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Bioaffinity Adsorption Behavior of Several Enzymes onto β-Cyclodextrin–Chitosan

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ABSTRACT

In this study, specific and nonspecific adsorption and desorption of α-amylase, lactase, and amyloglucosidase (AMG) enzymes onto the β-cyclodextrin (CD)–chitosan system were investigated. α-Amylase and lactase enzymes were adsorbed onto the chitosan-only and chitosan-spacer gels, but very little adsorption was observed in the case of the chitosan gels with CD. Furthermore, AMG enzyme showed a high degree of interaction with CD immobilized on chitosan. Therefore, the interaction between the AMG and CD molecules can be explained by the fact that there is true biospecific adsorption. Desorption experiments also confirm this theory because the elution of AMG was much more difficult than the elution of lactase and α-amylase from the CD matrix.

INTRODUCTION

Affinity chromatography is a purification method based on biospecific adsorption of a particular enzyme on an affinity ligand. The ligand is attached onto the matrix using a spacer arm, and it is used to keep the ligand away from the matrix so that the active site of the ligand is available to the sample.

In this study β-cyclodextrin (CD) was selected as a ligand since earlier studies showed that amyloglucosidase has an affinity for cyclodextrin mol-

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ecules (1). β-CD was attached onto the chitosan gels with an EDGE (ethylene glycol-diglycidyl ether) spacer. In some adsorption operations the affinity matrix itself and the spacer may show nonspecific adsorption (2). The most ideal case is to obtain biospecific adsorption only between the protein molecules and the ligand, avoiding the nonspecific interactions.

Therefore we investigated the nonspecific and biospecific adsorption of chitosan matrices. To do this, a series of adsorption–desorption experiments with α-amylase, lactase, and amyloglucosidase (AMG) enzymes were conducted using chitosan only, chitosan with spacer, and β-CD–chitosan gels under different pH and ionic strength environments.

EXPERIMENTAL

Adsorbents were chitosan gels-only, chitosan gels with a spacer and β-CD–chitosan of different sizes (200, 500, and 600 μm), and supplied from Japan Organo Co. β-Cyclodextrin was immobilized onto the chitosan matrix by introducing the spacer EDGE. AMG, α-amylase, and lactase enzymes were the products of Novo Nordisk, Japan.

Methods

Batch experiments were conducted to measure the nonspecific and biospecific adsorption of AMG, α-amylase, and lactase on chitosan only, chitosan with spacer, and β-CD–chitosan matrices.

Effect of pH. AMG (4.0 mg/mL), α-amylase (3.1 mg/mL), and lactase (0.35 mg/mL) were used to measure the effect of pH by using gels equilibrated with different pH buffers between 2.5 and 8.0. Protein concentrations and enzyme activities of the free solution were measured after 24 hours of incubation at 25°C. Partition coefficients were calculated as described before (3).

Effect of Ionic Strength. AMG enzyme samples (0.1 mL; 4.0 mg/mL) were prepared in 6.0 mL of acetate buffers of different ionic strengths (0–1.0 M NaCl) at pH 4.5 and added to 0.6 mL of gel. These samples were left at 25°C in a shaking water bath for 24 hours. After adsorption was complete, samples were taken, assayed, and the partition coefficients were calculated.

Determination of Adsorption Isotherms. These experiments were done in well-stirred batch solutions at 25°C (gel/liquid ratio = 0.1) at pH 4.0 and 4.5. The acetate buffer contains 2.0 M NaCl at different concentrations of AMG enzyme.

Desorption of the Enzymes. Gels with adsorbed protein were washed with the initial buffer solution and left for desorption with different pH buffer solutions contain 0.5, 1.0, and 2.0 M NaCl.

pH Stability of α-Amylase Enzyme. Diluted α-Amylase enzyme solution (0.5 mL) was incubated for 24 hours at 25°C in 5.0 mL of different buffer solutions (KCl–HCl, glycine–HCl, phosphate, borate, glycine–NaOH, acetate, citrate, Tris–HCl) in the pH range of 2.0 to 12.0. After the incubation period the residual activities were measured under standard assay conditions. The stability of an enzyme is expressed as the percent activity remaining after 24 hours at the corresponding pH at 25°C, based on the original activity.

Analytical Methods

Measurement of Protein Concentrations. The Lowry protein method (4) was used to estimate the specific activities of the enzyme solutions.

