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Synthesis and characterization of starch-poly(methyl acrylate) graft copolymers using horseradish peroxidase

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ABSTRACT

Horseradish peroxidase (HRP)-mediated graft polymerization in the presence of hydrogen peroxide (H_2O_2) and acetylacetone (Acac) has been successfully applied to the synthesis of starch-poly(methyl acrylate) (PMA). The graft copolymer was characterized by Fourier transform infrared (FT-IR), elemental analysis, nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR), and differential scanning calorimetry (DSC). FT-IR, elemental analysis and NMR confirmed that methyl acrylate (MA) was grafted onto starch successfully. DSC results showed the graft reaction had changed the crystalline regions of the gelatinized starch. The effects of pH, MA content, HRP dosage, incubation temperature and time on grafting percentage (GP) and grafting efficiency (GE) were also investigated. The GP and GE under optimal conditions reached 30.21% and 45.13%, respectively.

1. Introduction

Starch is a naturally occurring high molecular weight compound, which has a wide range of applications in food and chemical industry and as a raw material in other industrial processes because it is available abundantly and has various properties. However, starch has some inherent limitations in its properties which make it unsuitable for some specific applications, thereby restricting its widespread use. Therefore, modification of starch using either physical or chemical methods is necessary in order to improve the properties of starch-based materials (Brockway & Moser, 1963; Kiatkamjornwong, Mongkolsawat, & Sonsuk, 2002). In recent years, attention has been particularly focused on chemically modified starches due to their varied properties, reproducibility, biodegradability and low-cost (Liu & Su, 2005). Starch can be chemically modified by reactions such as hydrolysis, etherification, grafting, dextrinization and other methods (Meshram, Patil, Mhaske, & Thorat, 2009). These modifications help to overcome the above mentioned limitations and improve the thickening, binding, gelling, adhesive and film forming properties of starch, thereby widening the scope of its applications in different fields such as agriculture, medical, textile (Gao, Yu, & Wang, 1998). Recently, a large number of researches have reported grafting of some vinyl monomers onto starch using chemical initiators, like ceric ammonium nitrate (Athawale & Rathi, 1997), potassium permanganate/citric acid (Mostafa, 1995), potassium bromate-thiourea, (El-Rafie, Zahran, El-Tahlawy, & Hebeish, 1995) and ammonium persulfate (Zhang et al., 2014). It should be noted that although the chemical methods for the graft polymerization of starch have many advantages such as high catalytic efficiency and less triggering time, it still has some disadvantages such as complexity associated with the modification process, which makes it difficult to control the reaction. Moreover, some chemical initiators are responsible for environmental pollution and also degrade the starch during modification. Thus, it is essential to find an eco-friendly alternative for these traditional initiators. With rapid developments in biological science, it has been realized that some inorganic initiators can be replaced by natural enzymatic catalysts (Shogren, Willett, & Atanu, 2009). It has been proved that enzymatic catalysis is a powerful method for graft polymerization reaction in material modification (Hollmann & Arends, 2012; Nyanhongo, Kudanga, Prasetyo, & Guebitz, 2011). Thus, the modification of starch via enzymatic catalysis constitutes a green method of improving properties of starch and widening its scope of applications.

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Table 1
Elements content of soluble starch and grafted starch.

Samples (Theoretical value)	C (%)	H (%)	O (%)
Soluble starch	40.300 (44.444)	6.649 (6.173)	53.051 (49.383)
Grafted starch (GP = 7.50%)	41.800 (45.332)	6.593 (6.242)	51.607 (48.635)
Grafted starch (GP = 30.21%)	43.280 (47.082)	6.720 (6.359)	50.000 (46.559)

