Carbohydrate Polymers 84 (2011) 775–780

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Polydopamine coated magnetic-chitin (MCT) particles as a new matrix for enzyme immobilization

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form 9 February 2010
Accepted 19 March 2010
Available online 27 March 2010

Keywords:
Magnetic-chitin particles (MCT)
Dopamine
Polydopamine
α-Amylase
Immobilization

ABSTRACT

Chitin was used as a protective and dispersive matrix for the preparation of magnetic granules in the co-precipitation process. The easily prepared magnetic-chitin (MCT) particles with average size about 1.5 μm were modified with dopamine to be an effective enzyme immobilization matrix. Dopamine was self-polymerized and coated onto MCT, offering adherent surface for enzymes. A starch hydrolysis enzyme, α-amylase can be easily immobilized on the polydopamine coated MCT (DMCT). Glutaraldehyde treatment can further enhance the enzyme immobilization efficiency on DMCT particles. The surface immobilized α-amylase demonstrated a comparable starch hydrolysis rate as the free enzyme. However, the relative activities are greater than free enzyme over wider pH and temperature ranges. The magnetically retrievable immobilized α-amylase retained over 70% of its original activity after six times of repeated use.

1. Introduction

Carbohydrate biopolymers such as cellulose, starch and chitin (CT) have been demonstrated as vital natural materials in the fields such as biology, chemistry and biotechnology. Among these polymers, CT is the second most abundant biopolymer on earth, after cellulose. CT is non-toxic and biodegradable mucopolysaccaride, which is the component of arthropods, insect exoskeletons and fungal cell walls. CT is made of β (1→4) linked 2-acetamido-2-deoxy-β-D-glucose units. CT and chitosan are frequently compared with cellulose and structurally differ only at C_2 carbon position. Although CT has been produced minimum 10^{10} tonnes per annum (Stankiewicz, Briggs, Evershed, Flannery, & Wuttke, 1997), less attention has been paid on its application and exists as an unconsumed natural resource. Due to strong intra- and intermolecular hydrogen bonding provide rigid polymer structure, which offer inaccessible structure configuration for many solvents and reagents. N-acetyl group exists in C_2 carbon position of CT restricts the direct usage of the polymer. Deacetylation of the acetamido groups of CT produces the commercially important chitosan. Chitosan has been investigated as extensively as cellulose for industrial and laboratorial applications (Khor, 2001; Ravi Kumar, 2000).

Dopamine, which contains the catechol and amine functional groups recently has been compared as the marine adhesive protein mimics and has been showed to be a successful adhesive compound which can be simply attached onto various surfaces (Lee, Scherer, & Messersmith, 2006; Lee, Dellatore, Miller, & Messersmith, 2007). At typical marine pH environment, simple oxidative self-polymerization of dopamine (2 mg/ml 10 mM Tris, pH 8.5) was considered to create the reactive quinones and free radicals. Dopamine quinone holds electron-donating amine groups and electron-deficient ring. Deprotonation of amine groups leads the cyclization reaction and followed by oxidation to form dopaminochrome. This product is readily converted to 5,6-dihydroxyindole, which then develops black and thin adherent polydopamine film. Exact dopamine polymerization, physical and chemical have not been well understood. However, polydopamine layer was believed to have numerous active surface functional groups such as amino, imino and catechol, which may be particularly beneficial for secondary reactions (Lee et al., 2006, 2007; Waite, 2008). Recently, Lee, Rho, and Messersmith (2009) also showed potential of polydopamine surface for the immobilization biomolecules.

By taking advantage of the specific affinity interaction between chitin and lysozyme, chitin dissolved in ferric solution was co-precipitated to form magnetic-chitin (MCT) particles as an easily retrievable affinity adsorbent for the isolation of lysozyme from egg white (Safařik, 1991). Since most of the amine groups at C_2 carbon positions of chitin subunits are N-acetylated, chitin does not provide any other easily activated functional groups can be used for biomolecules immobilization. To have adherent polydopamine form on the surface of MCT may enable MCT as an effective and con-

