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Immobilization of β-cyclodextrin in chitosan beads for separation of cholesterol from egg yolk

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Abstract

Immobilization of β-cyclodextrin to chitosan beads (Ch-BCD) by cross-linking with 1,6-hexamethylene diisocyanate (HMDI) reagent has been successfully developed and demonstrated that it possessed excellent capacity for cholesterol adsorption from egg yolk. Under optimum conditions, a maximum β-cyclodextrin (BCD) loading of 0.43 g/g-chitosan was obtained. The experimental data on cholesterol adsorption fitted well in the Langmuir isotherm equation with a maximum adsorption capacity (N_m) of cholesterol 0.33 g cholesterol/g-adsorbent. 92% of the cholesterol was removed from a 30-fold diluted yolk solution at 25 °C using 1% (w/v) Ch-BCD in 2 h. On the other hand, 96% of adsorbed cholesterol could be dissociated from the Ch-BCD using 95% ethanol at 50 °C. In addition, the Ch-BCD retained 84% adsorption capacity after 12 reuses.

Keywords: β-Cyclodextrin; Chitosan; Cholesterol; Adsorption; Egg yolk

1. Introduction

Elevated cholesterol levels in blood are a major risk factor for developing coronary heart disease (Chait et al., 1993; Gurr, 1992). A high dietary cholesterol intake can elevate its level in the blood considerably. Several methods such as extraction with solvents (Borges, Martucci, & Muller, 1996), supercritical fluid extraction (Bradley, 1989; Mohamed, Saldana, Socantaype, & Kieckbusch, 2000), steam distillation (Arul, Boudreau, Makhlouf, Tardif, & Grenier, 1988) and treatment with cyclodextrin or saponin (Chang, Oh, & Kwak, 2001; Yen & Tsui, 1995) have been adopted to reduce cholesterol. Among these methods, removal of complex cholesterol by β-cyclodextrin has been widely implemented in the dairy industry at lower operation costs compared to other methods. β-Cyclodextrin (BCD) is a cyclic oligosaccharide with glucose units linked by α-(1,4) bonds. BCD has apolar cavity in the center of the molecule and has the capability of forming an inclusion complex with various compounds such as cholesterol (Yen & Tsui, 1995). The various compounds inside the cavity of a cyclodextrin molecule produces a change in the electronic environment of the atoms of the cyclodextrin. Based on ^{13}C NMR stoichiometric considerations and studies of rotational nuclear Overhauser effect spectroscopy (ROESY), structural models have been proposed in literatures (Alvarez-Parrilla et al., 2002; Singh, Cabrer, Alvarez-Parrilla, Meijide, & Tato, 1999). It also has the advantages of non-toxicity (Rao et al., 2000), edibility, non-hygroscopic and chemical stability (Nagatomo, 1985). Therefore, it is widely used for removing cholesterol from lard (Yen & Tsui, 1995), cream (Ahn & Kwak, 1999), egg yolk or whole egg (Ravichandran & Divakar, 1998; Smith, Awad, Bennink, & Gill, 1995). However, commercial BCD is expensive and its wastage during the process also leads to environmental problems. Therefore, BCD recycling needs to be emphasized. Immobilization of BCD on a suitable support facilitates its separation from the reaction medium and reuse.

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Chitosan [poly(1,4-β-D-glycopyrano-samine)] which is a highly antimicrobial (Muzzarelli et al., 1990) and biocompatible natural material (Lehr, Bouwstra, Schacht, & Junginger, 1992) can be considered as a suitable support for BCD immobilization. Because of its high biocompatibility and easy biodegradability, chitosan has been used as a raw material in medical applications such as an immunosuppressant (Chang, Puryear, Funkhouser, Newton, & Cairney, 1996), surgical sures (Chandy & Sharma, 1990), and as a matrix for drug delivery systems (Chiou, Wu, Huang, & Chung, 2001). Chitosan has also been used as a matrix for immobilization of several enzymes (Betigeri & Neau, 2002; Noda, Furuta, & Suda, 2001; Song, Babiker, Usui, Saito, & Kato, 2002) since it possesses several reactive functional groups. Immobilization to chitosan also has the advantages of high stability, recoverability and reutilization. Chitosan powder has been used to make chitosan–BCD complex as a novel adsorbent matrix for cholesterol (Sreenivasan, 1998). However, this method of making chitosan–BCD complex would lead to severe chitosan swelling due to strong reaction and lead to recovery difficulties after cholesterol adsorption.