Measurement of AMG Activity. AMG activities were measured by using the method of Novo Nordisk (5). Enzyme solutions (1.0 mL) were incubated with 1.0 mL preheated substrate solution (2% maltose substrate in 0.1 M acetate buffer, pH 4.3) at 25°C for 30 minutes. After stopping the reaction with 3.0 mL of 1.66 M Tris buffer, pH 7.6, the reducing sugar formed was assayed using the glucose kit produced by Boehringer Mannheim (Cat. no. 124036), and the degree of hydrolysis was calculated according to Novo's analytical method. One unit of glucoamylase activity was defined as the amount of enzyme which at standard conditions splits 1.0 μmol maltose per minute at 25°C and pH 4.30.

Measurement of α-Amylase Activity. α-Amylase activities were measured using the Wako amylase iodo-starch method (Code no. 274-04009). Enzyme solutions (0.02 mL) were incubated with 1.0 mL of preheated substrate buffer solution (0.25 mol/L phosphate buffer, pH 7.0, containing 40 mg/dL soluble starch) at 37°C for 7.5 minutes. Incubation was terminated by the addition of a color reagent which reacts with any starch that escaped hydrolysis by amylase in the specimen. The amount of starch hydrolyzed is calculated by subtracting the residual amount and is directly proportional to the activity of amylase in the specimen. The hydrolysis degree was calculated according to the Wako manual. One unit is the amount of amylase activity in 100 mL of serum that catalyzes the hydrolysis of 10 mg of starch in 30 minutes at 37°C.

RESULTS AND DISCUSSION

Effect of pH

Effect of pH for AMG

The partitioning behavior of AMG adsorption on chitosan only, chitosan with spacer, and β-CD–chitosan gels was studied at 25°C. All the chitosan

gels used here have the same particle size (600 μm). The partition coefficients for all kind of adsorbents are affected by pH in the 3.0 to 8.0 range. The maximum value of the partition coefficients for the AMG/chitosan-only system is obtained around pH 4.5, whereas this value is 4.2 for the chitosan matrix with spacer. As reported in our earlier publication (3), AMG adsorption on β-CD–chitosan gels is at its maximum at pH 4.0. A similar trend of AMG adsorption for all matrices with pH was obtained, and at pH 3.0 and 8.0 almost no adsorption took place. At pH 6.0 the partition coefficients of β-CD–chitosan gels are much bigger than those of chitosan only and chitosan gels with spacer.

The AMG enzyme is adsorbed by different matrices. To understand whether there is real biospecific binding between CD and AMG, similar experiments were conducted for α-amylase and lactase enzymes.

Effect of pH on the Adsorption of Contaminating Proteins

There are two reasons for using α-amylase and lactase enzymes in these experiments. First, to examine the binding relation between CD and AMG by comparing the results obtained here. Second, these enzymes are mainly present in the fermentation broths of AMG by *Aspergillus niger*. The experimental results will help us to select the optimal conditions for the elution of AMG separately from the majority of contaminating proteins.

Figures 1 and 2 show the effect of pH on α-amylase and lactase adsorption, respectively, using chitosan only, chitosan with spacer, and β-CD–chitosan gels (600 μm) at 25°C.

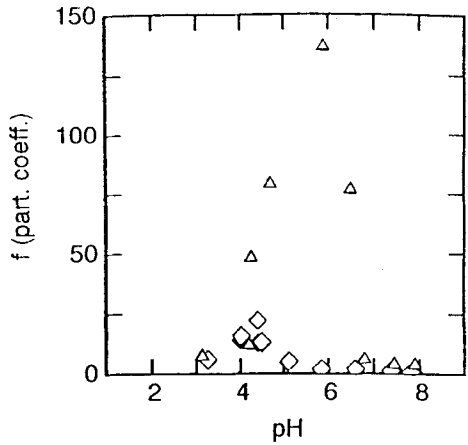

FIG. 1 Effect of pH for α-amylase enzyme adsorption on chitosan gels: (△) chitosan only, (◆) chitosan with spacer, (◇) β-CD–chitosan.

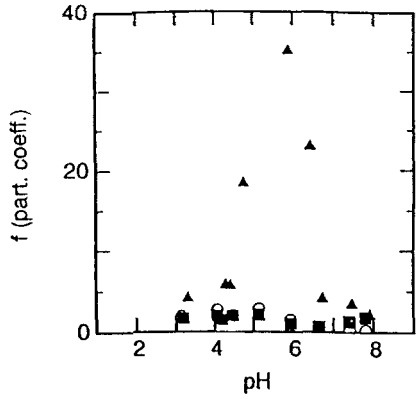

FIG. 2 Effect of pH for lactase enzyme adsorption on chitosan gels: (▲) chitosan only, (■) chitosan with spacer, (○) β-CD–chitosan.