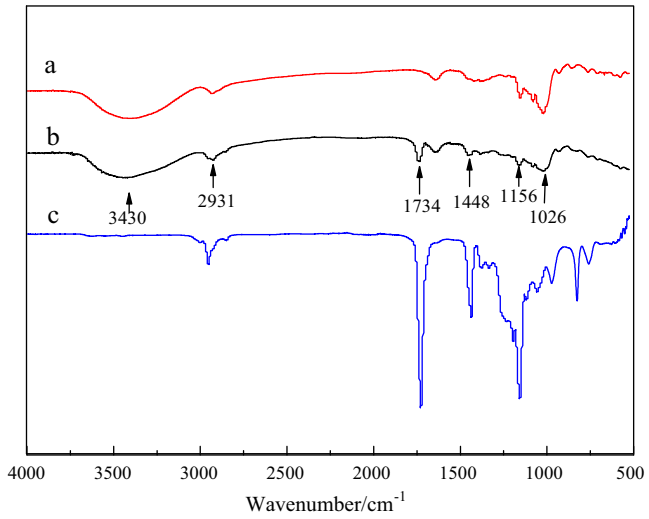

Fig. 1. FT-IR spectra of the soluble starch (a), graft copolymer (b) and PMA (c).

Horseradish peroxidase (HRP, EC1.11.1.7), an oxidoreductase obtained from the roots of horseradish, is a very popular and efficient biocatalyst, which has been used to catalyze free radical polymerizations of phenols (Ikeda, Sugihara, Uyama, & Kobayashi, 1996) and anilines (Bruno et al., 1995; Teixeira, Lalot, Brigodiot, & Maréchal, 1999). It has been reported that HRP can catalyze the oxidative coupling of many substrates like phenols and aromatic amines in the presence of hydrogen peroxide in aqueous, nonaqueous and interfacial media (Bhanu & Gross, 2000). Derango et al. were the first to demonstrate the potential use of HRP and other oxidases as catalysts in the free radical polymerization of vinyl monomers (Derango, Chiang, Dowbenko, & Lasch, 1992). Bhanu et al. studied HRP-catalyzed acrylamide polymerizations in an aqueous medium both in presence and in absence of surfactants. Also, polymerization in concentrated emulsions using sorbitan monooleate as the emulsifier was also reported (Bhanu & Gross, 2002). Emery et al. reported that the free radical polymerization of acrylamide in water could be initiated by HRP/H_2O_2/2,4-pentanedione (Emery, Lalot, Brigodiot, & Marechal, 1997). The graft polymerization of starch and acrylamide in an aqueous solution using an initiator system comprising of HRP/H_2O_2/2,4-pentanedione was reported by Shogren et al. (2009). The results showed that polyacrylamides were bonded to the starch skeleton to form branched chains with low molecular weights in the presence of HRP/H_2O_2. In addition to this, Lv et al. showed that different monomers like dimethyl diallyl ammonium chloride (DMDAAC), p-hydroxybenzoic acid and resorcinol could be grafted onto starch in the system initiated by HRP/H_2O_2 (Lv, Gong, & Ma, 2012; Lv, Gong, Yan, & Hou, 2012; Lv, Sun, Zhou, Liu, & Ding, 2014).

It is well-known that the viscosity of native starch slurry is unstable and the film made from starch is brittle, which greatly limits its industrial applications. In the present work, HRP-mediated polymerization of starch and methyl acrylate (MA) has been studied, with an aim to graft flexible polymers onto starch chains. Besides this, this is an innovative method to substitute traditional chemical initiators by HRP in the starch modification process.

The polymerization of soluble starch with MA was characterized by FT-IR, elemental analysis, ^{1}H NMR, ^{13}C NMR, and DSC. The effects of the reaction conditions on graft copolymerization were also investigated.

2. Materials and methods

2.1. Materials

Soluble starch (≤13% water content) and hydrogen peroxide (30% (w/v)) were provided by Sinopharm Chemical Reagent Co (Shanghai, China). Horseradish peroxidase was obtained from Aladdin Reagent Ltd. (Shanghai, China). All the other chemicals used were commercially available and were of analytical purity.

2.2. Horseradish peroxidase assay

2 mL HRP (0.01 mg/mL), 2 mL H_2O_2 (30% (w/v)) and 25 mL gallic acid were added (0.01 g/mL) to a 100 mL conical flask containing 20 mL phosphate buffer solution and then placed in an incubator shaker and allowed to react for 6 h at 30 °C. Then, 10 mL of anhydrous ether was poured into the reaction mixture to extract the product. The ether extract was used for analysis. The activity of HRP was measured using an UV/Vis spectrophotometer by monitoring the absorbance of the prepared solution at a wavelength of 420 nm and expressed in units defined as follows:

$$\text{HRP (U/mg)} = E_{420} \times 8.5 \times E_w \quad (1)$$

where E_{420} is the absorbance at a wavelength of 420 nm, 8.5 is the weight of the oxidized product that was extracted with 100 mL anhydrous ether. The absorbance at the wavelength of 420 nm is 1.000, and E_w is the weight of the enzyme.

