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The separation of GMP from milk whey using the modified chitosan beads

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Abstract The cationic property of chitosan was used to adsorb glycomacropeptide (GMP) molecules from milk whey proteins. β-cyclodextrin was immobilized to native chitosan beads by cross-linking with 1,6-hexamethylene diisocyanate (HMDI). The resultant modified beads (CS-HMDI-BCD) presented superior adsorption affinity and capacity towards GMP. At pH 3.0, 90.23% of GMP was adsorbed with a maximum adsorption capacity corresponding to 12.87 mg of sialic acid/g-adsorbent. Desorption experiments showed that the modified beads could be regenerated and used in many cycles without significant decreases in the capacity and selectivity.

Keywords Chitosan · β-cyclodextrin · Glycomacropeptide · Adsorption · Milk whey

1 Introduction

Glycomacropeptide (GMP), which is released from κ-casein by the action of chymosin during cheese making (Eigel et al. 1984), is an acidic C-terminal glycopeptides containing 64 amino acid residues. GMP lacking aromatic amino acids (phenylalanine, tyrosine, and tryptophan) is useful in the diet for patients with phenylketonuria, an important innate error in phenylalanine metabolism. It also contains sugars such as N-acetylneuraminic acid (sialic acid), galactose, and N-acetylgalactosamine (Abd ElSalam et al. 1996). Due to its biological activities (Dziuba and Minkiewicz 1996) and potential as an ingredient for functional food and pharmaceuticals, great attention has been given to GMP isolation and purification in recent years.

GMP has been purified from sweet whey by different techniques including ultrafiltration (Chu et al. 1996), gel chromatography (Nakano and Ozimek 2002), hydrophobic interaction chromatography (Nakano and Ozimek 2000b), and ion exchange chromatography (Doultani et al. 2003; Nakano and Ozimek 2000a; Silva-Hernandez et al. 2002). Heat treatment, ethanol precipitation, and trichloracetic acid precipitation were usually combined with the above methods as pretreatment to improve the purity (Li and Mine 2004; Saito et al. 1991; Thoma et al. 2006). With anion exchange chromatography, GMP can be separated from other major whey protein with relatively high yield and purity due to its lower isoelectric point (Nakano et al. 2004). Thus, this technique is considered as one of the most practical approaches for large scale production of GMP. The major issue of this method, however, is the cost and efficiency of the adsorbents used.

Chitosan (CS), a polysaccharide comprising copolymers of glucosamine and N-acetyl-glucosamine, is a derivative of the naturally abundant bio-polymer, chitin. In acidic condition, its cationic property makes it a good anionic exchanger towards GMP. Thus, CS (Casal et al. 2005; Nakano et al. 2005) and its derivatives treated with epichlorohydrin (Nakano et al. 2004) have been utilized in the separation of GMP. However, the adsorption capacity and selectivity of those CS materials are generally low.

β-cyclodextrin (BCD) is a cyclic oligosaccharide with glucose units linked by α-(1,4) bonds. BCD has a polar cavity in the molecule and has the capability of forming inclusion complex with various compounds (Ahn and Kwak 1999; Chiu et al. 2004; Prabaharan and Mano 2006). The

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major contamination of whey in the separation of GMP is the lipid. The unique combination of the hydrophobic bucket of BCD and anion exchange capacity of CS is expected to enhance the adsorption selectivity/capacity towards GMP. Moreover, immobilization of BCD onto CS may also improve the stability, recoverability and reutilization of the adsorbents.

In the present study, β-cyclodextrin was immobilized onto CS by using 1,6-hexamethylene diisocyanate (HMDI) as the spacer and used for the selective adsorption of GMP from sweet whey. The structure characterization, adsorption selectivity, and the re-generation of the modified beads are presented and discussed.

2 Experiments and methods

2.1 Preparation of chitosan beads

2 wt% of CS powder was dissolved in 1% (v/v) acetic acid solution. The CS solution was filtered and pumped through a syringe needle (27G) drop-wisely into 1 M NaOH solution under stirring to form spherical gel beads (with the diameter ~2 mm). The beads were rinsed with tap water until neutral. Then the beads were washed with ethanol and followed by deionized (DI) water. The beads were then freeze-dried overnight for further application.

