Immobilization of α-Amylase onto Chitosan and Its Amino Acid Condensation Adducts

M. A. Abd El-Ghaffar, M. S. Hashem

Department of Polymers and Pigments, National Research Center, Dokki, Egypt

Received 24 February 2008; accepted 27 July 2008
DOI 10.1002/app.29292
Published online 23 January 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: α-Amylase from *Bacillus subtilis* was immobilized on insoluble chitosan and its amino acid (L-glutamic acid and 4-aminobutyric acid) condensation adducts with the direct covalent attachment method and with glutaric dialdehyde (GDA) as a crosslinking agent. The immobilization process was carried out at 25°C and pH 6.9, and the maximum retained activity was obtained with 3 mg of α-amylase. The properties of the immobilized α-amylase were investigated and compared with those of the free α-amylase. For the assays carried out via the crosslinking method at 25°C and pH 6.9, the retained activities were found to be 68.59, 97.36, and 79.50% for chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid crosslinked with 1% GDA, respectively. The immobilized α-amylase had better stability and higher retained activities with respect to the pH, temperature, and storage stability than the free α-amylase. In the repeated-use experiments, the α-amylase immobilized with chitosan–GDA (1%) retained about 46.45% of its original activity after 25 uses. In contrast, the activities of α-amylase immobilized on chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) did not change after 11 and 8 uses, respectively. The retained activities after 25 uses were 79 and 71% with respect to the original activity for the aforementioned carriers. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 112: 805–814, 2009

Key words: biopolymers; enzymes; chitosan

INTRODUCTION

Chitosan, a poly(*N*-acetylglucosamine), is a transformed oligosaccharide obtained by the deacetylation of chitin, and it is the second most abundant biopolymer after cellulose.[1] Commercially available chitosan is obtained from crustaceans and has been used in a wide variety of applications. Its membrane has several uses, including food processing, protein purification, and skin replacement technology.[2] It has also been widely used as a support for enzyme immobilization because it offers many advantages, including low cost, good biocompatibility, low nonspecific adsorption, and ease of fabrication into various forms (powders, gel beads, fibers, capsules, and membranes).[3] Enzymes such as catalase,[4] tyrosinase,[5] dextranase,[6] and β-galactosidase[7] have been immobilized on commercial crustacean chitosan.

α-Amylase (EC 3.2.1.1), which is mainly used as a thinning agent in starch hydrolysis, is widely applied in the food, paper, and textile industries.[8] All α-amylases are calcium metalloenzymes, binding at least one calcium ion per monomer unit; for example, *Bacillus subtilis* α-amylase strongly binds four calcium ions. The calcium ions impart resistance to pH, temperature, proteolysis, and denaturation by urea and heat. The stability arises from the contribution of the calcium ions to the enzyme's tertiary structure; they probably compensate for the lack of disulfide bonds.[9] The immobilization of α-amylase on mainly water-insoluble carriers seems to be the most promising way to obtain more stable and reusable forms of enzymes.[10]

This study was designed to investigate the immobilization of α-amylase from *B. subtilis* on chitosan and its amino acid (L-glutamic acid and 4-aminobutyric acid) condensation adducts. The immobilization process was carried out via covalent attachment and crosslinking methods. The study was also focused on the determination of the retained activity of free α-amylase and α-amylase immobilized on the aforementioned supports under different conditions (pH, temperature, and storage). The reusability of the immobilized α-amylase was also studied.

EXPERIMENTAL

α-Amylase (EC 3.2.1.1 from *B. subtilis* with an activity of 402 U/mg), Folin reagent (2N), and anhydrous sodium dihydrogen phosphate were purchased from Fluka (Taufkirchen, Germany). Chitosan flakes with an 85% degree of deacetylation were obtained from Sigma (St. Louis, MO). 4-Aminobutyric acid, glutaric dialdehyde (GDA; 25 wt % solution in water), and bovine serum albumin were obtained from Aldrich

Correspondence to: M. A. Abd El-Ghaffar (mghaffar50@yahoo.com).

(St. Louis, MO). Starch was obtained from Merck (Darmstadt, Germany). An α-amylase kit was obtained from Biomerieux (Basingstoke, United Kingdom). L-Glutamic acid and maltose were obtained from BDH (Poole, United Kingdom). 3,5-Dinitrosalicylic acid (DNSA) was obtained from Panreac (Barcelona, Spain). Anhydrous dibasic sodium phosphate was obtained from El-Gomhouria Co. (Cairo, Egypt).

Modification of chitosan

Chitosan and each of the amino acids (L-glutamic acid and 4-aminobutyric acid) were mixed in stoichiometric amounts (equimolar ratios) and subjected to a condensation reaction with a Dean–Stark apparatus in the presence of xylene until the calculated amount of water was separated. Chitosan amide products were separated by filtration, washed several times with methanol and hot distilled water, dried in an electric oven at 50°C, and weighed. The nitrogen content for chitosan and its amino acid adducts was determined with the Keldal method.[11]

Preparation of GDA supports

Chitosan and chitosan–amino acid condensates (amide adducts; 0.1 g) were added to GDA solutions (10 mL) of different concentrations (0.1, 0.5, and 1 mL) in 100 mL of a phosphate buffer (pH 6.9); they were kept in a shaking water bath for 4 h at 25°C and left at that temperature overnight. The activated supports were separated and washed three times with 5 mL of a phosphate buffer (pH 6.9).

Immobilization of α-amylase

Covalent attachment method

Chitosan and chitosan–amino acid condensates (amide adducts; 0.1 g) were added to the α-amylase solution (1, 2, 3, 4, or 5 mg) in 1 mL (0.02*M*) of a phosphate buffer (pH 6.9), and the immobilization reaction was carried out for 24 h at 25°C in a shaking water bath. The precipitates were filtered, and the unbound enzyme was removed by three washes with 5 mL of a phosphate buffer. The immobilized α-amylase polymer samples were stored at 4°C until use.