Then pH of phosphate buffer solution and the incubation temperature in the HRP activity assay were optimized. The pH value was optimized by measurement of activities at pH values ranging between 5 and 9 at 30 °C. For each of the temperatures studied (20, 30, 40, 50 and 60 °C), the optimum pH of phosphate buffer solution, was chosen. The incubation procedure was the same as that described for pH optimization.

2.3. Preparation of copolymers

Soluble starch (2.5 g, dry weight) and 90 mL water were placed in a three-necked, round-bottomed flask and gelatinized by stirring for 10 min at 100 °C and then cooled to room temperature. Then 10 mL of 0.1 M potassium phosphate buffer, pH 7.0, 3.0 g MA, and specific amounts of Acac and HRP were successively added to the three-necked flask under a nitrogen atmosphere and stirring. Next, 2.0 mL of H_2O_2 (0.3%) was added dropwise within 1 h. Finally, the reaction mixture was stirred for 5 h at 30 °C. After this, the reaction mixture was poured into 200 mL of absolute ethanol to precipitate the product (Lv et al., 2014; Shogren et al., 2009).

The product was poured into 200 mL of absolute ethanol, stirred for 24 h at room temperature, followed by centrifugation at 4000 rpm for 10 min to remove the unreacted MA and other reagents and to get a mixture of the grafted copolymer and homopolymer. The mixture was weighed after it was dried to constant weight under vacuum at 50 °C and extracted with acetone for 12 h to obtain the grafted starch. The homopolymer was gained from the acetone used in extraction by volatilization. And the

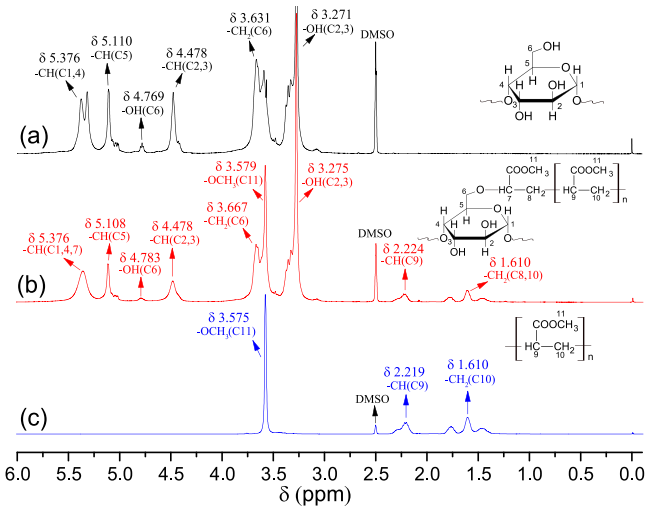

Fig. 2. ^{1}H NMR spectra of soluble starch (a), grafted starch (b) and PMA (c).

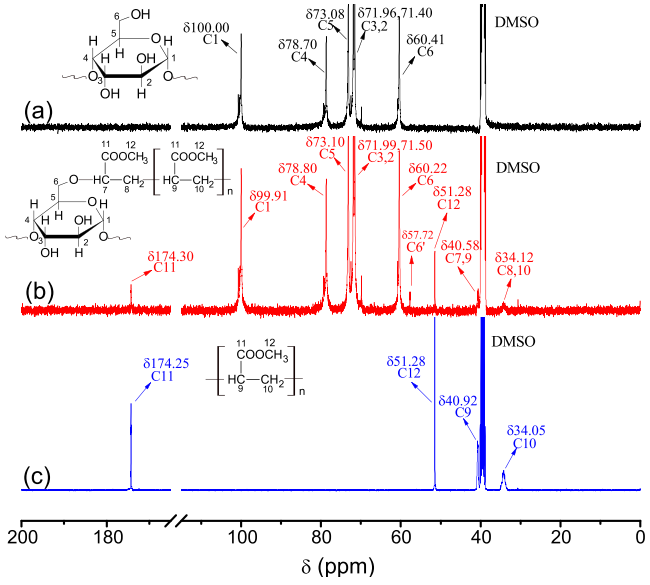

Fig. 3. ^{13}C NMR spectra of soluble starch (a), grafted starch (b) and PMA (c).

gelatinized starch was prepared under the same conditions as the preparation of the graft copolymers without HRP/Acac/H_2O_2.

