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W. E. Baille[a]; W. Q. Huang[b]; M. Nichifor[c]; X. X. Zhu[a]
[a] Département de Chimie, Université de Montréal, Montréal, Canada [b] Institute of Polymer Chemistry, Nakai University, Tianjin, China [c] Petru Poni Institute of Macromolecular Chemistry, Iasi, Romania

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FUNCTIONALIZED β-CYCLODEXTRIN POLYMERS FOR THE SORPTION OF BILE SALTS

W. E. Baille, W. Q. Huang,† M. Nichifor,‡ and X. X. Zhu*

Département de Chimie
Université de Montréal
C. P. 6128, Succursale Centre-ville
Montréal, QC, H3C 3J7, Canada

Key Words: Polymer Sorbents, Cyclodextrin, Bile Salts, Bile Acids, Binding, Inclusion Complex

ABSTRACT

Poly(β-cyclodextrin) (PCD) resins were prepared by a crosslinking reaction of β-cyclodextrin with different amounts of epichlorhydrin. Some hydroxyl groups of these polymers were functionalized with alkyl quaternary ammonium groups. The polymers were tested for their ability to bind several bile salts (including the sodium salts of cholic acid, glycocholic acid, and chenodeoxycholic acid), individually and competitively, from phosphate buffer solutions. In all cases, the aminated PCD resin had a higher binding capacity for bile salts. The binding of chenodeoxycholate by the resins was much more effective than that of cholate and its conjugate, which indicates the importance of the host cavity size relative to that of the guest molecules. The degree of hydrophobicity of bile acids also plays a role in their binding by PCD resins. The variable temperature studies indicate

*Author to whom correspondence should be addressed. E-mail: julian.zhu@umontreal.ca

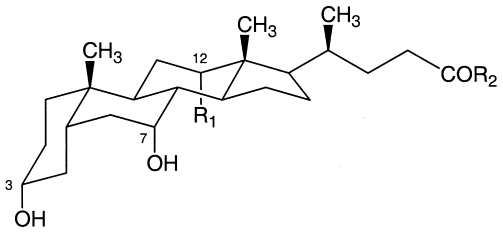
†Present address: Institute of Polymer Chemistry, Nankai University, Tianjin, China

‡Present address: Petru Poni Institute of Macromolecular Chemistry, Aleea Grigore Ghica Voda 41 A, 6600 Iasi, Romania

that the electrostatic interactions become weaker at higher temperatures while the hydrophobic interactions are not as much affected.

INTRODUCTION

Bile salts are amphiphilic molecules that assist in the digestion of fats by the formation of micellar aggregates. The chemical structure of some of the bile acids is shown in Figure 1. They all possess a hydrophobic steroid skeleton to which are attached hydrophilic groups, in particular, hydroxyl groups and the side-chain carboxyl group. They are synthesized biologically in the liver from cholesterol and stored in the gall-bladder, secreted in jejenum when needed for fat digestion and reabsorbed in the terminal ileum. The enterohepatic circulation of bile salts can be interrupted by oral administration of polymeric sorbents [1-2]. This can lead to the lowering of serum cholesterol levels, hence a reduction of the risk of cardiovascular diseases [1-4]. However, the binding of bile salts by the currently used polymeric sorbents, such as cholestyramine (Questran), is based on an ion-exchange mechanism and therefore shows little discrimination in the binding [5]. Other anionic species are also bound by the polymer resins, thereby depleting certain drugs [6-8] and essential nutrients such as vitamins. Moreover, the amount of bile acid anions bound to cholestyramine in the small intestine decreases along the digestive tract [8-9]. Consequently, large doses (8-24 g/day) of cholestyramine are required to be clinically effective



Bile Acid	R_1	R_2
Cholic acid	OH	OH
Glycocholic acid	OH	$NHCH_2COOH$
Chenodeoxycholic acid	H	OH

Figure 1. The chemical structure of the bile acids used in the binding studies.

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[9, 10], having as results poor patient compliance, high costs and therapeutic failures. Hence, there is a continuous need for new polymeric resins with a better selectivity and affinity for bile acids.

