding at active PG enzyme sites. Another study showed that the optimum pH of PG immobilized on calcium alginate was 4.5 [45]. The optimum pH of PG purified from *Aspergillus flavus* was 5.0 [46].

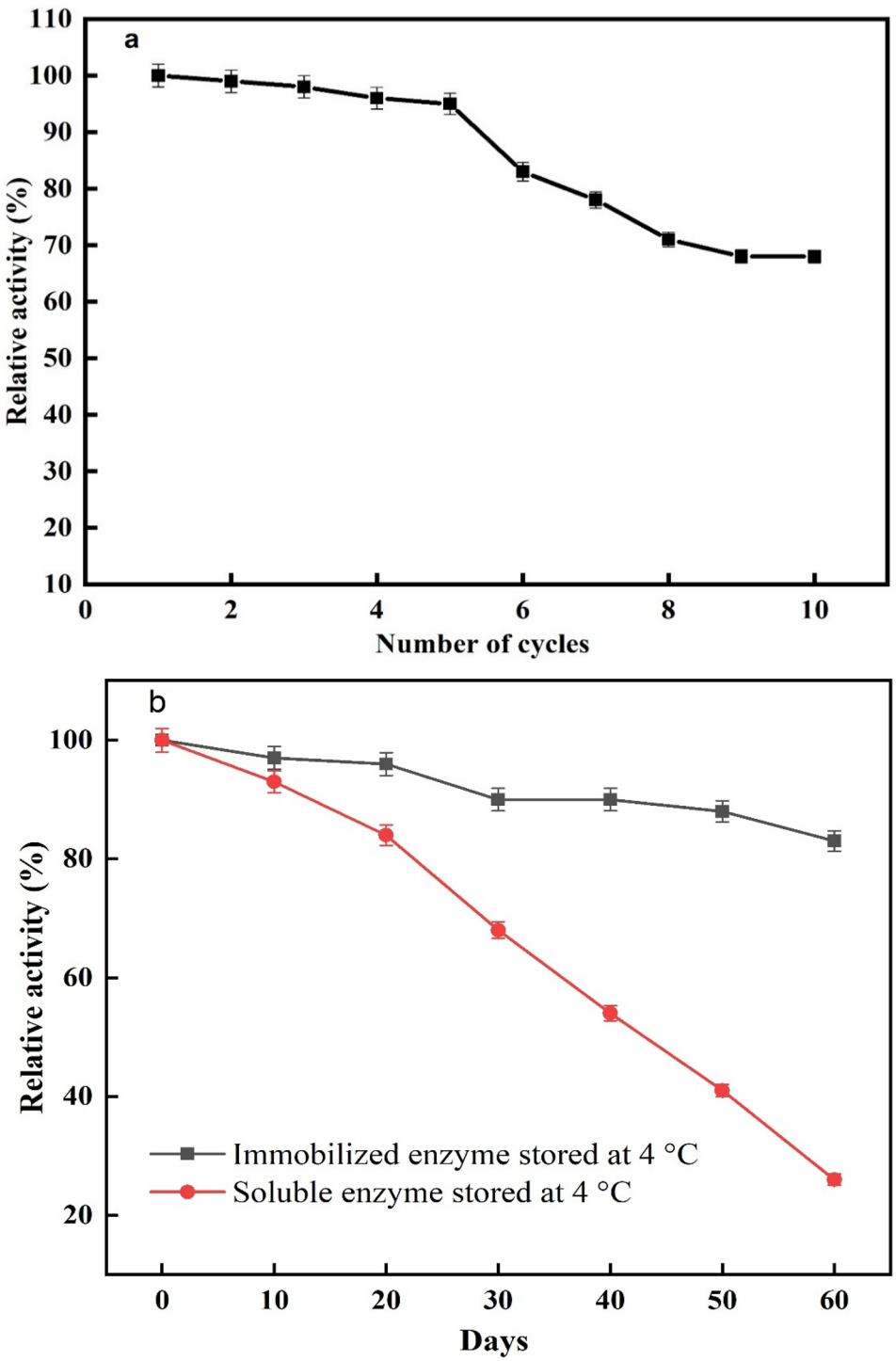


Fig. 7 Reuse of immobilized PG (**a**), storage stability of free and immobilized PG at 4 °C (**b**). (means ± S.D, n = 3)

