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Beta-cyclodextrin complexing to reduce antibiotic residue in milk (2004)

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BETA-CYCLODEXTRIN COMPLEXING TO REDUCE ANTIBIOTIC RESIDUE IN MILK

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Summary

Various percentages (1.2% to 13%) of β-cyclodextrin (β–CD) were added to water or pasteurized whole milk to study β-CD crystallization patterns. Influential factors such as crystallization time (4 to 12 h), crystallization temperature (45° vs. 72°F), and centrifugation speed (25 to 3000 × *g*) were investigated. Optimized crystallization conditions were verified in antibiotic-tainted raw milk samples via the enzyme-linked receptor-binding assay and by solids partitioning. In water, β-CD precipitate increased significantly as β-CD concentration and crystallization time increased, but was independent of the centrifugation speed. In pasteurized whole milk, precipitate increased as β-CD concentration, crystallization time, and centrifugation speed increased. The best β-CD precipitation conditions in milk were designated as: 9.1% β-CD concentration (w/w), 4 hour crystallization time, 45°F, and centrifugation at 25 × *g* for 10 minutes. Eleven cows were treated with cephapirin sodium or cephapirin benzathine. Milk was obtained 12 h later and treated with β-CD. As β-CD concentrations increased, precipitates increased, and the corresponding supernatants showed reduced concentrations of antibiotics when tested by enzyme-linked receptor-binding assay. Results indicated that β-CD may have the potential to reduce the residues of cephapirin sodium or cephapirin benzathine in antibiotic-tainted raw milk.

(Key Words**:** β-cyclodextrin, Cephapirin Sodium, Cephapirin Benzathine, Milk.)

Introduction

Cyclodextrins are chemically and physically stable molecules formed by the enzymatic modification of starch. β-Cyclodextrin (β-CD) is a cyclic oligosaccharide having a cavity at the center of its molecular arrangement that allows for inclusion-complexing with various compounds. Of all the cyclodextrins, β-CD has limited aqueous solubility of 1.85 g/100 mL at 77°F. Previous work has shown that β-CD can bind cholesterol from various food sources, such as milk, and thus, lower the cholesterol content in the liquid portion of the food.

Antibiotics are used to treat bacterial infections in humans and animals. Bovine mastitis is an inflammatory and highly communicable udder disease, which is often treated with the administration of antibiotics. The most common antimicrobials used in lactating cows are the group known as β-lactams (e.g., cephalosporins and penicillins). Antibiotics can enter the raw milk supply when milk from antibiotic-treated cows is inadvertently mixed with that from healthy cows. If humans sensitive to these drugs consume the antibiotic-tainted milk, they may exhibit allergic reactions that range from mild skin rashes to life-threatening anaphylaxis. Studies showed that penicillin may produce allergic reaction when its concentration is as small as 10 ppb in milk. In the United States, testing of each incoming milk shipment is mandatory; in 2002 alone, the United States disposed of 85.5 million lb of milk because of the presence of antibiotics. As of now, no tested and proven method

has been developed to remove these potentially harmful compounds from milk.

The β-Lactam drugs have structures similar to other organic compounds such as cholesterol (molecular size: Cephalosporin - 478.7; Cholesterol - 414.7). β-CD complexes may be precipitated under certain conditions, such as heating then cooling, to remove unwanted compounds from liquid solutions. The test hypothesis was to study the crystallization pattern of β-CD in water and to determine whether β-CD can complex and precipitate unwanted antibiotic residues in raw milk.

Experimental Procedures

Pharmaceutical grade β-CD (Wacker Biochem Corp. Adrian, MI), pasteurized whole milk (Kansas State University dairy plant, Manhattan, KS), and raw milk (Kansas State University Dairy Teaching and Research Center, Manhattan, KS) were obtained.

Solutions were made with 20 g of solvent (pasteurized whole milk or deionized distilled water) and a variety of concentrations (1.2, 2.4, 4.8, 9.1, or 13%) of β-CD (w/w). Solutions were stirred continuously at 500 rpm for 10 minutes at 140°F to allow for complete dissolution of β-CD.

