Grafted Pectin with Glycidyl Methacrylate for Multi-Sites Urease Immobilization

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Abstract: Pectin poly glycidyl methacrylate copolymer (PGMA-*g*-pectin) was prepared *via* emulsion polymerization technique, characterized and used as multi-sites enzyme immobilization system. Urease, an enzyme model in this study, was sequentially immobilized onto the prepared carrier *via* both the carboxyl and epoxy groups. The structure and surface morphology of the prepared copolymer, before and after immobilization, were characterized by FT-IR and SEM. Both the amount of bounded urease and its relative activity were measured. A higher activity (about 68.4%) was measured for urease bounded to PGMA-*g*-SA activated with 10 mg of 1-[3-(dimethylamino) propyl]-3-ethylcarbo diimide hydrochloride (EDC) at pH 5 for 3 h. The various parameters affecting the potency of urease immobilization process (e.g. activation and immobilization time, pH and the concentration of EDC and urease) were investigated. The basic characteristics (optimum pH and temperature, thermal stability, storage stability and reusability) of the immobilized enzyme were also determined. The results showed that the immobilized urease maintained its excellent performance in detecting urea in 20 measurements and retained 70 % from its original activity after 60 days of storage at 4 °C.

Keywords: PGMA-g-Pectin, Emulsion polymerization, Urease immobilization, Multi-sites enzyme immobilization system.

1. INTRODUCTION

Urease is a highly efficient catalyst for the hydrolysis of urea to ammonia and carbon dioxide. Urea is the main toxic metabolic product, and removal of its excess is a major problem for patients suffering from renal failures. Urease applications include determination of urea content in blood, urine, alcoholic beverages, natural water, and environmental wastewaters. Moreover, the most effective way of removing urea from aqueous solutions is the utilization of immobilized urease onto insoluble carriers [1-3]. A great number of natural and synthetic polymers have been specially designed for enzyme immobilization such as chitosan [4,5], alginate [6,7], cellulose [8,9], as well as binary copolymers of methyl methacrylate with glycidyl methacrylate and 2-hydroxyl ethyl methacrylate [10].

Pectins are natural polysaccharides composed mainly of (1→4) linked α-D-galactopyranosyluronic (galacturonic) acid units, containing some of carboxyl groups in the methyl ester form. They are found in the cell walls of plants and their chief commercial sources are peels of apples, limes, and oranges. Commercial pectins are divided according to the degree of esterification (DE) of the galacturonic acid subunits that are methyl esterified. High-methoxyl (HM) pectins have DE ≥ 50 or more, while low-methoxyl (LM) pectins have DE < 50 [11-13].

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However, the greatest challenge found in the use of pectin in the enzyme immobilization technology is to overcome its solubility in aqueous medium. An alternative to reduce the high solubility of polysaccharides is to chemically modify them without affecting their biodegradability by the grafting emulsion polymerization [14].

To the best of our knowledge, the immobilization of enzymes especially urease onto pectin grafted with PGMA is not established. The main task of the current research is to modify pectin *via* emulsion polymerization with glycidyl methacrylate (GMA) to develop optimized multi-sites enzyme immobilization system based on (PGMA-*g*-pectin) through introducing epoxy groups in the polysaccharide structure. These epoxy groups will later react through free radicals and generate polymeric chains and produce polysaccharide hydrogels [15,16]. Then, urease will selectively immobilize through epoxy and carboxyl groups on PGMA grafted pectin.

2. EXPERIMENTAL

2.1. Materials

Urease (EC 3.5.1.5 from jack beans), Pectin (low methoxyl content ≈ 7%,), 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma. Urea and Sodium alginate (SA) were obtained from Fluka. Glycidyl methacrylate (GMA), bovine serum albumin (BSA) and ethylene diamine tetra acetic acid (EDTA) were purchased from Aldrich. Nessler's reagent, Folin reagent and

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ammonium peroxy disulfate (APS) were obtained from Merck. Double distilled water (DDW) was used in all experiments. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Methods

2.2.1. Preparation of PGMA-g-Pectin Hydrogels

For the synthesis of the PGMA-*g*-pectin hydrogels, an emulsion polymerization technique was employed using SA and APS as an emulsifying agent and initiator, respectively. The polymerization was carried out in a three necked flask (100 ml) fitted with a condenser and a thermometer. The system also had a nitrogen inlet and was stirred with a magnetic stirrer. SA (0.1 g), Pectin (1 g) and DDW (50 ml) were added into the reaction vessel and heated to 65°C while flushing nitrogen throughout the solution. Then APS (0.15 g) and different amounts of GMA were added and the reaction ingredients were stirred vigorously at 65°C for 4 h. The prepared stable emulsion was transferred to Petri dish till gel formation. The PGMA-*g*-pectin hydrogel was cut into small pieces with cubic style followed by rinsing in dichloromethane to remove any residual of the unreacted monomer and PGMA homo-polymer. Then, the hydrogel pieces were immersed in ethanol for 7 days for complete removal of unreacted monomer. Finally, the hydrogel pieces were collected and stored at room temperature for further uses.