In Fig. 1, partition coefficients of the α-amylase enzyme for the chitosan-only gel are quite high. pH values between 4.5 and 6.5 especially favor α-amylase adsorption; the maximum adsorption is obtained at pH 6.0. The partition coefficients for gels with spacer and β-CD are smaller.

Maximum lactase adsorption by chitosan beads was obtained at pH 4.5–6.5 (which was the same for α-amylase) (Fig. 2). The highest partition coefficient value was around 35 at pH 6.0. Lactase adsorption was negligible for gels with spacer and β-CD.

Both lactase and α-amylase enzymes show very little adsorption below pH 4.0 on any kind of matrice. The pH stability of lactase is reported to be good between pH 6.0 and 8.0 at 40°C for 3 hours (5). α-Amylase is stable between pH 5.0 and 8.0, but below pH 4.0 the activity is lost very quickly (5) at 50°C over 4 hours.

In our study, the pH stability of α-amylase was studied at 25°C for 24 hours; the results are given in Fig. 3. As seen from this figure, the α-amylase enzyme is quite stable at pH 5.5 to 7.5. Below pH 4.0 the enzyme loses around 80% of its activity, probably due to acid denaturation. Above pH 8.0, this enzyme is very unstable.

Effect of Ionic Strength

Figure 4 shows the effect of ionic strength on the adsorption of AMG on chitosan gels at pH 4.5 at 25°C. The partition coefficient decreases sharply with a small increase in ionic strength for chitosan-only gels and gels with spacer. At very low ionic strength values the partition coefficient is very high (around 130) for chitosan-only gel. Partition coefficients reach

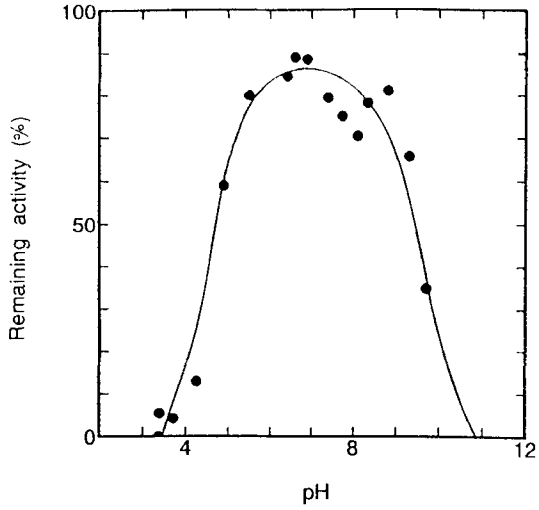

FIG. 3 Stability of α-amylase enzyme.

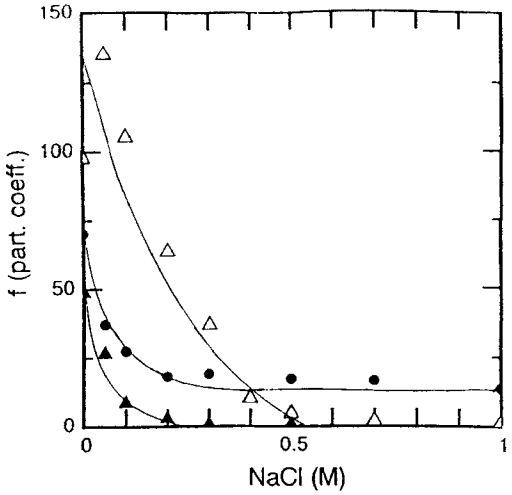

FIG. 4 Effect of ionic strength on the adsorption of AMG on chitosan gels: (△) chitosan only, (▲) chitosan with spacer, (●) β-CD–chitosan.

zero values at 0.5 M and higher NaCl concentrations. With β-CD–chitosan, at zero ionic strength and a partition coefficient value of 70, 0.2 M and higher NaCl concentrations cause nearly a 70% decrease. Even at very high ionic strength levels, partition coefficients do not reach zero, which means nonspecific interactions (electrostatic, etc.) are excluded by the addition of salt, and the adsorption of AMG on β-CD is biospecific.

Chitosan matrix with spacer was strongly affected by increasing ionic strength. NaCl concentrations 0.2 M and higher yield no adsorption. It should be pointed out that the amount of adsorbed protein for chitosan-only gels is much higher than for gels with spacer. This is probably due to the presence of the spacer which suppresses most of the adsorption caused by chitosan beads.

Figure 5 shows the effect of pH on partitioning at a fixed high ionic strength value (0.3 M NaCl). As seen, at an ionic strength value of 0.3 M the highest partitioning was observed at pH 3.0. The partition coefficient decreased monotonously to its lowest value at pH 8.0.