The graft copolymer was hydrolyzed with 100 mL hydrochloric acid (1.0 mol/L) in a water bath at 98 °C for 10 h (The degree of hydrolysis of the copolymer was tested using a solution of I_2-KI). Then, the mixture was neutralized with a solution of sodium hydroxide (1.0 mol/L) and washed until the washings were free of Cl^- s (The washings were tested using a solution of $AgNO_3$). The product obtained in the last step was dried to constant weight under vacuum at 50 °C, in order to obtain the polymer grafted onto soluble starch.

2.4. Characterization

2.4.1. Grafting percentage and grafting efficiency

The degree of grafting was reflected by the GP and GE. GP indicates the amount of PMA grafted onto the soluble starch. GE expresses the ratio of insoluble PMA grafted onto the soluble starch

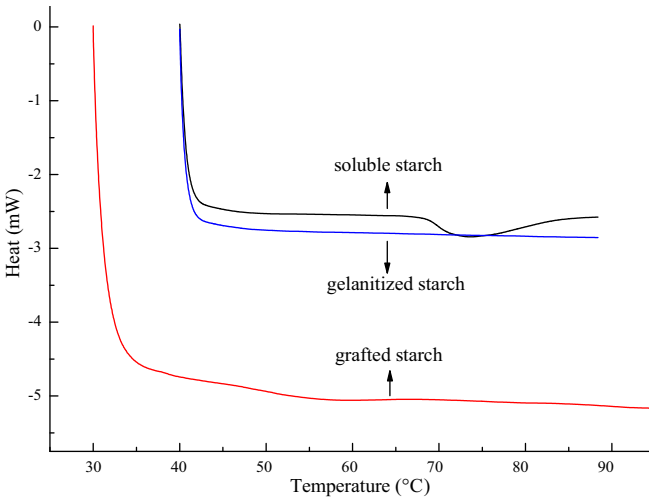

Fig. 4. DSC curves of the soluble starch, gelatinized starch and grafted starch.

to the total PMA. The GP and GE were calculated by using following equations:

$$GP\ (\%) = \frac{M_2 - M_0}{M_2} \times 100 \quad (2)$$

$$GE\ (\%) = \frac{M_2 - M_0}{M_1 - M_0} \times 100 \quad (3)$$

where M_0 is the weight of the soluble starch taken; M_1 is the combined weight of the graft copolymer and the homocoplymer formed; M_2 is the weight of the graft copolymer (Guo et al., 2015).

2.4.2. Optimization of the graft copolymerization

The pH was optimized by testing at pH values of 5.0, 6.0, 7.0, 8.0, and 9.0 using MA 3%, HRP 0.4% and H_2O_2 0.6% in phosphate buffer at 40 °C for 6 h. The MA concentration was optimized using MA concentrations of 1, 2, 3, 4, and 5% at an optimum pH for the grafting reaction. The other grafting conditions were the same as those described for pH optimization. For optimization of enzyme concentration the enzyme concentrations tested were 0.1, 0.2, 0.3, 0.4 and 0.5% at optimum conditions of pH and MA concentration. For optimizing the temperature, the temperatures tested were 20, 30, 40, 50 and 60 °C using optimum an enzyme concentration. The incubation procedure was the same as described for the optimization of enzyme concentration. For optimization of the reaction time, the reaction times chosen were 3, 4, 5, 6 and 7 h, the other reaction conditions being optimum, based on the above experiments.

2.4.3. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra were recorded on a Nicolet iS10 FT-IR spectrometer (Thermo Fisher Scientific, USA) using the ATR technique. The spectra were recorded in the absorption mode in the range of 4000–500 cm^{-1} with a 4 cm^{-1} resolution, using KBr pellets made from a mixture of polymer (1 wt%) and KBr.