Some solutions were held at 72 ± 2°F for 5 or 10 h to allow for precipitation. After crystallization, solutions were transferred into 50-mL centrifuge bottles and centrifuged at 25, 50, 100, 250, 500, 1000, 2000, or 3000 × *g* for 10 minutes at 22 ± 2°F. Other solutions were placed in capped centrifuge bottles immediately after mixing and heating, and were transferred to an ice bath (32 ± 2°F) to achieve a solution temperature less than or equal to 50°F within 20 minutes. After 20 minutes in the ice bath, centrifuge bottles were transferred to storage (45°F) and maintained for 4, 5, 8, or 12 h. Solutions held at refrigerated conditions were centrifuged at 25 × *g* for 10 minutes. Total solids for β-CD, raw milk, pasteurized whole milk, supernatants, and precipitates were performed as outlined in standard methods.

Antibiotic-infused milk was obtained from K-State Dairy Teaching and Research Center 12 h after antibiotic administration. Two different β-lactam drugs (10 mL), cephapirin sodium or cepharpirin benzathine, were infused into all four quarters of 5 or 6 cows, respectively. Milks were tested by using an enzyme-linked receptor-binding assay (ELRBA) approved by the FDA for the rapid assay for presence of antibiotic in milk. Antibiotic-tainted milk was diluted with raw, bulk tank milk to obtain ratios that might occur when milk from an antibiotic-treated cow was inadvertently mixed with normal milk. Milk samples were then treated with β-CD, heated to 72°F, cooled, refrigerated, and then centrifuged for 10 minutes at 25 × *g*. Supernatants were retrieved and tested for antibiotic presence with the ELRBA test.

Three experiments were conducted. In the first experiment, the crystallization pattern of β-CD in water as a function of centrifugation speed and β-CD solution concentration was studied. A simple 2-factorial design was used with various β-CD concentrations (1.2 to 13.1%), and centrifugation speeds (1000 to 3000 × *g*). Crystallization patterns were evaluated by monitoring β-CD in the precipitate and supernatant. The second experiment focused on the effect of centrifugation speed on the precipitate recovery of β-CD in water solutions. A simple 1-factorial design comparing five centrifugation speeds (25, 50, 100, 250, and 500 × *g*) at a single β-CD concentration (9.1% w/w) was used. The same experiment was repeated with pasteurized whole milk as the diluent. In the third experiment, crystallization patterns of β-CD in water and pasteurized whole milk at 45°F as a function of holding time were determined by using a randomized block design with replication as

the blocking factor and holding time (4, 5, 8, and 12 h) as the main factor, keeping a constant concentration of 9.1% β-CD. Finally, to validate our data, antibiotic-tainted milk was obtained from 11 cows and was treated with β-CD and subsequently analyzed for the presence of antibiotics.

Results and Discussion

The main effects and interaction of β-CD and centrifugation speed were significant for total solids in the precipitate and in the supernatant. Table 1 summarizes average total solids for the precipitate and supernatant. The results illustrate the solubility characteristics of β-CD. Table 1 clearly shows that, at a β-CD concentration of 1.2% (less than the solubility limit), no solids were recovered in the precipitate. In contrast, at the greater concentration of 2.4%, a small amount of solids (11.4%) was recovered in the precipitate. When concentrations exceeded the β-CD solubility range, greater amounts of total solids were recovered in the precipitate (Table 1). For the 9.1% solution, precipitate recovery was independent of the centrifugation speed.

When considering the final objective of using β-CD to complex β-lactam antibiotic, and subsequently precipitate the complex, two conditions were thought to be important: 1) choose a β-CD concentration that is sufficient to complex with β-lactam; and 2) use conditions to maximize β-CD+β-lactam complex precipitation with minimal expenditures of energy and cost. Table 1 indicates that the greatest precipitate recovery occurred at the 13% concentration, but the difference between the 9.1% and 13% concentration was relatively small (70% vs. 75%). Therefore, a 9.1% concentration of β-CD was selected for further work to maximize complexing and recovery of the contaminant in the precipitate.

To verify whether centrifugation speed affected precipitate recovery, a range of centrifugation conditions (25 to 3000 × g) were selected. Results from various centrifugation speeds (Figure 1 and Table 2) illustrate that recovery of precipitate was independent of speed when water was the diluent. When milk was the diluent, amount of precipitate increased as centrifugation speed increased, indicating that the centrifugation procedure caused some of the milk solids to precipitate. To minimize milk solids in the precipitate, the slowest centrifugation speed (25 × g) was selected for further experiments.