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0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.03.036

venient carrier for biomoleucles immobilization which will widen the biotechnological application of chitin. Dopamine has been used to form a stable, robust anchor on the surface of iron oxide to immobilize functional molecules to the magnetic nanoparticles (Xu et al., 2004). However, the results that dopamine will quickly facilitate the degradation of the iron oxide nanoparticles have also been reported recently (Shultz, Reveles, Khanna, & Carpenter, 2007). Therefore, in this work, chitin was used as protective and dispersive coating on the magnetite co-precipitated. Dopamine was employed and induced to self-polymerize on the surface of MCT so that biomolecules can be easily immobilized. To further enhance the immobilization yield, glutaraldehyde (GA) was employed as a coupling agent to modify the polydopamine coated MCT particles. α-Amylase was used as a model enzyme to study the effect of polydopamine coating and GA treatment on enzyme immobilization. The prepared CT-based particles were characterized and the efficiency of α-amylase immobilization was studied by determining the starch hydrolysis rate. The activity and reusability of α-amylase immobilized on the abovementioned matrix under different pH and temperature were also examined.

2. Materials and methods

2.1. Materials

Hydrochloric acid (HCl, 37%), ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), dopamine (2-hydroxy tyramine hydrochloride) and 3,5-dinitrosalicylic acid (DNS) were purchased from Acros (NJ, USA). Sodium hydroxide (NaOH) was purchased from Fisher scientific (NJ, USA). GA (25%), CT (α-form from crab shells) and α-amylase (*Bacillus* sp.) were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of polydopamine coated magnetic-chitin (DMCT)

Chitin (3 g) was added into 80 ml of 6 M HCl and slowly stirred for 48 h at 4 °C. The dissolved CT was filtered and the supernatant is mixed with 50 ml of 2:1 stoichiometric ratio of $FeCl_3{\cdot}6H_2O$ (2.70 g) and $FeSO_4{\cdot}7H_2O$ (1.39 g). Slow addition of 1.5 M NaOH formed fine magnetic particles (Safaŕik, 1991). The resulting MCT particles were collected through neodymium–iron–boron magnet block (Magtech Co., Taipei, Taiwan) and thoroughly washed with distilled water until neutral pH. Continuous over-night stirring of MCT with dopamine (2 mg/ml, 10 mM, pH 8.5 Tris buffer) produced DMCT particles (Lee et al., 2007). Self-oxidized or polymerized dopamine was expected to form adherent polydopamine coating on the surface of the MCT particles. The unadhered polydopamine particles were removed by rinsing resulted dark black particles with water. Multifunctional groups present in the polydopamine surface were further modified with GA (DMCT-GA) by using 1 g of DMCT particles with 10 ml of 8% (v/v) GA at 40 °C for 2 h. The particles were collected and washed several times with ethanol followed by water and stored at 4 °C.

2.3. Characterization of MCT, DMCT and DMCT-GA

Surface morphology of MCT, DMCT and DMCT-GA particles were examined using field emission scanning electron microscopy (FESEM) JEOL JMF-6390 model equipped with an energy dispersive X-ray (EDX) analyzer. FTIR spectrums were obtained by using a FTIR spectrophotometer (FTIR FTS-3500 series, Bio-Rad Digilab). Thermogravimetric analysis (TGA) was performed by employing Diamond-TG/DTA (Perkin-Elmer) over the temperature range of 40–700 °C at the heating rate of 20 °C per min under the flow of N_2 at the rate of 30 ml/min. The size distribution of MCT was measured by dynamic light scattering apparatus (Otsuka, Photal LPA-3000/3100). Magnetic properties of MCT-based particles were determined by superconducting quantum interference devices (SQUID) (SQUID, MPMS-7 Quantum Design).