2.4.4. Elemental analysis

C, H and O elements of the graft copolymer and soluble starch samples were determined using a Vario ZL III elemental analyzer (Elementar, Germany).

2.4.5. NMR analysis

The ^{1}H NMR and ^{13}C NMR spectra of 20–50 mg purified samples dissolved in 450–500 μL of deuterated DMSO were recorded by using tetramethylsilane (TMS) as an internal standard on a Bruker Avance III spectrometer (Bruker, Germany) at an operating frequency of 400 MHz.

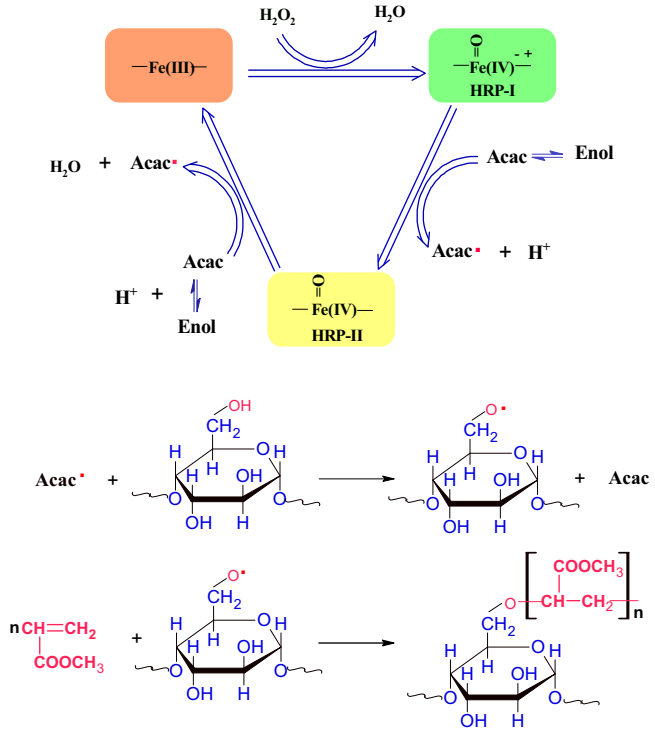

Fig. 5. Catalytic synthesis process of the enzymatic reaction.

2.4.6. Differential scanning calorimetry (DSC)

DSC experiments were performed using a differential scanning calorimeter TA-Q200 (Waters, Shanghai) with an N_2 flow rate of 20 mL/min. The temperature was varied from 40 to 90 °C at a heating rate of 10 °C/min.

3. Results and discussion

3.1. FT-IR analysis

To demonstrate grafting reaction, the comparison of FT-IR spectra of soluble starch, graft copolymer, and PMA are shown in Fig. 1. The FT-IR spectrum of soluble starch (Fig. 1a) showed the characteristic absorption peaks at 3430 cm^{-1}, 2931 cm^{-1} and a triplet peak at 1156 cm^{-1} which are ascribed to the O—H stretching, C—H stretching and C—O—C stretching vibrations of the starch molecule, respectively (Zhang et al., 2014). The absorption peak at 1026 cm^{-1} is a characteristic of the glucose ring in the structure of starch. As shown in Fig. 1b, the characteristic peaks appearing at 3430 cm^{-1}, 2931 cm^{-1} and 1026 cm^{-1} can be ascribed to the O—H stretching vibration, C—H stretching vibration and to the glucose ring of the starch, respectively, which is consistent with the spectrum of soluble starch (Fig. 1a). The peaks appearing at 1734 cm^{-1} and 1448 cm^{-1} (Fig. 1b) are due to —COO^- stretching vibration in PMA. In contrast to these peaks, characteristic of the carbonyl functional group at 1734 cm^{-1} and 1448 cm^{-1}, are observed in the FTIR spectrum of PMA (Fig. 1c). These results suggest formation of PMA-grafted starch using MA and starch.