2.2.2. Urease Immobilization

2.2.2.1. Immobilization of Urease *via* Carboxyl Groups

Dried PGMA-*g*-pectin (100 mg) was added to an activated solution of EDC (10 mg) dissolved in 5ml phosphate buffer (pH 5, 0.02M). The pH of the solution was adjusted to 5.0 with 0.1M HCl. The solution was shaken gently in a water bath for 1 h at 30°C. The activated PGMA-*g*-pectin was collected and washed three times with 3 ml of phosphate buffer (pH 5, 0.02M). The activated PGMA-*g*-pectin was added to 5ml (pH 5, 0.02M) phosphate buffer solution and 5ml (contain 1 mg/ml enzyme) of urease solution was added. The pH of the solution was again adjusted to 5.0 with 0.1M HCl. The solution was shaken in a water bath for 4 h at 30°C. PGMA-*g*-pectin pieces loaded with urease were collected and washed three times with 3 ml of phosphate buffer (pH 5, 0.02M) till no enzyme found. The same procedure was made without EDC to show urease was not immobilized from carboxyl and epoxy groups and/or immobilized noncovalently at pH 5.

2.2.2.2. Immobilization of Urease *via* Epoxy Groups

Dried PGMA-*g*-pectin (100 mg) was added to 5ml phosphate buffer solution (pH 8, 0.02M) and 5ml (contain 1 mg/ml enzyme) of urease solution was added. The solution was shaken at 30°C for 4 and 24 h. PGMA-*g*-pectin pieces immobilized with urease were collected and washed three times with 3 ml of phosphate buffer (pH 8, 0.02M) till no enzyme found.

2.2.2.3. Sequential Immobilization of Urease onto PGMA-*g*-Pectin

Firstly, immobilization of urease was realized from carboxyl groups according to Section 2.2.2.1. Occurred PGMA-*g*-pectin pieces immobilized with urease were added to 0.1M sodium citrate at 4°C. The solution was dialyzed against (pH 8, 0.02M) phosphate buffer solution at 4°C to remove citrate molecules. Secondly, 5ml (contain 1mg/ml enzyme) of urease solution was added to dialysate and the immobilization reaction from epoxy groups was carried out according to Section 2.2.2.2.

2.3. Testing

2.3.1. Grafting Percentage of PGMA

Dried PGMA-*g*-pectin (50 mg) was dissolved in 10 ml distilled water containing $Na_2S_2O_3.5H_2O$ (2 mmol) and acetic acid (1 mmol) and 10 ml acetone was added. The solution was incubated at 40°C for 20 min, cooled and titrated with 0.1N NaOH. Grafted PGMA was determined with standard curve against GMA standard solution [17]. In addition, the grafting percentage of PGMA was calculated gravimetrically according to equation (1):

$$\text{Grafting percentage (G) (\%)} = (W_G - W_o) \times 100 / W_o \quad (1)$$

where W_G mass of grafted polymer and W_o is the initial mass of polymer i.e Difference between the grafted pectin mass and its initial mass before grafting multiplied by 100 and divided by the initial mass.

2.3.2. Determination of Immobilized Urease Content

Urease content in the PGMA-*g*-pectin hydrogel was determined according to the Lowry method [18] by using BSA as a standard. The quantity of immobilized enzyme was calculated by subtracting the enzyme recovered in the combined washings of the urease-polymer network from the enzyme used for immobilization.

2.3.3. Urease Activity Assay

The activities of free and immobilized urease were determined using the Nessler's method [19]. Free (400

µg) or immobilized (100 mg) urease was kept in a test tube and 1 mL of phosphate buffer (pH 7.5) containing 150 mM urea and 5 mM EDTA were added and incubated at 30°C for 30 min. 0.3 mL of 0.68N sulfuric acid was added and a total volume of up to 5 mL was made. Then 1 mL of the above solution was treated with 1 mL of Nessler's reagent and the total volume was made up to 10 mL with DDW. The color produced was determined spectrophotometerically at 480 nm. The amount of ammonia liberated from the urease-catalysed hydrolysis of urea was calculated by comparing the absorbance with a standard curve from ammonium sulfate solution.

One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1µmol urea per minute at 37°C under standard assay conditions.

2.3.4. Properties of Free and Immobilized Urease

2.3.4.1. Optimum pH

The effect of pH on the activity of free and immobilized urease was assayed in the acetate buffer (0.1M) in the pH range 3.0–6.0 and in the phosphate buffer (0.1M) in the pH range 7.0–11.0 by using the above mentioned standard activity assay procedure.

2.3.4.2. pH Stability

The pH stabilities of free and immobilized urease were compared in the acetate and phosphate buffers (0.1M) between pH 3.0 and 11.0. Free and immobilized enzymes were incubated at these buffer solutions for one hour. Both forms of urease were assayed using the standard assay conditions.

2.3.4.3. Optimum Temperature

The effect of temperature on the activities of both free and immobilized urease was studied between 30 and 90°C and assayed under standard assay conditions.

2.3.4.4. Thermal Stability

The thermal stability of the free and immobilized urease was evaluated by measuring the residual activity of urease exposed to various temperatures between 30 and 90°C, in phosphate buffer (0.1M, pH 7.5), for 15 min. The remaining activities were expressed as relative to the original activities assayed at 25°C.

2.3.4.5. Storage Stability and Reusability

The immobilized urease was stored in a dry state at 4°C for 60 days. Its activity was measured at specific time intervals. The immobilized urease was repeatedly used for hydrolysis of urea, for reusability evaluation, under standard assay conditions. After each activity assay the samples were washed with the buffer and stored in a dry state until the next assay.