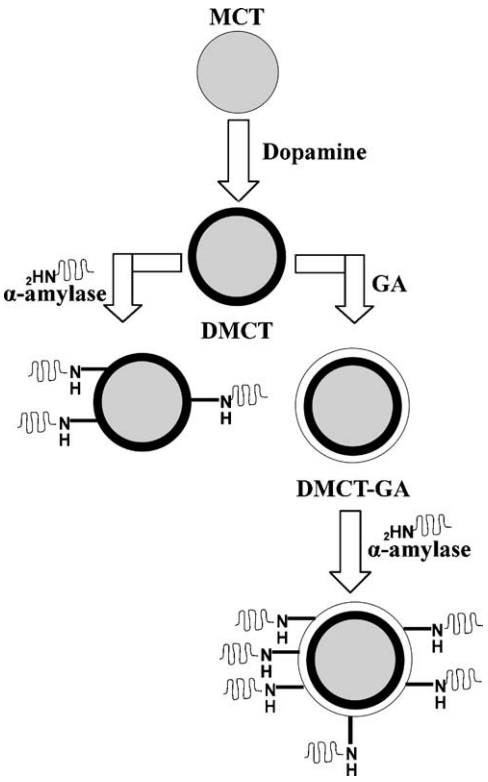

Fig. 1. Schematic diagram of polydopamine coating and α-amylase immobilization on magnetic-chitin microparticles.

2.4. Immobilization of α-amylase onto polydopamine coated MCT

The α-amylase immobilization process is demonstrated in Fig. 1. Polydopamine coated MCT (DMCT or DMCT-GA) were equilibrated in 50 mM, pH 6.5 phosphate buffer for 30 min. About 1 g of DMCT or DMCT-GA was added to the α-amylase solution (80 mg/l in 20 ml pH 6.5 50 mM phosphate buffer) and the immobilization reaction was carried out at 4 °C for 24 h in a rotary shaker. The particles were collected by external magnet and the unbound enzyme was washed thoroughly with phosphate buffer. The immobilized α-amylase particles were stored in phosphate buffer at 4 °C until use.

2.4.1. Activity of the free and immobilized α-amylase

Immobilized and free enzyme reactions were based on the same amount of α-amylase for starch hydrolysis. Hydrolysis reaction was carried out in 20 ml 50 mM, pH 6.5 phosphate buffer containing 1% starch (w/v) with constant shaking at 30 °C. Aliquots of 0.1 ml were taken at every 10 min and added to 0.5 ml of DNS reagent. The resulting solution was incubated in a boiling water bath for 5 min. After dilute with DI water, the amount of hydrolysis products (reducing sugar) was measured spectrophotmetrically at 540 nm with maltose as the standard. All the data points for reducing sugar concentration are an average of duplicated measurements.

2.4.2. Optimum conditions for α-amylase activity

The optimum pH of free and immobilized α-amylase was determined as the relative activity after incubation in 50 mM phosphate buffer of pH 5.5–8.0 containing 1% starch (w/v) for 10 min at 30 °C. For determining the effect of temperature on the activity of the free

Table 1
Energy dispersive X-ray (EDX) of elemental composition of MCT, DMCT and DMCT-GA.

Elements	MCT	DMCT%	DMCT-GA
C	20.63	19.40	19.81
N	0.09	6.00	5.34
O	18.76	21.30	22.02
Fe	60.52	53.29	52.83

and immobilized α-amylase, enzyme is incubated at various temperatures (40–70 °C) in 1% starch (w/v) solution for 10 min and the reducing sugar released was measured.

2.4.3. Reusability of immobilized α-amylase

To monitor the reusability of the immobilized enzyme, α-amylase was exposed in 1% starch (w/v) starch solution for 10 min at 30 °C. The immobilized enzyme was collected by external magnet and was washed with phosphate buffer. The steps were repeated to obtain the relative activity of immobilized α-amylase.

3. Results and discussion

3.1. Characterization of enzyme immobilization matrix

The surface morphology of MCT, DMCT and DMCT-GA were studied by FESEM. As shown in Fig. 2a, MCT is composed of porous nano-sized particles aggregate. When treated with dopamine, the surface morphology of MCT did not change much but with slightly blurred outline (Fig. 2b). Probably, the polydopamine coating blurred the outline of nano-sized particles aggregate. EDX analysis (Table 1) confirmed the presence of the magnetite in MCT and DMCT by the significant increase of Fe content. Besides, the presence of polydopamine coating was also verified by the increase of nitrogen content on the DMCT. GA treatment seems to increase the size of granule on DMCT (Fig. 2c) probably due to the cross-linking effect of GA. The actual size distribution of MCT in solution was measured by a particle size analyzer. As show in Fig. 3, MCT has a very broad size distribution from 0.5 up to 4 μm with an average about 1.5 μm.