To enhance the crystallization rate of β-CD and maintain the milk at refrigerated conditions, solutions of β-CD with water or raw milk were held at 45°F for 4, 5, 8, or 12 h. Results indicated that the main effects and interactions of β-CD holding time were significant for the total solids in the precipitate. In contrast, for the supernatant solids, the 9.1% solution did not show a difference. Table 3 shows the results of total solids in the supernatant and precipitate portion for raw milk or water. As the holding time increased, total solids in the precipitates of the milk also increased (P<0.05; Table 3). At 4 h, raw milk had minimum solids in the precipitate. Precipitate recoveries in milk exceeded the amount of added β-CD (at 12 h), indicating that milk solids were being precipitated with β-CD (Table 3). The difference of total solids in the water and milk precipitate was reported as loss of milk solids in milk precipitate. A holding time of 4 h was considered best for raw milk and was used as a standard holding time in future experiments to minimize losses of milk solids in the precipitate.

To test that hypothesis raw milk was obtained from mastitic cows 12 h after treatment with either cephapirin sodium or cephapirin benzathine. For brevity, results from two cows are presented herein. Initial results indicated that antibiotic presence was high, so various amounts of β-CD were added to the raw milk, dissolved, and precipitated, and the resultant milk supernatant was tested for the presence of antibiotics. The ELRBA values

indicated that as β-CD concentration increased, ELRBA value decreased, and some to the point of the milk being considered negative for residue (Table 4). This study provides some evidence that the β-CD may be able to complex and then precipitate unwanted β-lactam drugs in raw milk.

In water, β-CD precipitate increased as β-CD concentration increased, but the amount of precipitate was independent of centrifugation speed. In raw milk, the total solids in the precipitate increased with the increased β-CD concentration and with crystallization time. Optimal conditions for the β-CD precipitation in milk was 9.1% β-CD concentration (w/w), 4 h crystallization time at 45ºF, and centrifugation at 25 × *g* for 10 minutes. Results from ELRBA indicated that β-CD has the potential to reduce the residues of cephapirin sodium or cephapirin benzathine in antibiotic-tainted raw milk.

Table 1. Total Solids in Precipitate and Supernatant from Solutions of De-ionized Distilled Water and 1.25 to 15% (0.25 to 3 g) β-Cyclodextrin, Centrifuged at 1000, 2000, or 3000 × *g* for 10 Minutes

	Precipitate[1], g		
β-cyclodextrin	1000 × *g*	2000 × *g*	3000 × *g*
0.25 g (1.2%)	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}
0.50 g (2.4%)	0.06 ± 0.01^{f}	0.06 ± 0.02^{f}	0.05 ± 0.02^{f}
1.00 g (4.8%)	0.51 ± 0.02^{d}	0.48 ± 0.02^{e}	0.47 ± 0.03^{e}
2.00 g (9.1%)	1.39 ± 0.02^{c}	1.39 ± 0.01^{c}	1.39 ± 0.02^{c}
3.00 g (13%)	2.21 ± 0.02^{b}	2.27 ± 0.01^{a}	2.27 ± 0.01^{a}
	Supernatant[1], g		
β-cyclodextrin	1000 × *g*	2000 × *g*	3000 × *g*
0.25 g (1.2%)	0.21 ± 0.01^{k}	0.21 ± 0.01^{k}	0.22 ± 0.01^{k}
0.50 g (2.4%)	$0.38 \pm 0.02^{e,f}$	$0.37 \pm 0.02^{f,g,h}$	$0.38 \pm 0.01^{e,g}$
1.00 g (4.8%)	$0.46 \pm 0.01^{a,b,c}$	0.48 ± 0.03^{a}	$0.47 \pm 0.02^{a,b}$
2.00 g (9.1%)	$0.45 \pm 0.02^{b,c}$	$0.43 \pm 0.02^{c,d}$	$0.37 \pm 0.02^{f,g,i}$
3.00 g (13%)	$0.40 \pm 0.01^{d,e}$	$0.35 \pm 0.01^{h,i,j}$	0.34 ± 0.01^{j}

[a,b,c,d,e,f,g,h,i,j,k]Means having different superscript letters within columns and rows for either precipitate or supernatant differ ($P<0.05$).
[1]Mean and standard deviations (n = 3).