The results of pH, temperature, reusability and storage stability of free and immobilized urease were presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

2.4. Characterization

FT-IR spectra were recorded on a FT-IR spectrophotometer (Nicolet 670, range from 4000-400 cm^{-1}, USA) using KBr pellets. Surface morphology was visualized by scanning electron microscopy (JXA-840A Electron probe microanalyzer, JEOL, Japan) using an accelerating voltage of 30 KV after coating with gold film using S150A Sputter Coater (Edwards, England). The enzyme content and the activity of free and immobilized urease were measured using double-beam Spectrometer (Shimadzu UV-2401 PC, Japan).

3. RESULTS AND DISCUSSION

The popularity of urease in immobilized enzyme techniques stems its analytical usefulness in the determination of urea, its specificity, and its stability [20,21]. Support material, which plays an important role in the utility of an immobilized enzyme, should be readily available, non-toxic and in insoluble form so the resulting biocatalyst can be reused several times, thus lowering the cost [22,23].

Pectin is a natural hydrophilic biopolymer, predominant in the middle lamella of plant cell walls, providing cell-cell adhesion and mechanical strength [24,25]. Grafting of pectin with PGMA using emulsion polymerization technique could be done for the reduction of the hydrosolubility of pectin *via* the formation of insoluble PGMA-*g*-pectin hydrogel. Various concentrations of pectin (0.5–5 wt %) were reacted with a fixed concentration of GMA (0.25 mol). We found that when the pectin concentration was higher than 1 wt %, the solution became extremely viscous. In fact, we were unable to stir the solutions having a high pectin concentration (5 wt %). Furthermore, we found that the use of a low pectin concentration resulted in the production of deformed hydrogels. The optimal pectin concentration was identified as 1 wt %. Accordingly a series of hydrogels could be made with this specific pectin concentration and various GMA concentrations (0.25–1 mol) without

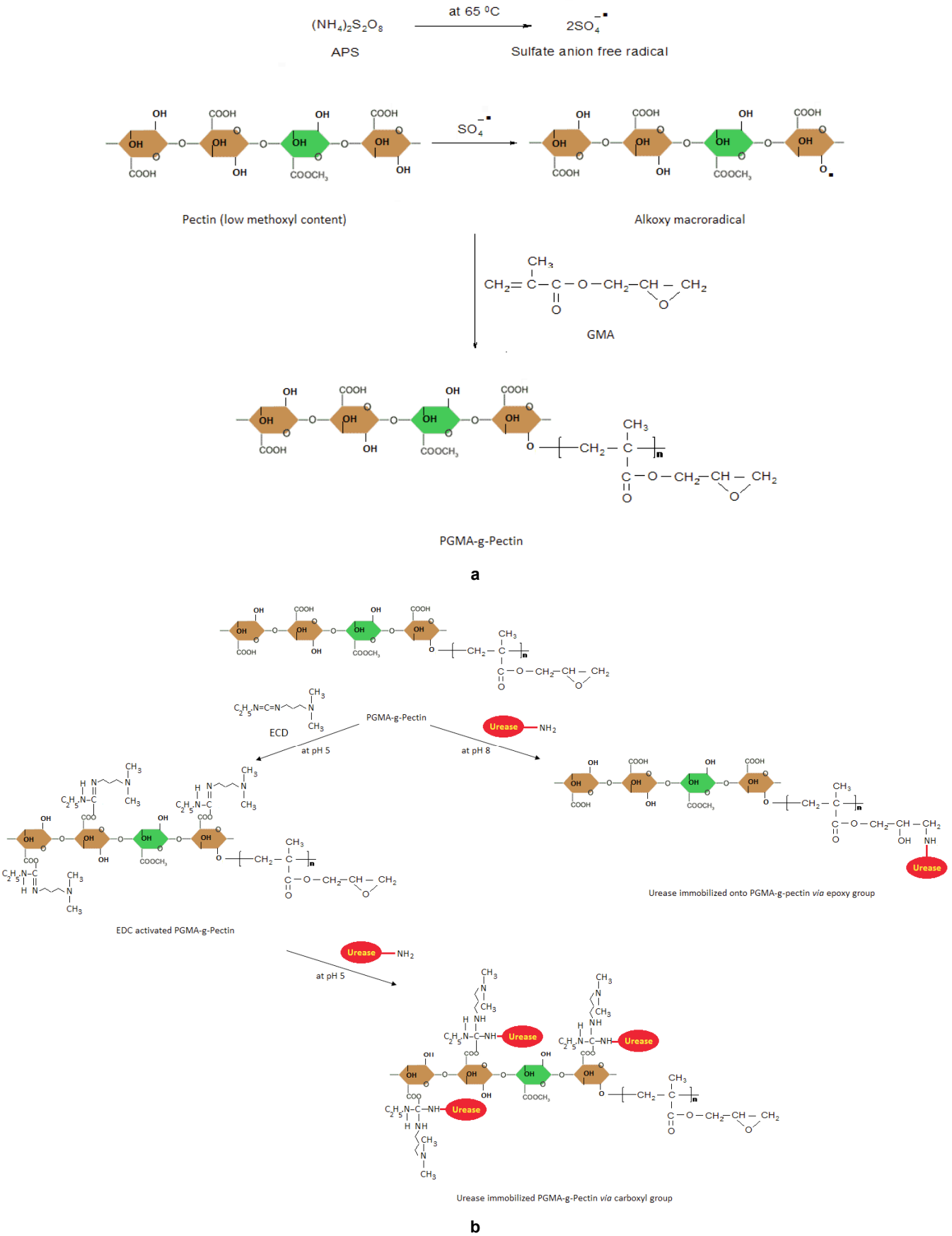

Figure 1: Schematic representation of (**a**) the grafting reaction mechanism of the PGMA on pectin and (**b**) the immobilization of urease onto PGM-*g*-pectin *via* carboxyl and epoxy groups.

any problem. The tailored insoluble hydrogel was investigated as multi-sites enzyme immobilization system with urease as an enzyme model.