In the present study, we immobilized β-cyclodextrin onto chitosan beads by cross-linking with 1,6-hexamethylene diisocyanate (HMDI) reagent and used for cholesterol adsorption from egg yolk. The behavior of cholesterol adsorption was investigated using the Langmuir isotherm. Desorption studies were also carried out for the recovery of Ch-BCD for reuse.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation of 92%; molecular weight 310 kDa) used in this study was provided by Kiotek (Hsinchu, Taiwan). HMDI, BCD, stannous 2-ethylhexanoate, anthrone, cholesterol (95% purity), *N*,*N*-dimethylformamidum (DMF) and toluene were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). All other chemicals used were of LC grade.

2.2. Preparation of chitosan beads

3% (w/v) chitosan powder was completely dissolved in 1% (v/v) acetic acid. This solution was extruded through a syringe needle (27G) into a coagulant bath consisting of 1 N sodium hydroxide solution containing 26% (v/v) ethanol under stirring to form spherical gels. The solution was allowed to stand for 3 h and the spherical gels were removed by filtration and rinsed with deionized (DI) water until neutrality. Finally, the beads were dried at room temperature for more than 24 h for further application.

2.3. Immobilization of β-cyclodextrin

The method of covalent binding was used for immobilization of BCD to chitosan beads. In this process, HMDI acts as a spacer between the BCD and chitosan. One gram of chitosan beads was placed in 25 ml of a mixture of toluene and HMDI containing 5% (v/v) of HMDI. The mixture was magnetically stirred at room temperature after adding few drops of stannous 2-ethylhexanoate which catalyzed the immobilization reaction. After stirring for 40 min, the supernatant was discarded and the chitosan beads were dried using nitrogen gas. The chitosan beads with bound HMDI were then placed in 25 ml of DMF solution containing 2% (w/v) BCD. The mixture was magnetically stirred for 1 h at room temperature after adding few drops of stannous 2-ethylhexanoate. The supernatant was decanted and the Ch-BCD beads were washed several times with DI water followed by ethanol and finally with DI water before freeze drying.

2.4. Quantitative analysis of β-cyclodextrin

β-Cyclodextrin (BCD) concentration was estimated spectrophotometrically by a method similar to that used for the estimation of reducing sugars by DNS (Nakamura & Horikoshi, 1976). One ml of sample solution containing BCD was added to 2 ml of 0.1 N hydrochloric acid and 1 ml of 0.1% (w/v) anthrone reagent. The mixture was heated at 100 °C for 30 min to hydrolyze the BCD, The mixture was cooled and OD_{530} was measured in a UV/VIS spectrophotometer (Beckman DU-530, USA). The BCD binding on chitosan was calculated as the difference between the BCD concentration in the solution before and after immobilization.

2.5. Cholesterol adsorption studies

Isothermal adsorption studies were conducted at 25 °C using 1 g of Ch-BCD and varying concentrations (0–10 g/L) of cholesterol in 100 ml of ethanol (95%). The extent of adsorption was calculated on the basis of the difference between the cholesterol concentrations in the solution before and after adsorption. A method similar to that reported by Grizard, Sion, Bauchart, and Boucher (2000) was used for the estimation of cholesterol. The cholesterol concentration was determined by HPLC (Shimadzu LC9A, Japan) using a Hichrom C18 reverse phase column (Hichrom, Reading, UK) at 35 °C. A mobile phase consisting of solvents A (methanol) and B (hexane/isopropanol, 4:5, v/v) was used. Concentration of cholesterol was obtained from a standard curve of cholesterol in chloroform solution.

2.6. Cholesterol removal from yolk

In order to reduce the resistance to mass transfer during adsorption, the yolk was initially diluted to 10-, 20- and 30-fold with DI water. One percent (w/v) Ch-BCD was added to the diluted solutions in a beaker to carry out adsorption. 0.5 ml of yolk solution was pipetted out from the beaker at predetermined intervals of time and the cholesterol concentration was determined after adding 9.5 ml of chloroform. The percentage of removal was calculated on the basis of the difference between cholesterol concentrations in the yolk solution before and after adsorption.