2.2 Immobilization of β-cyclodextrin

BCD was immobilized onto CS by cross-linking with HMDI reagent in two reaction steps with 2-ethylhexanoate as the catalyst (Chiu et al. 2004). About 0.2 g of dry CS beads was placed in 10 ml of dimethylformamide (DMF) solution containing 2.5% (v/v) of HMDI. The mixture was magnetically stirred at room temperature after adding few drops of stannous 2-ethylhexanoate. After stirring for 1 hour, the supernatant was discarded and the CS beads were dried in a vacuum oven. The interim CS beads bound HMDI (CS-HMDI) were then placed in 10 ml of DMF solution containing 1% (g/L) BCD. The mixture was stirred for 1 hour at room temperature after adding few drops of the same catalyst. The supernatant was decanted and the final beads (CS-HMDI-BCD) were washed with DI water followed by ethanol washing. The beads were then washed with DI water again before freezing drying.

2.3 Characterizations

FTIR spectroscopy of the native CS beads, interim CS-HMDI beads, and the final beads (CS-HMDI-BCD) were measured on a NICOLET 5700 FTIR spectrometer with the attenuated total reflectance (ATR) mode. The scanning electron microscope (SEM) was used to observe the porous structure of the beads. The pore volume and surface area of the beads were determined by N_2 adsorption isotherm at 77 K.

2.4 Preparation of whey protein powder

The CS RT-90^{TM} native whey protein isolate was the kind gift of ANF innovations Pty, Australia. Whey solution was prepared by 10 g of milk whey in 200 ml of DI water. The non-dialyzable fraction of the whey solution was obtained by dialyzing under running water for 24 hours using a 6000–8000 molecular weight cut-off membrane. The fraction retained in the dialysis tube (non-dialyzable fraction of sweet whey) was freeze-dried and stored at 4°C.

2.5 Assay of GMP

The simple acidic ninhydrin assay specific to sialic acid (Yao et al. 1989) was used here for the rapid and sensitive determination of GMP. Sialic acid was an effective indicator to determine the presence of GMP in the whey because GMP is account for approximately 80% of total sialic acid in the whey. The method was also proven to be as effective as HPLC method in the determination of adulteration of whey in milk (Fukuda et al. 2004).

An acidic ninhydrin reagent of Gaitonde was prepared by 5 g of ninhydrin, 120 ml of glacial acetic acid and 80 ml of 37% HCl (w/v). The reaction mixture containing identical volume of acid ninhydrin reagent, glacial acetic acid, and sample solution was heated for 10 min in a boiling water bath. After that the mixture was rapidly chilled in tap water and a stable color (yellowish) would appear. The acidic ninhydrin solution was shown to be specific for sialoglycoproteins with stable color development at 470 nm, at which other asialoglyproteins cannot be detected.

2.6 Adsorption experiments

Two types of adsorbents, native CS beads and the BCD grafted beads were compared in adsorption of GMP from acidic to alkaline conditions. The pH of the adsorption medium, adjusted by the concentrated HCl or NaOH solutions, varied from 2.5 to 8.5. In a typical adsorption experiment, concentrated protein mixture was diluted to various concentrations and added with 0.05 g of adsorbents. Then the system was stirred at the rate of 200 rpm for 24 h at room temperature. The initial and final concentrations of GMP in the liquid phase were determined at 470 nm by UV/Vis spectrophotometer (NICOLET EVOLUTION 500) with the acidic ninhydrin reagent. As the composition of whey protein was complicated, the initial and final concentrations of other proteins in the liquid phase were determined at 280 nm.

The effect of incubation time on the uptake of GMP was also studied. 0.05 g of the BCD modified beads was mixed with 10 ml of DI water and 200 µl of concentrated protein mixture and stirred at the rate of 200 rpm. At different time intervals, the sample compositions were determined by UV/Vis. All experiments were conducted in triplicates.