The proposed reaction mechanism between GMA and pectin is summarized in Figure **1a**.

3.1. Characterization of Hydrogels

3.1.1. FT-IR Spectroscopy

Figure **2** gives the FT-IR spectra of pectin, PGMA-g-pectin, PGMA-*g*-pectin-urease, and urease. The FT-IR spectra of pectin (Figure **2a**) exhibited a broad band at 3405 cm^{-1} assigned to stretching of –OH groups; a band at 2940 cm^{-1} assigned to the aliphatic C–H vibrational stretching of CH_3; bands at 1744 and 1627 cm^{-1} which are assigned to C=O stretching of esterified carboxylic groups ($–COOCH_3$) and free carboxylic groups (–COOH), respectively. In addition, bands at 1451, 1110 and 1021 cm^{-1} assigned to $–CH_2$, –CH–OH stretching of aliphatic cyclic secondary alcohol and –CH–O–CH– stretching, respectively.

The presence of new bands in the PGMA-*g*-pectin spectrum (Figure **2b**) at 932, 873, and 749 cm^{-1} are attributed to the epoxide group of PGMA which characterizes the chemical modification of pectin with PGMA. The presence of these peaks also indicates that the grafting process did not affect the retention of epoxide groups. The band at 1724 cm^{-1} region of the PGMA-*g*-pectin spectrum is attributed to the axial deformation of C=O conjugated ester groups.

The immobilization of urease is also confirmed by FT-IR analysis. All stretching and bending vibration modes of the urease are observed in the spectra of the various hybrid samples (Figure **2d**). The bands pointed at 1650 and 1547 cm^{-1} correspond to the stretching and deformation mode of the C=O (amide I) and N–H (amide II), respectively. The bands between 1303 and 1245 cm^{-1} can been assigned to the C–N and N–H vibrations (amide III). The bands at 1443 and 1404 cm^{-1} are due to C-H vibrations of the CH_2 group. The characteristic infrared feature of the hydrotalcite-like structure also appears (ν_{OH}: 3371 cm^{-1}, δ_{HOH}: 1650 cm^{-1}, $\nu_{M–O}$: 858– 657 cm^{-1} and $\delta_{O–M–O}$: 427 cm^{-1}). The vibration bands of the immobilized urease are at the same wavelength position as the free one (Figure **2c**), suggesting that the molecular structure of the urease seems to be preserved after being immobilized onto PGMA-*g*-pectin hydrogel. These results are in accordance with the previous results [26-29].

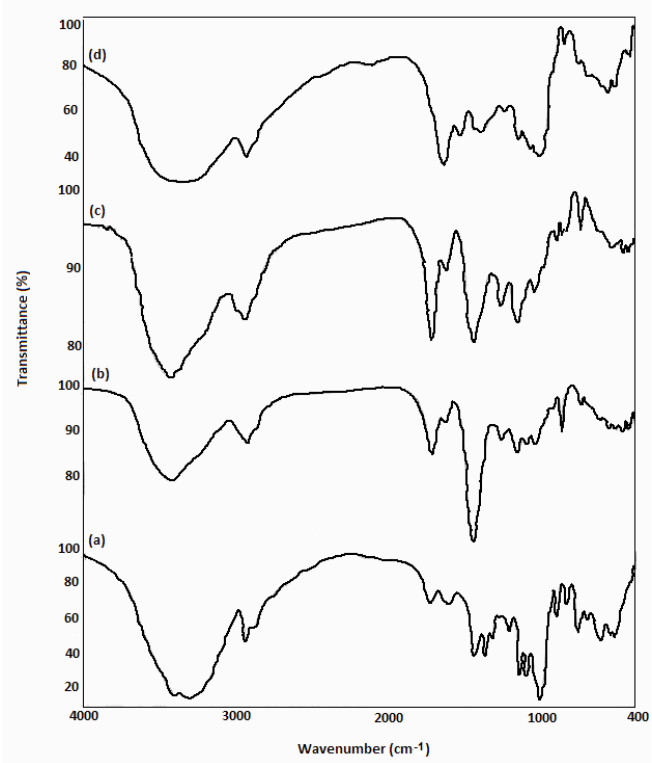

Figure 2: FT-IR of (**a**) Pectin, (**b**) PGMA-*g*-Pectin, (**c**) PGMA-*g*-Pectin-Urease, and (**d**) Urease.