2.7. Cholesterol dissociation from Ch-BCD

The behavior of cholesterol dissociation from Ch-BCD–Cholesterol complex under varying agitation rates (0–125 rpm) and temperature (25–70 °C) was also investigated. One gram of the complex was added to 50 ml ethanol (95%) and agitated for 30 min to carry out desorption. After 30 min, 0.5 ml ethanol was pipetted out from the beaker and the cholesterol concentration was determined by HPLC after diluting with 9.5 ml chloroform. All the data presented corresponds to the average of triplicate measurements.

3. Results and discussion

3.1. Immobilization of β-cyclodextrin

1,6-Hexamethylene diisocyanate (HMDI) is generally utilized as a strong cross-linker of amino or hydroxyl groups since it possesses two isocyanate groups (—N=C=O). The hypothetical illustration of BCD immobilized to chitosan using HMDI as a cross-linker is shown in Fig. 1. Under the suitable conditions (pH < 6), the hydroxyl groups of chitosan reacts with an isocyanate to form a *urethane* product (–NH–CO–O–) due to the transfer of proton from hydroxyl to nitrogen atom of isocyanate. In addition, isocyanate also reacts with hydroxyl groups of BCD to form a product the same with *urethane* (Wade, 1999). It is assumed that the cross-linking of the hydroxyl groups of chitosan with HMDI resulted in chitosan–HMDI complex, which then binds with the hydroxyl groups of BCD to form Ch-BCD. HMDI can not bind to amino groups of chitosan due to the lower affinity for amino group as compared to hydroxyl groups under low pH value (Wade, 1999). Above facts supported the presumed structure of BCD cross-linked to chitosan by HMDI. The efficiency of immobilization depends on the concentrations of HMDI and BCD, time of cross-linking and immobilization, and reaction temperature. The effect of these variables on BCD binding was investigated and the reaction conditions were optimized. Under optimum conditions, a maximum BCD loading of 0.43 g/g-chitosan was obtained using 5% (v/v) HMDI with a cross-linking time of 40 min and using 2% (w/v) BCD for immobilizing for 1 h at 25 °C.

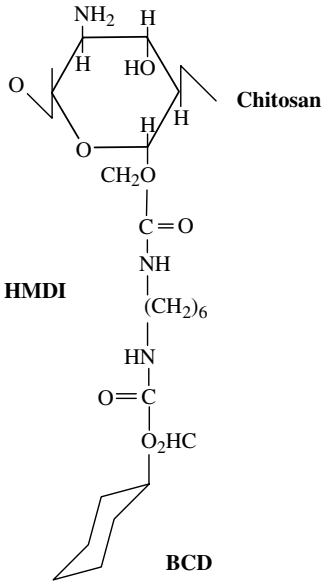

Fig. 1. Hypothetical illustration of BCD immobilized to chitosan using HMDI as a cross-linker.

3.2. Isothermal adsorption studies

The extent of adsorption of cholesterol as a function of the initial concentrations of cholesterol at 25 °C is shown in Fig. 2. The amount of cholesterol adsorbed on Ch-BCD increased with increasing initial concentrations of cholesterol up to 4 g/L. However, further increase in the initial cholesterol concentration did not show any increment in the adsorption cholesterol. From Fig. 2, the maximum cholesterol adsorption capacity of Ch-BCD was found to be about 0.3 g/g Ch-BCD.

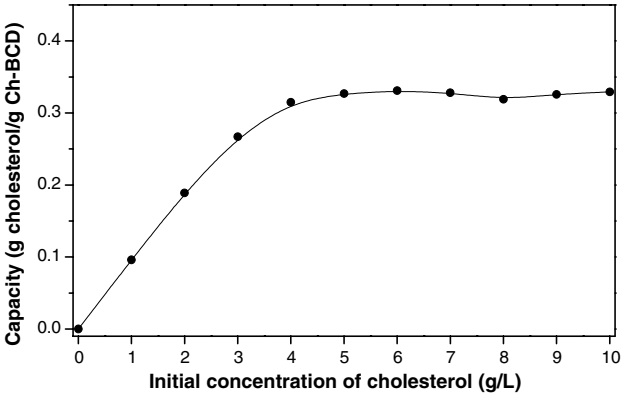

Fig. 2. Amount of cholesterol adsorbed on Ch-BCD as a function of the initial concentration at 25 °C.