Crosslinking method

The previous crosslinker supports (0.1 g) were added to the α-amylase solution (1, 2, 3, 4, or 5 mg) in 1 mL (0.02*M*) of a phosphate buffer (pH 6.9), and the immobilization reaction was carried out for 4 h at 25°C in a shaking water bath. The precipitates were filtered off, and the unbound α-amylase was removed by three washes with 5 mL of a phosphate buffer. The immobilized α-amylase polymer samples were stored at 4°C until use.

Determination of the amount of the immobilized α-amylase

The Lowry method[12] was used to determine the α-amylase content in solution. After the immobilization process, the supernatant and washing solutions were collected. The α-amylase concentration was determined by a comparison with the standard curve constructed with bovine serum albumin of known concentrations. The amount of immobilized α-amylase was determined from the initial amount of α-amylase present in the α-amylase coupling solution by subtraction of the final total amount of α-amylase present in the remaining coupling solution.[13] The coupling yield (%) of the α-amylase was then calculated from the amount of α-amylase coupled onto the polymeric carriers by the initial total amount of the α-amylase present in the coupling solution according to the following equation:

$$\alpha - \text{Amylase coupling yield}(\%) = \frac{\text{Amount of } \alpha - \text{amylase coupled}}{\text{Amount of } \alpha - \text{amylase introduced}} \times 100$$

Assay of the α-amylase activity

The activity of α-amylase was measured by two methods.

First, the activities of free and immobilized α-amylase were assayed by the methods of Bernfield[14] and Raviyan et al.[15] The activities were determined by the incubation of α-amylase with 1% starch in a phosphate buffer (0.02*M*, pH 6.9) at 25°C for 3 min; then, the reaction was terminated by the addition of DNSA. The amount of reducing sugar (maltose) was measured at 540 nm with maltose as the standard. The specific activity of free and immobilized α-amylase was calculated according to the following equation:

$$\text{Units/mg} = \frac{\text{Micromoles of maltose released}}{3(\text{minutes}) \times \text{mg of } \alpha\text{-amylase in reaction mixture}}$$

The retained activity yield of the α-amylase immobilized on the polymeric matrix was calculated according to the following equation:

$$\text{Retained activity yield}(\%) = \frac{\text{Specific activity of immobilized α-amylase}}{\text{Specific activity of free α-amylase}} \times 100$$

Second, the activities of free and immobilized α-amylase were measured by the enzymatic method. The amylase unit is defined as the amount of enzyme that, under the conditions of the enzymatic procedure, will hydrolyze 10 mg of starch in 30 min to a stage at which no color is given with iodine.[16–18] The specific activity of free and immobilized α-amylase was calculated according to the following equation:

$$\text{α-Amylase units/100 ml} = \frac{\text{OD control} - \text{OD test}}{\text{OD blank}} \times 600$$

where OD is the optical density.

The retained activity yields of the α-amylase immobilized on the polymeric matrix were calculated according to the following equation:

$$\text{Retained activity yield}(\%) = \frac{\text{Specific activity of immobilized α-amylase}}{\text{Specific activity of free α-amylase}} \times 100$$

All activity measurement experiments were carried out at least twice (generally three times), and the relative standard deviations were found to be less than 1%.

Effect of pH on the activity of free and immobilized α-amylase

The effect of pH on the enzyme activity was investigated in the range of 2.9–9.9 for both free and immobilized α-amylase.

Effect of temperature on the activity of free and immobilized α-amylase

The effect of temperature on the α-amylase activity was investigated in the range of 30–100°C for both free and immobilized α-amylase.

Storage stability of free and immobilized α-amylase

This experiment was carried out to determine the stabilities of free and immobilized α-amylase after storage in a phosphate buffer (0.02*M*, pH 6.9) at 4°C for 90 days. The residual activities were then determined as described previously, and the activity of each preparation was expressed as a percentage of its residual activity versus its initial activity.

Reusability of the immobilized α-amylase

To evaluate the reusability of the immobilized α-amylase, the polymeric supports were washed with water and a buffer after use and then suspended again in a fresh reaction mixture to measure the enzymatic activity.

Techniques

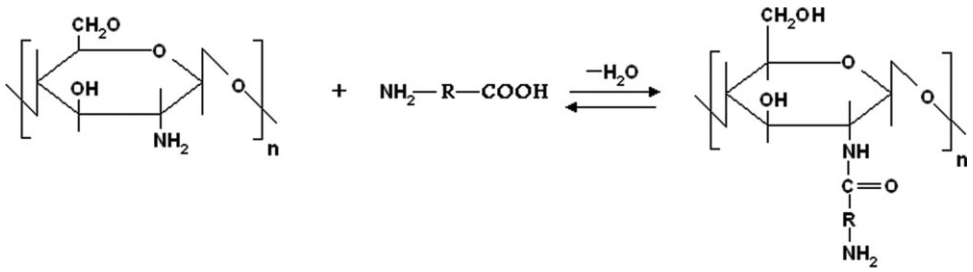
Fourier transform infrared (FTIR) spectra were measured with an FTIR spectrometer (model 670, Nicolet, Waltham, MA; from 400 to 4000 cm^{-1}); thermogravimetric analysis (TGA) was performed with a TGA 7 series apparatus (PerkinElmer, Norwalk, CT). The shapes of the particles were scanned with scanning electron microscopy (SEM; JXA-840A electron probe microanalyzer, JEOL, Tokyo, Japan), and ultraviolet–visible spectra were measured with a Shimadzu (Duisburg, Germany) UV-2401 PC double-beam spectrometer.