Adsorption Isotherms

Adsorption isotherms were generated for AMG enzyme using chitosan only, chitosan with spacer, and β-CD–chitosan gels. Table 1 gives the Langmuir parameters, and Fig. 6 shows adsorption isotherms of chitosan gels at pH 4.5 and 25°C.

From Figure 6 it is possible to compare the differences in the maximum adsorption capacity and dissociation values (at a fixed pH, temperature,

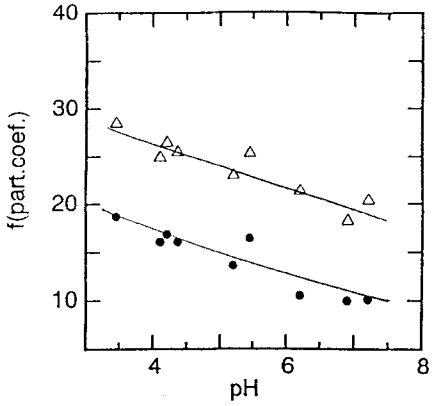

FIG. 5 Effect of pH on the AMG/β-CD–chitosan (600 μm) adsorption using 0.3 M NaCl (△, enzyme; ●, protein).

TABLE 1
Adsorption Isotherm Parameters for Chitosan Gels

	q_m (mg/mL gel)	K_p (mg/mL solvent)
Chitosan spacer (600 μm)	48	0.46
Chitosan only (600 μm)	286	2.8
β-CD–chitosan (600 μm)	111	1.9
Chitosan only (200 μm)	225	1.85
β-CD–chitosan (200 μm)	151	2.5
β-CD–chitosan (500 μm)	120.5	2.0

and ionic strength) for chitosan gels with or without spacer and β-CD. There is some degree of adsorption in all cases. The highest adsorption value was caused by chitosan beads. The same gel with spacer gives the lowest adsorption values (both q_m and K_p values are the smallest). The spacer probably suppresses the adsorption caused by chitosan beads.

The isotherm parameters estimated for the β-CD–chitosan matrix are median, as seen in Table 1.

It is known that nonspecific ionic interactions can be greatly reduced using salt concentrations around 0.5 M NaCl, but sometimes 2.0 or 3.0 M salt is needed (6). Therefore, we also studied the adsorption isotherms

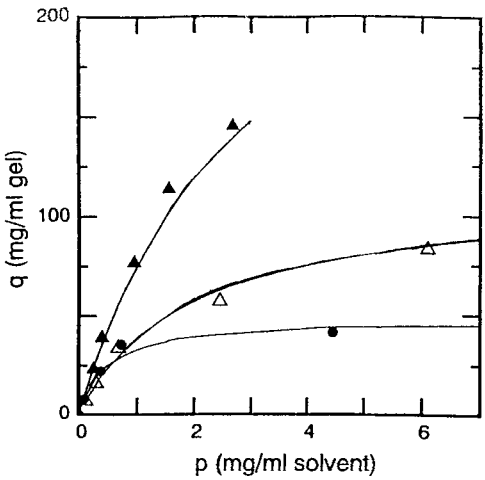

FIG. 6 Adsorption isotherms for AMG enzymes on chitosan gels (600 μm), pH 4.5, 25°C: (▲) chitosan only, (●) chitosan with spacer, (△) β-CD–chitosan.

TABLE 2
Adsorption Isotherm Parameters for Chitosan Gels in the Presence of 2.0 M NaCl (pH 4.0, 25°C)

β-CD–chitosan	q_m (mg/mL gel)	K_p (mg/ml solvent)
200 μm	115	6.4
500 μm	125	5.5
600 μm	111	7.16

using high ionic strength solutions to exclude the electrostatic interactions and to quantify the biospecific adsorption with an estimation of the isotherm parameters (q_m and K_p) which apply in chromatography.

Langmuir model parameters and isotherms of the AMG/chitosan system under high ionic strength conditions (2.0 M NaCl, pH 4.0, 25°C) are shown in Table 2 and Fig. 7.

Experimental results show that while β-CD–chitosan matrices interact with AMG, chitosan only and chitosan with spacer do not adsorb AMG at all in the presence of 2.0 M NaCl (data not shown). This may explain why the adsorbed protein was easily eluted from matrices without CD using ionic strength buffers, and why desorption was not successful with these eluants for the chitosan matrix with CD, as we will see in the next section.