3.1.2. Hydrogels Morphology

The effect of the content of GMA on the morphology of PGMA-*g*-pectin hydrogel is examined using SEM. Figure **3** clearly illustrates the microstructure of the resulting gels changes with increasing GMA content. For 0.25 mol of GMA the spongy structure with large pore size is observed (Figure **3a**). By increasing the ratio of GMA (0.5 mol) the pore size is reduced and the heterogeneity in the surface is noticed. The best homogeneity between pectin and PGMA was achieved at 0.75 mol of GMA with suitable pore size. The surface morphology becomes more compact with cracks and the pores nearly disappear when the ratio of GMA reaches 1 mol (Figure **3d**).

In addition, the urease immobilization onto PGMA-*g*-pectin hydrogel is also proved by SEM micrographs. Figure **3e**, **f**, and **g** show the filling of hydrogel pores with urease particles.

3.1.3 Determination of Percentage Grafting GMA in PGMA-g-Pectin

Determination of the percentage of GMA was carried out according to the method mentioned in Section 2.3.1. The data are illustrated in Table **1**. As observed from the results, the percentage of grafting increased by increasing the GMA ratio till 0.75mol then the percentage decreased. The results of titration and

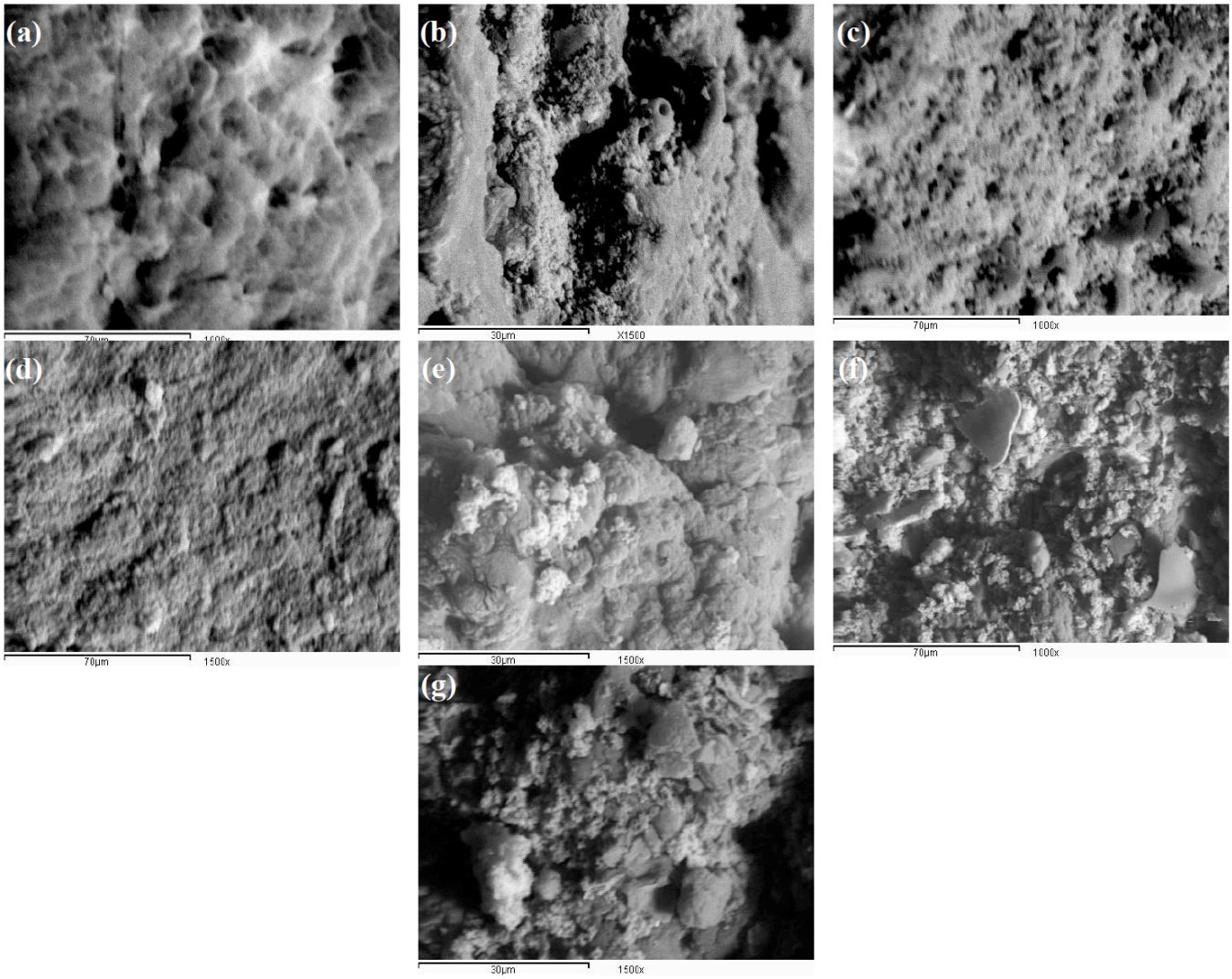

Figure 3: SEM microphotographs of (**a**) PGMA-*g*-Pectin hydrogel (o.25 mol GMA), (**b**) PGMA-*g*-Pectin hydrogel (o.5 mol GMA), (**c**) PGMA-*g*-Pectin hydrogel (o.75 mol GMA), (**d**) PGMA-*g*-Pectin hydrogel (1 mol GMA), (**e**) PGMA-*g*-Pectin-Urease (o.75 mol GMA, 1 mg urease), PGMA-*g*-Pectin-Urease (o.75 mol GMA, 5 mg urease), and (**g**) PGMA-*g*-Pectin-Urease (o.75 mol GMA, 10 mg urease).