TGA curves of CT and MCT were exhibited in Fig. 4. Thermogram of CT showed two decomposition stages. First one could be assigned to water evaporation. The second stage was due to the degradation of CT structure (Paulino, Simionato, Garcia, & Nozaki, 2006). Approximately, 23% of original weight of CT remained after heating at 700 °C. As expected, a much higher residual weight about 75% of original MCT was observed in the thermogram that witnessed the presence of magnetite. Polydopamine coated MCT particles showed an extra decomposition stage in the range of 630–680 °C, which indicates the presence of polydopamine layer on the surface (Fei et al., 2008). Compare to DMCT, slightly higher stability in the first decomposition stage for the GA treated DMCT particles can be observed. On the other hand, total residual mass of DMCT-GA particles was about 2.8% less than the polydopamine coated particles.

The magnetic properties of the CT-based magnetic particles were studied by SQUID at 30 °C. Fig. 5 exhibits the magnetization hysteresis loops of the samples. The saturation magnetization was measured to be 27.95 and 16.57 emu/g for DMCT and DMCT-GA particles, which is much less than the MCT particles (47.38 emu/g). Probably, the nonmagnetic polydopamine coating increased the mass of MCT which leads to a significant decrease of the saturation magnetization and the following GA treatment decreases the value further. Each of the magnetization curves crossed the zero point in the SQUID study, which indicates that the as-prepared CT-based magnetic particles are superparamagnetic.