The amount of GMP uptake at equilibrium, Q_e, was calculated using (1):

$$Q_e = (C_0 - C_e)V/W \tag{1}$$

where C_0 and C_e are the initial and final sialic acid concentration in liquid phase, respectively. V is the volume of solution, and W is the weight of adsorbent. The adsorption behavior of GMP at different pH and in different incubation time was explained by the adsorption percentage of GMP (the ratio of the sialic acid in solid phase to the initial sialic acid in liquid phase). The adsorption behavior of whey proteins were determined by the ratios of their overall absorbance at 280 nm.

The GMP adsorption kinetics was also measured during batch experiments at different time intervals, and was studied with different mathematical models. The pseudo-first-order kinetic model of Langergren and Svenska is:

$$\log(Q_e - Q_t) = \log Q_e - \frac{k_1}{2.303}t \tag{2}$$

and the pseudo-second-order kinetic model is:

$$\frac{t}{Q_t} = \frac{1}{k_2 Q_e^2} + \frac{1}{Q_e}t \tag{3}$$

where k is the rate constant, t is time, and Q is the amount adsorbed. The subscript, e, refers to the equilibrium state.

2.7 Regeneration of the adsorbents

The regeneration of BCD-HMDI-CS beads was investigated through several adsorptions-desorption cycles. About 0.05 g of adsorbents carrying GMP and whey proteins was added to 10 ml of sodium chloride solution (1 M) in water and agitated for 2 h at a rate of 200 rpm for the desorption. The concentrations of GMP and other whey proteins in liquid phase were determined with the essay described previously. All the data presented correspond to the average of triplicate measurements.

3 Results and discussion

3.1 Immobilization of β-cyclodextrin

1,6-hexamethylene diisocyanate (HMDI) has two isocyanate groups (–N=C=O). The hydroxyl group of chitosan reacted with an isocyanate to form a urethane structure (–NH–CO–O–) and formed the interim CS-HMDI beads. In the second step, the other isocyanate group reacted with hydroxyl group of BCD to form the same urethane structure and thus formed the final CS-HMDI-BCD beads. HMDI cannot react with amino groups of CS due to its lower affinity for amino group as compared to hydroxyl groups under low pH value (Chiu et al. 2004). The above mechanisms and the presumed chemical structure of CS-HMDI-BCD beads are shown in Fig. 1. The amino groups are retained as the major binding sites for GMP.

3.2 Characterization of BCD-HMDI-CS beads

The surface areas of native CS beads and the modified beads, which were obtained from N_2 adsorption at 77 K, were found to be similar at 38.43 and 35.47 m^2/g, respectively. Their structures were exemplified in the SEM images shown in Figs. 2. It is seen that the surface of the modified beads (Fig. 2b) is smoother and less porous than the native ones (Fig. 2a). This might be due to the high loading of BCD at the exterior surface. However, the inner (cross-sectional) structure of the modified beads is highly porous (Fig. 2c). The less porous nature of the exterior surface of the modified beads could present diffusion barriers to larger protein molecules and result in high selectivity for GMP.

The ATR-FTIR spectra of the native CS beads, interim beads, and BCD modified beads were shown in Figs. 3, respectively. Compared with the native CS beads (A), interim beads (B) demonstrated the presence of the methylene C–H asymmetric stretch band at 2931.8 cm^{-1}, the methylene C–H bend band at 1478.5 and 1461.3 cm^{-1}, the aliphatic polyurethane C=O band at 1253.5 cm^{-1}, and the isocyanate asymmetric stretch band at 2288.7 cm^{-1}. They confirmed the introduction of HMDI, the formation of the CS-HMDI, and the existence of the left –NCO groups. At around 3300 cm^{-1}, two peaks combined to one peak at 3315 cm^{-1}, which was assigned to the aliphatic secondary amine stretch or aliphatic primary amine stretch while the O–H bend band at 3296.2 cm^{-1} disappeared. The hydroxyl group bend band has much weakened and shifted to 1303.1 cm^{-1}. These evidence indicated that most of the hydroxyl groups reacted with –NCO groups. However, the amide I band and amide II band also shifted to lower wave numbers of 1618 and 1518.3 cm^{-1}, respectively. This led us to infer that some amine groups were also involved in the reaction with –NCO groups. The spectrum of the BCD modified beads (C) was similar to that of interim beads because both BCD and CS have some similar groups. When BCD was immobilized onto CS, the FTIR spectra of most groups of BCD were covered by the similar groups of CS. However,