RESULTS AND DISCUSSION

Modification of chitosan and its amino acid condensation adducts

Stoichiometric amounts of condensation reactants of chitosan (0.025 mol) were calculated for 1 unit with either L-glutamic acid (0.025 mol) or 4-aminobutyric acid (0.025 mol). The reactions were carried out with a Dean–Stark apparatus until the calculated amounts of water were separated (≈0.5 mL). These results were similar to those obtained by Esparza and Gomez,[19] who reacted chitosan with glycine, L-lycine, isoleucine, and L-glutamic acid in the presence of a dehydrating agent (concentrated H_2SO_4). These results were confirmed by the data obtained for the nitrogen content: 6.99, 10.06, and 13.19 for chitosan, chitosan–L-glutamic acid adducts, and chitosan–4-aminobutyric acid adducts, respectively. It can be seen from Scheme 1 that the chitosan and chitosan–amino acid adducts (chitosan/aminobutyric and chitosan/L-glutamic acid adducts) had free amino groups (—NH_2) that reacted directly with the carboxylic terminal residue in α-amylase.

The reaction of chitosan directly with α-amylase was similar to the reactions carried out by Abdel-Naby et al.,[6] Arica et al.,[20] and Li et al.,[21] but they used dextranase, invertase, and lipase, respectively. These data confirm that the immobilization process occurred by a covalent method, and this agrees with our assumption concerning the possible reaction mechanisms between chitosan and chitosan–amino acid adducts with α-amylase, which are illustrated



Scheme 1 Possible reaction mechanism between chitosan and the amino acids (R = L-glutamic acid or 4-aminobutyric acid).

in Schemes 2 and 3. On the other hand, the literature is scanty concerning the reaction between α-amylase and chitosan–amino acid condensation adducts.

The amino groups in chitosan and chitosan–amino acid adducts reacted with the crosslinker GDA, and then α-amylase was bound to them. The reaction of chitosan and GDA was similar to those reactions carried out by Gamze and Senay,[1] Çetinus and Öztop,[4] Wu et al.,[22] and Juang and Min-Yun.[23] The proposed reaction mechanisms for chitosan and chitosan–amino acids with GDA and α-amylase are summarized in Scheme 4.

There is another possibility for chitosan amino acid adducts in the case of glutamic acid, which has two carboxylic groups to react with another molecule of α-amylase.

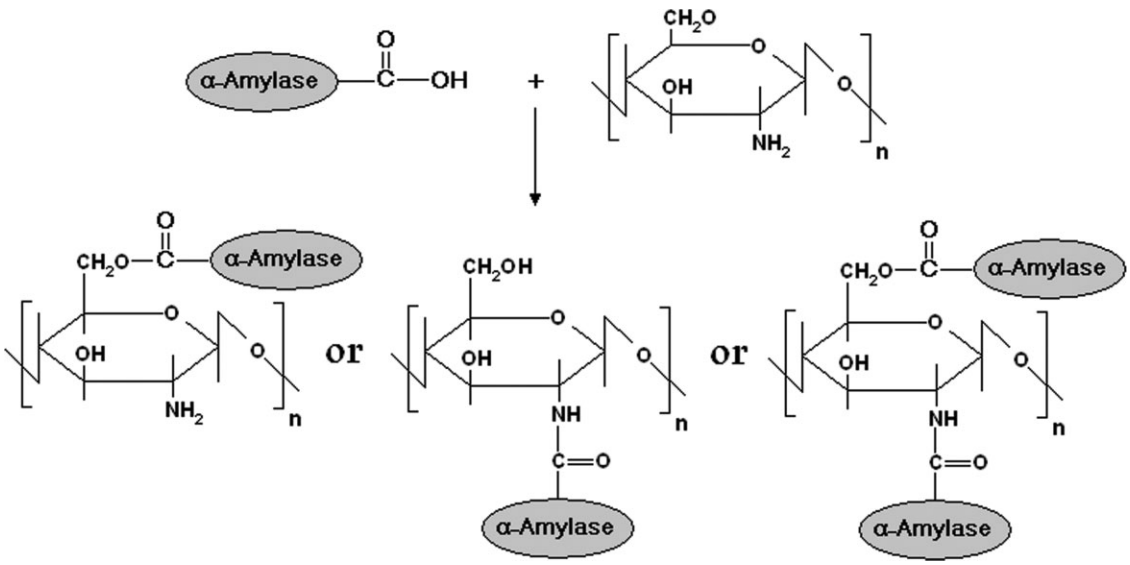
Characterization of chitosan and its amino acid condensation adducts

FTIR studies

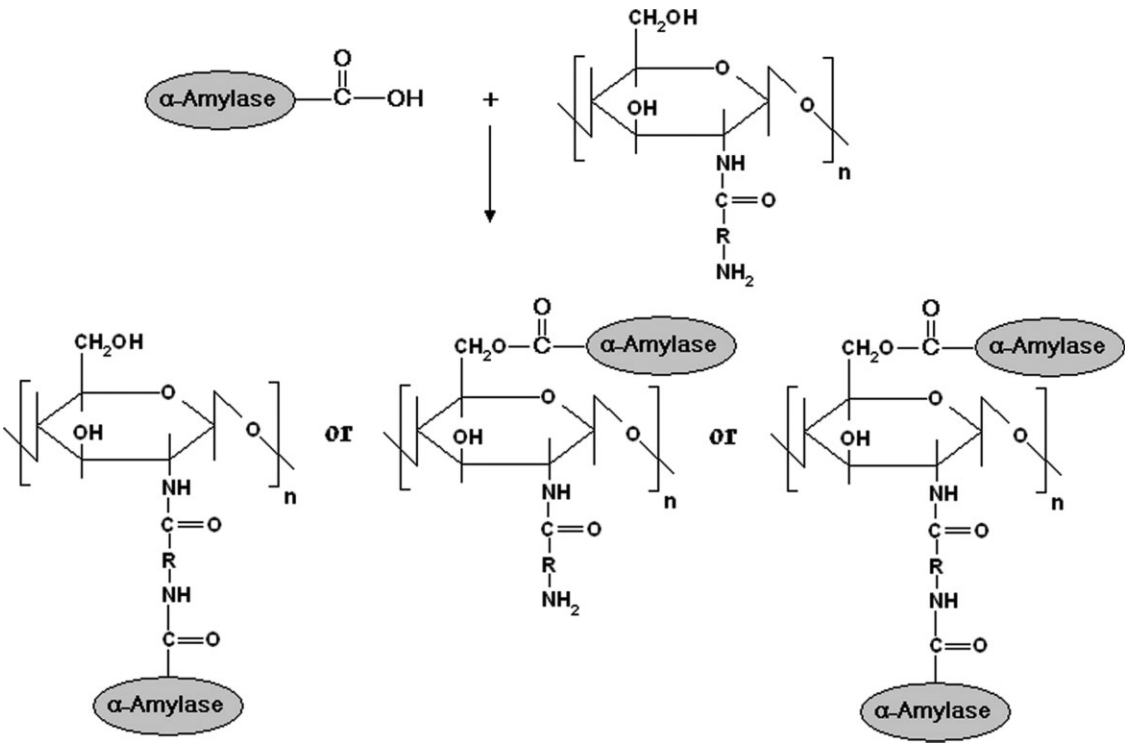
The FTIR spectra of the chitosan and its amino acid condensation adducts are shown in Figure 1. There are significant peaks at 1029 and 1149 cm^{-1} characteristic of the saccharide structure and two other absorption bands at 3353 and 3200 cm^{-1} characteristic of the amino group. Also, Figure 1 shows peaks at 1576 and 1591 cm^{-1}, which indicate the formation of amide bonds from the condensation reaction of chitosan with L-glutamic acid and 4-aminobutyric acid, respectively. The FTIR spectra of the products formed from the condensation reaction of chitosan and its amino acid adducts with GDA are shown in Figure 2. There are significant bands at 1626, 1633, and 1630 cm^{-1}, which can be attributed to the characteristic peak of C=N for chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid condensates, respectively, with GDA.