Cyclodextrins (CDs) are macrocyclic oligosaccharides that have found important applications in molecular recognition studies. The structure of these molecules resemble a truncated cone with cavities of different sizes depending on the number of glucosidic units, which varies from 6 to 9 [11]. The interior of the cavities is rather hydrophobic while the exterior remains hydrophilic [11]. These characteristics were used to trap molecules of a comparable size and have been exploited for applications as agrochemicals, pharmaceuticals, chemical processing agents, etc. [12]. Since CDs can form inclusion complexes and exhibit size specificity in the binding of bile acids [11-15], they have been used in chromatographic separations of different kinds of bile salts [11, 16-19]. It has been also reported that some CDs or their derivatives can act as serum cholesterol level depressants [13, 20]. For the same reasons, resins based on cyclodextrins were prepared by reaction of β-CDs with epichlorhydrin to form gel-like solids that are insoluble but swellable in water [21-23]. We have also prepared some β-cyclodextrin polymers and showed that they can be effectively used in the binding of bile acids [24].

The aim of the present work is to study the binding of bile salts by the polymerized β-cyclodextrin resins and their aminated derivatives. Isotherms for the binding of various bile acid anions in aqueous buffer solutions at different temperatures were obtained and analyzed by the use of Langmuir equation. Competitive binding experiments were carried out to determine the selectivity of these sorbents. Kinetics of the binding were also investigated.

EXPERIMENTAL

Materials

β-Cyclodextrin (cyclohepataamylose) (β-CD), epichlorhydrin (EP), sodium cholate (NaC), sodium glycocholate (NaGC) and sodium chenodeoxycholate (NaCDC) were purchased from Sigma. The purity of the bile salts was verified by reverse-phased HPLC, and the products were used as received.

Preparation of Polycyclodextrin Resins

The poly(β-cyclodextrin) (PCD) resins were synthesized by crosslinking β-CD with EP by the use of suspension polymerization techniques [21-23].

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When the EP/β-CD ratio is low (e.g., less than 4:1), the resulting polymer tends to remain soluble in water. To obtain insoluble crosslinked polymers, different quantities of EP were added to an aqueous solution of β-CD (38-47 wt%). The mixture was stirred at 50°C and an aqueous solution of NaOH (10-12.5 mL, 40%) was added slowly to the flask, followed by stirring at the same temperature for 25 minutes. Paraffin oil (250 mL) was then added and the suspension was vigorously stirred for 20 hours. The reaction mixture was cooled to room temperature and filtered, and paraffin oil was removed by extensive washing with ethanol. The dried polymer as spherical particles (beads) was sieved and the fractions with the mesh size lower than 400 (m were retained for the binding studies. The introduction of pendant amino groups was performed by tosylation of the PCD and subsequent treatment with trimethylamine [25, 26]. All the polymers were characterized by solid state NMR spectroscopy as described previously [24]. The general structure of prepared PCDs is depicted in Figure 2 and their characteristics are listed in Table 1. The degree of swelling is expressed as the

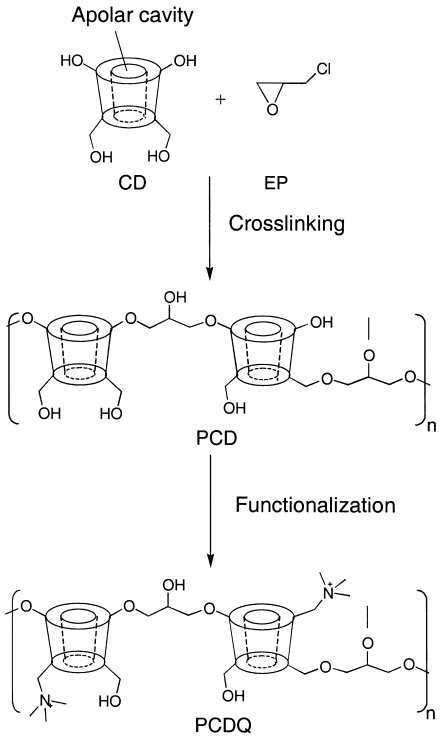

Figure 2. Synthesis of the crosslinked and aminated β-cyclodextrin resins.

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TABLE 1. Characteristics of the Poly(β-cyclodextrin) Resins

Resin	Crosslinking* [EP]/[β-CD]	Amino Group Content (mmol/g)	Degree of Swelling (g H2O/g)
PCD1	20	-	2.34
PCD2	14	-	4.32
PCDQ	15	0.1	5.32

*Expressed as the molar ratio of EP to β-CD in the reaction mixture.

amount of water uptake at equilibrium (in g/g dry resin). The degree of functionalization (expressed as mmol of functional groups/g dry resin) was determined by the nitrogen content obtained from elemental analyses. The number obtained is rather low, representing about one functional group for every fifth β-CD unit. An error of ±10% is estimated because of the hygroscopic nature of the PCD resins.