In order to gain more insight into the phenomenon of cholesterol adsorption by Ch-BCD, the adsorption data was fitted into the Langmuir and Freundlich equations. To test the applicability of the Langmuir single site isotherm, the data in Fig. 2 were replotted in Fig. 3 in the form suggested by the following equation:

$$C/N = C/N_m + 1/(N_m \times K_L),$$

where N is the amount of cholesterol adsorbed by Ch-BCD at the equilibrium concentration (g/g), C the equilibrium or final concentration of cholesterol (g/L), N_m the maximum adsorption at monolayer coverage (g/g), and K_L is the adsorption equilibrium constant (L/g). The data could be fitted into a straight line (correlation coefficient = 0.9998). From the slope and intercept of the line, the values of N_m and K_L were determined as 0.33 g/g and 19.79 L/g, respectively.

The experimental data was also fitted in the Freundlich equation, which can be expressed as follows:

$$\mathrm{Log}N = 1/K_F \times \mathrm{Log}C + \mathrm{Log}P,$$

where N and C are same as described above, P denotes the adsorption capacity (g/g), and K_F represents the adsorption intensity. The isotherm for cholesterol adsorption based on the Freundlich equation is shown in Fig. 4. The plot showed a slight but definite curvature which indicated that the experimental data could not fit into the Freundlich equation well. The values of P and N as obtained from the slope and intercept were 0.26 g/g and 5.04 L/g, respectively, with a correlation coefficient of 0.8832.

A comparison of Figs. 3 and 4 indicated that the adsorption of cholesterol on Ch-BCD could be very well represented by the Langmuir model. The Langmuir adsorption model is generally regarded as being more sensitive than the Freundlich model in describing sorption phenomena. This is because of the assumptions concerning the Langmuir model, especially, that the substrate (such as cholesterol) is homogeneous with respect to the bonding energy of sites, monolayer adsorption, and that no desorption takes place (Campbell & Davies, 1995). Therefore, Langmuir model has been adopted by several researchers to describe the phenomenon of cholesterol adsorption (Kim, Kim, & Byun, 2001; Palit & Moulik, 2001).

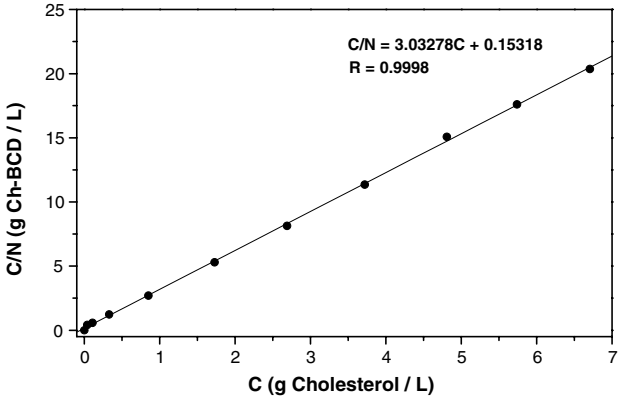

Fig. 3. Adsorption isotherm (25 °C) of cholesterol on Ch-BCD, based on the Langmuir equation.

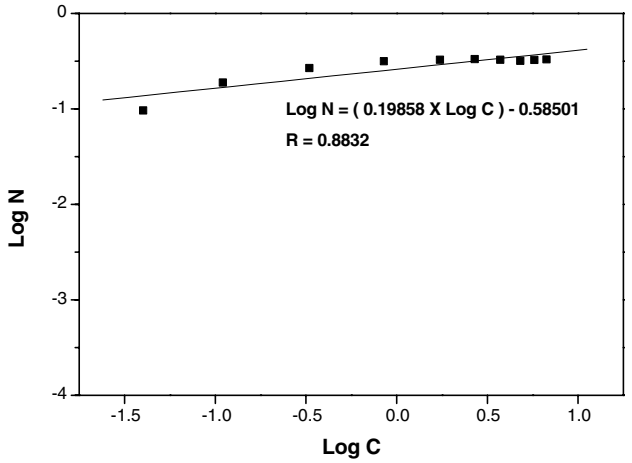

Fig. 4. Adsorption isotherm (25 °C) of cholesterol on Ch-BCD, based on the Freundlich equation.