The Lineweaver–Burk plot was applied to evaluate the kinetic parameters of free and immobilized PG. The K_m and V_{max} values for immobilized PG were lower than those for free PG (Fig. 8b). This may be due to improvement in the enzyme structure following ionic interaction with the matrix support that increased the thermal stability of the PPyAgNp/Ca-alginate-PG. The K_m is relevant to the enzyme and substrate affinity. After immobilization, the affinity of the enzyme to the substrate increased by 1.38-fold. The catalytic efficiency (V_{max}/K_m) of immobilized PG was higher than that of the free form (11.06 and 14.48, respectively). This result suggests that the enzyme on the PPyAgNp/Ca-alginate surface has more accessible potential active sites, thus increasing PG's affinity to the substrate. It demonstrates that immobilized enzymes can tolerate higher temperatures than free form [47, 48]. Changes

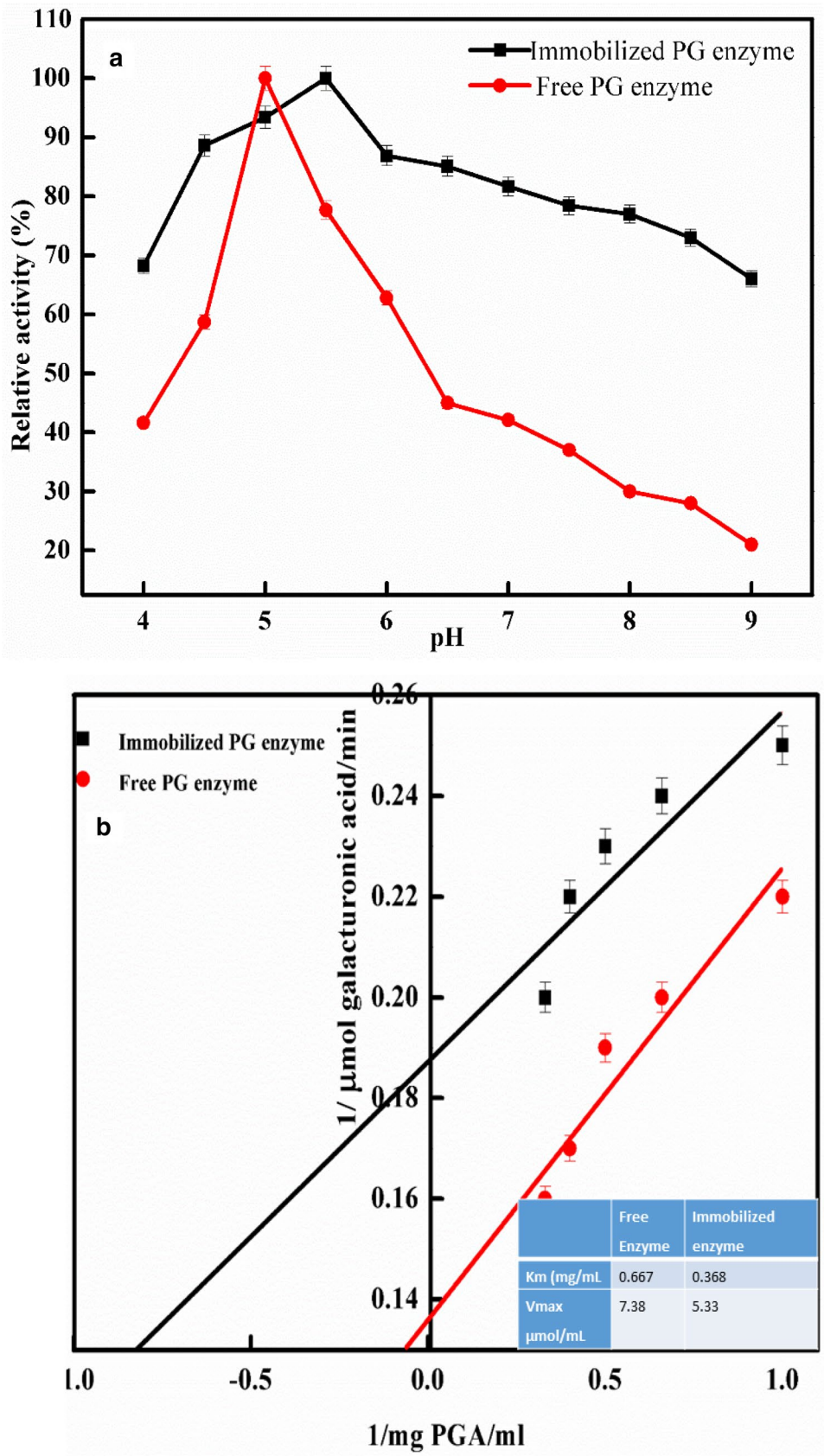


Fig. 8 Effect of pH (**a**), and kinetic parameters (**b**) of free and immobilized PG (means ± S.D, n = 3)