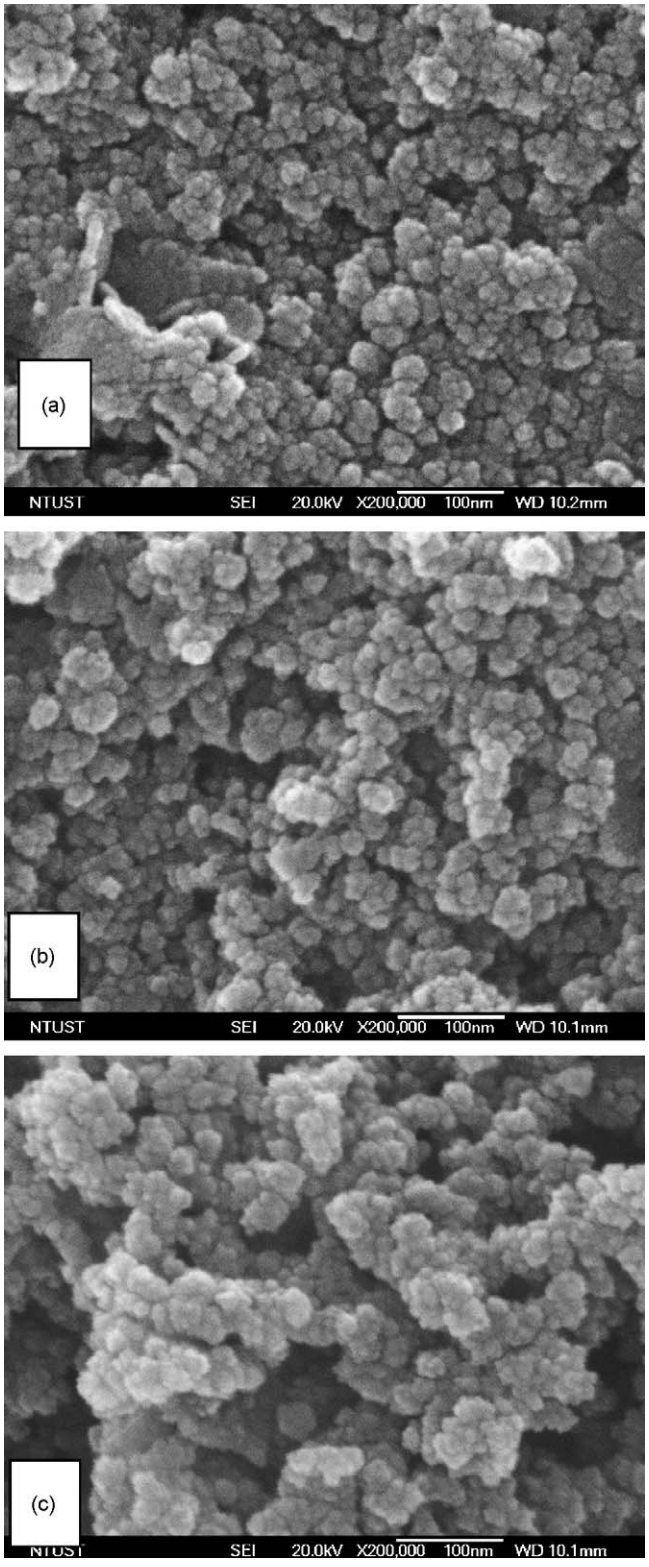

Fig. 2. Field emission scanning electron microscopy (FESEM) images of magnetic-chitin particles MCT (a), DMCT (b) and DMCT-GA (c).

The surface chemical nature of modified CT particles was further demonstrated by FTIR. Fig. 6 exhibits the FTIR spectrum of MCT, DMCT and DMCT-GA particles. Band around 1640–50 cm^{-1} corresponds to the amide I stretching of C=O in CT molecule. Characteristic peak at 1650 cm^{-1} represents the stretching vibrations of acetylated amino groups present in the CT (Cardenas,

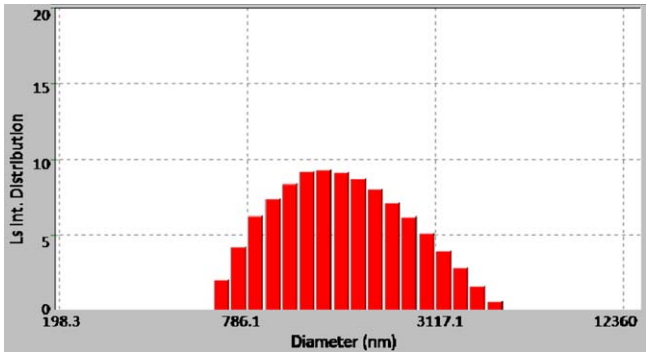

Fig. 3. The size distribution of magnetic-chitin (MCT) measured by Otsuka dynamic light scattering.

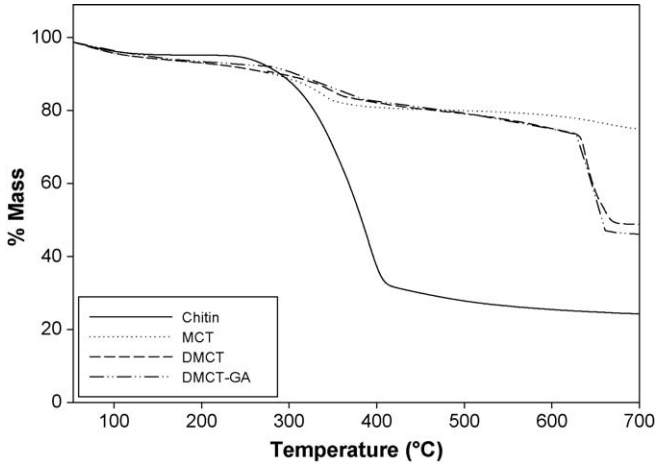

Fig. 4. Thermogravimetric (TG) curves of CT, MCT, DMCT and DMCT-GA particles.

Cabrera, Taboada, & Miranda, 2004). The band at around 600 cm^{-1} was the characteristic band to confirm the presence of the magnetic particles. Dopamine treatment created several bands in the range of 1000–1600 cm^{-1}. Adsorption peaks found in the range of 1450–1620 cm^{-1}, which were belong to the C–C vibration of benzenes ring and N–H bending of the polydopamine structure. Typical peak around 1480 cm^{-1} resulting from the aromatic C–H bending and might be the aliphatic primary amine vibration (Gutièrrez-Tauste, Domènech, Domingo, & Ayllon, 2008; Ge, Tan, Xie, Ma, & Yao, 2009). Signals around 1280 cm^{-1} might be corresponding to the C–O stretching and primary amine vibration from polydopamine coated surface. GA modification usually gives C=N vibration band at around 2300 and 1631 cm^{-1}. However, imine bands might have superimposed with other DMCT characteristic bands (Adriano, Filho, Silva, Giordano, & Gonçalves, 2005; Ge et al., 2009).