Sorption Studies

The procedure used for sorption studies was previously described [27]. The bile salt solutions were prepared in an aqueous phosphate buffer (5.0 mM KH_2PO_4-NaOH, pH 7.4). The concentration of the stock solutions was about 3-4 mM. For the competitive binding studies, equimolar amounts of selected bile salts were dissolved in the buffer to prepare the stock solution (total combined concentration of bile salts about 3-4 mM). The sorption solutions were prepared by diluting the stock solution with the buffer to the desired concentrations (0.4-4 mM).

Usually 10 mg of the polymer resin were used for the binding experiments. Depending on the binding capacity, 5-20 mL of the salt solution was used to ensure the accuracy of the HPLC analysis after binding. With stirring at different temperatures (10, 25, 37 and 50°C), binding equilibrium was reached within 3 hours in all cases.

A reverse-phased HPLC system (Waters 600 pump, equipped with a waters 410 differential refractometer, Waters 700 automatic injector and a NovaPak C-18 column) was used for the analysis of the bile salt concentrations, before and after sorption. The details of the HPLC analytical method have been described elsewhere [28]. The uncertainty of the sorption data was estimated to be ≤5%, based on replicated experiments. A least-square non-linear curve-fitting

procedure was used in the treatment of the experimental data by the Langmuir equation.

RESULTS AND DISCUSSION

Binding Kinetics

The time required to reach the equilibrium for the sorption of a bile salt on the PCD resins was determined by kinetic studies carried out at different temperatures. PCD2 and PCDQ resins and sodium cholate were used in these studies. The sorption equilibrium for PCD2 was reached in about 20 minutes, while that for PCDQ was reached in about 40 minutes irrespective of the temperature of the experiment. However, 3 hours were allowed for all the subsequent binding experiments.

Effects of Crosslinking and Functionalization

In order to determine the effect of the degree of crosslinking and the effect of functionalization of the PCD resins, they were studied for the binding of sodium glycocholate. The characteristics of the PCD resins are listed in Table 1. As shown in the table, the degree of swelling of the resins in water depends on the degree of crosslinking of the samples. The functionalization of the PCD resin with quaternary ammonium groups increases also the swellability (Table 1).

The isotherms in Figure 3A show that the amount of NaGC bound per gram of resins increases continuously with increasing equilibrium concentration of the bile salt. In the range of concentration studied here, the most heavily crosslinked PCD1 has the lowest binding capacity. A higher degree of crosslinking obviously hinders the interaction between the hosts (β-CD cavities) and the guests (glycocholate anions), thereby lowering the binding capacity of the resin. The comparison of PCD1 and PCD2 resins with PCDQ (aminated resin) indicates that the quaternary ammonium functional groups have a greater effect on the binding of the bile acid anions, obviously due to supplementary electrostatic interactions between cationic groups and glycocholate anions.

The Langmuir equation could be used in the non-linear fitting of the experimental results and can be written as:

$$Q = \frac{Q_m K C_{eq}}{1 + K C_{eq}} \tag{1}$$

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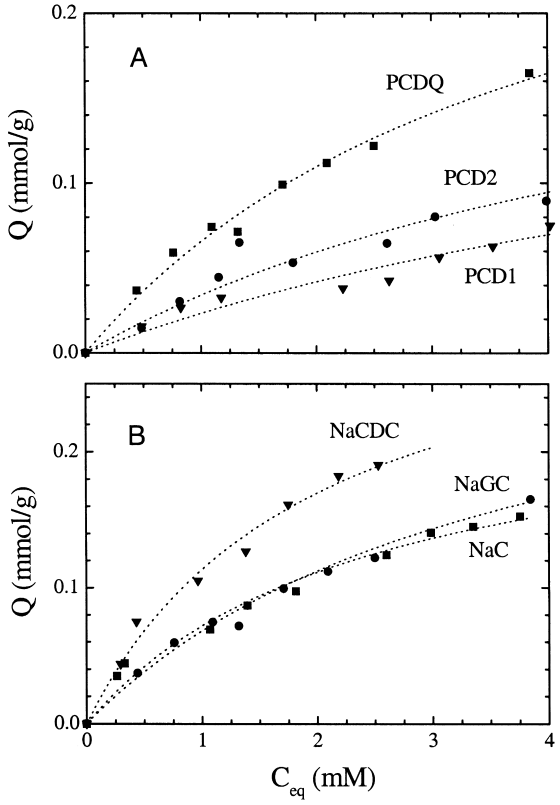