TGA

TGA was performed for chitosan and its amino acid condensation adducts. It is shown in Figure 3 that there was a small loss in the weight of the samples (7.25 and 9.59%) from 50 to 233.63 and 200°C for chitosan and chitosan–4-aminobutyric acid, respectively, after which a sharp loss took place up to 325.44 and 333.33°C (the weight losses were 41.606 and 43.488%) for chitosan and chitosan–4-aminobutyric acid, respectively. After that, gradual



Scheme 2 Possible reaction mechanism between chitosan and α-amylase via the covalent attachment method.



Scheme 3 Possible reaction mechanism between chitosan–amino acid adducts and α-amylase via the covalent attachment method (R = L-glutamic acid or 4-aminobutyric acid).

decomposition took place up to 625.44 and 590.00°C (the weight losses were 51.324 and 43.774%) for chitosan and chitosan–4-aminobutyric acid, respectively, and this was followed by complete decomposition. In the case of chitosan–L-glutamic acid, gradual decomposition took place from 75 up to 471.13°C (the weight loss was 59.563%), as shown in Figure 3; another sharp decomposition occurred up to 680.6°C (the weight loss was 39.878%), followed by complete decomposition.

SEM

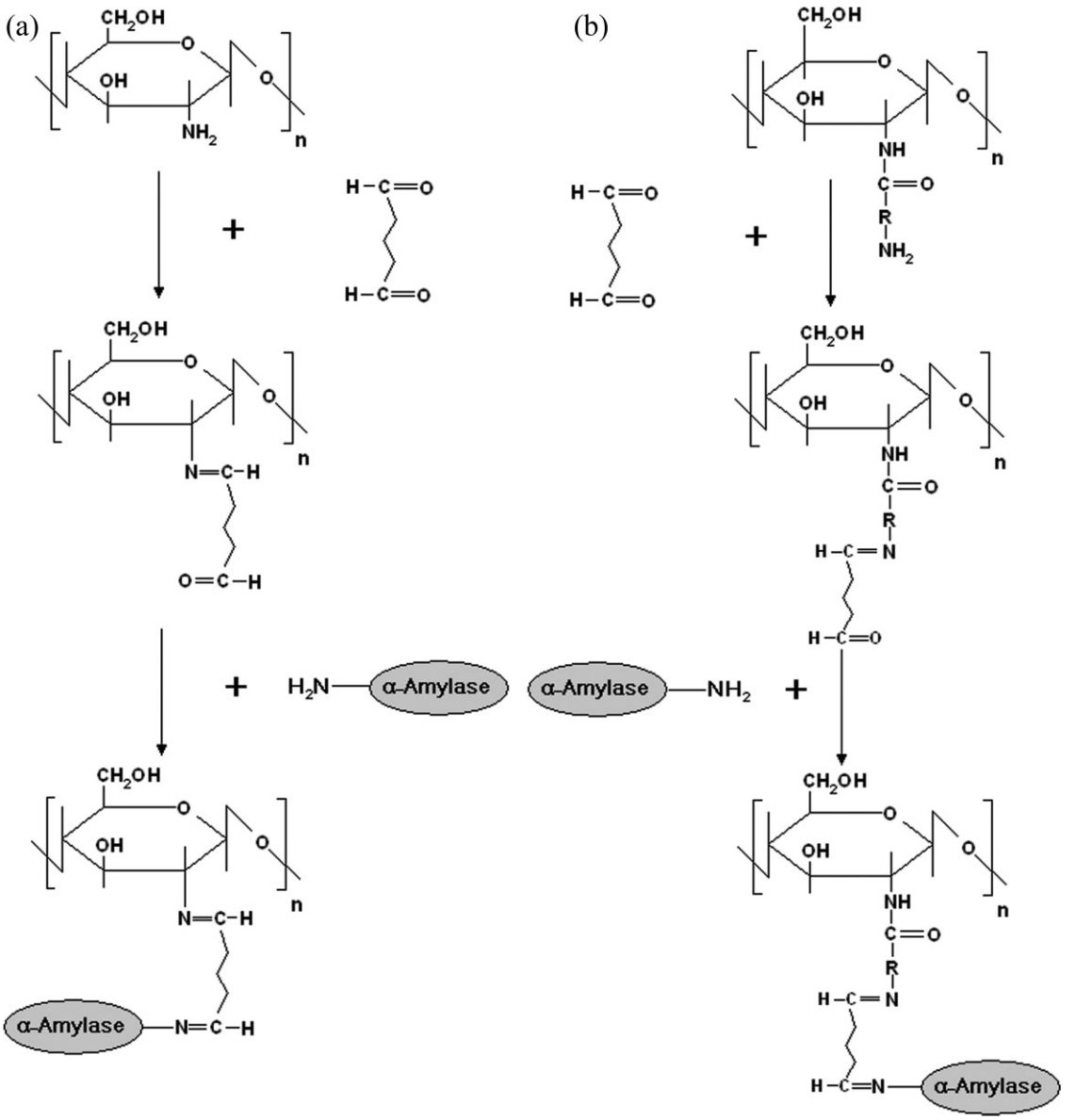
SEM micrographs of chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid show the morphology of these polymers before and after α-amylase immobilization. Figure 4(a) shows that the unimmobilized chitosan had a flaky surface morphology, and after the immobilization process, the chitosan surface became fibrous, as shown in Figure 4(b). On the other hand, Figure 4(c,e) shows that the surface morphology of chitosan–L-glutamic acid and chitosan–4-aminobutyric acid before α-amylase immobilization was flaky, and after the immobilization process, their surfaces became smooth, as shown in Figure 4(d,f). All these surface morphology observations demonstrated that α-amylase was successfully immobilized on chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid. The changes occurred in the texture of the polymer surfaces before and after enzyme immobilization, as confirmed by morphology observations; this confirmed that α-amylase was successfully immobilized on chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid.