Theoretically, 1 mol of BCD will complex with 1 mol of a compound (Hedges, 1998). Therefore, the maximum capacity of cholesterol adsorption should be 0.33 g/g (molecular weights of cholesterol and BCD are 386.7 and 1135 Da, respectively). However, larger molecular weight compounds may complex with more than one cyclodextrin molecule (Hedges, 1998). In our study, 0.43 g of BCD was immobilized on 1 g of chitosan beads and the maximum capacity (N_m) of cholesterol adsorption was 0.33 g/g. This result showed that 1 mol of BCD has complexed with more than 2 mol of cholesterol. A similar case is also suggested by Sreenivasan (1998). It might be due to the chelation of more cholesterol molecules in the gap between chitosan and BCD thereby causing an increased cholesterol adsorption. A comparison of the efficiencies of cholesterol adsorption using various adsorbents reported in the literature is listed in Table 1. The cholesterol adsorption capacity of 0.33 g/g achieved with Ch-BCD in this study is higher than that obtained by using other adsorbents. In addition, this method has the lowest energy consumption since the reaction is carried out at room temperature.

3.3. Cholesterol removal from yolk

The residual cholesterol in yolk as a function of adsorption time for different dilution ratios between yolk and water at 25 °C is shown in Fig. 5. The efficiency of cholesterol removal in terms of % cholesterol removed increased with the ratio between yolk and water. This could be attributed to the decreased resistance to mass transfer in dilute solutions of yolk. 92% of the

Table 1
Comparison of the efficiency of cholesterol removal by different adsorbents

Adsorbent	Reaction time	Temperature (°C)	Cholesterol removal (mg/g-matrix)	References
Alumina/methane	6 h	40	3	Mohamed et al. (2000)
Alumina/CO_2	4 h	40	2.4	Mohamed, Neves, and Kieckbusch (1998)
Terpolymers	24 h	37	17	Sellergren, Wieschemeyer, Boos, and Seidel (1998)
BCD	10 min	50	92	Awad and Smith (1996)
BCD	40 min	50	43.8[a]	Mine and Bergougnoux (1998)
Ch-BCD	50 min	25	330	This study

[a] Cholesterol is from low density lipoprotein.

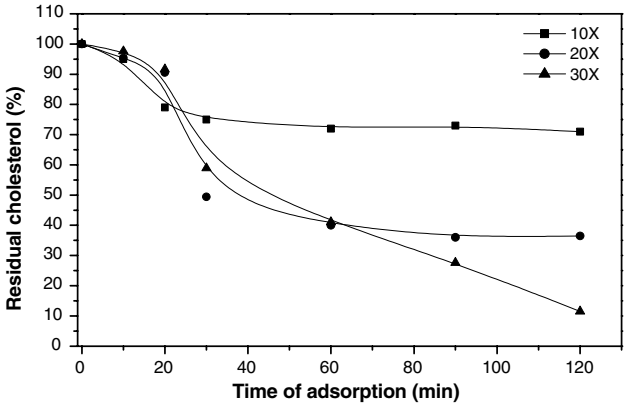

Fig. 5. Cholesterol adsorption from egg yolk solutions at (■) 10-fold; (●) 20-fold; (▲) 30-fold dilutions at 25 °C.

cholesterol in the yolk could be removed with 1% (w/v) of Ch-BCD in 2 h. Even though, the efficiency of adsorption might be enhanced by further dilutions, the increased water content would make the subsequent drying process to produce cholesterol free yolk powder uneconomical.

A comparison of the efficiency of cholesterol removal from different dietary sources using free BCD reported in the literature is given in Table 2. The cholesterol removal efficiency of 92% achieved in this study using Ch-BCD is comparable to that obtained by other researchers by using the free BCD. The higher cholesterol removal efficiency coupled with the lower percentage of adsorbent used (1%) makes Ch-BCD an ideal candidate for cholesterol adsorption. Moreover, the Ch-BCD can be regenerated and recycled.