3.2. Elemental analysis

The elemental analysis of soluble starch and grafted starch are presented in Table 1. It can be seen that the C and O contents of grafted starch show obvious trends with changes in GP. The C and O contents of MA are 55.81% and 37.21%, respectively. The contents of C and O in soluble starch are 40.30% and 53.05%, respectively. It can be seen in Table 1, that the C and O contents of grafted starch lie between those of soluble starch and MA. With the increase of GP, the measured values of the C content increase and the measured values of the O content decrease, consisting with the change trends of the theoretical values. However, all the measured values of the H, O content are higher than the theoretical values. The errors between the measured values and the theoretical values were caused by water in the samples. These results further indicate that PMA chains have been successfully grafted onto the starch chains, and the graft reaction had obviously changed the element compositions of the starch.

3.3. ^1H NMR analysis

^1H NMR spectra of soluble starch, grafted starch and PMA are shown in Fig. 2. The ^1H NMR spectrum of soluble starch is showed in Fig. 2a, which is consistent with previous study (Vargha & Truter, 2005). In addition to retaining the characteristic peaks of soluble starch, the new peak appears at 1.160 ppm in Fig. 2b, which is caused by the proton (—CH_2) of the grafted side chains. And the peak appearing at 2.224 ppm is attributed to the proton (—CH) of the grafted side chains, which is consistent with previous studies (Morgan, Michael, Daniel, Christian, & Robert, 2011; Mou, Li, Wang, Fei, & Liu, 2012). Furthermore, the obvious difference between the soluble starch and grafted starch appears at 5.376 ppm, which is caused by the proton (—CH) in the first unit of the grafted side chains. This difference illustrates that the graft reaction increases the chemical shift of the proton (—CH) in the first unit of the grafted side chains because the O could decrease the shielding effect of the proton (—CH) in the first unit. Thus, the difference is the forceful proof that PMA has been linked to the starch successfully. All the split peaks of the ^1H NMR spectrum of PMA (Fig. 2c) appear in the ^1H NMR spectrum of the grafted starch. Based on the ^1H NMR results, the grafting reaction between MA and starch can be confirmed. This result is also consistent with the results of FT-IR and element analysis as described above.

3.4. ^{13}C NMR analysis

^1H NMR analysis provides a powerful indication that the graft reaction had taken place successfully. In order to further confirm that PMA had been grafted onto the molecular chains of the starch, ^{13}C NMR spectra of soluble starch, grafted starch and PMA were tested. As shown in Fig. 3a, the ^{13}C NMR spectrum of soluble starch presents the low field peaks at δ = 100.00 ppm due to anomeric carbons (C1), the peaks at δ = 78.70 ppm due to C4 (Zhang et al., 2014). In addition, the peaks in the range of 71–73 ppm are attributed to carbon atoms (C2, 3, 5) connected with —OH groups. The peaks at δ = 60.41 ppm belong to the carbon atoms (C6) of the CH_2OH in glucose (Lan, Yu, Chen, Zou, & Simon, 2010; Zou et al., 2012).

The ^{13}C NMR spectrum of grafted starch is shown in Fig. 3b. The peaks of carbon atom C1–C5 can be clearly identified for grafted starch, which is consistent with the chemical shifts of soluble starch. However, some new peaks can be observed after grafting. The peaks at δ = 174.30 ppm represent the carbon atoms (C11) of ester groups in the grafted side chains (Lv, Gong, & Ma, 2012; Lv, Gong, Yan, et al., 2012; Mou et al., 2012; Vargha & Truter, 2005). The peaks appear at δ = 40.58 ppm and 34.12 ppm are responsible for carbon atoms (—CH—CH_2—)n units in the grafted chains (Pal, Nasim, Patra, Ghosh, & Panda, 2010; Sen, Kumar, Ghosh, & Pal, 2009). The peaks at δ = 51.28 are attributed to C12 in the grafted chains. It should be noted that a new peak is observed at δ = 57.72 ppm, which is close to the peak for C6 at δ = 60.22 ppm. This new peak at δ = 57.72 ppm was caused by the shielding effect of a part of C6 linked to the grafted chain, which is a powerful evidence to support