Table 1: Effect of Amount of Initial GMA According to Gravimetric and Titrimetric Methods

Amount of initial GMA (mg)	Amount of GMA grafted to 100 mg pectin (mg)[a]	Amount of GMA grafted to 100 mg pectin (mg)[b]	G (%)
177	15.8	16.5	91.2
355	32.3	33.8	93.1
533	51.5	52.6	97.6
710	60.4	61.2	85.6

[a]gravimetric method.
[b]titrimetric method.

gravimetric methods were compared and the results were in accordance.

3.2. Urease Immobilization

The amounts and percentages of bound urease to PGMA-*g*-pectin with different ratios of GMA content are shown in Table **2**. It was observed that the amount of bound urease is increased with increasing the GMA ratio up to 0.75 mol of GMA after that ratio, the amount of immobilized urease decreased. This behavior can be illustrated by the morphology of the prepared hydrogels as discussed in section 3.1.2. It was seen in Figure **3a**

Table 2: Effect of Amount of GMA on the Immobilized Urease Content and Activity

GMA (mol)	Bound Urease (mg)	Bound Urease (%)	Retained Activity (%)
0.25	1.5	33.1	38.6
0.5	3.2	64.8	56.8
0.75	4.3	85.2	68.4
1	0.5	10.2	18.2

that the large pores of PGMA-*g*-pectin (0.25mol GMA) led to a rapid loss of most urease content during washing and tests followed the immobilization reaction. The loss in urease content decreased with increasing the molar ratio of GMA which can be attributed to the less porosity and very narrow micropores present in PGMA-*g*-pectin (0.5mol GMA) hydrogel, but the morphology still not homogeneous (Figure **3b**). The maximum urease content was found in case of PGMA-*g*-pectin (0.75mol GMA) at which the structure morphology became homogeneous with very narrow pores (Figure **3c**). In this case the narrow porosity structure retarded the urease to leave the carrier. The PGMA-*g*-pectin (1mol GMA) hydrogel has a very dense morphology nearly without any pores which led to low immobilized urease content (Figure **3d**). From these results and observations it was found that the suitable formed hydrogel for immobilization reaction is PGMA-*g*-pectin (0.75mol GMA) which was selected in all the immobilization reactions and tests.

In the immobilization process, urease was immobilized onto PGMA-*g*-pectin *via* epoxy group and carboxyl group of PGMA and pectin, respectively. Urease was immobilized from carboxyl group of pectin *via* its activation with EDC at pH 5 and then immobilized from epoxy group of PGMA at pH 8. This model of immobilization process order allows immobilizing more than one enzyme onto the same carrier (multi-sites enzyme immobilization system).

The proposed reaction mechanism of urease immobilization onto PGMA-*g*-pectin *via* carboxyl and epoxy groups is summarized in Figure **1b**.

3.2.1. Urease Immobilization via Carboxyl Groups

The selection of appropriate pH, activation time and the concentration of EDC are important in minimizing detrimental effects during the activation of the PGMA-*g*-pectin carrier prior to immobilization process. For this purpose, the activation of the carboxylic groups on PGMA-*g*-pectin carrier with EDC was carried out within the pH range from 4 to 7. The optimum pH of activation reaction was found at pH 5. The effect of activation time on the catalytic performance of the carrier is shown in Table **3**. It corresponds to the reaction time between the carboxylic groups on the PGMA-*g*-pectin carrier and the EDC coupling agent. The activity of the PGMA-*g*-pectin carrier using EDC was in the maximum value when the activation reaction carried out for 3 h. This represents the saturation of the surface functional groups (carboxyl) with EDC molecules. Beyond 4 h, not only the reaction between EDC and the surface completed but also hydrolysis of EDC occurred which resulted in a reduced activity [30]. Urease activity for pH 5 was lower than other pH values assays but the activity was enough for immobilization. The activated PGMA-*g*-pectin with different concentrations of EDC was also investigated. The effect of EDC concentrations and the optimal one is shown in Table **3**. It was found that the optimal concentration of EDC is 10 mg/ml, where the bounded urease was 85.7 % and

Table 3: Effect of Different pH, Activation Time, and Concentration of EDC on Immobilization Process

pH of activation			Activation Time			EDC conc.		
pH	Bound Urease %	Retained activity %	Time (h)	Bound Urease %	Retained activity %	Conc. (mg)	Bound Urease %	Retained activity %
4	46.7	40.6	1	33.9	21.3	1	32.6	10.7
5	85.2	68.1	2	61.5	50.7	5	60.4	30.5
6	65.1	59.5	3	85.6	68.5	10	85.7	68.4
7	39.5	50.2	4	40.7	48.4	50	95.2	31.6
						100	99.3	25.5

Table 4: Effect of Immobilization Time and Urease Concentration on Immobilization Process

Immobilization Time			Urease conc.		
Time (h)	Bound Urease %	Retained activity %	Conc. (mg)	Bound Urease %	Retained activity %
1	27.8	25.9	1	96.7	43.1
2	50.3	44.4	5	85.4	68.2
4	85.1	68	10	47.8	31.6
24	96.6	18.4			

its retained activity 68.4 %. At 1 mg, amount of EDC was low according to other concentrations and urease was bounded to pectin at lower grade. In the mean while, at 50 and 100 mg of EDC more urease was immobilized according to bounded enzyme results. In this case, urease was bounded to pectin from multi-point and 3D structure of urease was changed. So the activity of immobilized urease was decreased [31,32].