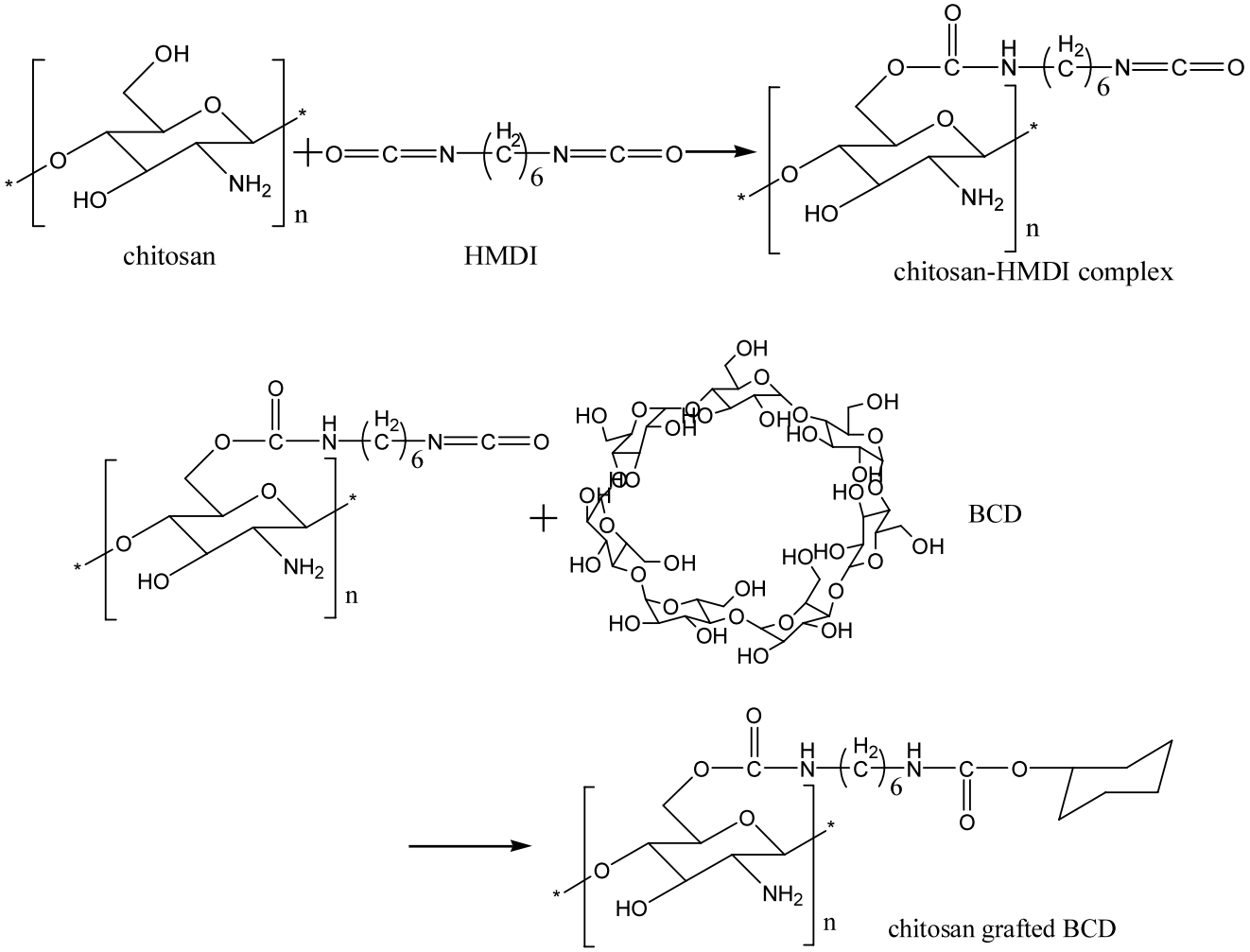

Fig. 1 The illustration of BCD immobilized to chitosan using HMDI as a spacer

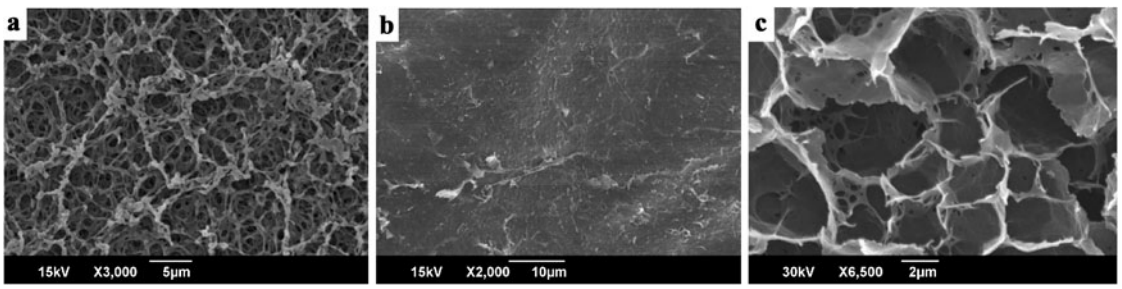

Fig. 2 SEM images of: (**a**) native CS beads; (**b**) surface and (**c**) interior of BCD-HMDI-CS beads

the characteristic peak of the α-pyranyl vibration of BCD at 946.5 cm^{-1} appeared in (C). The absorption band at 1417.4 cm^{-1}, which was related to $-NH_2$, confirmed the presence of $-NH_2$ after the modification. The disappearance of the isocyanate asymmetric stretch band at 2288.7 cm^{-1} indicated the absence of –NCO groups on the modified beads.

3.3 GMP adsorption studies

3.3.1 Effect of pH

The effect of pH on the adsorption of GMP and other whey proteins was illustrated in Figs. 4. It appeared that the native CS beads (Fig. 4a) adsorb both GMP and other whey proteins with relatively high percentage (capacity) from acidic

to alkaline conditions, but with poor selectivity towards GMP. This is in disagreement with the observation of Casal et al. (2005), who reported that CS solution could selectively precipitate GMP from casein hydrolysate, possibly due to the different forms of CS. In case of the modified beads (Fig. 4b), a peak value for the GMP selectivity (90.23%) was observed at pH 3.0.

It has been known that protein adsorption is dependent on the *isoelectric* points, or pI. GMP, which is a heterogeneous peptide, may present different pI values depending on its source and composition. Silva-Hernandez (2002) demonstrated that the pI of bovine GMP was below 3.8. Nakano and Ozimek (2000a) found that the optimum pH value ranged from 2.5–4.0 for GMP purification on an anion-exchange chromatography with DEAE-Sephacel. The pK_a value of sialic acid is 2.6 and a higher content of sialic acid could result in a lower pI value of GMP. When the pH is higher than the pI of GMP, stronger electrostatic interaction will take place between the positively charged CS and the negatively charged GMP. When the pH is lower than the pI of GMP, repulsive force will set in, as both CS and GMP are now positively charged. The inclusion of the selective bucket of BCD will further complicate this adsorption process, as it may involve the hydrophobic interaction, shape recognition, and physical adsorption. As the result, the adsorption selectivity of other whey proteins was affected by pH to a smaller magnitude of 30%∼ 35%, as indicated in Fig. 4b. Therefore, the pH value of 3.0, which gave the highest selectivity and good capacity towards GMP, was selected as the separation condition for GMP.