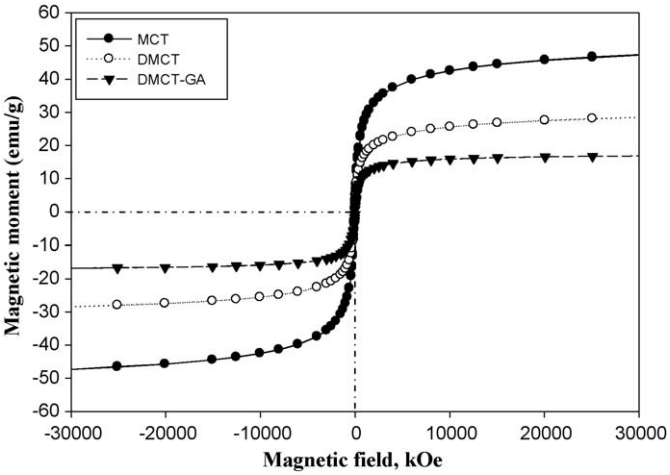

Fig. 5. Hysteresis loops of MCT, DMCT and DMCT-GA particles measure at 300 K.

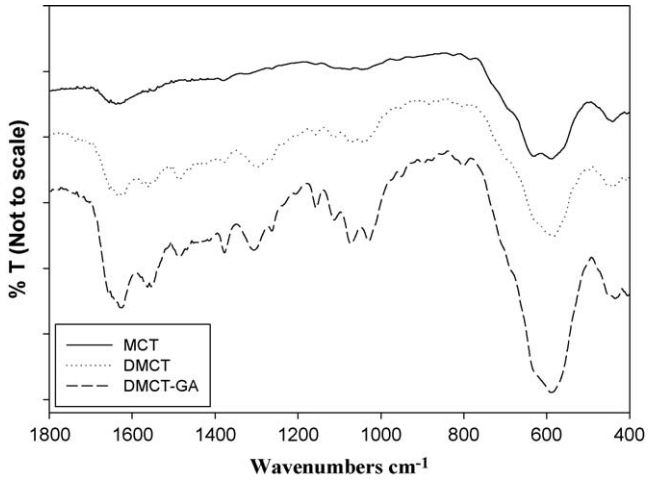

Fig. 6. FTIR spectra of MCT, DMCT and DMCT-GA particles.

3.2. α-Amylase immobilization

The α-amylase immobilization efficiency on DMCT or DMCT-GA was determined by measuring their starch hydrolysis rates. The starch hydrolysis rate was monitored by measuring the amount of reducing sugars released with respected to time (Konsoula & Liakopoulou-Kyriakides, 2006; Konieczna-Molenda, Kochanowski, Walaszek, Bortel, & Tomasik, 2009). As shown in Fig. 7, the reducing sugars releasing rate starts to slow down after 20 min for all the α-amylase preparations. In the rest of reaction time, the immobilized α-amylase on DMCT-GA showed the best performance that the reducing sugar released is about 10% higher than that of free α-amylase and 15% higher than that of immobilized on DMCT. For example, at the end of 80 min reaction, the reducing sugars released were 0.90, 0.80 and 0.71 mg/ml for DMCT-GA, free α-amylase, and DMCT, respectively. Evidently, α-amylase was effectively immobilized on CT-based magnetic particles. Even though exact nature