α-Amylase immobilization

The immobilization of enzymes onto insoluble polymeric supports has been a topic of active research in the field of enzyme technology and is essential to their applications in industrial processes. A large number of enzymes have been successfully immobilized with very high activity yields on appropriate supports. The selection of support materials and the method of immobilization are very important for carrying out the desired enzymatic reaction. In this study, a large variety of natural supports were used as carriers for α-amylase immobilization via covalent attachment and crosslinking methods.

Covalent attachment method

Chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid (0.1 g) were used for immobilization of α-amylase [1–5 mg (402–2010 U)] in a phosphate buffer (pH 6.9) at 25°C for an incubation time of 24 h. The maximum amount of α-amylase activity was reached with 3 mg (1206 U). The effect of the α-amylase concentration on the immobilization with



Scheme 4 Possible immobilization mechanism of α-amylase on (a) chitosan and (b) chitosan–amino acid adducts crosslinked with GDA (R = L-glutamic acid or 4-aminobutyric acid).

the previously mentioned supports is shown in Table I. The retained activities were 60.00, 89.17, and 68.00% for chitosan, chitosan–L-glutamic acid, and chitosan–4-aminobutyric acid, respectively.

Crosslinking method

Chitosan–GDA (0.1, 0.5, or 1%), chitosan–L-glutamic acid–GDA (0.1, 0.5, or 1%), and chitosan–4-aminobutyric acid–GDA (0.1, 0.5, or 1%; 0.1 g) were used for the immobilization of α-amylase [1–5 mg (402–2010 U)] in a phosphate buffer (pH 6.9) at room temperature for an incubation time of 4 h. The maximum amount of α-amylase activity was reached with 3 mg (1206 U). The effect of the concentration of α-amylase on its immobilization on the previously mentioned supports is shown in Table II. The retained activities were 68.59, 97.36, and 79.50% for chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%), respectively. It can be observed from the previous results that the higher values of the retained activities of α-amylase immobilized on chitosan and its amino acid condensation adducts were at the GDA concentration of 1%.

Parameters affecting α-amylase activity

The activities of free α-amylase and α-amylase immobilized on chitosan and its amino acid condensation adducts were calculated by the measurement of the absorbance of the liberated maltose at 540 nm;

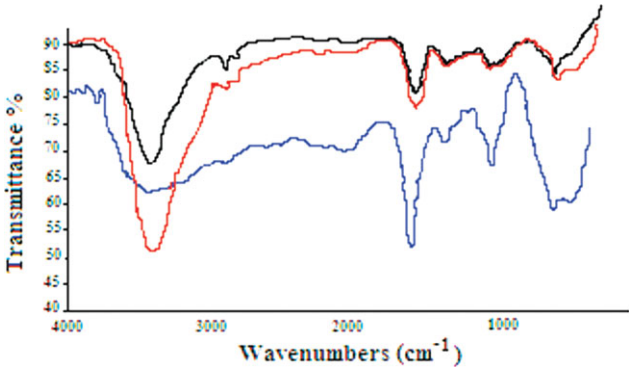


Figure 1 FTIR spectra of chitosan (black line), chitosan–L-glutamic acid (blue line), and chitosan–4-aminobutyric acid (red line). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

we took into account that the retained activities of the α-amylase immobilized on chitosan and its amino acid condensation adducts after the immobilization process would be the initial activities (relative activity = 100%) in the subsequent experiments. Effects of pH values and temperatures, as well as storage stability and repeated-use capability, were examined.