3.4. Cholesterol dissociation from Ch-BCD

In order to regenerate the spent Ch-BCD beads for reuse, it is necessary to desorb the cholesterol from the beads. The extent of desorption depends on temperature, solvent used and the speed of agitation (Kwak, Sun, Ahn, & Kwon, 2001). The effect of agitation speed and temperature on efficiency of desorption are shown in Table 3. The results indicated that the efficiency of cholesterol dissociation increased with increasing the agitation speed and temperature up to 75 rpm and 50 °C. About 96% desorption of cholesterol could be desorbed from 1 g of Ch-BCD in 30 min using 50 ml of 95% ethanol. Ethanol was selected as the preferred solvent as it is non-toxic and less expensive. A highest cholesterol dissociation of 82.5% with an agitation rate of 100 rpm at 50 °C for 1 h and using acetic acid/butanol mixture as solvent has been reported (Kwak et al., 2001). In comparison to this, a higher regeneration of the adsorbent could be achieved with Ch-BCD beads using ethanol as solvent and at an agitation rate of 75 rpm at 50 °C.

Table 2
Comparison of the efficiency of cholesterol removal from dietary sources using BCD

Adsorbent	Target	Adsorbent used (%)	Cholesterol removal (%)	References
BCD	Yolk	14	95	Kohlrausch, Cully, and Schmid (1996)
BCD	Cream	15	98	Ahn and Kwak (1999), Kwak, Ahn, and Lee (2000)
BCD	Lard	5	90	Yen and Tsui (1995)
BCD	Milk	1	94	Kwak et al. (2000)
Ch-BCD	Yolk	1	92	This study

Table 3
Effect of agitation speed and temperature on the efficiency of cholesterol dissociation

Temperature (°C)	Speed of agitation (rpm)					
	0	25	50	75	100	125
25	21.3	45.7	49.7	86.1	87.2	87.4
30	24.5	53.2	59.8	89.7	89.8	90.1
40	29.1	61.4	74.2	92.4	92.4	92.6
50	35.2	64.2	76.5	96.1	96.3	96.3
60	36.4	64.7	78.2	96.3	96.4	96.5
70	36.9	65.3	78.8	96.4	96.4	96.5

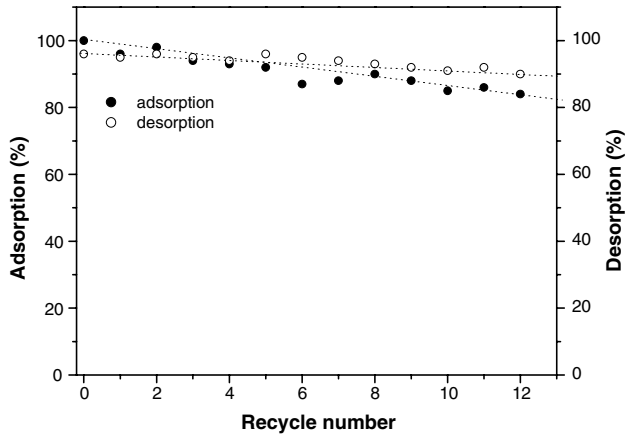

Fig. 6. Effect of recycle on cholesterol adsorption from yolk (•) and desorption from Ch-BCD (○).

The effectiveness of the recycled Ch-BCD in removing cholesterol was also examined. The effect of recycle number on the adsorption and desorption of cholesterol from yolk is shown in Fig. 6. The percentage of cholesterol adsorption and desorption decreased with increasing recycle number. After 12 reuses, the efficiency of cholesterol adsorption and desorption decreased to 84% and 90%, respectively. Literature reports (Kwak et al., 2001) indicated that only 75% cholesterol adsorption efficiency could be retained by free BCD after 10 reuses. In contrast, Ch-BCD could retain 85% adsorption efficiency after 10 reuses.

4. Conclusions

An immobilization technique to cross-link BCD to chitosan beads by chemical binding has been developed and demonstrated that it possessed excellent cholesterol adsorption capacity. Langmuir equation could be used to describe the cholesterol adsorption phenomena by Ch-BCD. The maximum cholesterol adsorption achieved was 0.33 g/g Ch-BCD (N_m). By using the Ch-BCD for the cholesterol adsorption from yolk, 92% of cholesterol could be removed with 1% Ch-BCD in 2 h and at a yolk to water ration of 1:30. In the case of desorption, 96% of the cholesterol in the Ch-BCD was dissociated using 95% ethanol with an agitation rate of 75 rpm at 50 °C. After 12 reuses, the Ch-BCD retained a cholesterol adsorption and desorption ability of 84% and 90%, respectively. The present study could also point to the feasible application of the Ch-BCD adsorbents to remove cholesterol for other industries.