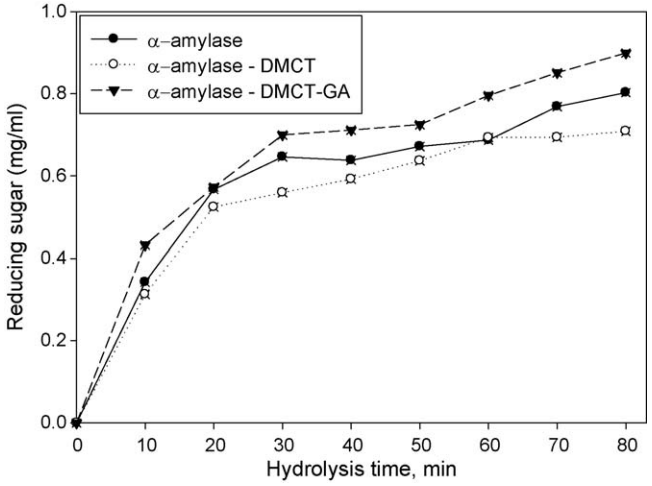

Fig. 7. Time course of starch hydrolysis using free α-amylase, α-amylase immobilized onto DMCT and DMCT-GA particles.

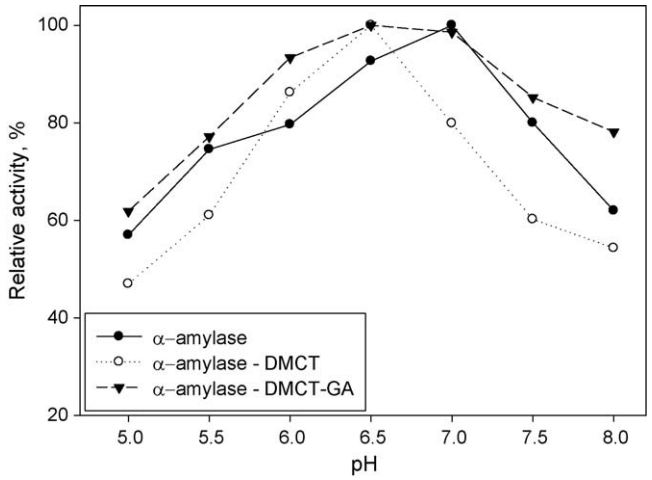

Fig. 8. pH profiles of the free and immobilized α-amylase.

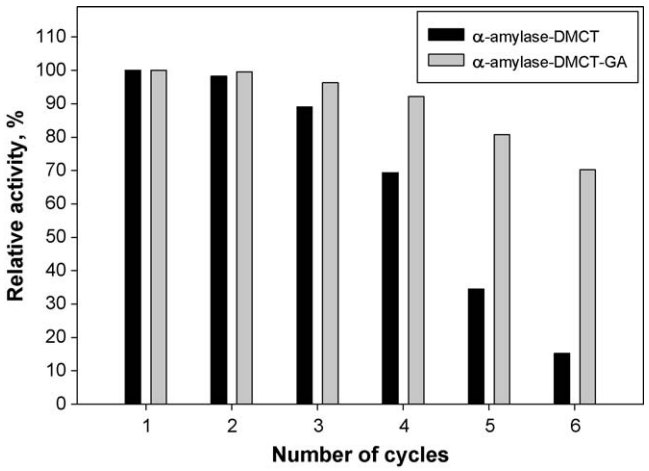

Fig. 10. Reusability of immobilized α-amylase.

of the polydopamine surface is unknown, it was demonstrated to be an effective matrix for biomolecules immobilization (Lee et al., 2009). Self-oxidation of dopamine generated quinone groups in polydopamine layer, which will actively take part in nucleophilic addition reaction with –SH and $–NH_2$ of amino acid residues of protein that leads to α-amylase being immobilized on DMCT. On the other hand, DMCT-GA with aldehyde groups on the surface, which has long been demonstrated as very effective enzyme immobilization functional groups; can further improve α-amylase immobilization efficiency that resulted in a higher reducing sugars release from starch hydrolysis than that of DMCT.

Fig. 8 displays the effect of reaction pH on relative activities of α-amylase in immobilized and free forms. The immobilization of α-amylase increased its reaction pH range in the direction of both acidic and alkaline ranges have been noticed previously (Bryjak, 2003; Chang & Juang, 2005; Kahraman, Bayramo lu, Kayaman-Apohan, & Güngör, 2007). Both free and immobilized α-amylase show characteristic bell-shaped pH profile curves with a minor shift of the optimum pH. As shown in Fig. 8, the optimum pH for free α-amylase and α-amylase immobilized on DMCT matrixes were around 7.0 and 6.5, respectively. This indicates the optimum pH for the immobilized enzyme shifted towards slightly acidic pH. Enzyme immobilized on DMCT and DMCT-GA particles exhibited optimum pH value at 6.5, agreeing with the previous report of α-amylase covalently immobilized on the polymer surface (Tümtürk, Aksoy, & Hasırcı, 2000). The pH profile of the enzyme immobilized on DMCT-GA particles was broader range than other systems, which indicates DMCT-GA particles maintain the enzyme activity in a wider pH range. The optimum pH slightly shift toward acidic pH for the immobilized α-amylase might be due to the amine or imines groups of polydopamine surface near α-amylase active site which can diminish of proton concentration.