Effect of pH

The change in the optimum pH depends on the charge of the enzyme and/or matrix. This change is useful in understanding the structure–function relationship of the enzyme and for comparing the activity of the free and immobilized enzymes as a function of pH. The pH dependence of the immobilized α-amylase activity was compared with that of the free enzyme in the pH range of 2.9–9.9 at 25°C. Figure 5 shows the effect of pH in the range of 2.9–9.9 at 25°C on the relative activity of α-amylase in the immobilized and free forms. The maximum ac-

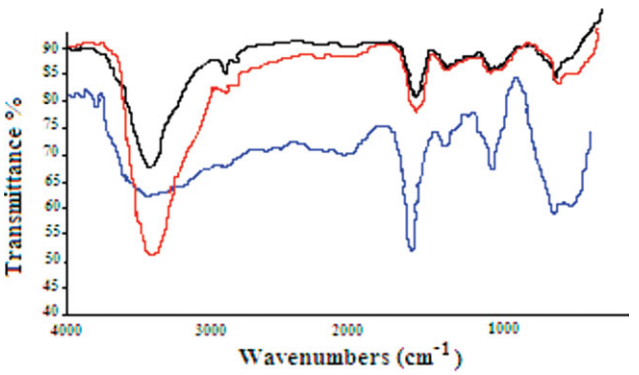


Figure 2 FTIR spectra of chitosan–GDA (1%) (black line), chitosan–L-glutamic acid–GDA (1%) (blue line), and chitosan–4-aminobutyric–GDA (1%) (red line). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

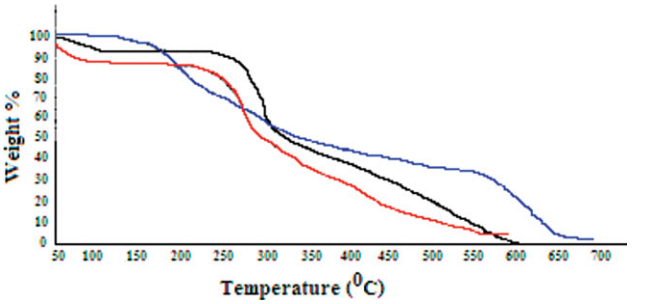


Figure 3 TGA of chitosan (black line), chitosan–L-glutamic acid (blue line), and chitosan–4-aminobutyric acid (red line). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tivity was at pH 6.9 for free α-amylase and α-amylase immobilized on chitosan–GDA (1%) and at pH 7.9 for α-amylase immobilized on chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%). Accordingly, the medium became basic according to the free amino group of the enzyme. Furthermore, the pH profiles of the immobilized α-amylase displayed significantly improved stability on both sides of the optimum pH values in comparison with that of the free form, and this means that the immobilization method preserved the enzyme activity in a wider pH range. It is illustrated in Figure 5 that, at pH 9.9, the retained activities of the free α-amylase and α-amylase

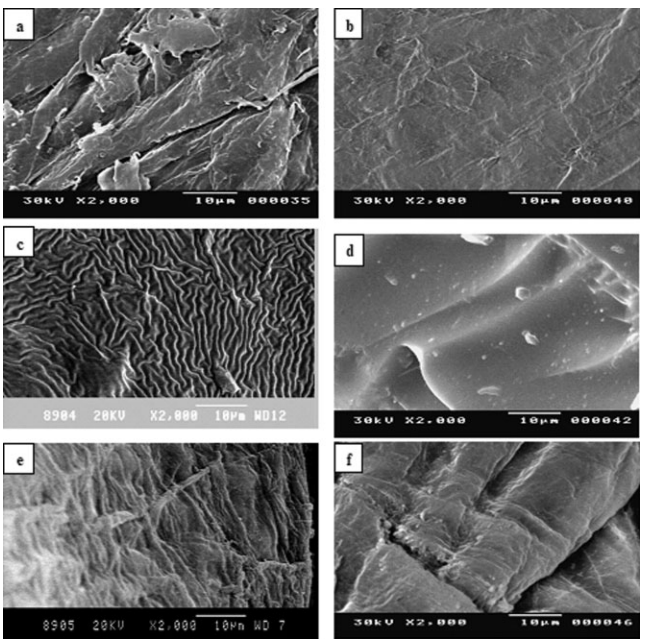


Figure 4 SEM of chitosan and its amino acid condensation adducts before and after α-amylase immobilization via the crosslinking method: (a) chitosan, (b) chitosan–GDA (1%)–α-amylase, (c) chitosan–L-glutamic acid, (d) chitosan–L-glutamic acid–GDA (1%)–α-amylase, (e) chitosan–4-aminobutyric acid, and (f) chitosan–4-aminobutyric acid–GDA (1%)–α-amylase.

TABLE I
Effect of the Concentration of α-Amylase on Its Immobilization on Chitosan and Its Amino Acid Condensation Adducts via the Covalent Attachment Method

Added enzyme (mg)	Immobilized enzyme (mg/0.1 g of support)			Retained activity (%)		
	A	B	C	A	B	C
1	0.50	0.69	0.50	50.30	69.05	50.14
2	1.09	1.57	1.19	54.61	78.60	59.81
3	1.80	2.67	2.04	60.00	89.17	68.00
4	2.25	3.09	2.32	56.31	77.31	58.00
5	2.57	3.51	2.45	51.32	70.20	48.91

A = chitosan; B = chitosan–L-glutamic acid; C = chitosan–4-aminobutyric acid.

immobilized on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%) were 27.34, 72.81, 90.40, and 85.23%, respectively. Meanwhile, at pH 2.9, the retained activities of the free α-amylase and α-amylase immobilized on the aforementioned copolymers were 4.81, 55.13, 66.00, and 57.43%, respectively; this enhanced the immobilization process from an economic point of view.

Effect of temperature

The upper temperature for enzyme activity is governed by the limits of enzyme stability. It is well known that an immobilized enzyme is more resistant to heat and denaturing agents than the free form.[24] Figure 6 shows the effect of temperature on the relative activities of free and immobilized α-amylase in the range of 30–100°C at pH 6.9. The free α-amylase exhibited a temperature optimum of 45°C, and this shifted to 50°C for α-amylase immobilized on chitosan–GDA (1%) and to 55°C for α-amylase immobilized on chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%). Also, it was observed that the temperature profile of the immobilized α-amylase was slightly broader than that of the free one as a direct result of the changes in the physical and chemical properties of the enzyme. The covalent bond formation via amino groups of the immobilized α-amylase might also reduce the conformational flexibility and result in a higher activation energy for the molecule to reorganize the proper conformation for binding to the substrate.[25–30] These results demonstrate the positive role of the carriers in protecting the α-amylase activity at higher temperatures. It is illustrated in Figure 6 that the free α-amylase lost all its activity at 70°C, whereas at 100°C, the retained activities of the α-amylase immobilized on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%) were 0, 55.76, and 50.16%, respectively, with respect to the original activity.