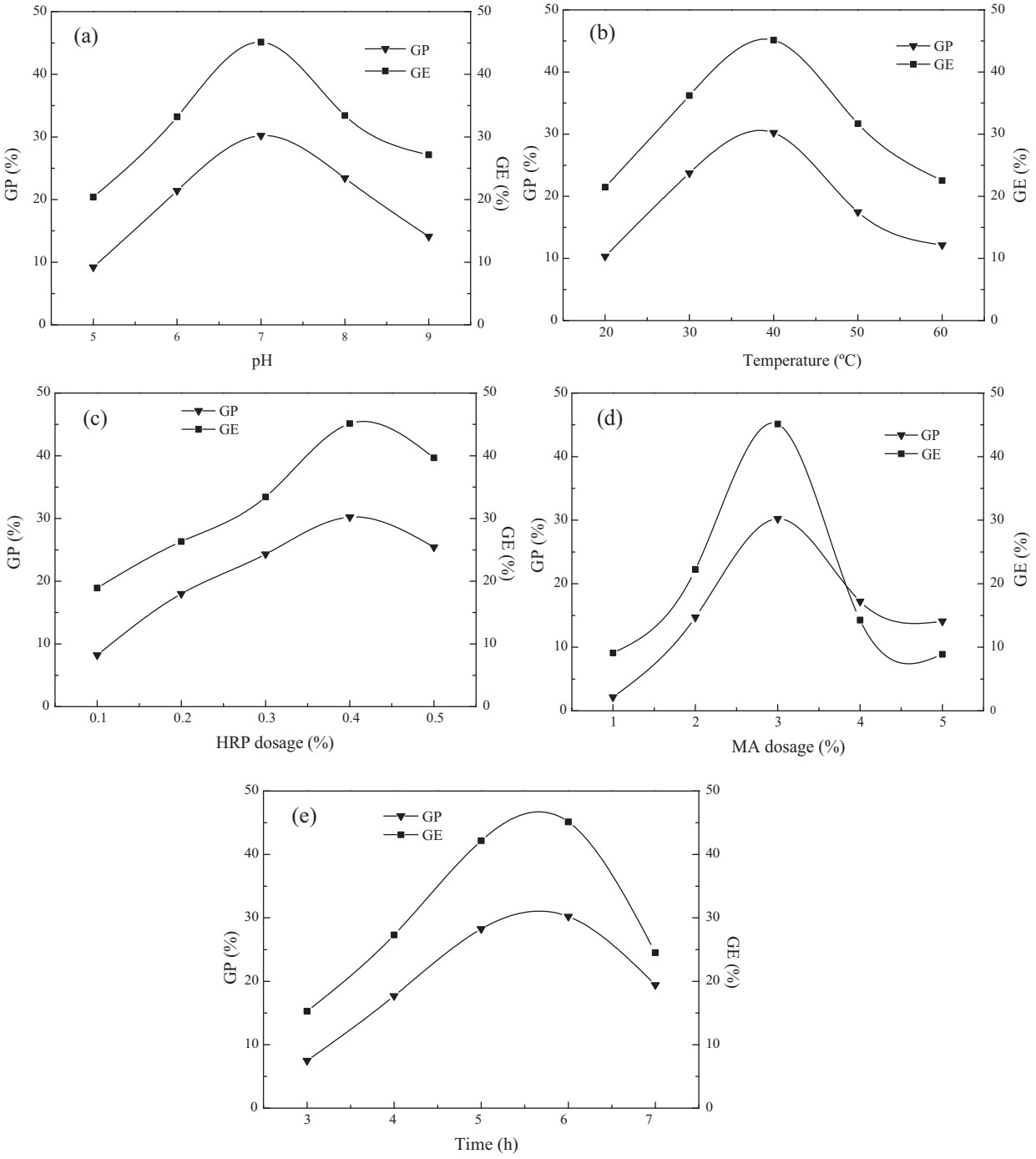

Fig. 6. Optimization of the process parameters: pH (a), incubation temperature (b), HRP dosage (c), MA dosage (d), and incubation time (e).

that PMA had been grafted onto the hydroxyl groups linked to C6 (Zhang, Xu, & Wang, 2008), corresponding to the results of ^{1}H NMR (Fig. 2). The chemical shift of the proton (—CH) in the first unit of the grafted side chains is different from the protons (—CH) in other units of the grafted chains, which forms a new peak at 5.376 ppm. According to the chemical shifts of the carbon atoms (C6′, C7) in Fig. 3b and the proton linked to C7 in Fig. 2b, it can be concluded that the covalent coupling of the oxygen free radicals linked to C6 and the free radicals of C7 occurred, forming the key structure of —CH_2—O—CH— in the grafted copolymers. The forming of this key structure illustrates that the PMA was connected with the glucose units of starch. In addition, the peaks shown in the ^{13}C NMR spectrum of PMA (Fig. 3c) can be observed in Fig. 3b. Therefore, the results of ^{13}C NMR indicate that the graft reaction had taken place successfully.