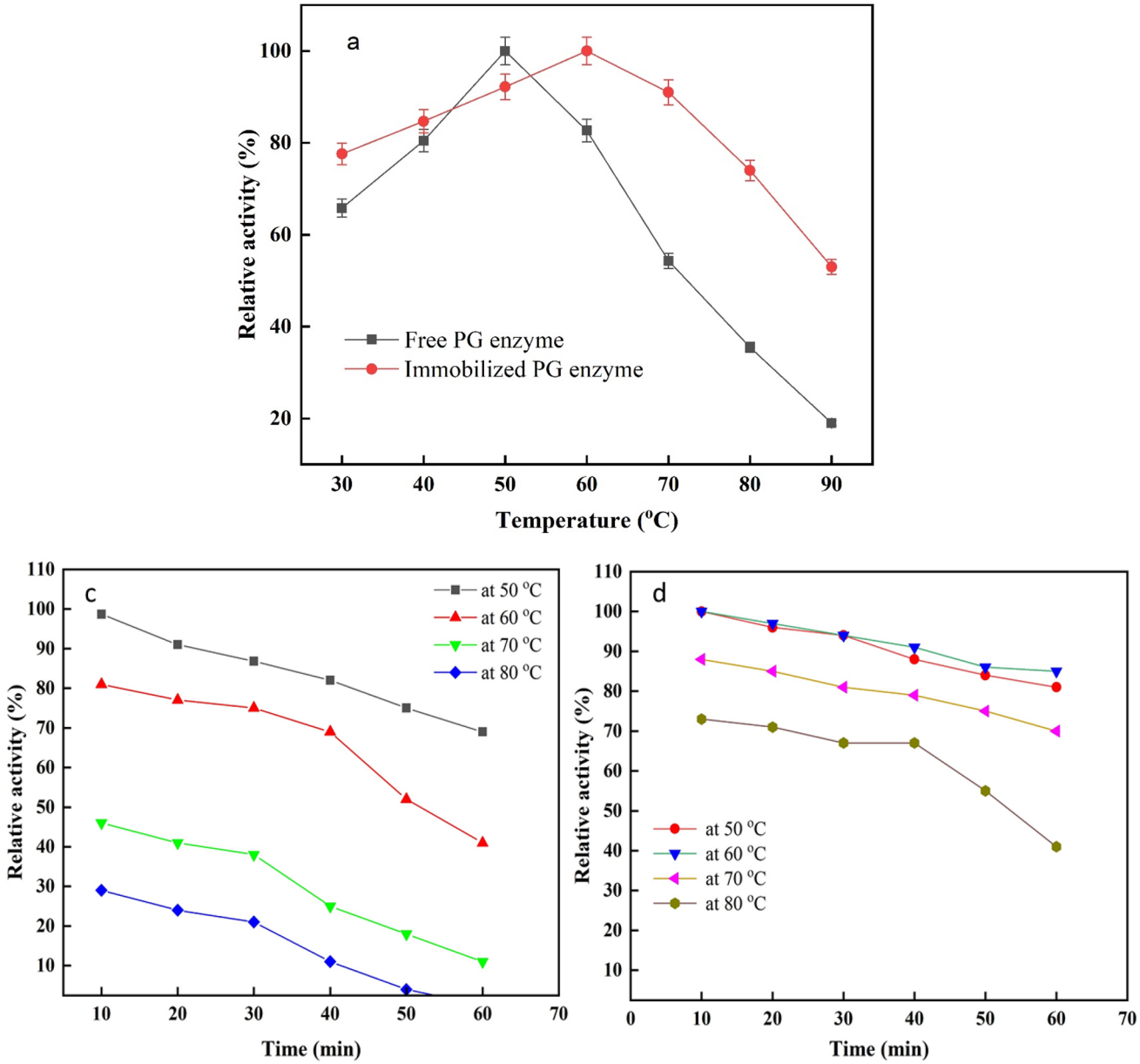


Fig. 9 Effect of temperature (**a**), Thermal stability of free PG (**b**), and immobilized PG (**c**) (means ± S.D, n = 3)

in the physical and chemical properties of immobilized PG cause temperature shifts. In addition, the ionic interaction via disulfide and/thiol groups of immobilized PG might decrease conformational flexibility, leading to the higher activation energy for the formation of the desired configuration, making the substrate more easily bind to the molecule [48]. Rehman et al. reported that the K_m and V_{max} values for polygalacturonase were 1.055 mg ml^{-1} and 11.4 mM min^{-1} after entrapment within the calcium alginate beads [49].