Storage stability

One of the most important parameters to be considered in enzyme immobilization is storage stability. The storage stability of the free and immobilized α-amylase was investigated at 4°C in the dry state by the measurement of the enzyme activities at certain time intervals, and the results are given in Figure 7. The enzyme activity was determined at 25°C in a phosphate buffer (pH 6.9). It was found that the α-amylase immobilized on chitosan–GDA (1%),

TABLE II
Effect of the Concentration of α-Amylase on Its Immobilization on Chitosan–GDA, Chitosan–L-Glutamic Acid–GDA, and Chitosan–4-Aminobutyric Acid via the Crosslinking Method

Added enzyme (mg)	Immobilized enzyme (mg/0.1 g of support)									Retained activity (%)								
	Chitosan–GDA			Chitosan–L-glutamic acid–GDA			Chitosan–4-aminobutyric acid–GDA			Chitosan–GDA			Chitosan–L-glutamic acid–GDA			Chitosan–4-aminobutyric acid–GDA		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	0.40	0.33	0.46	0.69	0.64	0.77	0.49	0.50	0.74	40.23	33.12	46.28	69.07	64.39	76.89	48.69	50.32	74.36
2	1.03	0.93	1.11	1.65	1.50	1.71	1.18	1.20	1.75	51.31	46.32	55.36	82.36	75.18	85.38	59.36	60.17	87.54
3	1.81	1.71	2.06	2.76	2.67	2.92	2.15	2.13	2.38	60.40	57.01	68.59	91.90	88.98	97.36	71.60	71.00	79.50
4	1.97	1.84	2.27	3.16	3.07	3.45	2.32	2.36	3.45	49.31	45.96	56.79	79.13	76.68	86.35	58.02	59.14	86.24
5	1.86	1.76	2.42	3.26	3.13	3.76	2.36	2.49	2.49	37.23	35.13	48.36	65.39	62.58	75.29	47.16	49.89	75.36

A = GDA (0.1%); B = GDA (0.5%); C = GDA (1%).

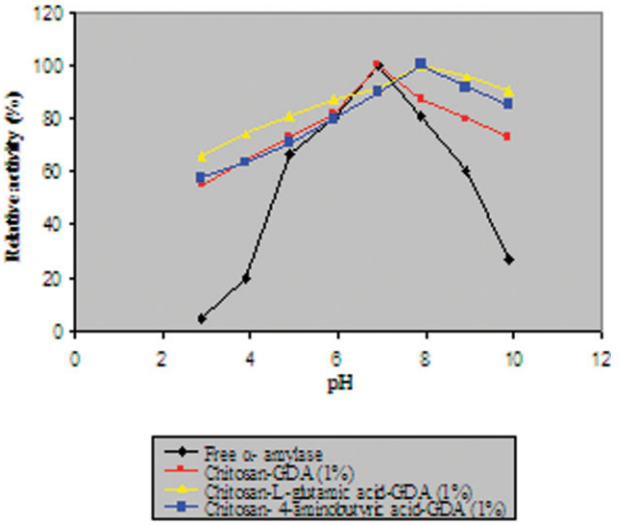


Figure 5 Effect of pH on the relative activity of free α-amylase and α-amylase immobilized on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

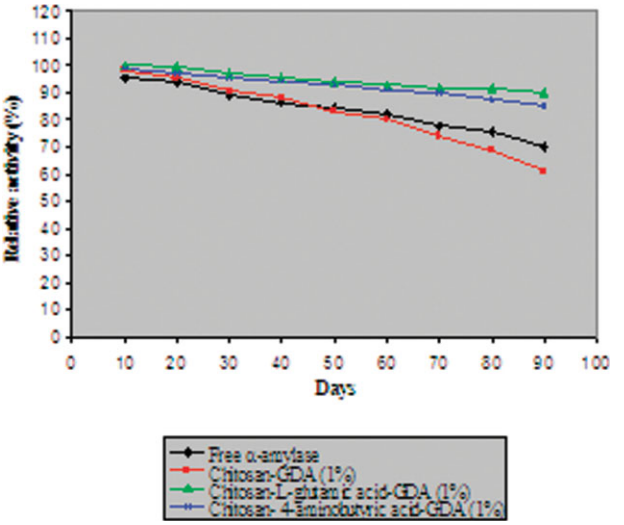


Figure 7 Storage stability of free α-amylase and α-amylase immobilized on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%) maintained values of 61.12, 90.05, and 85.24% with respect to the initial activity after 90 days.

Reusability

Reusing immobilized α-amylase is commercially very important, and increased stability can make the immobilized enzyme more advantageous than its free form.[31] Figure 8 shows the effect of repeated use on the activity of the immobilized α-amylase. The retained activity of the α-amylase immobilized on chitosan–GDA (1%) did not change after it was used 3 times, and 46.45% of its original activity was maintained after 25 uses. In contrast, the activities of α-amylase immobilized on chitosan–L-glutamic acid–GDA (1%) and chitosan–4-aminobutyric acid–GDA (1%) did not change after 11 and 8 uses, and 79 and 71% of the original activity were maintained after 25 uses. These results indicate that the immobilized α-

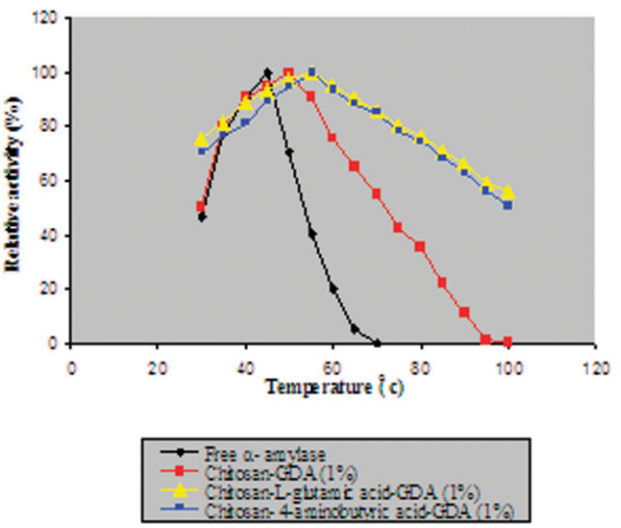


Figure 6 Effect of temperature on the relative activity of free α-amylase and α-amylase immobilized on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