Furthermore, the effect of immobilization time was investigated at 1, 2, 4 and 24 h and the optimum immobilization time was 4 h (Table **4**).

Effect of urease concentration at 1, 5 and 10 mg/ml was also investigated and the optimum concentration was 5 mg as mentioned in Table **4**. To the best of our knowledge, immobilization of urease was performed at room temperature, so 30°C was selected as the reaction temperature [32].

The ultimate goal of this study was to make pH selectivity as responsible target for immobilization reaction. To achieve this aim, the activation with EDC and the immobilization process were carried at pH 5. No urease activity was determined in absence of EDC at pH 5 *via* epoxy groups. This result was in agreement with the previous study [32].

3.2.2. Urease Immobilization via Epoxy Groups

Because the Epoxy group is very stabile, it can hydrolyze at extreme pH and accordingly the immobilization reaction *via* this group can occur at two steps. At the first step, enzyme is adsorbed to the carrier and in the second, epoxy group of PGMA-*g*-pectin reacts with amino group of urease to form covalent bound. We have chosen the immobilization reaction temperature to be 30°C and this was in accordance with the previous studies [33-35].

In enzyme immobilization *via* epoxy group, the most important parameter is the reaction time. It is clearly seen from Table **4** that the optimum reaction time is 4 h with higher retained activity and this result is very interesting because the previous reported results were more than 15 h with lower retained activity [34-37].

The reaction was occurred spontaneously and no chemical agent was used for immobilization, in addition, the retained activity (%) of immobilized urease onto PGMA-*g*-pectin was suitable for practical applications.

3.2.3. Dual Urease Immobilization

Firstly, urease was immobilized *via* carboxyl groups of PGMA-*g*-pectin at pH 5 with retained activity 68 %, and secondly, the enzyme was immobilized *via* epoxy groups of PGMA-*g*-pectin at pH 8 with total retained activity 65 %, selectively with a minor loss percentage.

3.3. Parameters Affecting Urease Activity

The activities of free and immobilized urease onto PGMA-*g*-pectin were calculated by measuring the absorbance of the librated ammonia at 480 nm, taking into account that the retained activities of the immobilized urease would be the initial activities (relative activity 100%) of them in the subsequent experiments. Effect of pH values and temperatures, as well as storage stabilities, and reusability were examined as observed in Figure **4**.

3.3.1. Effect of pH

The pH and thermal activity profiles of an enzyme can be altered due to its immobilization to the carriers [38-41]. Changes in optimum pH as well as a broadening of the pH and temperature stability have been demonstrated after immobilization process. The effect of pH on urease activity has been investigated by varying the pH of buffer from 3.0 to 11.0. The free urease shows its maximum activity at pH 7.0 whereas, the immobilized one shows its maximum activity at pH 5.8 (Figure **4a**). The shift in the optimum pH towards a lower pH value upon immobilization may be due to the reaction carried out through the free amino group of the enzyme. Accordingly the medium became acidic