Figure 3. Binding isotherms for bile salts in phosphate buffer solutions at 25°C. The bound amount of bile salt, Q, expressed as mmol per gram of resin, is plotted as a function of the equilibrium concentration of the bile salt (C_{eq}). (A) Binding isotherms of NaGC by three different PCD resins; (B) Binding isotherms for individual bile salts, NaC, NaGC and NaCDC, by the PCDQ resin.

where Q is the quantity of bile salt bound per gram of resin (mmol/g), Q_m is the maximum capacity of the resin for the bile salt, C_{eq} is the concentration of the bile salt at equilibrium (mM) and K is regarded as the binding constant (mM^{-1}). It is clear that Q_m represents the potential maximum binding capacity while K is an indication of the binding affinity. The isotherms in Figure 3 were fitted with equation 1 and good agreements with the experimental data were obtained. The values of Q_m and K obtained by the fitting procedure are listed in Table 2. It is interesting to note that the Q_m and K values for PCDQ resin are higher than those for PCD1 and PCD2 resins. These results show that the functionalized PCDQ resin has higher binding capacity and affinity for bile salts because of the

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TABLE 2. Potential Binding Capacity (Q_m), Binding Constants (K) and Apparent Free Energy Changes ($\Delta G°$) of the Binding of Bile Salts with Crosslinked and Aminated β-Cyclodextrin Resins in Phosphate Buffer Solutions at 25°C

System		Q_m (mmol/g)	K x 10^{-3} (M^{-1})	(G° (kJ/mol)
Polymer	Bile Salts			
Binding with Different Resins:				
PCD1	NaGC	0.21	0.13	-12.1
PCD2	NaGC	0.23	0.17	-12.7
PCDQ	NaGC	0.33	0.25	-13.7
Binding of Individual Bile Salts:				
PCDQ	NaGC	0.33	0.25	-13.7
	NaC	0.25	0.41	-14.9
	NaCDC	0.34	0.51	-15.5
Competitive Binding of Bile Salts:				
PCDQ	NaC	0.30	0.46	-15.2
	NaGC	0.31	0.27	-13.9

presence of the quaternary ammonium functional groups. Therefore, PCDQ resin was selected for all of the subsequent binding studies.

Binding of Individual Bile Salts

The sorption studies of PCDQ resin were carried out at 25°C for three bile salts: NaC, NaGC and NaCDC. The hydrophobicity of the three bile salts decreases in the order: chenodeoxycholate > cholate > glycocholate.

The binding isotherms are shown in Figure 3B and the results of fits to Equation 1 are listed in Table 2. The values of the binding constant and the apparent maximum binding capacity show that the PCDQ resin has a better affinity and a higher capacity for chenodeoxycholate than for the other two bile salts. This is consistent with the results of Tan *et al.*, which showed that the hydrophobic interaction is very important in the complexation between β-cyclodextrin and bile acid anions [29]. Furthermore, free energy changes show that the inclusion complexation of the PCDQ resin with chenodeoxycholate and is thermodynamically more favorable than those of the two other bile salts. As

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determined by NMR titration and molecular modeling studies [29], the presence of hydroxyl groups at position 12 of the steroid skeleton also has a marked effect on the formation of inclusion complex between bile salts and cyclodextrin. For example, the complex formation constants of both β-CD and β-CD hosts for glycochenodexoycholate are systematically higher than those for glyocholate [29]. The same explanation may also hold for the interaction between crosslinked PCDs and bile salts. However, the binding isotherms for glycocholate and cholate are rather similar under the same conditions (Figure 3B), indicating that the hydrophobicity is not the only determining factor and that the steric hindrance of the OH group at position 12 may cause difficulties in the complex formation process involving NaC and NaGC alike.