3.5. DSC analysis

The DSC curves of soluble starch, gelatinized starch and graft copolymer are presented in Fig. 4. The DSC curve of soluble starch shows an endothermic peak at 73 °C due to the existence of crystalline regions. With the increase of the temperature, the hydrogen bonds between molecular chains were damaged, which caused the decrease in crystalline regions in the interior of the starch granules. Compared to soluble starch, the DSC curve of the gelatinized starch appears smooth, without any endothermic peaks, which is

due to the disappearance of crystalline regions during the process of gelatinization. However, a weak endothermic peak appears at 55 °C in the DSC curve of the grafted copolymer, probably due to the realignment of the grafted PMA chains with the formation of a new crystalline region, which was caused by the formation of hydrogen bonds between the alkyl esters of the grafted PMA chains. Thus, the DSC illustrates that the graft copolymers were successfully prepared and the grafted chains had changed the crystalline regions of the gelatinized starch.

3.6. Catalytic mechanism and synthetic processes

Based on previous studies (Durand, Lalot, Brigodiot, & Marechal, 2000; Lalot, Brigodiot, & Marechal, 1999) and above conclusions, the catalytic mechanism and possible synthetic processes involved in this study are presented in Fig. 5.

3.7. Optimization of conditions for graft copolymerization

The optimization of conditions for enzymatic grafting, including pH of the phosphate buffer, incubation temperature, concentrations of MA and HRP, and the incubation time were optimized as shown in Fig. 6. Referring to the graph (Fig. 6a), it is evident that HRP activity and GP reached a maximum at a pH value of 7.0. With a temperature of 40 °C, maximum values of GP and GE were obtained, which can be seen from the curves for temperature (Fig. 6b). This can be attributed to the inactivation of enzymes at higher temperatures. Therefore, the optimum temperature for the copolymer formation was found to be 40 °C. Both the optimal results of pH and temperature were consistent with the results of pH and temperature on the activity of HRP enzyme (data not shown). From the curves of effects of HRP concentration (Fig. 6c) and MA concentration (Fig. 6d), the results showed that the GP increased greatly with the addition of MA until it reached its maximum value, with MA concentration of 3% and then decreased. This showed that increase in the concentrations of the monomer and HRP accelerate the homopolymerization reaction, rather than graft copolymerization after a certain limit, consequently decreasing the GP and GE. Finally, from the graph of incubation times (Fig. 6e), the optimum time was found to be 5.7 h. Prolonging the reaction further resulted in decrease in GP and GE, which is a result of depolymerization of parts of the copolymer. In conclusion, the optimum conditions of grafting copolymerization of MA onto starch are: 3% MA, 0.4% HRP, temperature 40 °C, pH 7.0, and time 5.7 h.

4. Conclusions

Modified starch is mainly prepared by graft copolymerization of monomers at reactive sites on the molecular backbone of starch for improvising the existing properties or for obtaining specific properties. In this study, a graft copolymer of starch with MA was synthesized using HRP/Acac/H_2O_2 system. The results of FT-IR, elemental analysis, ^{1}H NMR, ^{13}C NMR and DSC indicated that the target graft copolymers were formed by the catalysis of HRP. The present work showed that the graft copolymers of starch with MA were synthesized successfully by copolymerizing 2.5% of soluble starch with 3.0% of MA, using HRP (0.4%)/H_2O_2 (0.6%)/Acac (0.2%) at 40 °C and pH of 7.0 for 5.7 h. This environmentally friendly process provides an attractive alternative for modification of starch, thereby extending its applications in the industry.

Acknowledgments

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