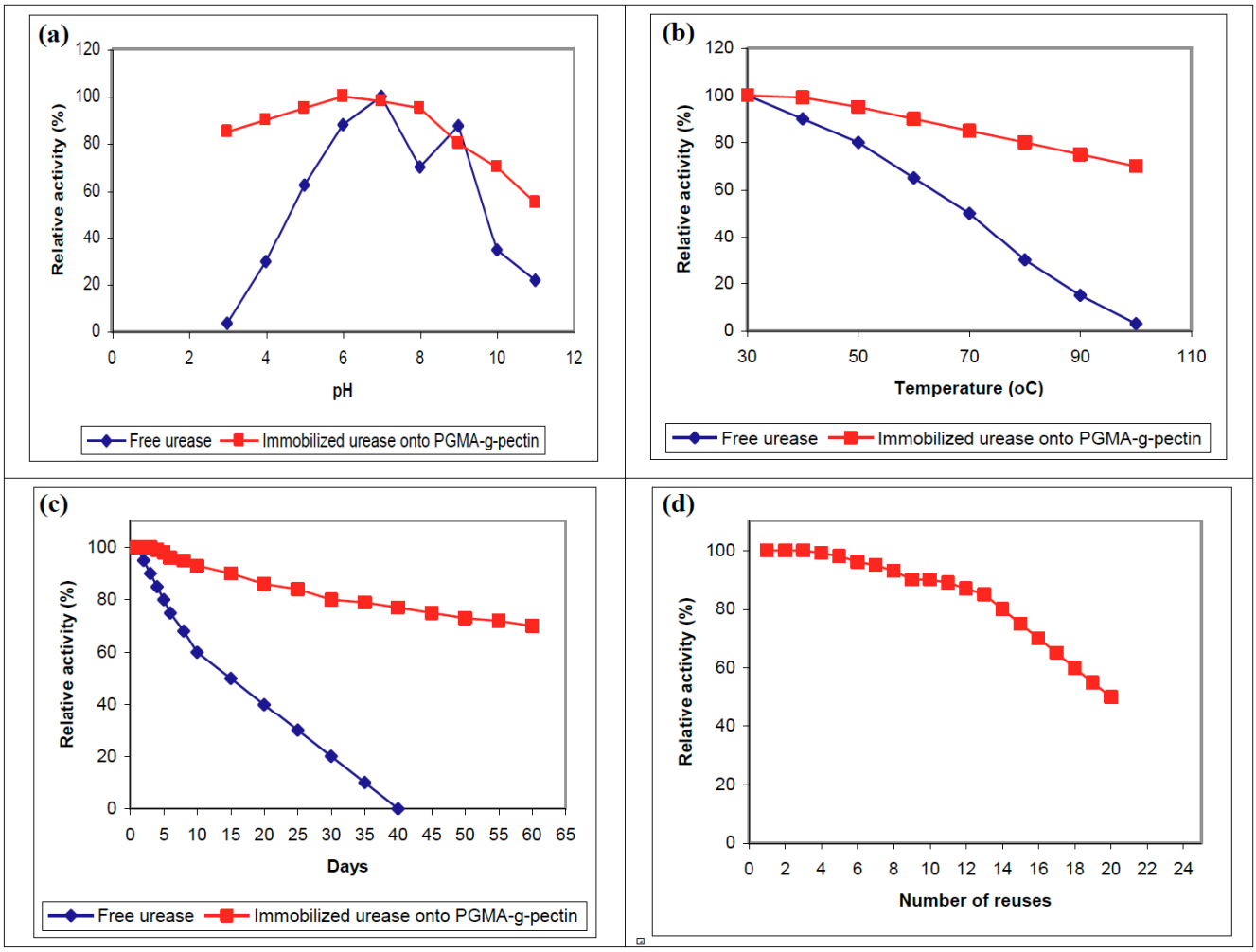

Figure 4: Studies of different parameters affecting urease activity: Optimum (**a**) pH and (**b**) temperature, (**c**) storage stability of free and immobilized urease onto PGMA-*g*-Pectin hydrogel at 4°C and (**d**) reusability of immobilized urease onto PGMA-*g*-Pectin hydrogel in repeated hydrolysis of urea.

according to the free and unreacted carboxylic groups of the enzyme and polymer carrier, respectively [42]. However, the immobilized urease shows almost equal relative catalytic activity with varying pH range from 3.0 to 9.0 (Figure **4a**), the relative catalytic activity of free urease was decreased at pH higher and lower than 7.0 (Figure **4a**). This means that the immobilization enhances enzyme stability at broader pH. Immobilized urease shows better activity in acidic pH as compared to the free one and the catalytic activity of immobilized enzyme is retained. This is due to the immobilized enzyme is less sensitive to pH changes than that of free urease [43]. Optimum pH values for immobilized urease on different carriers were reported in the ranges between 5 and 8 in the detailed review by Krajewska [44].

3.3.2. Effect of Temperature

In order to determine the effect of temperature on the free and immobilized urease, the catalytic activities were measured over a range of temperatures from 30 to 100°C. As seen in Figure **4b**, with increasing temperature the catalytic efficiency percentage of free urease decreases while immobilized one shows an increase in its catalytic activity percentage. At 100°C, the immobilized urease retained 70% of its original activity whereas the free one lost all. Hence, these results showed that immobilized enzyme has better thermal stability as compared to its free counterpart. The enhancement of thermal stability of immobilized urease is attributed to the covalent bonding between carboxyl groups of PGMA-*g*-pectin and urease, which may restrict the conformational change of urease through heating. The improved thermal enzymatic stability through binding onto PGMA-*g*-pectin has been ascribed to higher hydration strength of the enzyme on hydrogel. The presence of special water structuring properties of hydroxyl groups on hydrogel prevents the denaturation of urease. We conclude that this behavior is probably due to constraint induced by covalent

bonds that hinder denaturing by molecular relaxation [45,46].

3.3.3. Storage Stability

Free and immobilized urease were stored in dry state at 4°C, and the catalytic activity measurements were carried out for 60 days. The free urease lost all of its activity within 40 days, whereas the immobilized one lost about 23 % of its activity during the same time period as shown in Figure **4c**.

3.3.4. Reusability

For any applications based on immobilized enzymes, the feasibility of regeneration of the enzyme activity and consequent reuse of the biocatalyst are beneficial for its industrial productions [47]. The operational stability of immobilized urease onto PGMA-*g*-pectin was obtained by running measurements in the same day of immobilization process. Between each subsequent measurement the polymeric carrier washed with phosphate buffer solution and stored for 10 min. After 10 cycles the immobilized urease retained 90 % of its original activity, while 50 % loss was observed after 20 times of reuse (Figure **4d**). Therefore, the use of immobilized urease, in particular immobilized system, would lead to the continuous hydrolysis of urea without much loss of its activity. But free enzyme cannot be reused or recycled for next subsequent catalytic experiments.

CONCLUSIONS

It is obvious from the obtained results that the developed PGMA-*g*-pectin hydrogel was a suitable polymeric carrier for immobilization of more than one enzyme. The immobilization of urease *via* carboxyl and epoxy groups of PGMA-*g*-pectin depended on the pH selectivity. The immobilized urease has a good stability in a wide rang of pH and temperature over free one. Also, the immobilized urease can be used for continuous hydrolysis reaction of urea without serious effect on its activity, but the free one cannot be recycled.

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