Competitive Binding of Bile Salts

In order to determine the binding selectivity of the PCDQ resin for different bile salts, studies were made with equimolar mixture of cholate and glycocholate in phosphate buffer at 25°C and 37°C. The isotherms obtained are shown in Figure 4. At both experimental temperatures the binding of cholate is more favored than that of glycocholate. This difference becomes more obvious at higher equilibrium concentrations of bile salts and at higher temperature. This shows the importance of hydrophobicity in the binding. NMR studies on the complexation of β-cyclodextrin and bile salts [29, 30] suggested that the bile salt enters the β-cyclodextrin cavity via the smaller side chain with carboxylic acid groups. The glycine and taurine conjugates of a bile acid (such as glycocholate) have a longer but more hydrophilic side chain that can induce a significant decrease in the complex formation constant in comparison with the unconjugated bile acid (such as cholate) [30]. The free energy changes (Table 2) show that the inclusion complex formed by PCDQ resin and glycocholate is less favored than that formed by PCDQ resin and cholate. This result is also supported by the NMR study [30], which showed that the decomplexation rate of glycine conjugate with β-cyclodextrin is lower than that of the unconjugated form. These findings could explain the different binding behaviors of the individual and mixed bile salt solutions. In the presence of only one bile salt species, the balance between the complexation and decomplexation processes is the same for cholate and glycocholate, and their binding isotherms are similar. In the presence of both bile salts, the higher complexation rate of cholate results in higher binding efficiency of cholate by the PCDQ resin. This indicates that the hydrophobic CD cavities have a certain selectivity towards more hydrophobic bile acid anions interactions.

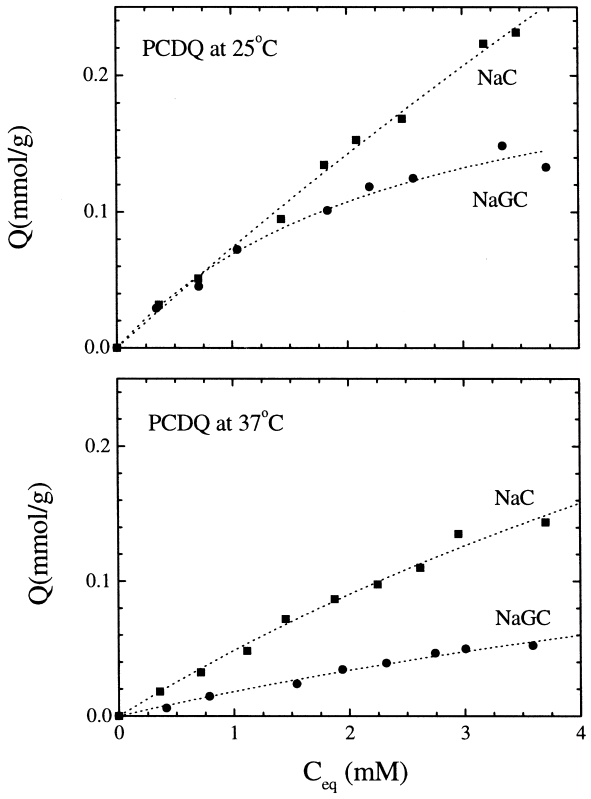

Figure 4. Isotherms for the competitive binding of bile salts by aminated β-cyclodextrin resin (PCDQ) at 25°C (top) and at 37°C (bottom).

Temperature Effect on the Binding of the Bile Salts

The influence of the temperature on the binding process was investigated by experiments carried out with PCD2 and PCDQ as sorbents and NaC as sorbate at four temperatures between 10 and 50°C. As shown in Figure 5, the binding capacity of both resins decreases with increasing temperature. The decrease is more obvious for PCDQ resin, especially at higher equilibrium concentration of the bile salt. The general trend is shown in Figure 6, where the values of Q_m and K are plotted as a function of reciprocal temperature (1/T). The formation constant K is always higher for PCDQ resin (Figure 6B), but the maximum capacity (Q_m) of PCD2 resin for the binding of cholate is shown to be constantly higher (Figure 6A), and the apparent activation energy is lower (Figure 6B).

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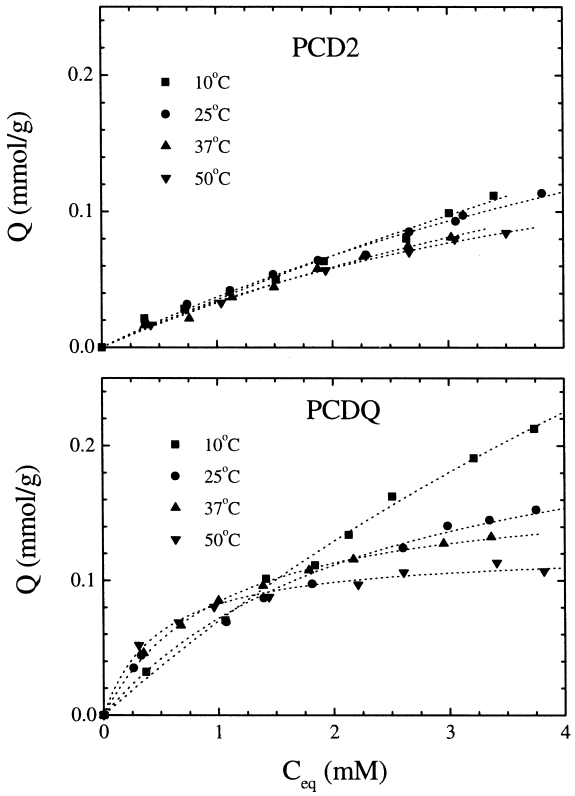