References

Ahn, J., & Kwak, H. S. (1999). Optimizing cholesterol removal in cream using β-cyclodextrin and response surface methodology. *Journal of Food Science, 64*, 629–632.

Alvarez-Parrilla, E., Cabrer, P. R., Singh, A. P., Al-Soufi, W., Meijide, F., Nunez, E. R., & Tato, J. V. (2002). Supramolecular linear conglomerates formed by β-cyclodextrin dimmers and sodium dexycholate. *Supramolecular Chemistry, 14*, 397–404.

Arul, J., Boudreau, A., Makhlouf, J., Tardif, R., & Grenier, B. (1988). Distribution of cholesterol in milk fat fractions. *Journal of Dairy Research, 55*, 361–372.

Awad, A. C., & Smith, D. M. (1996). Method for reduction of cholesterol in egg materials. US Patent No. 5484624.

Betigeri, S. S., & Neau, S. H. (2002). Immobilization of lipase using hydrophilic polymers in the form of hydrogel beads. *Biomaterials, 23*, 3627–3636.

Borges, S. V., Martucci, E. T., & Muller, C. O. (1996). Optimization of the extraction of cholesterol from dehydrated egg yolk using acetone. *Food Science and Technology-Lebensmittel-Wissenschaft and Technologie, 29*, 687–690.

Bradley, R. L. (1989). Removal of cholesterol from milk-fat using supercritical carbon dioxide. *Journal of Dairy Science, 72*, 2384–2840.

Campbell, L. S., & Davies, B. E. (1995). Soil sorption of caesium modeled by the Langmuir and Freundlich isotherm equation. *Applied Geochemistry, 10*, 715–723.

Chait, A., Brunzell, J. D., Denke, M. A., Eisenberg, D., Ernst, N. D., Franklin, F. N., Ginsberg, H., Kotchen, T. A., Kuller, L., Mullits, R. M., Nichaman, M. Z., Nicolosi, R. J., Schaefer, E. J., Stone, N. J., & Weidman, W. H. (1993). Rationale of the diet-heart statement of the American-Heart-Association report of the nutrition committee. *Circulation, 65*, 3008–3029.

Chandy, T., & Sharma, C. P. (1990). Chitosan as a biomaterial. *Biomaterials Artificial Cells and Artificial Organs, 18*, 1–24.

Chang, E. J., Oh, H. I., & Kwak, H. S. (2001). Optimization of cholesterol removal conditions from homogenized milk by treatment with saponin. *Asian-Australasian Journal of Animal Science, 14*, 844–849.

Chang, S. J., Puryear, J., Funkhouser, E. A., Newton, R. J., & Cairney, J. (1996). Cloning of a cDNA for a chitinase homology which lacks chitin-binding sites and is down-regulated by water-stress and wounding. *Plant Molecular Biology, 31*, 693–699.

Chiou, S. H., Wu, W. T., Huang, Y. Y., & Chung, T. Z. (2001). Effects of the characteristics of chitosan on controlling drug release of chitosan coated PLLA microspheres. *Journal of Microencapsulation, 18*, 613–625.

Grizard, G., Sion, B., Bauchart, D., & Boucher, D. (2000). Separation and quantification of cholesterol and major phospholipids classes in human semen by high performance liquid chromatography and light-scattering detection. *Journal of Chromatography B, 740*, 101–107.

Gurr, M. I. (1992). Dietary lipids and coronary heart disease: Old evidence, new perspective. *Progress in Lipid Research, 31*, 195–243.

Hedges, A. R. (1998). Industrial applications of cyclodextrins. *Chemical Reviews, 98*, 2035–2044.

Kim, K., Kim, C., & Byun, Y. (2001). Preparation of a dipalmitoylphosphatidyl-choline/cholesterol Langmuir – Blodgett monolayer that suppresses protein adsorption. *Langmuir, 17*, 5066–5070.

Kohlrausch, U., Cully, J., & Schmid, B. (1996). Process for the removal of cholesterol derivatives from egg yolk. US Patent No. 5498437.

Kwak, H.S., Ahn, J.J., & Lee, D.K. (2000). Method for removing cholesterol from milk and cream. US Patent No. 6110517.