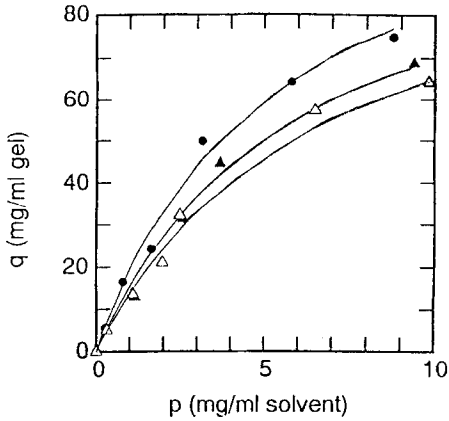

FIG. 7 Adsorption isotherms for AMG enzymes on β-CD–chitosan gels, pH 4.0, 25°C, 2.0 M NaCl: (▲) 200 μm, (●) 500 μm, (△) 600 μm.

Desorption

A series of desorption experiments were done using chitosan only, chitosan with spacer, and β-CD–chitosan matrices adsorbed with AMG. Table 3 summarizes the percentage yields for the elution using different ionic strengths and pH buffers.

As we reported earlier, chitosan only and chitosan with spacer show some degree of adsorption since chitosan beads are porous, but adsorbed enzyme can be easily eluted using 0.5 and 1.0 M NaCl in pH 4.5 buffer without any loss in activity. Desorption was achieved only with pH 8.0 borate buffer in the case of β-CD–chitosan, as reported elsewhere (3).

Desorption of α-amylase and lactase enzymes from all kinds of chitosan matrices was also studied. A NaCl concentration of 0.5 M gave 35, 45, and 42% lactase enzyme recoveries from chitosan only, chitosan with spacer, and CD matrices, respectively. Both enzymes were completely eluted from chitosan gels using higher ionic strength buffer solutions.

TABLE 3
Desorption Conditions for AMG from Chitosan Gels

	Desorption (%)		
	Partitioning	Enzyme activity	Elution buffer
Chitosan only (200 μm)	100	100	2 M NaCl, pH 4.5, acetate
Chitosan only (500 μm)	100	100	2 M NaCl, pH 4.5, acetate
Chitosan only (600 μm)	100	100	2 M NaCl, pH 4.5, acetate
β-CD–chitosan (500 μm)	35	30	0.5–2 M NaCl, pH 4.5, acetate
β-CD–chitosan (600 μm)	35	30	2 M NaCl, pH 4.5, acetate
Chitosan only (600 μm)	2	1	pH 5.0, acetate
Chitosan only (200 μm)	100	95	1 M NaCl, pH 4.5, acetate
Chitosan with spacer (600 μm)	100	96	0.5 M NaCl, pH 4.5, acetate
β-CD–chitosan (500 μm)	N.D.	39	0.5 M NaCl, pH 5.0, borate

CONCLUSIONS

Partition coefficients of AMG/chitosan gels were influenced by pH. The amount of adsorbed enzyme in the case of chitosan-only gels was larger than that of gels with CD. Chitosan gel with spacer also adsorbed enzyme, but less than the amount adsorbed by the gels with or without CD. The maximum adsorption values were obtained around pH 4.0–4.5 in all cases.

α-Amylase and lactase enzymes were strongly adsorbed by the chitosan-only matrix. Very little adsorption was observed with the spacer and β-CD gels. The optimum pH was found to be the same (around pH 6.0) for both α-amylase and lactase enzymes. The partition coefficients were higher for α-amylase enzyme than for lactase.

Ionic strength affected the phase equilibrium. First, an initial decrease of the partition coefficient was detected for all chitosan gels. Then, chitosan with spacer and chitosan-only gels showed no adsorption at NaCl concentrations of 0.2 and 0.5 M and higher, respectively. The partition coefficients remained steady at these ionic strength values for the AMG/β-CD–chitosan system.

Adsorption isotherms for the AMG/β-CD–chitosan affinity system with or without ionic strength were nonlinear and showed the Langmuir relationship. The maximum adsorption capacities were found for chitosan-only gels, whereas the minimum was found for chitosan with spacer without ionic strength. At 2.0 M NaCl concentrations, chitosan only and chitosan with spacer matrices showed no adsorption.

AMG enzyme was easily eluted from chitosan only and chitosan with spacer gels using low ionic strength buffer solutions. α-Amylase and lactase enzymes were washed from chitosan only, chitosan with spacer, and β-CD–chitosan under the same desorption conditions. Elution of AMG enzyme from β-CD–chitosan was achieved with pH 8.0 borate buffer. Even high ionic strength conditions did not help to elute the enzyme. Almost all the AMG activity was eluted at pH 8.0.

The experimental results of this study enable us to understand the nonspecific adsorption caused by chitosan beads and a spacer, the biospecific interaction between AMG and β-CD, and to select the optimum conditions for chromatographic separation.

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