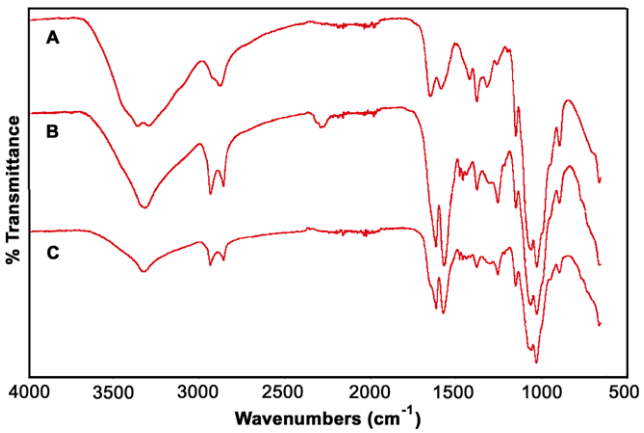

Fig. 3 FTIR spectra of (**A**) native CS beads, (**B**) interim beads, (**C**) BCD-HMDI-CS beads

3.3.2 *Equilibrium adsorption isotherm*

Figure 5 showed the apparent adsorption isotherm of GMP (dots) on the BCD modified beads and the fitting (line) of Langmuir isotherm, $Q_e = Q_m \times \alpha_L C_e/(1 + \alpha_L C_e)$. It was termed as the "apparent" isotherm because the system consisted of many other components which may also affect the GMP adsorption. The batch experiments were carried out at 298 K and pH 3.0. Repeated experiments found that 24 hours were enough for the system to reach adsorption equilibrium. The amount of GMP adsorbed was corresponding to that of sialic acid adsorbed, which was determined using the essay described in Sect. 2.6. The fitting results were listed in Table 1, with the R^2 of 0.9541 and R_L, $R_L = 1/(1 + \alpha_L C_e)$, of 0.094. The maximum monolayer capacity, Q_m , was found to be 12.87 mg/g (corresponding to sialic acid).

3.3.3 *Adsorption kinetics*

The adsorption kinetics of GMP on the BCD modified beads (at 298 K, pH 3.0) were investigated with the two rate equations [(2) and (3)]. Table 2 presents the derived optimal fitting parameters from the two kinetic models. Judging from

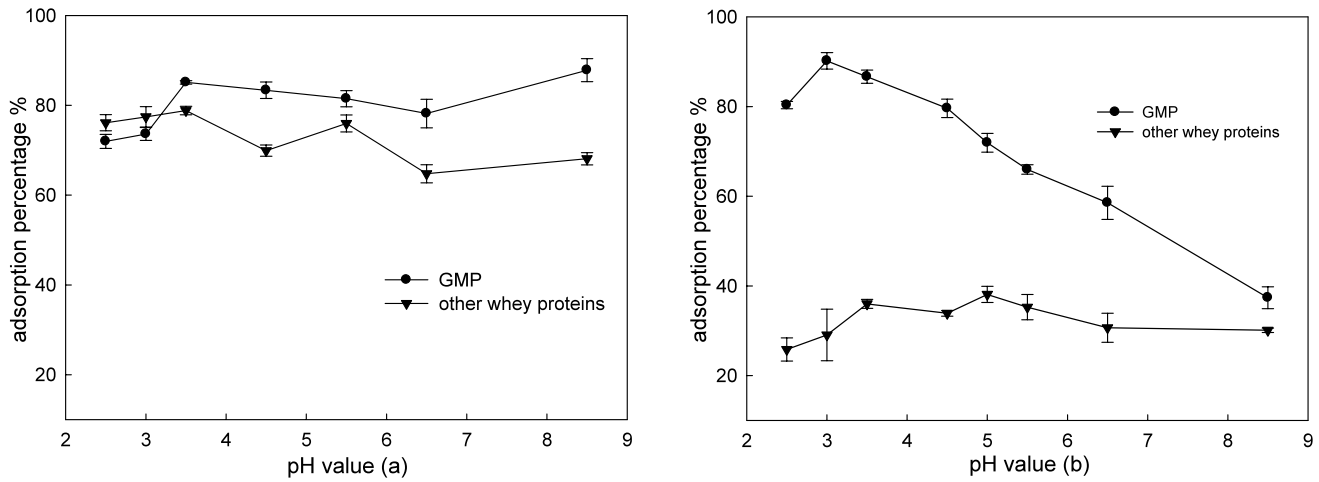

Fig. 4 Effect of pH on adsorption of GMP and other whey proteins. (**a**) native CS beads, (**b**) modified beads