Kwak, H. S., Sun, H. M., Ahn, J., & Kwon, H. J. (2001). Optimization of β-cyclodextrin recycling process for cholesterol removal in cream. *Asain-Australasian Journal of Animal Science, 14*, 548–552.

Lehr, C. M., Bouwstra, J. A., Schacht, E. H., & Junginger, H. E. (1992). In-vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. *International Journal of Pharmaceutics, 78*, 43–48.

Mine, Y., & Bergougnoux, M. (1998). Adsorption properties of cholesterol-reduced egg yolk low-density lipoprotein at oil-in-water interfaces. *Journal of Agricultural and Food Chemistry, 46*, 2153–2158.

Mohamed, R. S., Neves, G. B. M., & Kieckbusch, T. G. (1998). Reduction in cholesterol and fractionation of butter oil using supercritical CO_2 with adsorption on alumina. *International Journal of Food Science and Technology, 33*, 445–454.

Mohamed, R. S., Saldana, M. D. A., Socantaype, F. H., & Kieckbusch, T. G. (2000). Reduction in the cholesterol content of butter oil using supercritical ethane extraction and adsorption on alumina. *Journal of Supercritical Fluids, 16*, 225–233.

Muzzarelli, R., Tarsi, R., Filippini, O., Giovanetti, E., Biagini, G., & Varaldo, P. E. (1990). Antimicrobial properties of *N*-carboxybutyl chitosan. *Antimicrobial Agents and Chemotherapy, 34*, 2019–2023.

Nagatomo, S. (1985). Cyclodextrin-expanding the development of their formations and application. *Chemical Economy and Engineering Review, 17*, 28–34.

Nakamura, N., & Horikoshi, K. (1976). Purification and properties of cyclodextrins glucanotrasferase of an alkalophilic *Bacillus* sp. *Agricultural and Biological Chemistry, 40*, 935–941.

Noda, T., Furuta, S., & Suda, I. (2001). Sweat potato β-amylase immobilized on chitosan beads and its application in the semi-continuous production of maltose. *Carbohydrate Polymers, 44*, 189–195.

Palit, D., & Moulik, S. P. (2001). Adsorption behaviors of L-histidine and DL-tryptophan on cholesterol, silica, alumina, and graphite. *Journal of Colloid and Interface Science, 239*, 20–26.

Rao, P., Sujatha, D., Raj, K. R., Vishwanatha, S., Narasimhamurthy, K., Saibaba, P., Rao, D. N., & Divakar, S. (2000). Safety aspects of residual β-cyclodextrin in egg treated for cholesterol removal. *European Food Research and Technology, 211*, 393–395.

Ravichandran, R., & Divakar, S. (1998). Inclusion of ring a lot of cholesterol inside the β-cyclodextrin cavity: Evidence from oxidation reactions and structural studies. *Journal of Inclusion Phenomena and Molecular Recognition in Chemistry, 30*, 253–270.

Sellergren, B., Wieschemeyer, J., Boos, K. S., & Seidel, D. (1998). Imprinted polymers for selective adsorption of cholesterol from gastrointestinal fluids. *Chemistry of Materials, 10*, 4037–4046.

Singh, A. P., Cabrer, P. R., Alvarez-Parrilla, E., Meijide, F., & Tato, J. V. (1999). Complexation of 6-deoxy-6-(aminoethyl)amino-β-cyclodextrin with sodium cholate and sodium deoxycholate. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 35*, 335–348.

Smith, D. M., Awad, A. C., Bennink, M. R., & Gill, J. L. (1995). Cholesterol reduction in liquid egg-yolk using β-cyclodextrin. *Journal of Food Science, 60*, 691–696.

Song, Y., Babiker, E. E., Usui, M., Saito, A., & Kato, A. (2002). Emulsifying properties and bactericidal action of chitosan–lysozyme conjugates. *Food Research International, 35*, 459–466.

Sreenivasan, K. (1998). Synthesis and preliminary studies on a β-cyclodextrin coupled chitosan as a novel adsorbent matrix. *Journal of Applied Polymer Science, 69*, 1051–1055.

Wade, L. G., Jr. (1999). *Organic Chemistry*. USA: Prentice Hall International, pp. 822–836.

Yen, G. C., & Tsui, L. T. (1995). Cholesterol removal from a lard-water mixture with β-cyclodextrin. *Journal of Food Science, 60*, 561–564.