Table 2. Total Solids in Supernatant and Precipitate of De-ionized Distilled Water having 2g of β-Cyclodextrin and Centrifuged at 25, 50, 100, 250, or 500 × *g* for 10 Minutes

Force (× g)	Supernatant[1]	Precipitate[1]
25	0.40 ± 0.06[b]	1.36 ± 0.06[a]
50	0.41 ± 0.07[b]	1.36 ± 0.06[a]
100	0.35 ± 0.01[b]	1.40 ± 0.01[a]
250	0.39 ± 0.00[b]	1.35 ± 0.02[a]
500	0.39 ± 0.02[b]	1.39 ± 0.00[a]

[a,b]Means having different superscript letters within a column differ ($P<0.05$).
[1]Data represents mean ± SD (n = 3).

Table 3. Precipated Total Solids in De-ionized Distilled Water or Raw Milk Treated with 2 g of β-Cyclodextrin, Held at 45° for 4, 5, 8, or 12 Hours, and Centrifuged at 25 × g for 10 Minutes

Time	Raw milk[1]	Water[1]	Difference
4 hours	1.72 ± 0.24[b]	1.46 ± 0.03[d]	0.26
5 hours	1.98 ± 0.33[a,b]	1.48 ± 0.03[c]	0.50
8 hours	1.97 ± 0.31[a,b]	1.51 ± 0.03[b]	0.46
12 hours	2.03 ± 0.22[a]	1.53 ± 0.02[a]	0.50

[a,b,c,d]Means having different superscipts within a column differ ($P<0.05$).
[1]Mean ± SD.

Table 4. ELRBA Values of Antibiotic-tainted Raw Milk, Before and After Treatment with 0, 1, 2, or 4 g of β-Cyclodextrin

Antibiotic	ELRBA before treatment	Amount of β-Cyclodextrin, g	ELRBA after treatment
Cephapirin sodium	2.26 (positive)	0	2.19 (positive)
		1	1.35 (positive)
		2	0.93 (negative)
		4	0.89 (negative)
Cephapirin benzathine	1.26 (positive)	0	1.13 (positive)
		1	1.08 (positive)
		2	0.98 (negative)
		4	0.91 (negative)

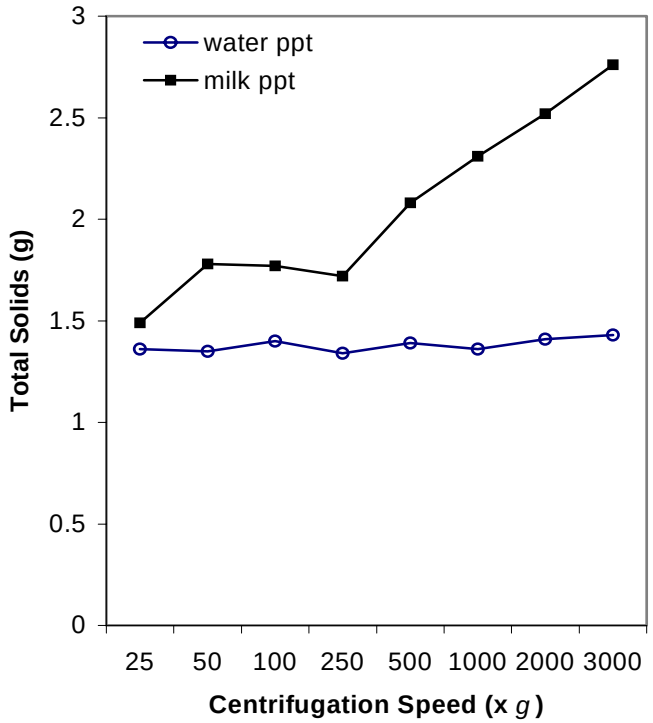

Figure 1. Total Solids in Precipitate of De-ionized Distilled Water and Milk Treated with 2 g of β-Cyclodextrin, Held at 72 ± 2°F, and Centrifuged at 25, 50, 100, 250, or 500 × g.