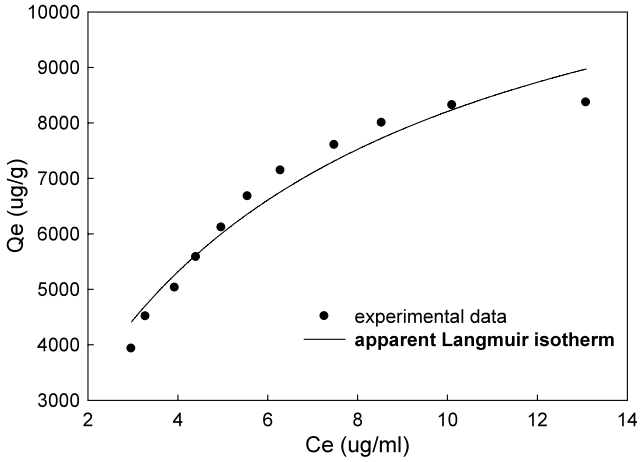

Fig. 5 The apparent GMP adsorption isotherms on the modified beads and the fitting of Langmuir equation

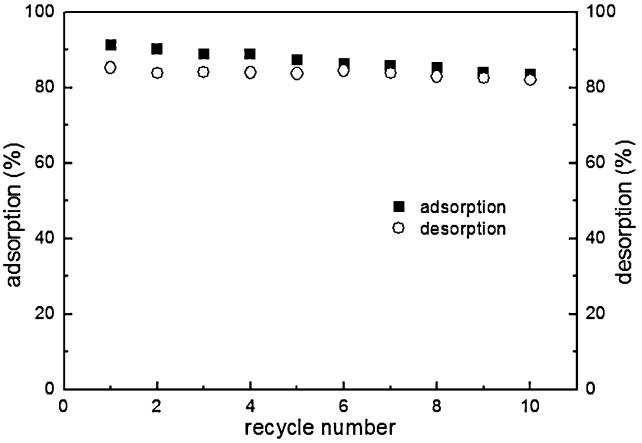

Fig. 6 The regeneration cycles of GMP adsorption/desorption on the modified beads

Table 1 The Langmuir parameters for the apparent GMP isotherm on BCD modified beads at 298 K

Q_{exp} (mg/g)	Langmuir isotherm		
	Q_m (sialic acid) (mg/g)	R_L	R^2
8.37	12.87	0.094	0.9541

Table 2 The kinetic parameters of different rate equations for the adsorption of GMP

Model	$Q_{e,exp}$ (sialic acid, mg/g)	$Q_{e,cal}$ (sialic acid, mg/g)	k	R^2
1st order	4.6	2.0	4.0×10^{-3}	0.9732
2nd order	4.6	4.1	9.8×10^{-6}	0.9997

the R^2, we see that the pseudo 2nd order model gave a slightly better fit than the 1st order rate equation (0.9997 vs 0.9732). However, both models should be deemed as being adequate for the fitting of the observed kinetics, considering that the system is multicomponent in nature and rate of GMP adsorption is dependent on such factors as pH, rate of stirring, and temperature, etc. It is difficult to identify the true nature of the adsorption kinetics of the system. The 2nd order rate equation might be used to model the observed kinetics of a batch system under similar condition.

3.4 Re-generation of the modified beads

For the practical separation process, the adsorbents must be re-generated easily and present a long life (or recycles numbers). The effect of re-generations on the adsorption and desorption of GMP on the BCD modified beads were shown in Fig. 6. We see that the percentage of adsorption to desorption (i.e. the selectivity) of each cycle decreased only slightly with the increased cycle number. After ten cycles, the selectivity decreased from 91.3% to 83.6% for adsorption and from 85.3% to 81.9% for desorption, respectively. This proves that the BCD modified beads present good regeneration performance with the proposed regeneration method (1 M NaCl solution, 2 hours).

4 Conclusions

β-cyclodextrin was successfully immobilized onto native chitosan beads by cross-linking reaction. It was demonstrated that, at pH$\sim$ 3, the β-cyclodextrin modified chitosan beads present higher selectivity and better adsorption capacity than native chitosan beads in the separation of GMP from the sweet whey. The modified chitosan beads also present good re-generation capability for practical applications.

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