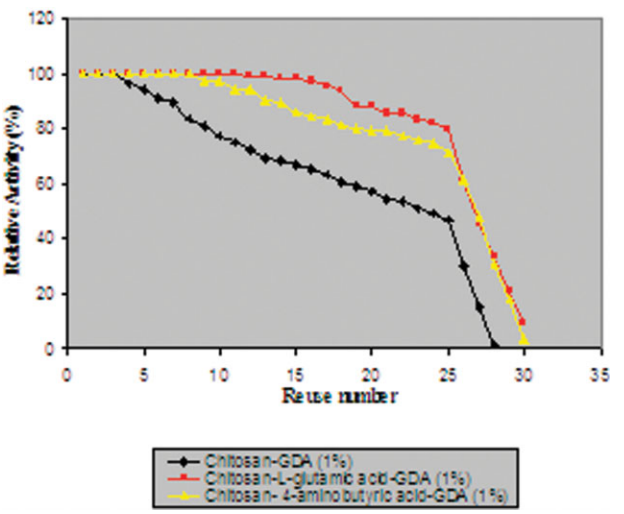


Figure 8 Reuse stability of α-amylase immobilized on chitosan–GDA (1%), chitosan–L-glutamic acid–GDA (1%), and chitosan–4-aminobutyric acid–GDA (1%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

amylase is relatively stable without a severe loss of activity after several repeated uses.

CONCLUSIONS

α-Amylase was successfully immobilized on chitosan and its amino acid condensation adducts. The immobilization process was carried out via covalent binding between the previous polymers and α-amylase and via a crosslinking method with GDA as a crosslinking agent. Our experiments showed that the latter method was effective for retaining high activity values. The α-amylase immobilized on chitosan and its amino acid condensation adducts promoted enzyme stability, and as a result, the α-amylase became more stable against pH, temperature, storage, and reuse in comparison with the free form.

References

1. Gamze, D.; Senay, C. Food Chem 2007, 100, 964.
2. Amorim, R.; Melo, E.; Carneiro-da-Cunha, M.; Ledingham, W.; Campos-Takaki, G. Bioresour Technol 2003, 89, 35.
3. Hsyue-Jen, H.; Po-Chih, L.; Wan-Jun, L. Biotechnol Lett 2000, 22, 1459.
4. Çetinus, S.; Öztop, H. Enzyme Microb Technol 2000, 26, 497.
5. Carvalho, G.; Alves, T.; Freire, D. Appl Biochem Biotechnol 2000, 84, 791.
6. Abdel-Naby, M.; Isamil, M.; Abdel-Fattah, A. Process Biochem 1999, 34, 391.
7. Shin, H.; Park, J.; Yang, J. Process Biochem 1998, 33, 787.
8. Zoe, K.; Maria, L. Enzyme Microb Technol 2006, 39, 690.
9. Hayrettin, T.; Aksoy, S.; Hasõrcõ, N. Food Chem 2000, 68, 259.
10. Reshmi, R.; Sanjay, G.; Sugunan, S. Catal Commun 2007, 8, 393.
11. Vogel, A. Textbook of Quantitative Inorganic Analysis, 3rd ed.; Longmans: London, 1961.
12. Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. J Biol Chem 1951, 193, 265.
13. Rosevaer, A. J Chem Technol Biotechnol 1984, 34, 127.
14. Bernfield, P. In Methods in Enzymology; Colowick, S.; Kaplan, N., Eds.; Academic: New York, 1955; Vol. I, p 149.
15. Raviyan, P.; Tang, J.; Orellana, L.; Rasco, B. J Food Sci 2003, 68, 2302.
16. Smith, B.; Roe, J. J Biol Chem 1949, 179, 53.
17. Smith, B.; Roe, J. J Biol Chem 1957, 227, 357.
18. Herbert, M.; Joaquin, I.; Albert, C.; Joseph, L. Clin Chem 1977, 23, 122.
19. Esparza, M.; Gomez, M. Macromol Symp 2003, 197, 277.
20. Arica, M.; Bayramoglu, G.; Akgol, S.; Bulut, A.; Denizli, A. Biochem Eng J 2003, 14, 117.
21. Li, Y.; Bai, Y.; Yang, Y.; Yi, L. J Biotechnol 2006, 125, 574.
22. Wu, W.; Hung, T.; Giridhar, R.; Chiou, S. J Mol Catal B 2003, 26, 69.
23. Juang, R.; Min-Yun, C. Enzyme Microb Technol 2005, 36, 75.
24. Kennedy, J.; Paterson, M. Polym Int 1993, 30, 71.
25. Ohtsuka, Y.; Kawaguchi, H.; Yamamoto, T. J Appl Polym Sci 1984, 29, 3295.
26. Strumeyer, D.; Constantinide, A.; Freundenberger, J. J Food Sci 1974, 39, 498.
27. Daniel, R. Enzyme Microb Technol 1996, 19, 74.
28. Lee, P.; Lee, K.; Siaw, S. J Chem Technol Biotechnol 1993, 58, 65.
29. Demircioglu, H.; Beyenal, H.; Tanyolaç, A.; Hasırcı, N. Polym Int 1994, 35, 321.
30. Arica, M.; Hasirci, V.; Alaeddinoglu, N. Biomaterials 1995, 15, 761.
31. Tien, C.; Chiang, B. Process Biochem 1999, 35, 377.