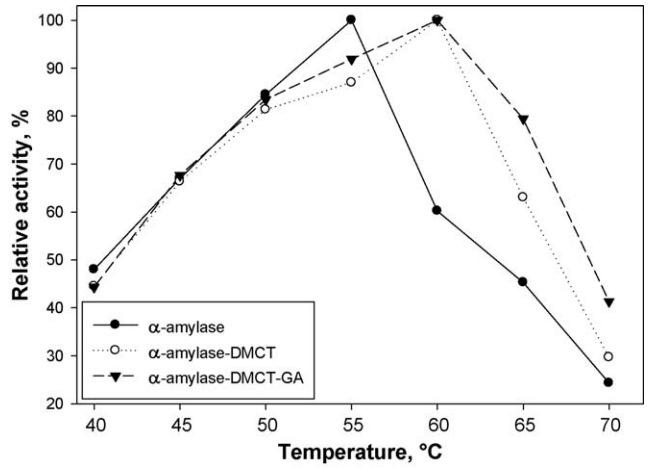

Fig. 9. The effect of temperature on the activity of free and immobilized α-amylase.

Fig. 9 explains the effect of temperature on the relative activities of free and immobilized α-amylase at pH 6.5 for hydrolyzing starch. As shown in Fig. 9, the optimum reaction temperature was found to be 55 °C for free enzyme. The optimum temperature for enzyme immobilized on DMCT, DMCT-GA shifted slightly to a higher value at about 60 °C. This optimum temperature shift could be explained as multipoint interactions between α-amylase and polydopamine coated surface. The immobilization of α-amylase stabilizes the rigidity of the enzyme, which is generally showed by higher temperature stability. It should be noted that the biocompatibility of the CT-based support could also play an important role on the stabilization of enzyme conformation.

Unlike free enzymes, immobilized enzymes on the magnetic particles can be quickly removed from reaction medium solutions by an external magnet. To investigate the reusability, the immobilized α-amylase (DMCT and DMCT-GA) was used for starch hydrolysis in pH 6.5 at 30 °C for 10 min. The activities of the first cycle were taken to be 100% relative activity and the hydrolysis reaction was repeated for 5 consecutive cycles. Fig. 10 demonstrates the changes of the activity of immobilized enzymes after multiple reusing by magnetic separation. It can be found enzyme immobilized on DMCT-GA particles was active and the holds over 70% of α-amylase activity after 6 repeated cycles. On the other hand, 90% activity of the enzyme immobilized on DMCT matrix was retained after three cycles. However, at the end of the 6th cycle, only 15% of activity was retained. Probably, the decrease in enzyme activity was caused by the denaturation and/or leakage of α-amylase from the DMCT matrix upon reuse. Evidently, GA on DMCT-GA particles can bind α-amylase more securely and leads to a more stable performance in repeated use.

4. Conclusion

A novel method for surface modification of CT has been developed. An easily recoverable and surface functionalized with adherent polydopamine magnetic-chitin microparticles was successfully used for immobilization of α-amylase. The immobilization efficiency can be further enhanced by pretreating polydopamine surface with glutaraldehyde. The optimum pH of the immobilized enzyme was shifted 0.5 pH unit to the acidic region. The immobi-

lized enzyme possesses starch hydrolysis efficiency in a wider pH and temperature range. It was also noticed that the immobilized α-amylase on glutaraldehyde treated polydopamine functionalized magnetic-chitin microparticles has superior durability, magnetic recovery and reusability. The above study concludes that the polydopamine coated CT particles could be used in other biotechnological applications.

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