In this study, the catalytic activity of free and immobilized PG was measured at different temperatures (Fig. 9a). The optimum temperature for free PG shifted was 50 °C, whereas that for PG immobilized on PPyAgNp/Ca-alginate was 60 °C. Several studies revealed that the immobilized enzymes can tolerate higher temperatures than free form [47, 48]. At temperatures from 50 °C to 90 °C, the activity of the free PG decreased significantly, while the activity of the immobilized enzyme decreased less at temperatures from 60 °C to 90 °C. The increase in immobilized PG activity can be related to the reduction in the alteration of the 3D structure of PG upon heating due to binding of the enzyme on the PPyAgNp surface and entrapment within calcium alginate. Compared to that of immobilized PG, the enzyme activity

of free PG decreased much further t temperatures greater than 50 °C because the enzyme was denatured, resulting in structural deterioration and decreased enzyme activity at increasing temperatures [50].

The thermal stability of free and immobilized PG was evaluated by preincubation of both enzymes at different temperatures ranging from 50 °C to 80 °C for various periods of time. After 24 h, aliquots were taken for determination of the residual enzyme activity. Compared to the free enzyme at varying temperatures, the immobilized enzyme was inactivated at a much lower rate (Fig. 9b, c). After 60 min at 50, 60, 70, and 80 °C, the immobilized PG maintained approximately 86%, 85%, 70%, and 41% activity, respectively, while the free enzyme displayed only 75%, 59%, 29%, and 6% residual activity under the same conditions. The increase in the thermal stability could be due to the stabilizing effect of the support system that limits thermal denaturation conformational changes. Rehman reported that the immobilization process increased the thermal stability of polygalacturonase [25].

3.9 The Wilcoxon Signed Ranks Statistic analysis

The Wilcoxon Signed Ranks Test results displays that there is statistically significant difference between relative activity (%) of free enzyme and relative activity (%) of immobilized enzyme across different conditions. The results have been demonstrated a significant difference between activity of free enzyme and immobilized enzyme during different pH values, temperatures and kinetics were ($P < 0.05$) (Tables 1S, 2S, 3S: Supplementary materials). In addition, there is difference of storage stability during days between free and immobilized PG at 4 °C (b) was ($P < 0.05$) (Table 4S: Supplementary materials).

4 Conclusion

Enzymes with distinct physicochemical characteristics and economical production for downstream applications have often been attractive for research projects. In the current study, three purification steps (ammonium sulfate precipitation and DEAE-Sepharose and Sephadex-150 columns) were successfully used to purify PG from Arabian balsam with a significant level of purification (tenfold). In various aspects of biological science, polymers play an important role, and in the current study, PPyAgNp/Ca-alginate was used to immobilize polygalacturonase by ionic bonding and trapping techniques with efficiency of 84.4%. The immobilized PG showed good stability in terms of reusability (68% of its initial activity after 10 cycles) and storage (remained 83% of its initial activity after storage for 60 days). The immobilization of PG on the carrier increased the thermal stability, pH value, and substrate affinity. In terms of reusability and immobilization efficiency, this approach might have beneficial applications in various textile and food industries. Furthermore, substantial research will be required to evaluate the viability of this method in different industrial processes.

Supplementary Information The online version of this article (https://doi.org/10.1007/s10562-021-03631-7) contains supplementary material, which is available to authorized users.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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Authors and Affiliations

Yaaser Q. Almulaiky[1,2] · Sami A. Al-Harbi[3]

✉ Yaaser Q. Almulaiky
yaseralmoliki@hotmail.com

[1] College of Science and Arts at Khulis, Department of Chemistry, University of Jeddah, Jeddah, Saudi Arabia

[2] Chemistry Department, Faculty of Applied Science, Taiz University, Taiz, Yemen

[3] Department of Chemistry, University College in Al-Jamoum, Umm Al-Qura University, Makkah, Saudi Arabia