Figure 5. Isotherms for the binding of NaC by PCD2 (top) and PCDQ (bottom) resins at four different temperatures.

These results are different from those observed for the binding of glycocholate on both resins at 25°C (Table 2). This difference could be assigned to the prevalence of different kind of binding mechanisms with the two resins. The binding on PCD2 resin seems to be controlled mainly by hydrophobic interactions, whereas the binding on PCDQ resin is controlled by combined electrostatic and hydrophobic interactions. Apparently, the temperature influences more the electrostatic interactions than the hydrophobic ones. But even at the higher temperatures, the amount of cholate bound by the functionalized PCDQ resin is still higher than that by PCD2. The electrostatic interaction is still the most important factor in the binding process, as it provides the adhesive force. This force, however, becomes less important as temperature rises. But the hydrophobic interactions are not as much affected by the temperature changes.

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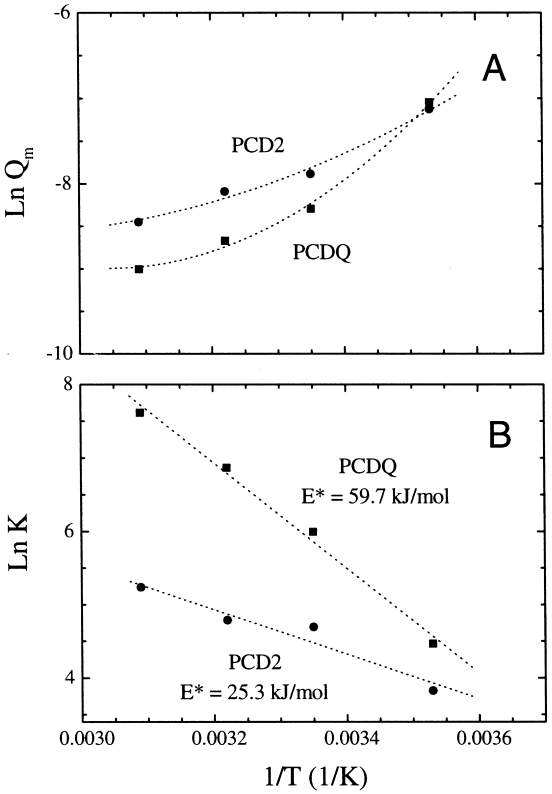

Figure 6. (A) Variation of maximum binding capacity (Q_m) with reciprocal temperature for the binding of NaC by PCD2 and PCDQ resins. (B) Arrhenius plots of the binding constant (K) for NaC by PCD2 and PCDQ resins. The apparent activation energy for the binding can be estimated from the slopes of the lines.

CONCLUSION

Crosslinked and aminated β-cyclodextrin resins were synthesized and tested for their ability to bind several bile salts, individually and from their mixture. We have found that a lower degree of crosslinking facilitates the formation of inclusion complex with bile salts. The introduction of pendant quaternary ammonium groups increases the binding affinity and capacity. In the individual and competitive binding studies, the PCDQ resin has shown a higher affinity for the more hydrophobic bile salts. The maximum binding capacity of PCD and PCDQ resins decreases with increasing temperature, but the temperature has a greater effect on the binding based on electrostatic interactions. Two conclusions

can be drawn from the results obtained under these experimental conditions: (1) The functionalized resin always has a higher binding capacity for the bile salts, which indicates the importance of electrostatic interactions in the binding process; (2) The polymer resins before and after functionalization always show a higher binding affinity for the more hydrophobic bile acid anions. The electrostatic interactions seem to provide the adhesion and driving force for the binding, while hydrophobic interactions play a secondary but important role in the binding of bile salts. In addition, the steric hindrance of a hydroxyl group of the steroid skeleton also affects the formation of inclusion complexes. The results of this study should help in the design and preparation of more efficient sorbent materials for biomedical and pharmaceutical applications.

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